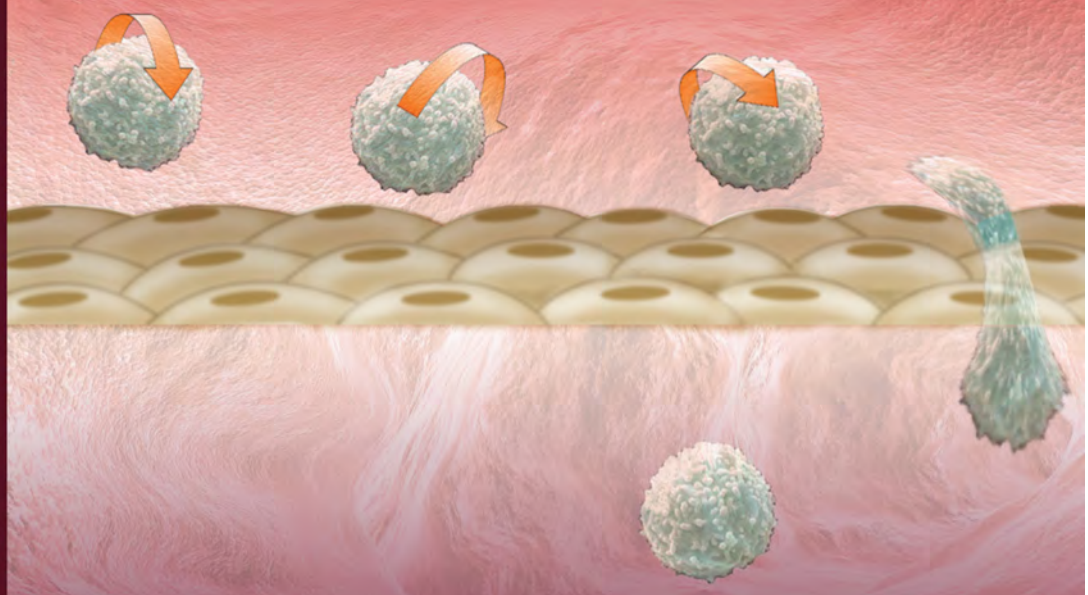
Mechanisms of Vascular Disease:

A Reference Book for Vascular Specialists



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Mechanisms of Vascular Disease

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A Reference Book for Vascular Specialists

Robert Fitridge
The University of Adelaide, The Queen Elizabeth Hospital, Woodville, Australia
Matthew Thompson
St George's Hospital Medical School, London, UK

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List of Contributors

David G Armstrong
The University of Arizona
Southern Arizona Limb Salvage Alliance
Tucson, AZ
USA

Vishwanath Biradar
Intensive Care Unit
The Queen Elizabeth Hospital
Woodville, SA
Australia

Matthew Bown
Department of Vascular Surgery
University of Leicester
Leicester
UK

Andrew W Bradbury
University Department of Vascular Surgery
Birmingham Heartlands Hospital
Birmingham
UK

Edward Choke
Department of Vascular Surgery
University of Leicester
Leicester
UK

Gillian Cockerill
Department of Clinical Sciences
St George's Hospital Medical School
London
UK

Prue Cowled
Department of Surgery
University of Adelaide
The Queen Elizabeth Hospital
Woodville, SA
Australia

Helen Daly
Royal Perth Hospital
Perth, WA
Australia

Mital Desai
University Department of Vascular Surgery
Royal Free Hospital
University College
London
UK

Robert F Diegelmann
Department of Biochemistry
Medical College of Virginia
Richmond, VA
USA

Timothy K Fisher
Rashid Centre for Diabetes and Research
Sheikh Khalifa Hospital
Ajmon
UAE

Robert A Fitridge
Department of Surgery
University of Adelaide
The Queen Elizabeth Hospital
Woodville, SA
Australia

Gail Gillespie
Royal Perth Hospital
Perth, WA
Australia

Jonathan Golledge
Vascular Biology Unit
School of Medicine & Dentistry
James Cook University
Townsville, QLD
Australia

George Hamilton
University Department of Vascular Surgery
Royal Free Hospital
University College
London
UK

Mark Hamilton
Department of Surgery
University of Adelaide
The Queen Elizabeth Hospital
Woodville, SA
Australia

Robert J Hinchliffe
St George's Vascular Institiute
St George's Hospital
London
UK

Richard D Kenagy
Department of Surgery
University of Washington
Seattle, WA
USA

Paul Kerr
Department of Pharmacology
University of Alberta
Alberta
Canada

Michael MD Lawrence-Brown
Curtin Health Innovation Research Institute
Curtin University
Perth, WA
Australia

Brian Lepow
The University of Arizona
Department of Surgery
Southern Arizona Limb Salvage Alliance
Tucson, AZ
USA

Kurt Liffman
CSIRO Material Science & Engineering and School of Mathematical Sciences
Monash University
Melbourne, Vic
Australia

Ian Loftus
Department of Vascular Surgery
St George's Hospital
London
UK

Mark J McCarthy
Department of Surgery and Cardiovascular Sciences
University of Leicester
Leicester
UK

Greg S McMahon
Department of Surgery and Cardiovascular Sciences
University of Leicester
Leicester
UK

Simon McRae
Adult Haemophilia Treatment Centre
SA Pathology
Adelaide, SA
Australia

Joseph L Mills
The University of Arizona
Southern Arizona Limb Salvage Alliance
Tucson, AZ
USA

Lyle Moldawer
Department of Surgery
University of Florida
Gainesville, FL
USA

John L Moran
Faculty of Health Sciences
University of Adelaide
The Queen Elizabeth Hospital
Woodville, SA
Australia

Stephen Nicholls
The Heart and Vascular Institute
Cleveland Clinic
Cleveland, OH
USA

Ian M Nordon
St George's Vascular Institiute
St George's Hospital
London
UK

Paul E Norman
School of Surgery
University of WA
Fremantle, WA
Australia

Karlheinz Peter
Baker IDI Heart & Diabetes Institute
Melbourne, Vic
Australia

Frances Plane
Department of Pharmacology
University of Alberta
Alberta
Canada

Janet T Powell
Imperial College
London
UK

Sandeep Prabhu
Baker IDI Heart & Diabetes Institute
Alfred Hospital
Melbourne, Vic
Australia

Rishi Puri
The Heart and Vascular Institute
Cleveland Clinic
Cleveland, OH
USA

Stephan A Schug
Royal Perth Hospital
Perth, WA
Australia

Gregory S Schultz
Department of Obstetrics and Gynaecology
University of Florida
Gainesville, FL
USA

Rahul Sharma
Baker IDI Heart & Diabetes Institute
Alfred Hospital
Melbourne, Vic
Australia

Guo-Ping Shi
Department of Cardiovascular Medicine
Brigham & Women's Hospital
Harvard Medical School
Boston, MA
USA

Michael Stacey
University Department of Surgery
Fremantle Hospital
Fremantle, WA
Australia

Ilija D Sutalo
CSIRO Material Science & Engineering
and Curtin Health Innovation
Research Instutute
Curtin University
Highett, Vic

Raymond Tam
Department of Pharmacology
University of Alberta
Alberta
Canada

Matthew Thompson
St Georges Hospital Medical School
London
UK

Martin Veller
Department of Surgery
University of Witwatersrand
Johannesburg
South Africa

Mauro Vicaretti
Department of Vascular Surgery
Westmead Hospital
Westmead, NSW
Australia

Matt Waltham
Academic Department of Surgery
St Thomas' Hospital
London
UK

Matthew L White
Vascular and Endovascular Surgery
University of Arizona
Tucson, AZ
USA

David P Wilson
School of Medical Sciences
Discipline of Physiology
University of Adelaide
Adelaide SA
Australia

Qingbo Xu
Department of Cardiology
Kings College
University of London
UK

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Abbreviation List

a1-PI	a1-protease inhibitor
5-HT	5-Hydroxytryptamine/Serotonin
AAA	Abdominal aortic aneurysm
AAS	Acute aortic syndrome
AAV	Adeno-associated viruses
ACE	Angiotensin converting enzyme
ACS	Acute coronary syndrome
ACS	Abdominal compartment syndrome
ACTH	Adrenocorticotropic hormone
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
ADP	Adenosine diphosphate
AIDS	Acquired immune deficiency syndrome
ALI	Acute lung injury
AMP	Adenosine monophosphate
AMPA	α-amino-3 hydroxy-5-methylisoxazole
ANA	Anti-nuclear antibody
ANCA	Anti-neutrophil cytoplasmic antibody
AOD	Aortic occlusive disease
AP1	Activated protein 1
APC	Activated protein C
APC	Antigen presenting cell
APLAS	Antiphospholipid antibody syndrome
ApoAI	Apolipoprotein AI
ApoE	Apolipoprotein E
APS	Antiphospholipid antibody syndrome
APTT	Activated partial thromboplastin time

ARDS	Acute respiratory distress syndrome
AT	Antithrombin
ATP	Adenosine triphosphate
AVP	Ambulatory venous thrombosis
β2-GPI	β2-glycoprotein Ib
bFGF	Basic fibroblast growth factor
BKCa	Large conductance calcium activated potassium channel
BMPs	Bone morphogenetic proteins
BMS	Bare metal stent
CAD	Coronary artery disease
CaM	Calmodulin
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin
cGMP	Cyclic guanine monophosphate
CD	Cluster of differentiation
CD40L	Cluster of differentiation 40 ligand
CEA	Carotid endarterectomy
CETP	Cholesteryl ester transfer protein
CFD	Computational fluid dynamics
CG	Cationized gelatin
CGRP	Calcitonic gene regulated peptide
CHD	Coronary heart disease
CI	Confidence interval
CIMT	Carotid intimal-media thickness
c-JNK	c-Jun N-terminal kinase
CK-MB	Creatinine kinase (Myocardial specific)
CNCP	Chronic noncancer pain
cNOS	Constitutive nitric oxygen synthase enzyme
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CROW	Charcot restraint orthotic walker
CRRT	Continuous renal replacement therapy

CRP	C-reactive protein
CRPS	Complex regional pain syndromes
CT	Computational tomography
CTA	Computed tomographic angiography
CTD	Connective tissue disorders
CTGF	Connective tissue growth factor
CYP	Cytochrome P450
CVD	Cardiovascular disease
CVI	Chronic venous insufficiency
DAG	Diacylglycerol
DES	Drug-eluting stent
DRG	Dorsal root ganglion
DNA	Deoxyribonucleic acid
DSA	Digital subtraction arteriography
DTS	Dense tubular system
DVT	Deep vein thrombosis
EC	Endothelial cell
ECM	Extracellular matrix
EDCF	Endothelium-derived contracting factor
EDH	Endothelium-dependent hyperpolarisation
EDS	Ehlers-Danlos syndrome
EET	Epoxyeicosatrienoic acids
ELAM-1	Endothelial-leukocyte adhesion molecule-1
ELG	Endoluminal grafts
ELISA	Enzyme linked immunosorbent assay
E_K	Equilibrium potential
E_M	Membrane potential
eNOS	Endothelial nitric oxide synthase enzyme
EPC	Endothelial progenitor cells
EPCR	Endothelial protein C receptor
ePTFE	Expanded polytetrafluoroethylene
ERK	Extracellular signal-regulated kinase
ESR	Erythrocyte sedimentation rate

ET	Essential thrombocytosis
ET-1	Endothelin 1
EVAR	Endovascular aortic aneurysm repair
EVLA	Endovenous LASER ablation
FDA	Food and drug administration
FDPs	Fibrin degradation products (soluble)
FGF	Fibroblast growth factor
FGF-2	Fibroblast growth factor 2
FMN	Flavin mononucleotide
FVL	Factor V Leiden
GABA	Gamma-aminobutyric acid
GABA B	Gamma-aminobutyric acid subtype B
G-CSF	Granulocyte colony stimulating factor
GMCSF	Granulocyte-macrophage colony stimulating factor
GP	Glycoprotein
GPCR	G-protein coupled receptor
GSV	Great saphenous vein
HDL	High density lipoprotein
HDL-C	High density lipoprotein cholesterol
HIF	Hypoxia inducible factor
HIT	Heparin induced thrombocytopenia
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HMG Co-A	Hydroxymethylglutaryl coenzyme-A
HMW	High molecular weight
HPETE	Hydroperoxyeicosatetraenoic acid
HETE	Hydroxyeicosatetraenoic acids
HR	Hazard ratio
hsCRP	High-sensitive C-reactive protein
HSP	Heat shock protein
HUV	Human umbilical vein
IAH	Intra-abdominal hypertension

IAP	Intra-abdominal pressure
IAPP	Intra-abdominal perfusion pressure
ICAM-1	Inter-cellular adhesion molecule-1
ICAM-2	Inter-cellular adhesion molecule-2
ICP	Intra-compartmental pressure
ICU	Intensive care unit
IFN	Interferon
IGF-1	Insulin-like growth factor-1
IHD	Ischemic heart disease
IL	Interleukin
IL-1	Interleukin-1
IL-1α	Interleukin-1 alpha
IL1-β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
ILT	Intraluminal thrombus
IKCa	Intermediate conductance calcium-activated potassium channels
IMH	Intramural haematoma
IMP	Inosine monophosphate
iNOS	Inducible nitric oxide synthase enzyme
IP(3)	1,4,5-inositol triphosphate
IRI	Ischemia reperfusion injury
IVIG	Intravenous pooled immunoglobulin
IVUS	Intravascular ultrasound
KGF	Keratinocyte growth factor
KGF-2	Keratinocyte growth factor-2
LAP	Latency associated peptide
LCS	Limb compartment syndrome
LDL	Low density lipoprotein
LDS	Loeys-Dietz syndrome
LLC	Large latent complex
LEC	Lymphatic endothelial cells

LFA-1	Lymphocyte function-associated antigen-1
LO	Lipoxygenase
LOX	Lysyl oxidase
LOPS	Loss of protective sensation
LPA	Lysophosphatidic acid
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LTGFBP	Latent TGF binding protein
MAC-1	Macrophage-1 antigen
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage-colony stimulating factor
MFS	Marfan syndrome
MHC	Major histocompatibility
MI	Myocardial infarction
MIP-1	Macrophage inflammatory protein-1
MLC_{20}	Myosin light $chain_{20}$
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MMP	Matrix metalloproteinase
MODS	Multiple organ dysfunction syndrome
MRA	Magnetic resonance angiography
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MRSA	Methicillin resistant *Staphylococcus aureus*
MRSE	Methicillin resistant *Staphylococcus epidermidis*
MRTA	Magnetic resonance tomographic angiography
MTHFR	Methylenetetrahydrofolate reductase
MT-MMP	Membrane-type MMP
MVPS	Mitral valve prolapse syndrome
NADPH	Nicotinamide adenine dinucleotide phosphate
NGF	Nerve growth factor

NFκB	Nuclear factor kappa B
NiTi	Nitinol
NJP	Non-junctional perforators
NMDA	N-methyl-D-aspartate
NNH	Number needed to harm
NNT	Number needed to treat
NO	Nitric oxide
NOS	Nitric oxide synthase enzyme
NSAID	Non-steroidal anti-inflammatory drug
NV	Neovascularisation
OCP	Oestrogen/progesterone contraceptive pill
OPN	Osteopontin
OPG	Osteoprotegerin
OR	Odds ratio
OxLDL	Oxidised low density lipoprotein
PAD	Peripheral arterial disease
PAF	Platelet activating factor
PAI	Plasminogen activator inhibitor
PAI-1	Plasminogen activator inhibitor-1
PAR	Protease activated receptor
PAR-1	Protease activated receptor-1
PAR-4	Protease activated receptor-4
PAU	Penetrating aortic ulcer
PC	Protein C
PCA	Poly (carbonate-urea) urethane
PCI	Percutaneous coronary intervention (angioplasty)
PCWP	Pulmonary capillary wedge pressure
PDGF	Platelet-derived growth factor
PDGFβ	Platelet-derived growth factor-β
PDS	Polydioxanone
PECAM-1	Platelet-endothelial cell adhesion molecule-1
PEDF	Pigment epithelium-derived factor
PES	Paclitaxel-eluting stent

PET	Positron emission tomography
PF4	Platelet factor 4
PGI_2	Prostacyclin
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
$PGEI_2/PGI_2$	Prostaglandin I_2
PGN	Peptidoglycan
PHN	Postherpetic neuropathy
PHZ	Para-anastomotic hyper-compliant zone
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PLC	Phospholipase C
PLOD	Procollagen lysyl hydroxylase
PMCA	Plasma membrane Ca^{2+} APTases
PMN	Polymorphonuclear leukocyte
POSS	Polyhedral oligomeric silsesquioxanes
PPAR	Peroxisomal proliferation activating receptor
PPI	Proton pump inhibitor
PRV	Polycythaemia rubra vera
PS	Protein S
PSGL-1	P-selectin glycoprotein ligand-1
PT	Prothombin time
PTCA	Percutaneous coronary angioplasty
PTFE	Polytetrafluoroethylene
PTS	Post-thrombotic syndrome
PUFA	Polyunsaturated fatty acid
PVI	Primary valvular incompetence
rAAA	Ruptured AAA
Rac	Ras activated cell adhesion molecule
RANTES	Regulated upon activation, normal T cell expressed and secreted
RAS	Renin angiotensin system
RCT	Randomised controlled trial

RF	Rheumatoid factor
RFA	Radiofrequency ablation
rhAPC	Recombinant human activated protein C
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RR	Relative risk
RSD	Reflex sympathetic dystrophy
S1P	Sphingosine-1-phosphate
SAPK	Stress-activated protein kinase
SCF	Stem cell factor
SCS	Spinal cord stimulation
ScvO2	Superior vena cava venous oxygen saturation
SDF-1	Stromal-cell-derived factor-1
SERCA	Sarco/endoplasmic reticulum CaATPases
SEP	Serum elastin peptides
SES	Sirolimus-eluting stent
SEPS	Subfascial endoscopic perforator surgery
SFA	Superficial femoral artery
SFJ	Sapheno-femoral junction
SIRS	Systemic inflammatory response syndrome
SKCa	Small conductance calcium-activated potassium channels
SLE	Systemic lupus erythematosus
SMA	Smooth muscle alpha actin
SMC	Smooth muscle cell
SMP	Sympathetically maintained pain
SNARE	Soluble N-ethylmaleimide-sensitive factor activating protein receptors
SNP	Single nucleotide polymorphisms
SNRI	Serotonin/Noradrenaline reuptake inhibitors
SPJ	Sapheno-popliteal junction
SPP	Skin perfusion pressure
SR	Sarcoplasmic reticulum
SSRIs	Selective serotonin re-uptake inhibitors
SSV	Small saphenous vein

SVT	Superficial thrombophlebitis
STIM1	Stromal interacting molecule 1
TαCE	TNFα converting enzyme
TAAD	Thoracic aortic aneurysm disease
TAD	Thoracic aortic dissection
TAFI	Thrombin-activatable fibrinolysis inhibitor
Tc-99 MDP	Technetium-99 methylene diphosphonate
TCA	Tricyclic antidepressant
TCC	Total contact cast
TCR	T-cell receptor
TENS	Transcutaneous electrical nerve stimulation
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGF	Transforming growth factor
TGF-α	Transforming growth factor-alpha
TGF-β	Transforming growth factor-beta
TGL	Triglycerides
Th	T helper
TIA	Transient ischemic attack
TIMP	Tissue inhibitors of metalloproteinase
TLR	Toll-like receptors
TNF	Tumour necrosis factor
TNF-α	Tumour necrosis factor-alpha
tPA	Tissue-type plasminogen activator
TRP	Transient receptor potential
TRPC	Transmembrane receptor potential canonical
TRPV1	Transmembrane receptor potential Vanilloid-type
TXA2	Thromboxane A2
uPA	Urokinase
UT	University of Texas
VCAM	Vascular cell adhesion molecule
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor

VEGF-R	Vascular endothelial growth factor receptor
VIP	Vasoactive intestinal peptide
VLA-1	Very late activating antigen-1
VOCC	Voltage operated calcium channels
VPT	Vibratory perception threshold
VSMC	Vascular smooth muscle cells
VTE	Venous thromboembolism
VV	Varicose veins
vWF	von Willebrand factor
XO	Xanthine oxidase

1 • Endothelium

PAUL KERR, RAYMOND TAM AND FRANCES PLANE

Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada

INTRODUCTION

The endothelium, first described over 100 years ago as an inert anatomical barrier between blood and the vessel wall, is now recognized as a dynamic organ with secretory, synthetic, metabolic, and immunologic functions. Forming a continuous lining to every blood vessel in the body, endothelial cells play an obligatory role in modulating vascular tone and permeability, angiogenesis, and in mediating haemostatic, inflammatory and reparative responses to local injury. To fulfil these roles the endothelium is highly dynamic, continuously responding to spatial and temporal changes in mechanical and biochemical stimuli. Such responsiveness is affected through receptors for growth factors, lipoproteins, platelet products and circulating hormones, which regulate changes in protein and mRNA expression, cell proliferation and migration or the release of vasoactive and inflammatory mediators.

All vascular endothelial cells have a common embryonic origin but show clear bed-specific heterogeneity in morphology, function, gene and protein expression, determined by both environmental stimuli and epigenetic features acquired during development. Thus, the endothelium should not be regarded as a homogenous tissue but rather a conglomerate of distinct populations of cells sharing many common functions but also adapted to meet regional demands.[1]

The continuous endothelial cell layer provides an uninterrupted barrier between the blood and tissues in the majority of blood vessels and ensures tight control of permeability of the blood-brain barrier. In regions of increased trans-endothelial transport such as capillaries of endocrine glands and the kidney, the presence of fenestrae, transcellular pores approximately 70 nm in diameter with a thin fenestral diaphragm across their opening, facilitate the selective permeability required for efficient absorption, secretion, and filtering. In hepatic sinuses, the presence of a discontinuous endothelium with large fenestrations (0.1–1 mm in diameter) lacking a fenestral diaphragm, provides a highly permeable and poorly selective sieve essential for transfer of lipoproteins from blood to hepatocytes.

Beyond these structural variations, endothelial heterogeneity is also manifest in regional differences in the release of vasoactive and inflammatory mediators, in response to changes in shear stress and hypoxia, and in expression of pro- and anti-coagulant molecules. For example, endothelial expression of the pro-thrombotic mediator von

Willebrand factor (vWF) is a function of endothelial cells found in vessels of discrete size and/or anatomic location. Similarly, the contribution of nitric oxide (NO) to endothelium-dependent vasodilation is far greater in large conduit arteries compared to small resistance vessels. These regional biochemical and phenotypic differences between endothelial cells extend to their susceptibility to injury in the face of cardiovascular risk factors such as hypercholesterolemia, diabetes and smoking and thus impact the function of the vasculature both in health and disease.

This chapter provides an overview of how the endothelium regulates four key aspects of cardiovascular homeostasis-vascular tone, angiogenesis, haemostasis and inflammation.

ENDOTHELIUM-DEPENDENT REGULATION OF VASCULAR TONE

Since the first report by Furchgott and Zawadski[2] of endothelium-dependent modulation of the contractile state of smooth muscle cells in the artery wall, it has become apparent that endothelial cells release a plethora of vasoactive factors in response to a wide range of mechanical and chemical stimuli. That many of these factors also modulate processes such as inflammation, cell adhesion and coagulation, highlights the crucial physiological role of the endothelium and why endothelial dysfunction is pivotal in the development of cardiovascular diseases such as atherosclerosis and hypertension. This section will focus on the four major pathways underlying endothelium-dependent modulation of vascular tone; NO, arachidonic acid metabolites, endothelium-dependent hyperpolarisation (EDH) and endothelin.

Nitric oxide

The first endothelium-derived relaxing factor described by Furchgott and Zawadski was subsequently identified as NO, a short-lived free radical synthesized from L-arginine by endothelial NO synthase (eNOS) and destroyed by reactive oxygen species (ROS). NO activates the haem-dependent enzyme, soluble guanylyl cyclase in surrounding smooth muscle cells, leading to formation of cyclic guanosine monophosphate (cGMP). Subsequent protein kinase G-mediated phosphorylation of a diverse range of target proteins such as large conductance calcium-activated potassium (BK_{Ca}) channels, RhoA, Rho kinase, transient receptor potential (TRP) channels, myosin light chain phosphatase and phospholamban, leads to smooth muscle cell relaxation and hence vasodilation.[3]

eNOS is a bidomain enzyme; an N-terminal oxygenase domain with binding sites for haem, tetrahydrobiopterin, oxygen and the substrate L-arginine supports the catalytic activity, and a C-terminal reductase domain binds nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN) and flavin adenine dinucleotide co-factors. Transfer of electrons from NADPH to flavins in the reductase domain and then to the haem in the oxygenase domain is required so that the haem iron can bind oxygen and catalyze the synthesis of NO from L-arginine. Binding of the ubiquitous calcium regulatory protein calmodulin (CAM) facilitates transfer of electrons from the reductase to the oxygenase domain and is critical for activation of the enzyme.

eNOS is constitutively expressed in all endothelial cells but regulation of the enzyme by physiological and pathophysiological stimuli occurs via a complex pattern of transcriptional and post-translational modifications. For example, both eNOS

mRNA and protein levels are increased by fluid shear stress via activation of a pathway involving both c-Src-tyrosine kinase and transcription factor NFκB. At the post-translational level, eNOS activity is highly regulated by substrate and cofactor availability as well as by endogenous inhibitors, lipid modification, direct protein-protein interactions, phosphorylation, O-linked glycosylation, and S-nitrosylation. Agonists at endothelial G-protein coupled receptors (GPCRs) such as bradykinin, and acetylcholine, elicit calcium-CAM-dependent NO production by via phospholipase C-mediated generation of inositol 1,4,5-trisphosphate (IP_3) and subsequent release of calcium from intracellular stores. However, activation of tyrosine kinase linked receptors such as the vascular endothelial growth factor (VEGF) receptor, and mechanical stimulation of the endothelium by shear stress, lead to phosphorylation of eNOS at Ser^{1177} to increase the calcium sensitivity of the enzyme so that it can be activated at resting calcium levels. Distinct kinase pathways can mediate eNOS phosphorylation; shear stress elicits phosphorylation of Ser^{1177} via protein kinase A whereas insulin and VEGF cause phosphorylation of the same residue via the serine/threonine protein kinase Akt. Conversely, phosphorylation of the enzyme at Tyr^{657} within the FMN domain or Thr^{495} within the CaM-binding domain, inhibit enzyme activity.[4]

Within endothelial cells, eNOS is targeted to invaginations of the cell membrane called caveolae, membrane microdomains enriched in cholesterol and sphingolipids, and defined by the presence of the scaffolding protein caveolin. Caveolae sequester diverse receptors and signaling proteins including GPCRs, growth factor receptors and calcium regulatory proteins such as CAM. Thus, targeting of eNOS to this region facilitates communication with upstream and downstream pathways. Within caveolae, caveolin-1 tonically inhibits eNOS activity, thereby limiting the production of NO; binding of calcium-CAM leads to disruption of the caveolin-1/eNOS interaction and increases eNOS activity. Other associated proteins such as platelet endothelial cell adhesion molecule-1 (PECAM-1), modulate eNOS activity by virtue of their function as scaffolds for the binding of signaling molecules such as tyrosine kinases and phosphatases.

A vast range of stimuli such as shear stress generated by the viscous drag of blood flowing over the endothelial cell surface, circulating hormones (e.g. catecholamines, vasopressin), plasma constituents (e.g. thrombin), platelet products (e.g. 5-HT) and locally-produced chemical mediaors (e.g. bradykinin) each evoke NO-mediated vasodilation. Release of endothelium-derived NO by such stimuli plays a critical role in mediating acute changes in local blood flow and tissue perfusion. Shear stress-stimulated NO production is central to exercise-induced increases in blood flow in skeletal muscle. Production of NO in response to 5-HT released from aggregating platelets, dilates coronary arteries thus preventing the clot from occluding the vessel. Mice lacking eNOS are hypertensive and infusion of L-arginine analogues, competitive inhibitors of eNOS, cause alterations in local blood flow and in systemic blood pressure, demonstrating the importance of endothelium-derived NO in long-term control of blood pressure and blood flow in vivo. In humans, elevated levels of an endogenous inhibitor of eNOS, asymmetric dimethylarginine, are associated with hypertension and increased cardiovascular risk.

In addition to its vasodilator actions, NO is now recognized as playing myriad other protective roles in the vasculature as a regulator of clot formation, inflammation and vessel repair. Loss of NO-mediated vasodilation, due to reduced expression or activity of eNOS and/or oxidative stress-mediated

reductions in NO bioavailability, is a hallmark of endothelial dysfunction associated with cardiovascular risk factors such as hypercholesterolemia, smoking, diabetes and obesity. Loss of NO tip the homeostatic balance in favour of vasoconstriction, proliferation, activation of platelets and blood clot formation, and inflammation. These pathological processes contribute to clinical manifestations such as hypertension, atherosclerosis and arterial thrombosis, which are associated with significant morbidity and mortality.

Metabolites of arachidonic acid: Arachidonic acid, released from cell membrane phospholipids by phospholipases, is metabolized by cyclooxygenase (COX), lipoxygenase (LO), and cytochrome P450 monooxygenase (CYP) enzymes to yield an array of endothelium-derived vasoactive factors.

Cyclooxygenases: COX enzymes metabolize arachidonic acid to endoperoxide intermediates which are then converted to a range of eicosanoids (e.g. prostacyclin; PGI_2, thromboxane A_2) through the actions of various synthases. Two isoforms of cyclooxygenase are found in the endothelium. The constitutively expressed COX-1 has long been regarded as vasculoprotective, the predominant product being PGI_2 which acts on prostanoid (IP) receptors to cause vasodilation and inhibition of platelet aggregation via activation of adenylyl cyclase and subsequent elevation of intracellular cyclic-adenosine monophosphate (cAMP). PGI_2 also inhibits platelet and lymphocyte adhesion to endothelium, limits vascular smooth muscle cell proliferation and migration, and counteracts the production of growth factors.

However, evidence is now emerging that GPCR-mediated activation of endothelial COX-1 can generate other products such as TXA_2 and PGH_2 which activate thromboxane (TP) receptors on smooth muscle cells and so function as endothelium-derived contracting factors (EDCFs). Stimulation of TP receptors elicit not only vasoconstriction but also proliferation of vascular smooth muscle cells, platelet adhesion and aggregation and expression of adhesion molecules on endothelial cells. COX-1 shows basal activity and is activated by endothelial GPCRs. A shift from production of endothelium-derived relaxing factors to COX-dependent EDCFs is implicated in endothelial dysfunction associated with ageing, diabetes and hypertension.[6]

COX-2 was first identified as an inducible form of the enzyme, regulated at the level of gene expression and associated with inflammation. However, it is expressed in some blood vessels in the absence of overt signs of inflammation and may be a major source of vasculoprotective PGI_2; hence the deleterious cardiovascular consequences seen in some patients treated with selective COX-2 inhibitors.[7]

Lipoxygenases: LO enzymes deoxygenate polyunsaturated fatty acids to hydroperoxyl metabolites. The three LO isoforms expressed in endothelial cells are 5-LO, 12-LO, and 15-LO, which correspond to the carbon position of arachidonic acid oxygenation. Each LO oxygenates arachidonic acid to form a stereospecific hydroperoxyeicosatetraenoic acid (HPETE). HPETEs are unstable and are reduced to the corresponding hydroxyeicosatetraenoic acids (HETEs). 5-LO is the initial enzyme in the synthesis of leukotrienes but 5-LO products do not seem to be involved in regulation of vascular tone. In contrast, products from the 12-LO and 15-LO pathways are vasoactive but show species and vessel variation in the responses they elicit. 12-HETE elicits relaxation of a number of peripheral arteries including human coronary vessels, but causes vasoconstriction in dog renal arteries. 15-HPETE and 15-HETE cause slight vasorelaxation at lower concentrations but contractions at higher concentrations mediated by activation

of TP receptors. Although vasoactive LO metabolites are produced by endothelial cells, elucidation of their physiological role has been hindered by the lack of selectivity of pharmacological inhibitors.

Cytochrome P450 monooxygenases: CYP enzymes add oxygen across the double bonds of arachidonic acid to produce four cisepoxides, 14,15-, 11,12-, 8,9-, and 5,6-epoxyeicosatrienoic acids (EETs). Two CYP enzymes have been cloned from human endothelium CYP2C8/9 and CYP2J2 both of which produce mainly 14,15-EET with lesser amounts of 11,12-EET. The latter are also the major EETs released from endothelial cells stimulated by GPCR agonists (e.g. acetylcholine, bradykinin) and physical stimuli such as cyclic stretch and shear stress. EETs are rapidly metabolized by esterification into phospholipids or hydration to dihydroxyeicosatrienoic acids by soluble epoxide hydrolase.

EETs are vasoactive causing vasoconstrictions in the lung but eliciting vasodilatation of systemic arteries via activation of iberiotoxin-sensitive, BK_{Ca} channels on the vascular smooth muscle cells. EETs are proposed mediators of EDH in systemic arteries, acting either as transferable factors that hyperpolarize and relax smooth muscle cells, or acting in an autocrine manner to cause hyperpolarisation of the endothelial cell membrane potential which is then spread to the underlying smooth muscle through gap junctions (see below).

An EET receptor on smooth muscle cells has not been identified but development of 14,15-EET analogues such as 14,15-epoxyeicosa-5Z-enoic acid has revealed strict structural and stereoisomeric requirements for relaxations suggesting a specific binding site or receptor and BK_{Ca} channel activation by EETs requires a G protein indicating that a GPCR for EETs exists.

Some EETs activate vascular TRP channels, non-selective cation channels that can mediate calcium influx. Endothelium-dependent flow-induced dilation is linked to 5,6-EET-mediated activation of vanilloid type 4, TRPV4, channels. Formation of a complex of TRPV4 with BK_{Ca} channels in smooth muscle cells may couple local increases in calcium due to activation of TRPV4 by EETs to membrane hyperpolarisation and vasorelaxation.[8] In contrast, endothelial stimulation by bradykinin or hypoxia is associated with activation of TRPC3 and TRPC6 channels. In addition to stimulating channel activity, EETs elicit the rapid intracellular translocation of TRP channels into caveolae, a process dependent on activation of protein kinase A by cAMP, and consistent with the activation of a GPCR.

In some models of endothelial dysfunction, reduced bioavailability of NO is counteracted by increased production of EETs which can maintain endothelium-dependent vasodilator responses. Thus, strategies aimed at enhancing production of endothelium-derived EETs or inhibiting their degradation, may represent a new therapeutic approach to endothelial dysfunction.

Endothelium-dependent hyperpolarisation (EDH): Observations of agonist-induced endothelium-dependent vasorelaxation which persisted in the presence of inhibitors of prostaglandin and NO synthesis and was accompanied by hyperpolarisation of the vascular smooth muscle cell membrane potential, led to identification of a third endothelium-derived relaxing factor, EDHF. Hyperpolarisation of the smooth muscle cells reduces the open probability of voltage-dependent calcium channels thus reducing calcium influx to cause relaxation. A range of agents have been proposed to account for the actions of EDHF including K^+ ions, EETs and C-type natriuretic peptide. However, in many arteries, endothelium-dependent hyperpolarisation of vascular smooth muscle (EDH)

actually reflects direct electrical coupling between endothelial and smooth muscle cells via myoendothelial gap junctions rather than the actions of a diffusible factor.[9]

Irrespective of the mediator, the initiating step in EDH-mediated vasorelaxation is activation of small- (SK_{Ca}) and intermediate–conductance (IK_{Ca}) calcium-activated potassium channels on endothelial cells. Inhibition of endothelium-dependent relaxation by a combination of SK_{Ca} and IK_{Ca} channel blockers is now regarded as the hallmark of EDH and has been documented in response to many agonists, in a wide range of blood vessels from a number of species.[10] SK_{Ca} and IK_{Ca} channels, activated by increases in intracellular calcium via CAM which is constitutively associated with the channels, are voltage-independent and thus can operate at negative membrane potentials close to the K^+ equilibrium potential.

The lack of selective inhibitors of EDH, aside from the SK_{Ca} and IK_{Ca} channel blockers, has hampered investigations of the physiological role of this pathway but it is now clear that EDH becomes progressively more important as a mediator of endothelium-dependent vasodilation with decreased vessel size. The importance of EDH as a regulator of blood flow and blood pressure in vivo is demonstrated by enhanced resistance artery tone and elevated systemic blood pressure seen in mice lacking endothelial SK_{Ca} or IK_{Ca} channels. Loss of EDH, due to changes in expression or activity of SK_{Ca} and/or IK_{Ca} channels, contributes to experimental hypertension and diabetes-related erectile dysfunction. In contrast, resistance of the EDH pathway to the deleterious actions of ROS may allow EDH-mediated vasodilation to be maintained in the face of reduced bioavailability of NO in atherosclerosis and heart failure. Thus, selective activation of endothelial SK_{Ca} and IK_{Ca} channels is a potential therapeutic avenue for the future.

Endothelin: Endothelins are a family of 21 amino acid peptides, of which there are three members (ET-1, ET-2, ET-3). Endothelial cells produce only ET-1; endothelin ET-2 is produced in the kidney and intestine, while ET-3 has been detected in the brain, gastrointestinal tract, lung and kidney. ET-1 is a potent vasoconstrictor inducing long-lasting vasoconstriction at a half maximum effective concentration in the nano molar range, at least one order of magnitude lower than values reported for other vasoconstrictor peptides such as angiotensin II.

ET-1 is produced constitutively by the endothelium but production is regulated at the level of gene expression; inflammatory factors such as transforming growth factor-β (TGFβ) and tumour necrosis factor-α (TNFα, insulin, and angiotensin II up-regulate ET-1 mRNA whereas NO, PGI_2 and shear stress cause down-regulation.) ET-1 is synthesized as a large protein, the pre-proET-1 (203 amino acids) that is cleaved to pro-ET-1 (39 amino acids) and then to ET-1 by ET-converting enzymes. The half life of ET-1 protein and mRNA is 4–7 minutes and 15–20 minutes, respectively, and the majority of plasma ET-1 (90%) is cleared by the lung during first passage.

The biological effects of ET-1 are mediated by two GPCR subtypes, ET_A and ET_B which have opposing effects on vascular tone. ET_A receptors present on vascular smooth muscle are responsible for the majority of ET-1 induced vasoconstriction; activation of phospholipase C increases formation of IP_3 and diacylglycerol, and the resultant increase in intracellular calcium and activation of protein kinase C cause vasoconstriction. ET_B receptors are mainly present on endothelial cells and play an important role in clearing ET-1 from the plasma in the lung. Activation of endothelial ET_B receptors induces vasodilatation by stimulating the release of

PGI_2 and NO. Inhibition of ET_B increases circulating ET-1 levels and blood pressure in healthy subjects demonstrating that although ET-1 is regarded as primarily a vasoconstrictor, ET_B-mediated vasodilation is physiologically important.[11]

ET-1 is not only a vasoactive factor. Acting via ET_B receptors, ET-1 modulates the expression and degradation of extracellular matrix (ECM) and thus plays a role in vascular remodelling. Acting via ET_A, ET-1 promotes smooth muscle proliferation contributing to neointima formation following vascular injury and to thickening of the arterial wall in pathological conditions such as pulmonary arterial hypertension, atherosclerosis and vein graft occlusion. As NO strongly inhibits the release of ET-1 from the endothelium and ET-1 attenuates NO-mediated dilation, ET-1 and NO are functionally closely interdependent and many of the cardiovascular complications associated with endothelial dysfunction are due to an imbalance in this relationship.

ANGIOGENESIS

Angiogenesis is the growth of new blood vessels as a result of endothelial cells sprouting from existing vessels. In adults, it is a protective mechanism initiated in response to tissue hypoxia and ischemia or injury. It is also a key process in pathological conditions such as the proliferative diabetic retinopathy and neovascularization of tumours and as such, inhibitors of angiogenesis have received considerable interest as potential therapeutic strategy. The angiogenic process depends on a complex transcriptional network coordinating production and release of numerous cytokines and growth factors. Recruitment and proliferation of bone marrow–derived endothelial progenitor cells to form new vessels (vasculogenesis) is a distinct but complimentary process which occurs simultaneously in ischemic and wounded tissue to augment perfusion.[12]

Angiogenesis requires a sequence of individual processes: degradation of ECM by metalloproteinase enzymes, proliferation and directional migration of endothelial cells to form endothelial tubes, maturation of new vessels by recruitment of pericytes (connective tissue cells) to stabilize endothelial sprouts and secrete ECM molecules to form the vascular basement membrane and apoptosis to prune back immature vessels into a vascular network.[13] The endothelial cells that sprout from the parent vessel, tip cells, possess long and motile filopodia that extend towards the source of pro-angiogenic growth factors and respond to other guidance cues to enable directional vessel growth. Endothelial cell migration requires the dynamic regulation of interactions between integrins and the surrounding ECM. Integrins are cell surface receptors which provide adhesive and signaling functions and link the actin cytoskeleton of the cell to the ECM at areas called focal adhesions. Phosphorylation of focal adhesion kinase, a cytoplasmic non-receptor tyrosine kinase, in response to pro-angiogenic signal molecules stimulates cell contraction, thus allowing cell movement on adhesive contacts. Subsequent integrin inactivation destroys the adhesive complex and allows detachment of the cell in its new location.

Cell-cell contacts between endothelial cells, essential for development of patent vessels, are mediated by cell surface receptors such as PECAM-1, a 130 kDa member of the immunoglobulin superfamily, which acts like a docking molecule to allow other proteins to provide further strength to vascular structures. Cadherins such as vascular endothelial cadherin are transmembrane proteins which provide weak adhesive cell-cell forces, further stabilized by catenins, intracellular proteins linking the cadherin cell surface molecule to the actin cytoskeleton.

Angiogenesis in response to hypoxia and ischemia is largely controlled by the transcription factor hypoxia-inducible factor-1 (HIF-1).[14] HIF-1 has multiple subunits; HIF-1α which is produced continuously but is rapidly degraded in the presence of oxygen and HIF-1β which is constitutively expressed. Under hypoxic conditions, HIF-1α degradation is inhibited and the stabilized protein translocates to the nucleus, where it dimerizes with HIF-1β and binds to hypoxia response elements on more than 60 HIF–responsive genes that function to enhance oxygen delivery and increase metabolism. Central angiogenic signals driven by increased HIF-1 activity include VEGF, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and angiopoietin. After injury, local platelets release TGFβ and PDGF, which stimulate vessel growth.

FGF and VEGF stimulate endothelial cell proliferation and migration. Their high affinity for heparan sulfate glycosaminoglycans on the endothelial cell surface facilitates binding to receptors and provides a reservoir of both factors in the ECM, which can be released during wounding or inflammation. VEGF stimulates endothelial replication and migration and increases vessel permeability facilitating extravasation of plasma proteins to form a provisional matrix for cell migration. PDGF is required for the recruitment and survival of pericytes for vessel stabilization and maturation. Angiopoietins have multiple effects the angiogenic process, particularly the interactions between endothelial cells, pericytes and the basement membrane. For example, angiopoietin-1 stimulates secretion of growth factors from endothelial cells, which in turn stimulate differentiation of surrounding pericytes into smooth muscle cells. Conversely, angiopoietin-2 is an antagonist of the actions of angiopoietin-1 and so acts as a naturally occurring inhibitor of angiogenesis. Overall regulation of angiogenesis is a balance between angiogenic versus angiostatic factors.

There is a fuller description of the angiogenic process in Chapter 6, which also deals with therapeutic angiogenesis.

HAEMOSTASIS

The endothelium plays a pivotal role in regulating blood flow by exerting effects on the coagulation system, platelets and fibrinolysis.[15] Under normal physiological conditions, the endothelium provides one of the few surfaces which can maintain blood in a liquid state during prolonged contact.

A key factor in blood clot formation is activation of the serine protease thrombin which cleaves fibrinogen, producing fragments that polymerise to form strands of fibrin. It also activates factor XIII, a fibrinoligase, which strengthens fibrin-to-fibrin links, thereby stabilising the clot and stimulates platelet aggregation. Heparan sulfate proteoglycan molecules provide an anti-thrombotic endothelial cell surface by serving as co-factors for antithrombin III, causing a conformational change that allows this inhibitor to bind to and inactivate thrombin and other serine proteases involved in the clotting cascade. The endothelium also prevents thrombin formation by expressing tissue factor pathway inhibitor which binds to clotting factor Xa. Tissue factor pathway inhibitor and antithrombin III both contribute to physiological haemostasis, and both show impairment in acquired thrombotic states. A third endothelial anti-coagulation mechanism is expression of thrombomodulin; binding of thrombin to cell surface thrombomodulin removes its pro-coagulant activity, and the thrombin-thrombomodulin complex activates protein C a vitamin K-dependent anticoagulant. Activated protein C, helped by its cofactor protein S, inactivates clotting factors Va and VIIa.

The anti-platelet properties of the endothelium are largely mediated by release of PGI_2 and NO. As in smooth muscle, PGI_2 inhibits platelet aggregation through the activation of IP receptors and activation of adenylyl cyclase whereas NO inhibits platelet adhesion, activation, secretion, and aggregation through a cGMP-dependent mechanism. NO inhibits agonist-dependent increases in intra-platelet calcium to suppress the calcium-sensitive conformational change in the heterodimeric integrin glycoprotein IIb– IIIa required for fibrinogen binding. NO also promotes platelet disaggregation by impairing the activity of phosphoinositide 3-kinase, which normally supports conformational changes in glycoprotein IIb–IIIa, rendering its association with fibrinogen irreversible. Should a blood clot form, fibrinolysis depends primarily on the action of plasmin, an active protease formed from its precursor, plasminogen, upon stimulation by tissue-type plasminogen activator.

Under physiological conditions, there is a haemostatic balance and in addition to these anti-thrombotic mechanisms, the endothelium also synthesises several key haemostatic components; vWF and plasminogen activator inhibitor-1 (PAI-1) being particularly important. PAI-1 is secreted in response to angiotensin IV, providing a link between the renin-angiotensin system and thrombosis. In addition to anti-coagulant activity, binding of thrombin to thrombomodulin accelerates its capacity to activate thrombin-activatable fibrinolysis inhibitor (TAFI) which cleaves fibrin and other proteins, resulting in the loss of plasminogen/plasmin and tPA binding sites and thus retarding fibrinolysis. Perturbations, such as those that may occur at sites of injury, inflammation or high hydrodynamic shear stress, tip this haemostatic balance in favour of a pro-thrombotic and anti-fibrinolytic microenvironment. Critical steps include loss of cell surface heparin proteoglycan molecules and increased expression of the transmembrane glycoprotein tissue factor (TF) which initiates coagulation by stimulating the activation of clotting factors IX and X, and pro-thrombinase, with subsequent fibrin formation. TF accumulates in experimentally injured vessels and accumulation in some atherosclerotic plaques likely accounts for their high thrombogenicity.

INFLAMMATION

Development of inflammatory reactions by the endothelium in response to injury or infection is critical for the maintenance and/or repair of normal structure and function of the vessel wall. However, excessive inflammatory reactions can lead to severe tissue damage and contribute to the development of atherosclerosis.

The interaction between endothelial cells and inflammatory cells such as leukocytes depends on the production of inflammatory cytokines (e.g. interleukin 8; IL-8) to attract leukocytes and expression of adhesion molecules (e.g. selectins) to facilitate their migration towards the site of infection. Loosely tethered leukocytes first roll over the endothelial surface, then arrest, spread, and finally migrate between endothelial cells to attach on to underlying ECM components.[16]

Leukocyte rolling involves endothelial adhesion molecules of the selectin family which transiently bind to carbohydrate ligands on leukocytes to slow passage through the blood vessel. E- and P-selectin are expressed only on the surface of activated endothelial cells whereas L-selectin is constitutively expressed on leukocytes and binds to ligands induced on the endothelium at sites of inflammation or on other leukocytes. The role of individual types of selectins in leukocyte rolling shows stimulus- and time-dependent variation. Immediate stimulation

of leukocyte rolling induced by histamine or thrombin depends on rapid expression of P-selectin, surface levels of this adhesion molecule declining after only 30 minutes. In contrast, TNFα stimulates delayed leukocyte rolling and adhesion to endothelial cells through the induction of E-selectin, surface levels of which peak after 12 hours and decline after 24 hours. Both E- and P-selectin are expressed on the surface of endothelial cells overlying atherosclerotic plaques, affirming the importance of these molecules in the development of atherosclerosis.

Firm adhesion of leukocytes is promoted by binding of chemokines such as IL-8 to leukocyte GPCRs resulting in rapid activation of β1 and β2 integrins to increase their affinity for adhesion molecules of the immunoglobulin superfamily, intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1). ICAM-1 is constitutively expressed on endothelial cells but levels are increased by stimuli such as TNFα peaking at 6 hours and remaining elevated for 72 hours. ICAM-1 mediates firm adhesion of blood cells by acting as a ligand for leucocyte beta2 integrins. VCAM, a ligand for integrins α4β1 and α4β7, principally mediates the adhesion of monocytes, lymphocyte, eosinophils, and basophils to the endothelial surface. Expression of VCAM-1 is induced by cytokines, oxidized low-density lipoproteins and ROS acting, as with induction of ICAM-1, primarily via NF-κB.

Migration of leukocytes through the endothelium requires the transient disassembly of endothelial cell junctions. Firm adhesion of leukocytes to the endothelium induces clustering of adhesion molecules like ICAM-1 and VCAM-1 triggering activation of intracellular signaling pathways which induce endothelial cell actin cytoskeleton and cell junction remodelling. The remodelling process involves numerous pathways including Rho GTPase signaling, protein phosphorylation and ROS generation but a key event is alteration of the dimerization of PECAM-1. PECAM-1 localizes to intercellular junctions of endothelial cells, forming homodimers linking two cells. Leukocytes also express PECAM-1 and the dissociation of PECAM-1 dimers between endothelial cells to form dimers between emigrating leukocytes and endothelial cells is critical for leukocyte migration.

CONCLUSIONS

The endothelium, once viewed as an inert physical barrier, is a dynamic secretory organ fulfilling numerous roles in the maintenance of cardiovascular homeostasis. Endothelial cells from different parts of the vasculature show highly differentiated functions as a consequence of both environmental stimuli and epigenetic modifications. Advances in defining many endothelial functions at the molecular level may lead to targeted therapies to alleviate chronic endothelial dysfunction associated with the progression of cardiovascular disease.

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2 • Vascular Smooth Muscle Structure and Function

DAVID P WILSON

Molecular Physiology of Vascular Function Research Group,
Discipline of Physiology, University of Adelaide, South Australia

INTRODUCTION

Smooth muscle has an important role in regulating the function of a variety of hollow organ systems including the: vasculature, airways, gastrointestinal tract, uterus and reproductive tract, bladder and urethra and several other systems. Smooth muscle has two fundamental roles: 1) to alter the shape of an organ and 2) to withstand the force of an internal load presented to that organ. In order to achieve these fundamental objectives smooth muscles have developed mechanisms of mechanical coupling, which enable the development of powerful and coordinated contractions at a relatively low energy cost. For example, smooth muscle in the gastrointestinal tract must undergo intermittent but coordinated phasic contractions to propel the bolus of food through the alimentary canal. Whereas in the airways and vasculature the smooth muscle is more often in various states of tonic contraction, but can be dynamically regulated to relax or contract in response to specific neuro-hormonal and haemodynamic signals. In keeping with the aims of this text, this chapter will focus on the principle mechanisms through which vascular smooth muscle functions.

SMOOTH MUSCLE (VASCULAR) STRUCTURE

Vascular smooth muscle cells have classically been envisaged as fusiform cells, on average 200 microns long × 5 microns in diameter, with a large central nucleus surrounded by an abundant array of endoplasmic reticulum and golgi apparatus, with the cytosol and plasma membrane tapering toward the poles. Although the dimensions of the vascular smooth muscle cell narrow toward their ends there is clear evidence that the end-to-end junctions coupling smooth muscle cells are complex and contain a significant number of membrane invaginations to provide increased surface area for both mechanical tight junctions and electrical coupling via gap junctions (Figure 2.1). Vascular smooth muscle cells do not contain the complex t-tubule/sarcoplasmic reticulum system common to striated muscles, but rather they contain a significant number of invaginations along the plasma membrane called caveolae, which serve a similar, albeit less developed role to increase the cellular surface: volume ratio. These specialized caveolae further provide a unique plasma membrane environment, which enables clustering of specific groups

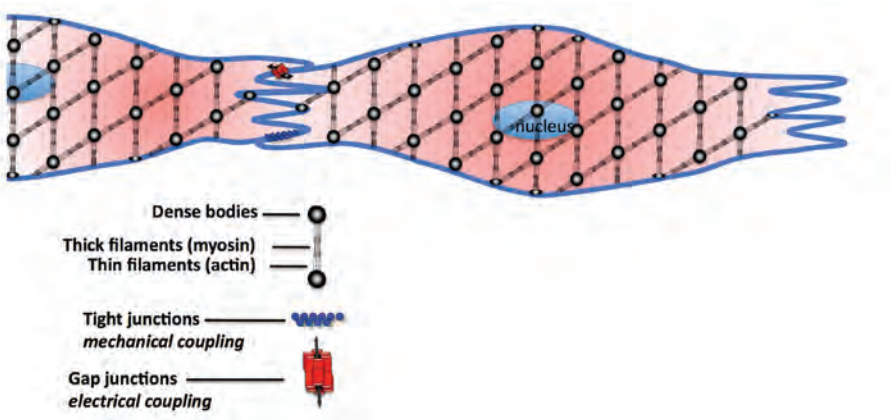

FIGURE 2.1: Highlights the fusiform shape of typical smooth muscle cells and the patterned array of actin myosin myofilaments across the cell. Smooth muscle cells have invaginations along their length to provide increased surface area for mechanical coupling via tight junctions and electrical coupling through Gap junctions. Dense bodies are thought to be similar to Z-disks found in striated muscle.

of ion channels and receptors important in cellular signal transduction.

In contractile vascular smooth muscle the endoplasmic reticulum has been modified to enable Ca^{2+} release and reuptake and has therefore been termed sarcoplasmic reticulum. In smooth muscle cells the sarcoplasmic reticulum/endoplasmic reticulum (SR/ER) complex comprises about 5% of the total cell volume, with a considerable amount of rough endoplasmic reticulum and golgi apparatus adjacent to the nucleus, which reflects the significant capacity that smooth muscle has for protein synthesis and secretion. The fact that vascular smooth muscle has a fundamental role in mediating pressure and flow in the vasculature is reflected in the abundance of cytoskeletal and contractile proteins expressed.

CYTOSKELETON

As with all eukaryotic cells the cytoskeleton is comprised of a network of many and various filamentous proteins, often formed by the polymerization of monomeric subunits. For example, monomers of alpha and beta tubulin self assemble into microtubules that function to provide static support to the cell and to enable motor protein mediated transport of cytosolic cargo and for chromosomal segregation during mitosis. The actin cytoskeleton and elements of the actin contractile myofilament are also generated from the polymerization of globular monomeric actin to form polymeric actin filaments. This process is dynamically regulated even within the time scale of contractile processes, i.e., as the smooth muscle slowly shortens it can actually synthesise and extend the length of the actin filaments.

CONTRACTILE MYOFILAMENT

The structure of the smooth muscle acto-myosin array is similar to striated muscle with several important differences:

1. there is no troponin complex in smooth muscle
2. contraction is regulated by Ca^{2+} calmodulin-dependent myosin light chain kinase (MLCK) mediated phosphorylation of the regulatory light chains of myosin, which enables actin

myosin interaction and cross bridge cycling
3. in the absence of Ca^{2+} and calmodulin (CaM), caldesmon interacts with actomyosin inhibiting the activity of myosin ATPase
4. the activity of myosin light chain phosphatase (MLCP) directly causes the dephosphorylation of myosin LC_{20} leading to relaxation
5. the actin: myosin ratio is higher in smooth muscle averaging 15:1 in vascular smooth muscle in comparison to 6:1 in skeletal or cardiac muscle. There are no intercalated disks or z-disks, however, dense bodies in smooth muscle are thought to be analogous to z-disks (Figure 2.2).

There are a variety of intermediate filament proteins but desmin and vimentin are particularly abundant in smooth muscle. In fact, desmin has been shown to be upregulated in several myopathies and during smooth muscle hypertrophy. As indicated once globular actin polymerizes into filaments they coil to form mature filamentous actin that then combines with tropomyosin to form a large actin-tropomyosin filament which together is arranged in side polar arrays with myosin II filaments (Figure 2.1). The myosin II thick filaments are composed of two 200kDa heavy chains, two 17kDa light chains and two phosphorylatable regulatory myosin light chains (LC_{20}). The two heavy chains coil together forming a 155nm rod, while the globular head contains the motor domain consisting of light chains and Mg^{2+} ATPase activity and the actin binding domain. The myosin is arranged in an anti parallel array that enables the myosin motors, on the heads of myosin molecules, to draw actin polymers along its length and effect shortening of the cell, so-called cross bridge cycling (Figure 2.2). MLCK, which is responsible for the Ca^{2+} calmodulin mediated phosphorylation of LC_{20} is actin associated while MLCP which removes the phosphoryl groups from LC_{20} is associated with myosin. (Figure 2.3)

FUNCTIONAL REGULATION OF VASCULAR SMOOTH MUSCLE: NEURONAL, HORMONAL, RECEPTOR MEDIATED

Smooth muscle from all hollow organs including blood vessels have been somewhat artificially categorized into either single unit smooth muscle or multiunit smooth muscle, when in reality they should likely be considered as a combination of both types. Nevertheless, historically, multiunit smooth muscle has been considered to be regulated primarily through autonomic sympathetic innervations, which release neurotransmitters from varicosities along the axon, rather than specifically coupling to individual cells. Consequently, neurotransmitters are required to diffuse anywhere from 5-100 nm to the adjacent smooth muscle membrane in order to activate their receptors. The activation of sympathetic nerves therefore causes membrane depolarization and activation of voltage dependent ion channels, the most prominent of which are the clinically relevant voltage operated Ca^{2+} channels (VOCC) of the $Cav_{1.2}$ family, also known as the long acting L-type Ca^{2+} channels. Due to the mechanism of membrane depolarization, this form of cellular activation has been termed electromechanical coupling. In contrast, single unit smooth muscles have very little innervation and are primarily activated by autocrine and paracrine hormones, including noradrenalin, adrenalin, and angiotensin II, all of which function through G-protein coupled membrane receptors. Receptor activation either triggers sarcoplasmic reticulum-mediated Ca^{2+} release or membrane

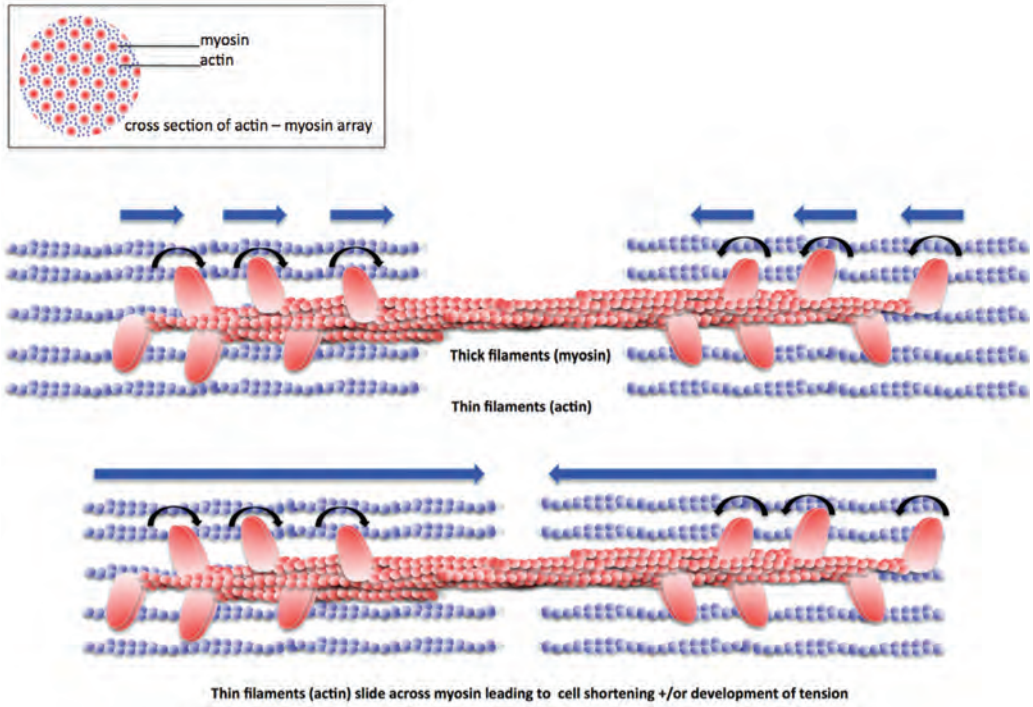

FIGURE 2.2: Illustrates the patterned array of actin and myosin in smooth muscle in both cross section and the longitudinal axis. Following phosphorylation of the light chains of myosin, actin and myosin interact followed by the synchronous sliding of actin across the myosin. The movement of actin filaments toward the center of the cell is driven by the Mg^{2+} ATPase activity in the myosin heads and results in cell shortening or development of tension.

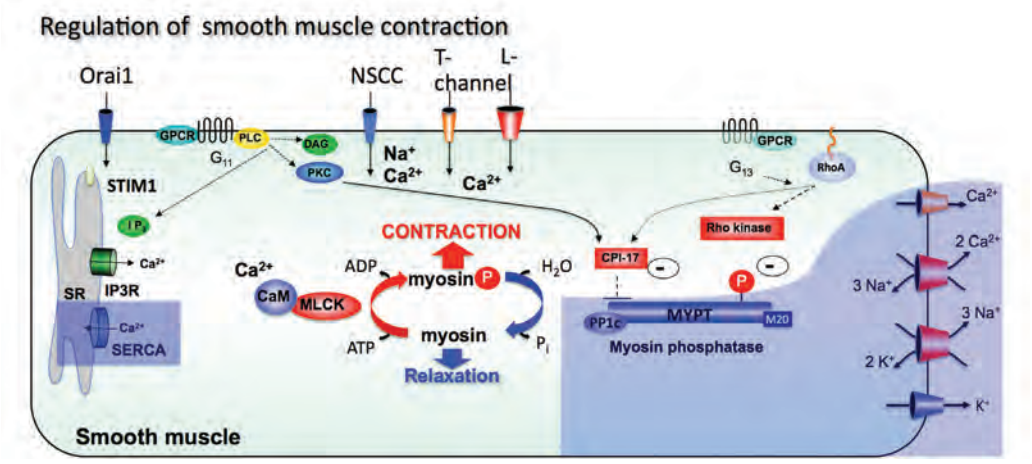

FIGURE 2.3: Illustrates the major pathways by which Ca^{2+} enters the cytosol, areas highlighted in blue indicate mechanisms by which Ca^{2+} exits that cell. Ca^{2+} entry activates Ca^{2+} CaM-dependent myosin light chain kinase leading to phosphorylation of myosin, leading to actin myosin interaction and contraction. Myosin phosphatase dephosphorylates myosin uncoupling actin-myosin favouring vasorelaxation. Various G-protein coupled receptors are capable of activating PKC/CPI-17 or RhoA/Rho kinases pathways which are capable of inhibiting myosin phosphatase further favouring vasoconstriction.

depolarization through the activation of ion channels which may include VOCC. Consequently this form of activation has been termed pharmacomechanical coupling. Experimental evidence also exists to suggest that like striated muscle, extracellular Ca^{2+} entry in smooth muscle can also activate SR Ca^{2+} release into the cytosol, so called Ca^{2+} induced Ca^{2+} release, although this appears to be a less common mechanism of Ca^{2+} entry. Once activated, single unit smooth muscle cells tends to contract in synchrony with neighbouring smooth cells, which are coupled through gap junctions. Gap junctions are composed of 6 connexin proteins, which are transmembrane spanning proteins which assemble to form a barrel-shaped connexon or hemichannel in the plasma membrane. When two hemichannels from adjacent cells assemble they form a functional gap junction that enables the movement of ions (electrical coupling) and small molecules between adjacent cells (Figure 2.1). Evidence indicates that at least some of the vascular smooth muscle cells in most vascular beds are electrically coupled via gap junctions and may even be coupled to the endothelium in a similar manner.

SMOOTH MUSCLE FUNCTION

As with other muscle types smooth muscle functions best when at its optimal resting length L_0, which provides the ideal balance of actin-myosin interaction and muscle shortening. Vascular smooth muscle cells that are stretched beyond L_0 have less than optimal overlap of actomyosin crossbridges and thus are unable to maintain or generate maximum force. In contrast, smooth muscle that is over shortened experiences increased internal resistance due to internal friction generated from having too many slowly cycling cross bridges. However, in smooth muscle the optimal length tension relationship is considerably more variable than that for skeletal or cardiac muscle, but appears to be directly related to the degree of phosphorylation of the regulatory light chains of myosin. The broader range of optimal resting length amongst smooth muscle may reflect the dynamic changes in load that exist within the vasculature and of the high actin:myosin ratio in smooth muscle relative to striated muscle. Interestingly, at the onset of stretch or pressurization smooth muscle responds via activation of stretch receptors (TREK and TRAK) that induce Ca^{2+} entry and consequent smooth muscle shortening, which directly offsets increased vascular wall stress. This is the important mechanism governing the phenomenon of autoregulation. It is important to note that although gastrointestinal smooth muscle appears relatively unaffected by significant stretch, more that 25% stretch often destroys the contractile properties of vascular smooth muscle.

MYOFILAMENT BASIS OF SMOOTH MUSCLE CONTRACTION AND RELAXATION

Motility studies using isolated actin and myosin proteins have identified that the duty cycle of the myosin head power stroke is 7-11 nm. Perhaps more important than the stroke length of the myosin is the activity of the myosin ATPase, which along with Ca^{2+}/CaM and the activation state of myosin light chain kinase and myosin light chain phosphatase, determines the extent and the rate of contraction of the vascular smooth muscle (Figure 2.3). Smooth muscle myosin ATPase hydrolyses ATP at a rate of ~0.16 moles ATP/mol myosin/second, which is several orders of magnitude slower than the skeletal muscle myosin ATPase which hydrolyses ~10-20 moles ATP/mol myosin/

second. In part the slower ATPase rate of the myosin head accounts for the vastly slower contraction rates of vascular smooth muscle compared with striated muscles. In addition, there are a variety of different isoforms of smooth muscle myosin II which when differentially expressed will increase or decrease the maximum rate of smooth muscle contraction. For example, so called foetal isoforms of smooth muscle myosin II have a slower rate of contraction. In addition, these "foetal" isoforms have been shown to be upregulated during extended hypoxia, and during phenotypic remodelling of smooth muscle from a contractile to synthetic or proliferative phenotype.

Smooth muscle contraction and relaxation

As mentioned, unlike cardiac and skeletal muscle, smooth muscle does not contain troponin and therefore is not subject to troponin mediated regulation of contraction. Smooth muscle contraction makes use of another Ca^{2+} binding protein called calmodulin (CaM), which when bound with Ca^{2+} mediates the activation of the actin bound myofilament enzyme, myosin light chain kinase (MLCK), which in turn phosphorylates the regulatory light chains of myosin (LC_{20}). Phosphorylation of the myosin LC_{20} is a critical step in smooth muscle contraction which causes a conformational change in the myosin head enabling the interaction of myosin with actin, cross bridge cycling, and contraction. Removal of cytosolic Ca^{2+} occurs through the activation of energy dependent plasma membrane Ca^{2+} ATPases (PMCA), Na^+/Ca^{2+} exchangers and sarco/endoplasmic reticulum CaATPases (SERCA) (Figure 2.3). However, since MLCK-mediated phosphorylation of the serine 19 of myosin LC_{20} generates a covalent bond, simply removing Ca^{2+} does not directly cause vasodilatation. However, myosin associated, myosin light chain phosphatase (MLCP), is responsible for the dephosphorylation of myosin LC_{20} and the consequent loss of smooth muscle acto-myosin interaction and the attenuation of cross bridge cycling (Figure 2.3). It is important to recognize that, simple removal of extracellular Ca^{2+} only stops the phosphorylation of LC_{20} and does not provide an explanation for subsequent smooth muscle relaxation since the LC_{20} remains phosphorylated. This also partially explains why Ca^{2+} channel blockers are less effective in attenuating pre-existing vascular spasm as opposed to preventing spasm.

ION CHANNELS IMPORTANT IN THE REGULATION OF SMOOTH MUSCLE FUNCTION

Regulation of cellular Ca^{2+}

There are vast arrays of ion channels, pumps, transporters and exchangers that are important in regulating ionic balance and smooth muscle membrane potential. Perhaps the best know are the electrogenic $3Na^+/2K^+$ ATPase and the voltage gated L-type Ca^{2+} channels but includes: Na^+/Ca^{2+} exchangers, plasma membrane Ca^{2+} ATPases (PMCA), which provide routes to extrude Ca^{2+} from the cytosol into the extracellular space, whereas the sarcoplasmic reticulum Ca^{2+} ATPases (SERCA) are important in removing Ca^{2+} from the cytosol back into the SR. In contrast, when the SR becomes depleted of Ca^{2+}, Ca^{2+} sensor proteins in the SR termed stromal interacting molecule 1 (STIM1) translocate to the plasma membrane and activate an ion channel called Orai1 which enables refilling of SR Ca^{2+} stores. In addition, a great deal of recent research has focused on the non-selective cation channels of the transmembrane receptor potential canonical (TRPC) family that are thought be involved

in regulating Na^+ and Ca^{2+} entry. Finally a series of potassium channels are involved in either re or hyperpolarisation of the plasma membrane and thereby have therapeutic potential in limiting extracellular Ca^{2+} entry through voltage operated Ca^{2+} channels and thereby limiting vasoconstriction.

Sources of cytosolic Ca^{2+} entry

Within the smooth muscle cell, Ca^{2+} enters the cytosol from the extracellular space or from the intracellular endoplasmic reticulum, which in muscle cells is termed the sarcoplasmic reticulum (SR). Within muscle the sarcoplasmic reticulum has become variously modified to affect Ca^{2+} release into the cytoplasm. Typically agents that activate the ryanodyne receptor such as caffeine or phospholipase C (PLC) derived inositol 1, 4, 5 tris phosphate (IP3) which activates the IP3 receptors (which are ion channels) in the SR cause Ca^{2+} to be released into the cytosol. Ca^{2+} entering the smooth muscle cell from the extracellular space does so through non-selective cation channels or selective Ca^{2+} channels, which may or may not be gated by voltage. To date the most important source of extracellular Ca^{2+} entry in vascular smooth muscle is mediated by the voltage dependent Ca^{2+} channels (VDCC). The primary VDCC are the long lasting Ca^{2+} channel, so called L-type or $Ca_v1.2$ channels, which are the clinical targets of the L-type channel blockers the dihydropryidines, phenylalkamines, benzothiazapenes. More recent evidence indicates that a second class of VDCC, the transient or T-type Ca^{2+} channels also known as the $Ca_v3.X$ family may be important in mediating Ca^{2+} entry in the microvasculature. As the name suggests VDCC are activated by a depolarization of the plasma membrane, which increases the open probability and overall Ca^{2+} conductance into the cell. In addition, a variety of non-selective cation channels have the capacity to conduct a variety of ions including Ca^{2+} and Na^+ into the smooth muscle cell but due to their low conductance are currently thought to be more important in regulating membrane potential and subsequent activation of plasma membrane VDCC (Figure 2.3).

Potassium channels

The insulin-dependent electrogenic $3Na^+/2K^+$ ATPase is important in establishing the resting membrane potential (E_M) of the vascular smooth muscle cell. However, the activation states of several types of K^+ channels in smooth muscle are also important in effecting membrane depolarization and hyperpolarisation and consequent smooth muscle contraction and relaxation, respectively. The inward rectifier K_{IR} channels become activated when the membrane becomes hyperpolarized and beyond the equilibrium potential for potassium (E_K) they transport more K^+ ions from the extracellular space into the cell thereby offsetting or rectifying the hyperpolarizing stimulus. However, there are few if any physiological conditions in which E_M is more negative than E_K, consequently, even the K_{IR} channels conduct a small outward hyperpolarizing K^+ current, and therefore along with the $3Na^+/2K^+$ ATPase may be important in mediating smooth muscle tone.

The K_V family of potassium channels as the name suggests are activated by depolarization and thus are thought to be an important control mechanism to hyperpolarize the smooth muscle cell following neural or hormonal-mediated depolarization. Agonists including histamine acting through the H1 receptor have been shown to block the 4-aminopyridine sensitive K_V channels in coronary arteries.

Physiologically K_{ATP} channels are activated by agents including, adenosine, calcitonin gene

regulated peptide (CGRP) and vasoactive intestinal peptide (VIP). The activation of K_{ATP} channels and hyperpolarization mediated vasodilatation is thought to be due in part to activation of adenylyl cyclase and subsequent cAMP dependent activation of protein kinase A. More recent evidence has also indicated that K_{ATP} channels become activated in a protein kinase C-dependent manner. However, perhaps more important is the fact that cytosolic ATP and ADP function to close and open K_{ATP} channels, respectively. This explains part of the mechanism underlying the finding that vasculature in ischemic tissue, containing high ADP: ATP levels extrude K^+ in an effort to hyperpolarize the membrane and effect vasodilatation. Both experimentally and clinically the so-called vasodilatory K^+ channel openers including pinacidil, cromakalim, diazoxide, and minoxidil activate K_{ATP} channels. Interestingly the antidiabetic sulfonylurea drugs, including glibenclamide actually inhibit K_{ATP} channels, enabling membrane depolarization and activation of VOCC in pancreatic beta cells enabling insulin release. Consequently overuse of sulfonourea drugs may therefore interfere with the efficacy of vascular K^+ channel openers or directly contribute to vasoconstriction.

The large conductance Ca^{2+} activated potassium channels (BK_{Ca}) channels are also voltage sensitive but the smaller conductance smK_{Ca} channels are less sensitive to voltage. This family of K^+ channels are activated by increases in cytosolic Ca^{2+} which occurs after agonist stimulation, membrane depolarization or stretch/pressure-dependent activation of Ca^{2+} entry and therefore is involved in that arm of the myogenic mechanism involved in hyperpolarization.

G protein coupled receptors (GPCRs) transduce signals from the autonomic nervous system and hormonal stimuli including bradykinin, noradrenalin, adrenaline, angiotensin II, endothelin-1, serotonin and thromboxane A_2. Many GPCRs exhibit divergent subcellular signalling mechanisms and there is increasing evidence for diversity of subcellular signalling amongst vascular beds (Figure 2.3). For example, angiotensin II can be generated both locally within smooth muscle cells and systemically through the renin angiotensin system (RAS). In smooth muscle the type I AngII receptors are prototypical G-protein coupled receptors which couple through $G_{q11.}$ Similarly endothelin-1 can be generated locally via endothelial cells, inflammatory cells or renal sources. Like Ang II, endothelin-1 makes use of G_{q11} in smooth muscle to activate PLC, releasing diacylglycerol (DAG) activating non selective cation channels which facilitates membrane depolarization and subsequent extracellular Ca^{2+} entry through VGCC. In addition, the activation of PLC generates IP3 causing SR-mediated Ca^{2+} release. DAG can also activate PKC leading to the phosphorylation of CPI-17 which specifically inhibits myosin phosphatase, favouring phosphorylation of the LC_{20} of myosin and vasoconstriction (Figure 2.3). However, in contrast to AngII, endothelin-1 also activates G_{13} coupled receptors activating the RhoA/ Rho associated kinase which leads to a direct inhibitory phosphorylation of myosin phosphatase, again favouring contraction (Figure 2.3).

ENDOTHELIAL REGULATION OF SMOOTH MUSCLE VASODILATATION

Nitric oxide is a potent vasodilator generated in the endothelium which has many and varied effects in the vasculature including attenuating: platelet adhesion and aggregation, cellular proliferation, and vasoconstriction. A common theme underlying the influence of nitric oxide is activation of guanylyl cyclase, formation of cGMP,

activation of protein kinase G and consequent activation of K^+ channels effecting K^+ removal from the cell leading to membrane hyperpolarization and consequent inactivation of VDCC favoring low intracellular Ca^{2+} and vasorelaxation. Cyclooxygenase activation in endothelial cells also leads to generation of prostaglandin PGI_2 which leads to receptor mediated activation of adenylyl cyclase, generation of cAMP, subsequent activation of PKA and inhibition of K^+ channels (Figure 2.4).

SMOOTH MUSCLE PROLIFERATION AND VASCULAR REMODELLING

In the normal adult vascular wall most vascular smooth muscle cells subserve a contractile function to directly modulate vasoconstriction and vasodilatation. However, during development, following injury or in the presence of growth factors and mitogens, including inflammatory cytokines and oxidized lipids, vascular smooth muscle can undergo phenotypic modulation. Vascular smooth muscle phenotypic modulation involves a partial down regulation of the proteins that activate the contractile apparatus in favour of the synthetic and proliferative cellular machinery i.e., the cell increases the abundance of; endoplasmic reticulum, ribosomes for protein synthesis and the density of the Golgi apparatus. So called synthetic vascular smooth muscle cells are therefore able to undergo very active protein and DNA synthesis, cell division, and in pathological settings are capable of taking up large amounts of oxidized and nonoxidized lipids which can contribute to lipid loading of vascular smooth muscle cells and the formation of so-called foam cells in the vascular wall. As the name foam cell suggests, under microscopic examination lipid laden smooth muscle cells appear much like foam. Synthetic vascular smooth muscle cells also secrete, external to the cell, a great deal of extracellular matrix including the proteins; collagen I, III, IV, and the proteoglycans, perlican, hyaluronan, laminin. Proliferative smooth muscle cells also secrete or associate with the membrane surface several, matrix metalloproteinases (MMPs) and their corresponding tissue inhibitors of matrix metalloproteases (TIMPs) to enable correct repair and remodelling of growing or damaged vessels. Evidence exists that a chronic excess of inflammatory cytokines and growth factors can cause dysregulation of both MMPs and TIMPs which can contribute to inappropriate vascular remodelling. This remodelling plays a significant role in the progression of vascular stenosis, restenosis following mechanical interventions, the progression of unstable atheroma, and aneurysmal dissection and rupture.

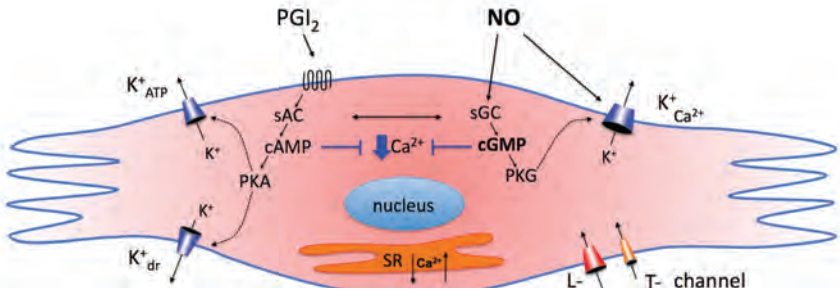

FIGURE 2.4: outlines the major influences of prostaglandin I_2 and nitric oxide (NO) in regulating the activation state of various K^+ channels leading to hyperpolarization of smooth muscle cells and vasodilatation.

At this point it is worth noting that proliferative smooth muscle cells have an attenuated response to vasoconstrictors and vasodilators, probably due to the down regulation of the contractile apparatus and certain elements of the subcellular signalling machinery that is involved in vasoconstriction. However, many of the ligands that normally lead to vasoconstriction, for example, noradrenalin, angiotensin II and endothelin also function to promote smooth muscle proliferation both in the context of cellular hypertrophy and hyperplasia. Platelet derived growth factor (PDGF), is a potent smooth muscle cell mitogen and growth stimulant and contributes to normal vessel repair while chronically elevated levels, for example, generated from unstable thrombus, can contribute to proliferative vascular disorders. Interestingly nitric oxide, in addition to functioning as a potent vasodilator also limits smooth muscle hyperplasia and hypertrophy, probably by limiting intracellular Ca^{2+} and associated Ca^{2+}dependent vascular proliferation.

SUMMARY

It is clear from the preceding that there is a dynamic interplay between cellular Ca^{2+} entry from the extracellular space mediated by membrane depolarization and the activation of voltage dependent Ca^{2+} channels. Extracellular Ca^{2+} entry can be offset by the activation of K^{+} channels through either endothelial nitric oxide-cGMP/PKG- or PGI2-cAMP/PKA-dependent mechanisms, both of which function to limit smooth muscle contraction and proliferation. However, the simple fact that L-type Ca^{2+} channel blockers and nitric oxide treatment are limited in their ability to effectively manage several disorders of hypercontractility suggests that the additional mechanisms including; SR Ca^{2+} release and regulation of myosin phosphatase are also important targets for future therapeutic development.

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3 • Atherosclerosis

GILLIAN COCKERILL[1], QINGBO XU[2]

[1]Department of Clinical Sciences, St George's Hospital Medical School, London, UK.
[2] Department of Cardiology, King's College, University of London, UK

INTRODUCTION

Atherosclerosis, the principal cause of heart attack, stroke, and peripheral vascular disease, remains a major contributor to morbidity and mortality in the Western World. Disease progression is slow, beginning in childhood and usually becoming clinically manifest in middle age or later. Although the aetiology of atherosclerosis is not fully understood, it is generally accepted that atherosclerosis is a multifactorial disease induced by the effects of various risk factors on appropriate genetic backgrounds[1]. Many risk factors, such as hypercholesterolemia, modified lipoproteins, hypertension, diabetes, infections and smoking have been identified in the development of atherosclerosis.

Atherosclerosis has been the focus of intense research for over 100 years. Since Anitschkow and Chalatow first reported that cholesterol can cause atherosclerosis, many investigators have intensively studied the role of blood cholesterol in the pathogenesis of atherosclerosis. Although formerly considered a bland lipid storage disease, new insights into the pathogenesis of atherosclerosis have emerged during the last decades, due to the progress of cellular and molecular approaches to the study of cell interactions in the arterial wall as well as alterations of lipid metabolism. These new insights were broadly summarized in three main theories, i.e. the 'response to injury',[2] 'oxidized low-density lipoprotein (LDL)', and 'inflammation'[1] hypotheses.

The response to injury hypothesis[2] relies on the concept that the primary cause of atherosclerosis is an injury to the arterial endothelium induced by various factors, i.e. smoking, mechanical stress, oxidized-LDL, homocysteine, immunological events, toxins, viruses, etc. The oxidized-LDL hypothesis postulates that LDL oxidized by various factors including endothelial cells, macrophages and smooth muscle cells of the arterial wall, plays a key role in the development of atherosclerosis. More recently, a widely accepted hypothesis is that atherosclerosis is an inflammatory disease, because recent advances in the basic science have established a fundamental role for inflammation in mediating all stages of this disease from initiation through progression and, ultimately, the thrombotic complications of atherosclerosis.[1] The aim of the present chapter is to summarize the data from a variety of research areas providing an overview of atherosclerosis focusing on mechanistic studies.

ATHEROSCLEROTIC LESIONS

The intima of large and medium sized arteries is composed of a monolayer of endothelial cells and matrix proteins and occasional smooth muscle cells in the subendothelial space (Figure 3.1a). The media of the vessel contains smooth muscle cells and the elastic lamina built by matrix proteins, while the main component of adventitia is connective tissue. With increasing age, the diseased arterial wall slowly thickens and develops focal lesions of lipid accumulation in the intima. These early lesions are known as fatty streaks and are thought to be the sites of predisposition to advanced lesions called atherosclerotic plaques or atheroma, which may lead to clinical symptoms in certain circumstances.

Fatty streaks

Fatty streaks are generally the lesion types found in children, although they may also occur in adults. These lesions represent the early changes of atherosclerosis and are recognized as an increase in the number of intimal macrophages filled with lipid droplets (foam cells). A larger lesion which can be grossly visible is characterized by layers of macrophage foam cells and lipid droplets within intimal smooth muscle cells and minimal coarse-grained particles and heterogeneous droplets of extracellular lipid (Figure 3.1b). With the progression of lesion development, intermediate lesions as described by pathologists, are the morphological and chemical bridge between fatty streaks and advanced lesions. These lesions appear in some adaptive intimal thickenings (progression-prone locations) in young adults and are characterized by pools of extracellular lipid in addition to other components of fatty streak lesions. The fatty streak is largely clinically benign, but is the precursor to later, clinically relevant lesions.

Plaque or atheroma

The advanced lesion, a dense accumulation of extracellular lipid, known as the lipid core, occupies an extensive but well-defined region of the intima.[3] No increase in fibrous tissue and complications such as defects of the lesion surface and thrombosis are present at this stage of disease. This atherosclerotic plaque is also known as atheroma (Figure 3.1c). The characteristic core appears to develop from an expansion and confluence of the small isolated pools of extracellular lipid that characterize atheroma. Between the lipid core and the endothelial surface, the intima contains macrophages, smooth muscle cells, lymphocytes and mast cells. Capillaries surround the lipid core, particularly at the lateral margins and facing the lumen. Frequently macrophages, macrophage foam cells, and lymphocytes are more densely concentrated in the lesion periphery. Much of the tissue between the core and the surface endothelium corresponds to the proteoglycan-rich layer of the intima, although infiltrated with the cells just described. Advanced lesions may or may not narrow the arterial lumen, nor be visible by angiography, nor produce clinical manifestations. Such lesions may be clinically significant even though the arterial lumen is not narrowed, because complications may develop suddenly.[3]

In addition, two types of atherosclerotic plaques, i.e. 'vulnerable' and 'stable' plaques, have been recognized.[4] Vulnerable plaques often have a well-preserved lumen, since plaques remodel outward initially. The vulnerable plaque typically has a substantial lipid core and a thin fibrous cap separating the thrombogenic macrophages bearing tissue factor (TF) from the blood. At sites of lesion

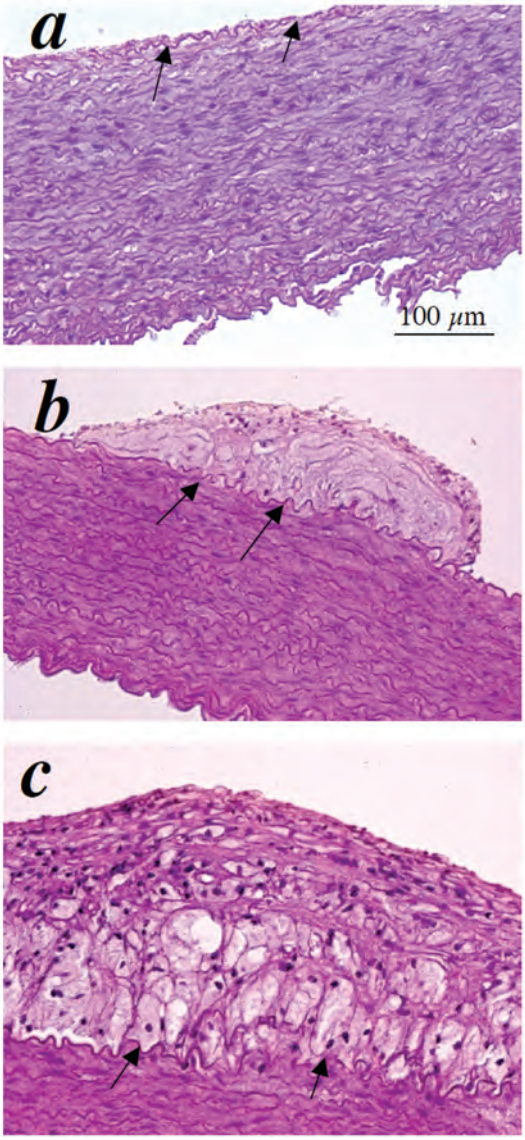

FIGURE 3.1: Sections of rabbit arterial wall. Rabbits were fed with a standard chow-diet (***a***) or cholesterol-enriched diet (0.2%) for 3 weeks (***b***) or 16 weeks (***c***). Their aortas were harvested and sections prepared and stained with hematoxilin and eosin. Arrows indicate the internal elastic lamina, the border between the intima and media of the arterial wall.

disruption, smooth muscle cells are often activated, as detected by their expression of the transplantation antigen HLA-DR. In contrast, the stable plaque has a relatively thick fibrous cap protecting the lipid core from contact with the blood. Clinical data suggest that stable plaques more often show luminal narrowing detectable by angiography than do vulnerable plaques, but with much less chance of rupture.

HYPERCHOLESTEROLEMIA AND OXIDISED-LDL

Accumulating evidence suggests a causal relationship between blood cholesterol and atherosclerosis. Blood cholesterol is carried by lipoproteins, including LDL, very low-density lipoprotein and high-density lipoprotein (HDL). LDL is believed to be 'bad' lipoprotein, while HDL is 'good' and plays a protective role in atherogenesis.[5] It is established that familial hypercholesterolemia related to increased LDL levels causes premature atherosclerosis and heart disease,[6] whereas non-genetic hypercholesterolemia is also associated with the development of atherosclerosis. The consensus of many trials using different cholesterol-lowering regimens indicate that for every 10% reduction in cholesterol level, the deaths of patients with coronary heart disease is reduced by at least 15%. It has been assumed that the reduction in adverse clinical events when plasma cholesterol levels are decreased is directly related to the magnitude of the cholesterol lowering. That assumption is supported by the fact that the benefit relates to the change in cholesterol level in much the same way whether the cholesterol lowering is achieved with diet or with drugs. These findings suggest that blood cholesterol exerts its role in the pathogenesis of atherosclerosis.

LDL can be modified by oxidation *in vivo* and *in vitro* and is detectable in the circulation as well as in atherosclerotic lesions. *In vivo*, the rate of production of oxidized-LDL in the arterial intima is a function of

the concentration of native LDL present. The mechanism whereby hypercholesterolemia and oxidized-LDL trigger events leading to the generation of early atherosclerotic lesions i.e. fatty streak (Figure 3.2) remains uncertain. Although rabbits and pigs were often used in studying this issue, the apolipoprotein (apo) E–deficient mouse[7] and the LDL receptor-deficient mouse have become preferred animal models. Deletion and overexpression of genes in animal models is now the gold standard for critically testing the relevance of candidate genes in atherogenesis. By using these models, it was observed that one of the earliest responses induced by hypercholesterolemia and oxidized-LDL is an increase in the expression of vascular cell-adhesion molecule-1 (VCAM-1), a key adhesion molecule for monocytes and T cells, on the endothelial surface lining the major arteries.[8] Oxidized-LDL is itself directly chemotactic for monocytes and T cells, and can also be cytotoxic for endothelial cells, mitogenic for macrophages and smooth muscle cells and stimulate the release of monocyte chemoattractant protein-1 (MCP-1) and monocyte colony-stimulating factor (M-CSF) from endothelial cells. The oxidative modification hypothesis has been extensively reviewed.[9]

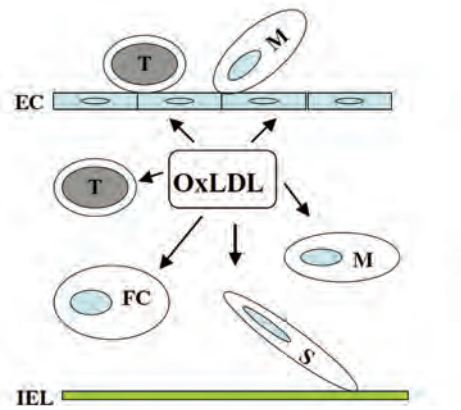

FIGURE 3.2: Schematic representation of the role of oxidized-LDL (oxLDL) in atherogenesis. Oxidized-LDL generated either locally or systemically stimulates endothelial cells (EC) expressing adhesion molecules, including ICAM-1, VCAM-1 and E-selectin, which are responsible for adhesion of blood mononuclear cells. Oxidized-LDL is a chemokine for T lymphocytes (T), monocytes (M) and smooth muscle cells (S), and promotes foam cell (FC) formation, which form the early lesion fatty streak.

Oxidized-LDL can account for the loading of macrophages with cholesterol. Here, monocytes undergo phenotypic modification and take up oxidized-LDL to become foam cells, loaded with multiple cytoplasmic droplets containing cholesterol esters.[10] Recently there has been considerable progress in identifying the components of oxidized-LDL that make it a ligand for scavenger receptors. Extensive degradation of the polyunsaturated fatty acid (PUFA) in the *sn*-2 position of phospholipids by oxidation seems to be essential. Moreover, oxidized-LDL and apoptotic cells compete for binding to macrophage scavenger receptor, indicating that oxidized phospholipids in the membranes of apoptotic cells are involved in their binding to macrophage scavenger receptors. Therefore, oxidized-LDL promotes foam cell formation that forms the earliest lesions in the intima, which may progress to advanced lesions in the presence of other pro-atherogenic factors (Figure 3.2).

High-density lipoproteins role in atheroprotection

It has been known for many years that the plasma concentration of HDL-C correlates inversely with the incidence of cardiovascular disease. The Framingham Heart Study showed that people whose HDL-C was less than 35 mg/dL (0.91 mmol/L) at the beginning of the study had a future coronary risk more than eight times that in subjects whose HDL-C concentration was greater that 65 mg/dL (1.68 mmol/L).[11] In the more

recent Prospective Cardiovascular Munster (PROCAM) Study, men with an HDL-C concentration of less than 35 mg/dL (0.91 mmol/L) at baseline were shown to have a four times greater risk, at six years, than men whose HDL-C concentration was greater than 35 mg/dL (0.91 mmol/L).[12] In both studies, the risk associated with lower plasma HDL-C concentration was independent of LDL-C concentration. HDLs have several properties that contribute to their ability to protect against the development of atherosclerosis.

The best known mechanism of atheroprotection relates to the ability of HDLs to promote efflux of cholesterol from foam cells. This process inhibits the progression of, and potentially promotes the regression of, atherosclerosis.[13] High-density lipoproteins can also inhibit the oxidative modification of LDLs and thus reduce their atherogenicity. The principle mechanism of this anti-oxidant function resides with the presence of para-oxonase enzyme residing in the HDL particle,[14,15] although the main apolipoprotein, apolipoprotein AI (ApoAI), has also been demonstrated to have anti-oxidant capacity.[16] Conceivably, the earliest observable cellular dysfunction in the normal blood vessel, leading to atherogenesis, is the expression of leukocyte adhesion molecules and chemokines. Many studies, both *in vivo* and *in vitro* have shown that HDLs can inhibit expression of endothelial cell adhesion molecules and MCP-1.[17-20] Endothelial dysfunction, and subsequent platelet activation and aggregation are key elements of the progression of atherosclerotic plaque formation. The ability of HDLs to be antithrombotic was demonstrated by the ability to induce prostacyclin (PGI_2) synthesis, via induction of cyclo-oxygenase 2,[21] and in addition to stimulate the generation of nitric oxide,[22] thus reducing the endothelial dysfunction that may precede the development of atherosclerosis.

HYPERTENSION AND BIOMECHANICAL STRESS

Hypertension is a well-established risk factor for atherosclerosis.[23] Clinical trials have shown that, in the highest quintile for diastolic pressure, hypertension still contributes significantly to the risk of atherosclerosis, even with the added risks of high cholesterol and smoking. Induction of hypertension in the Watanabe heritable hyperlipidemic rabbit showed a synergistic effect, causing intensification of atherosclerosis. The fact that atherosclerotic lesions preferentially occur in the areas where hemodynamic or biomechanical stress is altered, e.g. bifurcation of the arteries, supports the idea that hypertension exerts its role in the pathogenesis of atherosclerosis via altered mechanical stress to the vessel wall.

In vivo, the vessel wall is exposed to two main hemodynamic forces or biomechanical stress: shear stress, the dragging frictional force created by blood flow, and mechanical stretch, a cyclic strain stress created by blood pressure.[24] Shear stress stimulates endothelial cells to release nitric oxide and prostacyclin,[25] resulting in vessel relaxation and protection of vascular cells, whereas smooth muscle cells are stimulated by cyclic strain stress. In humans, atherosclerotic lesions occur preferentially at bifurcations and curvatures where hemodynamic force is disturbed, i.e. lower shear stress and higher mechanical stretch. Although veins do not develop spontaneous atherosclerosis-like lesions, accelerated atherosclerosis occurs rapidly in venous bypass grafts, which bear increased biomechanical forces due to alterations in blood pressure, i.e. vein (0-30 mm Hg) *vs.* artery (120 mm Hg). This finding supports the

hypothesis that mechanical stress could be a crucial factor in the pathogenesis of atherosclerosis.

The mechanism whereby mechanical forces are sensed by cells and transmitted through intracellular signal transduction pathways to the nucleus resulting in quantitative and qualitative changes in gene expression in the vessel wall is not fully understood. However, recent evidence indicates that mechanical stretch initiates intracellular signal pathways, especially mitogen-activated protein kinase (MAPK) cascades[26] which are thought to play a pivotal role in transmitting transmembrane signals required for cell proliferation, differentiation and apoptosis. MAPKs comprise a ubiquitous family of tyrosine/threonine kinases, and include extracellular signal-regulated kinases (ERKs), stress-activated protein kinases (SAPKs) or c-Jun NH_2-terminal kinases (JNKs), and p38 MAPKs.[27] They are highly activated or expressed in atherosclerotic lesions and vessel wall stimulated by acute hypertension.[28]

Biomechanical stress-induced cell death

While biomechanical force at physiological levels is essential to develop and maintain organic structure and function, at elevated levels mechanical stretch may result in cell death leading to pathological conditions. In recent years, however, it has become widely recognized that cell death, namely apoptosis, is not just a response to injury but a highly regulated and controlled process. Disturbances in the regulatory mechanisms of apoptosis often precede the development of atherosclerosis. Exploration of the molecular signalling mechanisms leading to mechanical stress-induced apoptosis in cardiovascular disorders has revealed the crucial role of apoptosis in the pathogenesis of atherosclerosis.[29] Recent data focussing on the molecular mechanisms of mechanical stress-induced apoptosis are summarised and the role of apoptosis in the development of atherosclerosis is highlighted.

Recently, the first mouse model of vein graft atherosclerosis was established by grafting autologous jugular vein or vena cava to carotid arteries in wild-type and apoE-deficient mice. In many respects, the morphological features of this murine vascular graft model resemble those of human graft atherosclerosis (Figure 3.3). Apoptosis occurred mainly in veins grafted to arteries, remaining unchanged in vein-to-vein grafts.[30] The veins grafted to arteries were subjected to increased biomechanical forces in the form of stretch stress due to blood pressure. When mouse, rat and human arterial smooth muscle cells cultured on a flexible membrane were subjected to cyclic strain stress, apoptosis was observed in a time- and strength-dependent manner. Mechanical stretch resulted in p38 MAPK activation. Smooth muscle cell lines stably transfected with a dominant negative rac, an upstream signal transducer, or overexpressing MAPK phosphatase-1, a negative regulator for MAPKs, completely inhibited mechanical stress-stimulated p38 activation, and abolished mechanical stress-induced apoptosis.[31] Interestingly, p53-deficient vein grafts had lower levels of apoptosis that correlated with increased atherosclerotic lesions.[32] The sudden elevation in mechanical forces could be a strong stimulus to the grafted vessel wall and may result in activation of intracellular signal pathways leading to gene expression and cell death. Thus, one of the earliest events in vein graft atherosclerosis is apoptosis, in which mechanical stress-induced p38-MAPK-p53 activation is, at least in part, responsible for transducing signals leading to apoptosis.

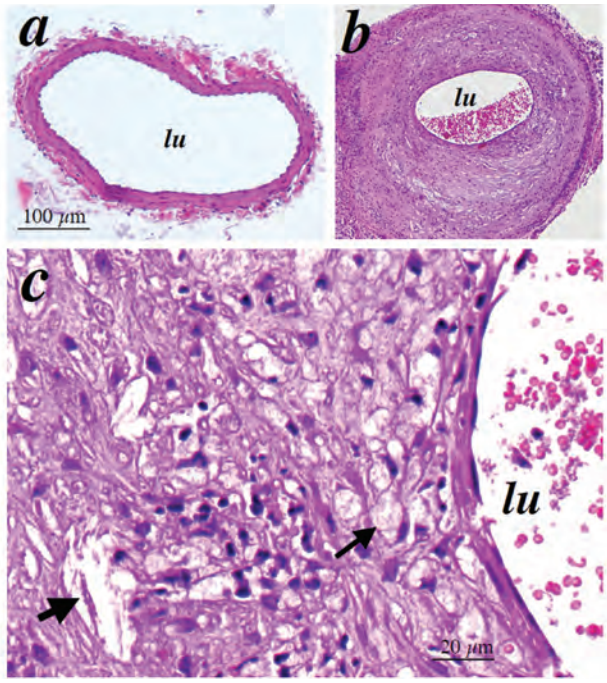

FIGURE 3.3: Hematoxilin and eosin-stained sections of arterialized mouse vein grafts. Under anesthesia, vena cava veins were removed and isografted into carotid arteries (of control mice)**(a)** of apoE-/- mice **(b)**. Animals were sacrificed 8 weeks after surgery, and the grafted tissue fragments fixed in 4% phosphate-buffered (pH 7.2) formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Panel **c** is a photograph of vein graft section with higher magnification. Smaller arrow indicates a foam cell, and larger one indicates cholesterol crystal structure. *lu* indicates the lumen of the vessel.

Biomechanical stress and inflammation

Vein graft atherosclerosis has an inflammatory nature characterized by mononuclear cell infiltration followed by smooth muscle cell proliferation. It has been postulated that biomechanical stress plays a role in adhesion molecule expression via MAPK signal transduction pathways, leading to NF-κB activation. Supporting this concept is the fact that neointimal lesions of vein grafts in intercellular adhesion molecule (ICAM)-1 -/- mice were reduced from 50% to 30% compared to wildtype controls. ICAM-1 is critical in the development of venous graft atherosclerosis. It has been established that exposure of endothelial cells to shear (mechanical) stress results in increased expression of ICAM-1 and monocyte chemotactic protein-1 (MCP-1) via activation of transcription factor NF-κB and AP-1. These molecules are essential for leukocyte-endothelial cell interaction and subsequently cell infiltration, characteristic of the early lesions of vein grafts that undergo elevated blood pressure. Interestingly, mechanical stress also leads to smooth muscle cells expressing ICAM-1 via activation of NF-κB. In animal models, smooth muscle cells express ICAM-1 which is associated with monocyte/macrophage accumulation in vein grafts. Smooth muscle cells of ICAM-1 -/- mice do not express ICAM-1 which is correlated with reduced early lesions.[33] Mechanical stress-induced adhesion molecules and chemokine expression in the vessel wall could be important for the inflammatory response.

Biomechanical stress-induced smooth muscle cell proliferation

It has been established that mechanical stress stimulates DNA synthesis and the proliferation of *in vitro* cultured smooth muscle cells. Hypertension increases mechanical force on the arterial wall up to 30%, resulting in marked alterations in signal transduction and gene expression in smooth muscle cells, which contribute to matrix protein synthesis, cell proliferation and differentiation.[23] Recently, several reports demonstrated that angioplasty resulted in stretching of the arterial wall leading to rapid activation of the MAPKs in the regenerating carotids.[34] The magnitude of Extracellular signal-regulated kinase p42 (ERK42) activation positively correlated with the degree of balloon injury to the arterial wall. *Ex vivo* stretching of the vessel wall also induced significant activation of ERK42 kinases. These findings suggested that the kinase activation in the early phase following injury may be due to mechanical stimulation of the vessel wall.

In cultured smooth muscle cells, mechanical forces evoked ERK activation followed by enhanced DNA-binding activity of transcription factor AP-1. Interestingly, physical forces rapidly result in phosphorylation of platelet-derived growth factor (PDGF) receptor,[35] epithelial growth factor receptor and vascular endothelial growth factor receptor. Thus, mechanical stresses may directly perturb the cell surface or alter receptor conformation, thereby initiating signalling pathways normally used by growth factors. Suramin, a non-specific PDGF inhibitor, has been shown to be a growth factor receptor antagonist that inhibits cell proliferation. When vein isografts in mice were treated *ex vivo* and *in vivo* with suramin, intimal lesions were reduced up to 70% compared to untreated controls. The mechanism of suramin-inhibited neointimal hyperplasia mainly involves inhibition of smooth muscle cell migration and proliferation via blocking PDGF receptor-MAPK-AP-1 signal pathways. Thus, research into biomechanical stress-regulated gene expression in atherosclerosis using these models could lead to a new therapeutic strategy in the treatment of this disease in humans.

INFECTIONS AND HEAT SHOCK PROTEINS

Risk factors, such as high blood cholesterol, hypertension and smoking only explain a proportion of the incident cases of all atherosclerosis. There is a body of evidence that microorganisms play a role in the pathogenesis of atherosclerosis and may be a primary risk factor in people who do not suffer from other established risk factors. Accumulating evidence suggests that infectious organisms reside in the wall of atherosclerotic vessels, including cyto–megalovirus (CMV) and *Chlamydia (C) pneumoniae*. Seroepidemiological studies demonstrate an association between the pathogen-specific IgG antibodies and atherosclerosis.[26,27] However, the data are inconsistent, with other studies showing no increased risk for atherosclerosis.[38,39] One possible explanation for this disparity is that infections contributing to atherosclerosis risk may depend, at least in part, on the host's response to the pathogen, i.e. inflammatory and immune reactions.

Infections

Several papers reviewing the infections, ie *C.pneumoniae, H.pylori* and CMV and atherosclerosis have been published[37] and these will be summarised. Saikku et al[40] was first to show a link between *C.pneumoniae* infection, coronary artery disease and atherosclerosis. Since then, many studies have

shown an association of *C.pneumoniae* with atherosclerosis. In vitro experiments have shown a preferential and specific attraction to and infection of macrophages, vascular endothelium and vascular smooth muscle, by *C.Pneumoniae*, thus resulting in their accumulation into atherosclerotic plaques. This is supported by studies of post-mortem specimens of vascular tissue which found a high correlation between the distribution of atherosclerosis and *C.pneumoniae*[41] and other organisms.

H.pylori, another gram negative bacteria which typically infects human gastric epithelial cells has been demonstrated in atherosclerotic plaques.[38] Sero-positivity to *H.pylori* was implicated as a risk factor in coronary heart disease (CHD) from the first report in 1994. However a meta-analysis[39] of 18 studies failed to show any correlation between sero-positivity against *H.pylori* and the presence or extent of CHD. Although the evidence supporting involvement of *H.pylori* in atherogenesis is not conclusive, it may be important to differentiate between virulent and avirulent strains of *H.pylori* to determine the effects on atherogenesis. Mayr et al[42] conducted a population based study and investigated the effects of CagA (cytotoxin-associated gene A) positive and CagA negative strains of *H.pylori*. This study concluded that there was an increased risk of atherosclerosis in individuals who were infected with CagA positive strains of *H.pylori*. Another group has obtained similar results, indicating the role of this strain in the pathogenesis of atherosclerosis.

Heat shock proteins

The role of HSPs in disease with regard to their physiological functions and pathological involvement have been described in many reviews on the subject. The HSP family of proteins is subdivided into groups based on their molecular weight (e.g. HSP60 is a 60kDa protein) and are produced by almost all cells and play an important role in the organism's general protective response to environmental and metabolic stresses (Table 3.1). They exist in all major cellular compartments. For example, HSP10, HSP60 and HSP75 are mainly located in mitochondria, while others are found in different compartments throughout the cell. They have important physiological functions, primarily as a molecular chaperone.[7] HSPs also appear to be important in preventing cellular damage during repair processes following injury. Evidence indicates that HSPs may be autoantigens in some circumstances.[43] HSP47, HSP60 and HSP70 have been identified as being involved in the pathogenesis of atherosclerosis.[44]

Infections and HSP expression

In a recent study, increased HSP60 was demonstrated on the endothelium, smooth muscle cells, and mononuclear cells of all atherosclerotic carotid and aortic specimens, whereas vessels with normal intima showed no detectable expression of this HSP. The level of HSP60 expression positively correlated with atherosclerotic severity.[45] Interestingly, chlamydial and human HSP60s have been shown to be co-expressed in atherosclerotic lesions. These data support the concept that elevated HSP expression in lesions may be induced by the pathogen *Chlamydiae species*. During its normal cycle generating infectious progeny, *Chlamydiae* express basal levels of HSP. During the lytic phases of chlamydial infection, host cells release their own HSP60, and also chlamydial HSP60 that has been produced by these microorganisms. Soluble HSP60 (sHSP) levels were significantly elevated in subjects with prevalent/incident carotid atherosclerosis and correlated to intima-media thickness independent of

TABLE 3.1: Heat shock protein families

Family	Members/other names	Physiological function	Pathological involvement
HSP10	HSP10, HSP17	Promotes substrate Release with HSP60	unknown
Small HSP	HSP20, HSP23 HSP27, HSP28	F-actin assembly, Molecular chaperones.	unknown
HSP40	HSP32, HSP40, HSP47	Guides protein folding, Binding and transport of collagen	Atherosclerosis
HSP60	HSP58, GroEL HSP60, HSP65 Grp58	Assemble polypeptides; Translocate proteins across membranes; Accelerate protein folding and unfolding	Atherosclerosis, Rheumatoid arthritis, Adjuvant arthritis, Diabetes mellitus, Systemic sclerosis.
HSP70	HSP68, Dnak. Hsc70, Hsx70 HSP72, HSP73 HSP75, Grp75 HSP78, Grp78	Molecular chaperone: Assembly and transport newly synthesized proteins; Fold or unfold polypeptides; Remove denatured proteins; Bind to specific polypeptides (e.g., p53); ATPase activity.	Atherosclerosis Tuberculosis, Leprosy, Filariasis,
HSP90	HSP83, HptG HSP87, HSP90-α grp94, HSP90-β	Bind to specific polypeptide receptors (e.g., glucocorticoid receptor).	Schistosomiasis, Systemic lupus erythematosus.

age, sex and other risk factors. Interestingly, sHSP60 was also correlated with anti-LPS, anti-*Chlamydia* and anti-HSP60 antibodies, inflammation markers and chronic infections.

Infections, sHSP and innate immunity

Infectious agents contribute to atherogenesis in a variety of ways. One mechanism is by triggering innate immune reactions leading to inflammatory responses. Innate immunity involves several different cell types, e.g. mononuclear phagocytes and endothelial cells. Both endothelial cells and macrophages express receptors that recognize molecular epitopes from a broad range of pathogens. These receptors include various scavenger and Toll-like receptors (TLRs). So far more than 10 human TLRs have been identified. A variety of bacterial and fungal components are known TLR ligands, including peptidoglycan for TLR2, LPS for TLR4, flagellin for TLR5, and unmethylated CpG (cytosine and guanine separated by a phosphate group, which links the two nucleotides together) motifs in bacterial

DNA for TLR9. It is possible that TLRs may be collectively responsible for detecting a large range of microbial pathogens. TLRs are evolutionarily conserved innate immune receptors that are shared by IL-1 receptor signalling to activate the NF-κB pathway and release inflammatory cytokines. TLR ligation therefore induces expression of a wide variety of genes such as those encoding proteins involved in leukocyte recruitment, production of reactive oxygen species, and phagocytosis. Activation of TLRs will also elicit the production of cytokines that augment local inflammation. Finally, TLR ligation may directly induce apoptosis, probably of key importance in the first line of defence.[46]

Expression of TLR4 in atherosclerotic plaques has been found, preferentially in lipid-rich and macrophage-infiltrated areas of lesions. *In vitro*, basal expression of macrophage TLR4 was shown to be up-regulated by oxidized-LDL. In addition, of the nine TLRs, expression of TLR1, TLR2, and TLR4 was shown to be markedly enhanced in human atherosclerotic plaques. A polymorphism or mutation of TLR4 was shown to be strongly correlated with the incidence and development of atherosclerosis in a large population study (Bruneck Study). Surprisingly, several groups reported that recombinant HSP60 and HSP70 from bacteria and humans specifically bind to TLR4 in macrophages, endothelial cells and smooth muscle cells. Recombinant HSP60 binding to the TLR4/CD14 complex of macrophages and endothelial cells led to activation of MyD88-NF-kB pathways. HSP70 and mycobacterial HSP65 have a similar binding activity to TLR4/CD14 that initiates MyD88-NF-κB signal pathways, suggesting that the TLR4/CD14 is a receptor for several HSPs that mediate the signal pathways leading to proinflammatory responses during infections.

In summary, infections with proatherogenic organisms may be important in individuals lacking additional risk factors as well as acting synergistically with established risk factors. In this process, HSP may be a link between infections and the pathogenesis of atherosclerosis. Infectious agents may exert their role by producing their own HSPs and inducing host production which could be released into blood. The soluble form of HSPs contact endothelial cells and immune cells where innate immune responses are initiated. Innate immune reactions to HSPs result in proinflammatory responses in the vessel wall. Together, infections via HSPs contribute to the development of atherosclerosis (Figure 3.4).

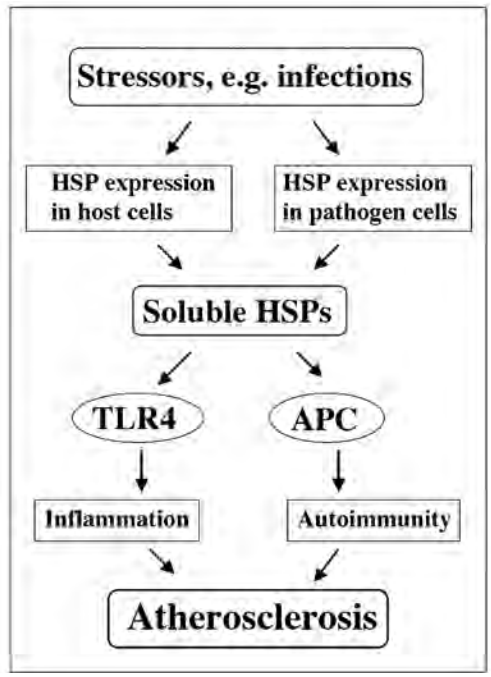

FIGURE 3.4: Schematic representation of the likely mechanism of action of heat shock proteins (HSPs) in the development of atherosclerosis in response to risk factors (stressor), e.g. infections. TLR, Toll-like receptor; APC, antigen-presenting cells.

IMMUNE RESPONSES

The contribution of immune responses to the pathogenesis of atherosclerosis has been recognized and much progress in this research field has been achieved through the participation of many investigators.[47] Involvement of the immune system in atherogenesis is supported by recent data, including the occurrence of granular deposits of immunoglobulins and co-distributed complement components, increased expression of C3b receptors (CR1) and C3b1 receptors (CR3) on macrophages within atherosclerotic lesions, but not in unaltered vessels. However, B cells are only found in very low numbers in various stages of atherosclerotic lesions, and the site of production for these immunoglobulins must, therefore, be sought elsewhere. Other than these humoral immune phenomena, it is now clear that T cells are among the first cells infiltrating the intima of arteries during the earliest stages of atherosclerosis, most probably before monocytes. A majority of these early T cells are $CD4^+$, HLA-DR^+ and interleukin-2 receptor+ (IL-$2R^+$), i.e. activated. Others have shown that T cells in late atherosclerotic plaques express the low molecular variant of the leukocyte common antigen (CD45RO) and the integrin very late activation antigen-1 (VLA-1). Hansson and his group analyzing the rearrangement of T cell receptor (TCR) genes in these latter cells derived from advanced lesions, showed that they represent a polyclonal population rather than displaying restricted T-cell receptor TCR usage. These findings support the role of the immune system in atherogenesis.

MHC class II antigen and T cells

Regardless of which antigen these lymphocytes may recognize, it seems improbable that endothelial cells (EC) which aberrantly express major histocompatibility complex (MHC) class II antigens act as primary antigen-presenting cells for T cell sensitization. MHC class II expression by EC occur concomitantly where T cells are found, and thus production of gamma-interferon (IFNγ), the major T-cell chemokine, is present in the intima directly beneath these areas. Therefore, it can be concluded that the expression of MHC class II molecules by endothelial cells represents a secondary rather than a primary phenomenon. The large majority of $CD3^+$ cells in the mononuclear infiltrate in atherosclerotic lesions expresses TCRα/β, but an unexpectedly high proportion also express TCRγ/δ. While the latter type of cell only constitutes approximately 1 % of leukocytes in peripheral blood, enrichment to 10 % or more within early atherosclerotic lesions has been observed. The majority of these latter cells express the TCRγ2 chain, i.e. resemble the TCRγ/δ+ population found in the intestinal mucosa. On the other hand, TCR Vγ9δ2+ cells characteristic of circulating TCRγ/δ+ cells are not proportionally increased in the intima. Finally, endothelial cells and leukocytes express and synthesise a variety of immunological-inflammatory mediators, occurring in atherosclerotic lesions. Among others, these include interleukin-1 (IL-1), tumour necrosis factor α (TNFα), lymphotoxin, IL-2, IL-6, IL-8, monocyte-chemotactic peptide-1 and IFNγ^1. Together, these molecules can modulate the local cellular immune response within emerging atherosclerotic lesions.

Oxidized-LDL as a candidate antigen

T cells isolated from human atherosclerotic plaques were shown to be specifically reactive to oxidized-LDL. One fourth of all CD4+ T cells cloned from human plaques recognized oxidized-LDL in an HLA-DR-dependent manner. Oxidized-LDL-specific T cells are

present in lymph nodes of apoE-KO mice, which have strong humoral as well as cellular immune responses to such modified lipoproteins. In humans, oxidized-LDL induces activation of a subset of peripheral T cells. In addition, antibodies to oxidized-LDL can be detected in atherosclerotic patients and are present in atherosclerotic lesions, suggesting that it is a quantitatively important antigen. The immune response to oxLDL plays a pathogenetic role in atherosclerosis because lesion progression can be inhibited by immunization or induction of neonatal tolerance to oxLDL. It seems paradoxical that both tolerization and hyperimmunization can reduce the extent of disease; this may be due to the different effector pathways activated by these two kinds of treatment.

HSP60 as a candidate antigen

As discussed above, HSPs have been implicated in activation of innate immune responses involved in the pathogenesis of atherosclerosis. Moreover, adaptive immune reactions to HSP60 have also been implicated in the development of atherosclerosis. In experimental models, rabbits immunised with HSP65/60 recorded induction of vascular inflammation, with endothelial activation and mononuclear cell adhesion demonstrated.[48] The developing lesions also contained T cells, and cell lines derived from such infiltrates exhibited anti-HSP60 reactivity. Anti-HSP60 antibodies occurred in peripheral blood, and immunization with HSP60 was found to increase fatty streak development in hypercholesterolemic rabbits and mice. In humans, antibodies to HSP65/60 are elevated in early and late atherosclerosis and may predict progression of atherosclerotic disease. Since heat shock proteins of humans and microbes are structurally and antigenically similar, it is possible that molecular mimicry between immune responses to microbial HSP and homologues expressed by vascular cells could account for the association between infections and atherosclerosis.[49] Based on these findings, Maron and co-workers provided the evidence that atherosclerotic lesions were reduced by nasal immunization with HSP65 in apoE-deficient mice, suggesting that atherosclerosis might be inhibited by vaccination against HSP65.[50]

β2-glycoprotein Ib as a candidate antigen

A third autoantigen, β2-glycoprotein Ib (β2-GPI), is present on platelets but may also be expressed by endothelial cells. Autoantibodies to β2-GPI are produced in several inflammatory disorders, in addition to atherosclerosis. The immune response to β2-GPI appears to be proatherogenic, because hyperimmunization with β2-GPI[116] or transfer of β2-GPI-reactive T cells aggravates fatty streak formation in LDLR-/- mice. The pathogenic mechanism by which β2-GPI acts remains unclear, but it may be related to this protein's capacity to bind phospholipids.

In summary, adaptive immunity powerfully modulates initiation and progression of atherosclerosis. Atherogenesis involves intercommunication between shared pathways involved in adaptive and innate immunity. Various established and emerging risk factors for atherosclerosis modulate aspects of immune responses, including lipoproteins and their modified products, HSPs, and infectious agents. As the molecular details become understood, new potential targets for therapies will doubtless emerge.

INFLAMMATION

It is generally accepted that atherosclerosis is an inflammatory disease,[1] because recent

findings have provided important links between all risk factors and the mechanisms of atherogenesis. Clinical studies have shown that this emerging biology of inflammation in atherosclerosis applies directly to human patients. Elevation of markers for inflammation predicts the outcome in patients with acute coronary syndromes.[4] In addition, low-grade chronic inflammation, as indicated by levels of the inflammatory marker C-reactive protein (CRP), prospectively defines the risk of atherosclerotic complications, thus adding to prognostic information provided by traditional risk factors. Certain treatments that reduce atherosclerosis risk also limit inflammation. For example, statins, used for lipid lowering,[4] have anti-inflammatory effects. Amongst the known inflammatory triggers on the vessel wall are known risk factors e.g. hypercholesterolemia, oxidized-LDL, hypertension, biomechanical stress and infection. These risk factors can directly or indirectly stimulate endothelial cells expressing adhesion molecules (VCAM-1, ICAM-1 and E-selectin) mediating subsequent mononuclear cell infiltration and foam cell formation in the subendothelial space.[1] In this section, some recent findings that have not been described above will be summarised.

C-reactive protein

C-reactive protein (CRP) is an acute-phase protein that is involved in inflammatory processes. It is made up of 5 identical subunits which are arranged in a pentagonal shape. CRP is predominantly synthesized by hepatocytes, but in response to inflammation, CRP expression can be found in atherosclerotic plaque, aortic endothelial cells, monocytes and vascular smooth muscle cells. Inflammatory cytokines induce CRP gene expression and statins have been shown to reduce CRP levels.[52]

Biasucci et al[53] studied a cohort of patients admitted with unstable angina and found that one half of this group had a persistently elevated CRP (>3mg/dL) at discharge. Those with elevated discharge CRP levels had a significantly elevated risk of recurrent unstable angina or MI during the subsequent 12 months. This group has also demonstrated that CRP elevation in individuals presenting with severe peripheral arterial disease was associated with an increased risk of MI, independent of other vascular risk factors.

CRP may be not only a marker of inflammation and atherosclerosis, it may also be an active component participating in atherogenesis. CRP can bind to lipoproteins and activate the complement system via the classical pathway. CRP deposits have been shown in the arterial wall early during lesion formation, which is co-localized with the terminal complement complex. This suggests that CRP may promote atherosclerotic lesion formation by activating the complement system and is involved in foam cell formation, which may be caused in part by the uptake of CRP-opsonized LDL.

CD40/CD40L

These antigens are ubiquitously expressed on the surface of endothelial cells, smooth muscle cells, macrophages, T lymphocytes, and platelets within human atheroma.[51] The proatherogenic functions of CD40 ligation include augmented expression of matrix metalloproteinases, procoagulant tissue factor (TF), chemokines, and cytokines. Indeed, interruption of CD40 signalling not only reduced the initiation and progression of atherosclerotic lesions in hypercholesterolemic mice *in vivo*, but also modulated plaque architecture in ways that might lower the risk of causing thrombosis. In addition to the 39-kDa cell membrane–associated form,

CD40L also exists as a soluble protein, termed sCD40L. Although lacking the cytoplasmic, the transmembrane region, and parts of the extracellular domains, this, the soluble form of CD40L, is considered to possess biological activity. Patients with unstable angina express higher sCD40L plasma levels than healthy individuals or patients with stable angina. Moreover, it was recently demonstrated that elevated plasma concentrations of sCD40L predict risk for future cardiovascular events. Although *in vitro* and *in vivo* studies established that CD40 signalling participates in atherosclerosis, the initial trigger for CD40/CD40L expression within atheroma may be regulated by oxidized-LDL. Thus CD40/CD40L may be a mediator in the inflammatory responses during the development of atherosclerosis.

SUMMARY AND PERSPECTIVES

Atherosclerosis is an inflammatory disease that is initiated by multiple risk factors, including hypercholesterolemia, oxidized-LDL, altered biomechanical stress, smoking and infections. Due to research achievements in recent decades, atherogenesis is no longer an inevitable consequence of aging—the statin revolution has left this in no doubt. Better control of hypercholesterolemia can clearly be achieved but many questions remain. For example, which factor is an initiator for the development of atherosclerotic lesions, and how do other factors participate in the disease process. Currently, atherosclerosis research is highly topical. The mystery of the molecular mechanisms in this disease will yield to the current multidisciplinary attack by academic institutions and the pharmaceutical industry using the powerful techniques of vascular biology and molecular approaches.

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4 • Mechanisms of Plaque Rupture

IAN LOFTUS

St George's Vascular Institute, London, UK.

INTRODUCTION

Atherosclerosis continues to cause considerable morbidity and mortality, particularly in the western world. While risk factors have been clearly identified, their precise roles in early atherogenesis are complex. The early development of the plaque is dependent upon interactions between damaged endothelial cells, vessel wall smooth muscle cells and circulating inflammatory cells mediated by the release of cytokines, growth factors and cell adhesion molecules. Plaque formation may represent a cell-mediated immune phenomenon, with a variety of potential antigenic agents identified. Shear stress and flow considerations also play a part.

Atherosclerosis begins in childhood, but it takes decades for atherosclerosis to evolve into the mature plaques responsible for the onset of ischaemic symptoms. Whilst plaque growth due to smooth muscle cell proliferation, matrix synthesis and lipid accumulation may narrow the arterial lumen and ultimately limit blood flow, uncomplicated atherosclerosis is essentially a benign disease. The final clinical outcome depends on whether a plaque becomes unstable, leading to acute disruption of its surface and exposure of its thrombogenic core to the luminal blood flow. The concept of a 'vulnerable plaque' was initially described in 1990[1,2] and though this initially gained wide acceptance, many authors now favour the broader concept of a 'vulnerable patient', whereby certain systemic and haematological conditions (e.g. relative hypercaogulability) must also be met before plaque rupture will result in symptomatic thrombosis.[3]

Mature atherosclerotic plaques are composed of a lipid core that is separated from the vessel lumen by a cap composed of fibrillar collagen. Disruption of this cap exposes the plaque's underlying thrombogenic core to the bloodstream, resulting in thromboembolism. This process of 'plaque rupture' is responsible for the majority of acute coronary syndromes (unstable angina, MI)[4-6,7] and ischaemic cerebral events (stroke, TIA, amaurosis fugax).[8-10]

Unravelling the complex biochemical and haemodynamic factors leading to plaque rupture is one of the greatest challenges facing contemporary medical research. The vital question in plaque pathogenesis is why, after years of indolent growth, life-threatening disruption and subsequent thrombosis should suddenly occur. Plaque stabilisation may prove to be an important clinical strategy for preventing the development of complications.[6] Identification of 'vulnerable plaques' (i.e., those most at risk of rupture)

and 'vulnerable patients' (i.e. those with predisposition to atherothrombotic occlusion) would allow pharmacotherapy to be targeted more effectively. Furthermore, a greater understanding of the mechanisms involved in plaque rupture will lead to improvements in preventative therapy.

EVIDENCE FOR THE 'PLAQUE RUPTURE' THEORY'

Coronary circulation

Evidence that plaque rupture leads to acute coronary syndromes has been provided from a number of sources. Early pathological studies using post-mortem specimens from fatal cases of acute myocardial infarction have revealed that virtually all cases of coronary thrombosis are related to rupture or fissuring of atheromatous plaques, along with evidence of distal embolisation.[7,11-13] Angioscopic findings in patients with stable angina have identified smooth atheroma within their coronary arteries, but disrupted irregular atheroma in the arteries of those with unstable angina.[14,15]

Radiological and histological studies have demonstrated that patients with a plaque morphology consisting of large lipid cores and thin fibrous caps are at increased risk of cardiovascular events.[16-18] In addition, these 'unstable' plaques are not necessarily the ones causing severely stenotic lesions.[19-21]

Cerebral circulation

A similar association between carotid plaque rupture and cerebrovascular events has been shown. In patients undergoing multiple TIAs or stroke progression, microemboli can be detected in the middle cerebral artery by transcranial Doppler.[10,22] Surface ulceration of carotid plaques seen on ultrasound imaging correlates well with symptoms[23] and echolucent (lipid-rich) plaques are at increased risk of causing future cerebrovascular events.

Early work utilising carotid plaques retrieved at carotid endarterectomy, highlighted the relationship between the presence of thrombus and the clinical status of patients.[24,25] This supported the theory that ischaemic attacks resulted from embolism rather than reduction in cerebral blood flow, particularly as few strokes occur in watershed areas.[26]

A number of subsequent studies demonstrated a relationship between the presence of intraplaque haemorrhage and patient symptoms.[27] Persson et al found that intraplaque haemorrhage appeared more frequently in symptomatic patients than asymptomatic patients,[28] while Lusby suggested a relationship between the onset of neurological symptoms and development of plaque haemorrhage.[29] Intraplaque haemorrhage may potentially arise *after* cap rupture, though it now seems most likely that it occurs *prior* to plaque breakdown[30] and may play an important role in disruption of the fibrous cap.

The most compelling evidence for an association between carotid plaque rupture and ischaemic cerebral events, is that carotid endarterectomy specimens removed from symptomatic patients are more likely to show histological evidence of rupture, compared to those from asymptomatic patients.[8,9] Van Damme and colleagues showed that 53% of complicated carotid plaques (intraplaque haemorrhage, haematoma, thrombus or ulceration) were symptomatic with a corresponding neurological deficit, compared to 21% of simple uncomplicated plaques.[31]

THE ROLE OF INDIVIDUAL COMPONENTS OF THE ARTERIAL WALL

A number of intrinsic and extrinsic factors have been identified that determine plaque vulnerability: the size and consistency of

the plaque core, the thickness and collagen content of the fibrous cap, and inflammation within the plaque. Further factors such as haemodynamic stress upon the plaque may ultimately contribute to cap disruption.

The evolution of a stable to unstable plaque with cap rupture and thrombosis can be outlined in the following simplistic terms (Figure 4.1): Endothelial damage allows passage of inflammatory cells and LDL into the vessel intima; free radicals are responsible for oxidation of the deposited LDL, and oxidized-LDL promotes cytokine and protease release from macrophages; proteases (in addition to other factors) degrade the fibrous cap causing disruption, allowing exposure of thrombogenic material to the blood; local thrombotic and fibrinolytic activity determine the degree of thrombus progression or dissolution.

Each component contributing to plaque rupture will be discussed in further detail. The relevant processes occur in the endothelium, the lipid core, the fibrous cap and the vessel lumen.

The endothelium

The origin of plaque destabilization can be traced back to endothelial dysfunction, or 'activation'. The endothelium is a single layer of highly specialised cells lining the vessel wall/lumen interface. It plays a vital role in modulating vascular permeability, perfusion, contraction and haemostasis. Leukocytes do not bind to normal endothelium. However, endothelial activation leads to the early surface expression of cell adhesion molecules, including VCAM-1, ICAM-1, E-selectin and P-selectin, which permit leukocyte binding. Many of the known atherosclerosis risk factors (e.g., smoking, hyperlipidaemia, hyperglycaemia, hypertension, hyperhomocysteinaemia) exert their damaging effects by causing endothelial activation.[32-37]

Activated endothelial cells express chemoattractant cytokines such as MCP-1, M-CSF, IL-1, IL-6 and TNF-α, as well as cell adhesion molecules. This pro-inflammatory environment, in conjunction with the altered permeability of the dysfunctional endothelium, mediates the migration and entry of leucocytes (mainly monocytes and lymphocytes) into the intima.[38-40]

The degree of endothelial dysfunction depends upon the balance between endothelial activation and endothelial 'passivation' (see Figure 4.2). Nitric oxide is the predominant molecule responsible for passivation, and the endothelium acts as an autocrine organ in its production.[41] Nitric oxide is an antioxidant, but has other plaque-stabilizing properties including reducing cell adhesion molecule expression,[42] platelet aggregation and SMC proliferation. Endothelial nitric oxide synthase, the enzyme responsible for nitric oxide production, is increased in people undergoing regular physical exertion, which may partly explain the benefits of exercise in atherosclerosis prevention.[43]

Endothelial cells are exposed to 3 different types of mechanical force. Hydrostatic forces (generated by the blood) and circumferential stress (generated by the vessel wall) are responsible for endothelial injury and activation. The third force is haemodynamic shear stress (generated by the flow of blood), which is inversely related to atherosclerosis formation – areas of high shear stress being relatively protected.[44] Despite the systemic nature of atherosclerosis, it is an anatomically focal disease with certain sites having a propensity for plaque formation. Arterial bifurcations exhibit slow blood flow, sometimes even bi-directional flow, resulting in decreased shear stress. The activity of endothelial nitric oxide synthase is decreased in these areas of non-laminar blood flow.[45,46] In addition, there is increased oscillatory and turbulent shear stress at bifurcations,

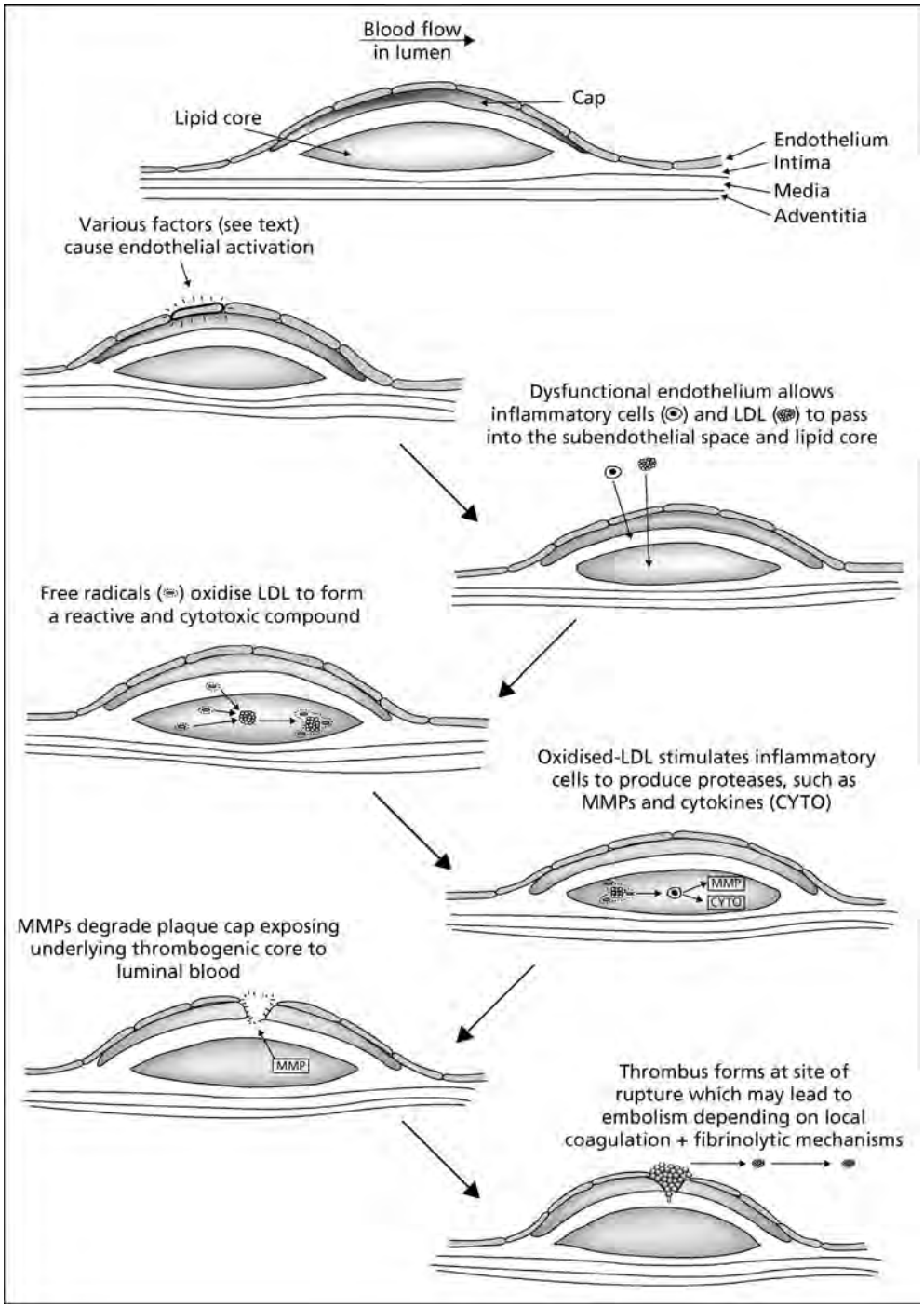

FIGURE 4.1: The stages of plaque rupture.

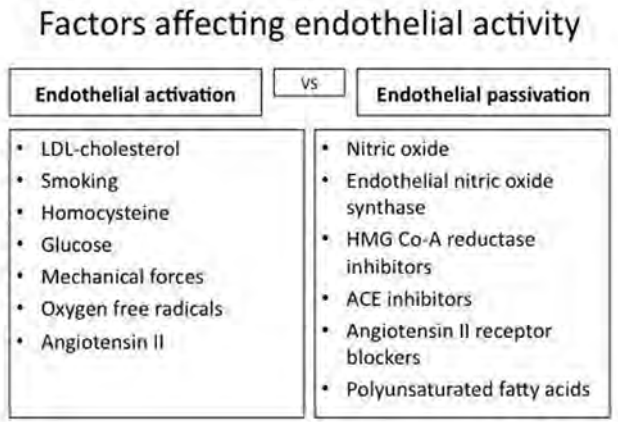

FIGURE 4.2: Factors affecting endothelial activity.

associated with an increase in oxygen free radical production[47] and monocyte adhesion.[48]

According to Laplace's law, the higher the blood pressure and the larger the luminal diameter, the more circumferential tension develops in the wall.[49] This phenomenon combined with a radial compression of the vessel wall may lead to excessive stress in vulnerable regions of the plaque, particularly the cap and shoulder.[50] For fibrous caps of the same tensile strength, those caps covering moderately stenotic plaques are probably more prone to rupture than those covering severely stenotic plaques, because the former have to bear a greater circumferential tension.[51]

The propagating pulse wave causes cyclic changes in lumen size and shape with deformation and bending of plaques, particularly those with a large soft plaque core. Eccentric plaques typically bend at the junction between the relatively stiff plaque and the compliant vessel wall.[52] The force applied to this region is accentuated by changes in vascular tone.

High blood velocity within stenotic lesions may shear the endothelium away, but whether high wall stress alone may disrupt a stenotic plaque is questionable.[4] The absolute stresses induced by wall shear are usually much smaller than the mechanical stresses imposed by blood and pulse pressure.[53]

It is clear that the endothelium is much more than an inert arterial wall lining. It is, in fact, a dynamic autocrine and paracrine organ responsible for the functional regulation of local haemodynamics. Factors that disturb this delicate balance are responsible for the initiation of a cascade of events eventually leading to plaque rupture.

The lipid core

The size and consistency of the atheromatous core is variable and critical to the stability of individual lesions, with a large volume lipid core being one of the constituents of the vulnerable plaque (Figure 4.3). It appears that the accumulation of lipids in the intima renders the plaque inherently unstable.

Although extremely variable, the 'average' coronary plaque is predominantly sclerotic with the atheromatous core making up <30% of the plaque volume.[54] The variability in plaque composition is poorly understood, with no relationship to any of the identified risk factors for atherosclerosis. Gertz and Roberts examined the histological composition of post-mortem plaques from 17 infarct-related coronary arteries.[55]

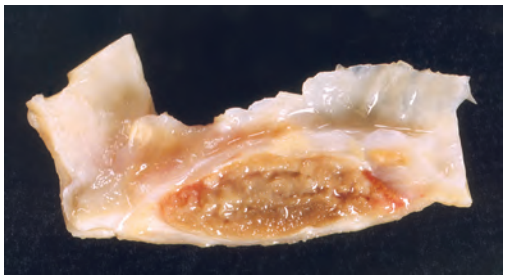

FIGURE 4.3: Longitudinal section of carotid plaque demonstrating a large volume lipid core

They found much larger proportions of the disrupted plaques to be occupied by atheromatous gruel in comparison to the intact plaques. Davies found a similar relationship in aortic lesions, with 91% of thrombosing plaques versus 11% of intact plaques exhibiting a lipid core that occupied >40% of the total plaque volume.[56]

Histological data regarding the necrotic core of carotid plaques is limited. There is, however, considerable evidence to link ultrasound-detected echolucent plaques (deemed to contain more soft or amorphous tissue) with symptomatology.[57,58] Feeley and colleagues demonstrated that symptomatic carotid plaques contained a significantly higher proportion of amorphous material than asymptomatic plaques,[59] with the lipid-rich core constituting 40% of overall plaque volume.[60]

LDL plays a more complex role in plaque instability than can be explained simply by the 'space-occupying' effect of accumulated lipid. A large core may produce a greater luminal narrowing, but plaque rupture sites are often characterized by 'outward remodelling' whereas those stenoses causing stable angina are more likely to be associated with 'inward remodelling'.[61] Indeed, it has been shown that in patients suffering acute coronary syndromes who had undergone angiography in the preceding months, the responsible lesion was recorded as causing a <70% stenosis in the majority of cases.[19,20,61] This is perhaps not surprising since, as mentioned earlier, a larger lumen places increased circumferential stress on the plaque, predisposing it to rupture.

As inflammatory cells cross the dysfunctional endothelium, cholesterol also enters in the form of LDL, and becomes trapped in the subendothelial space. This LDL is oxidized by free radicals creating a pro-inflammatory compound.[62] Oxidized-LDL is taken up by intimal macrophages – the process being mediated via receptors expressed on the macrophage surface,[63] although endocytosis of native LDL has also been demonstrated.[64] This process initially protects the surrounding smooth muscle and endothelial cells from the direct cytotoxic effects of oxidised-LDL, but leads to the formation of 'foam cells' (lipid-laden macrophages). Uptake of oxidized-LDL stimulates the expression of cytokines and proteolytic enzymes, propagating the cycle of inflammation.

The formation of a lipid core is a balance between LDL deposition of cholesterol in the damaged intima and removal by HDL (Figure 4.4). HDL and its carrier, apolipoprotein A-I, are responsible for so-called 'reverse cholesterol transport' – moving cholesterol from cells into the blood (from where it can be transferred to the liver for excretion in the bile).[65] However, it may also be capable of effecting lipid removal directly from the plaque, one of the possible explanations for plaque regression seen with increased HDL levels.[66] HDL may have other beneficial effects also, such as improving endothelial function,[67] decreasing cell adhesion molecule expression,[68] and inhibiting oxidation of LDL.[69]

In addition to the potential pro-inflammatory role of oxidised LDL, it has recently been proposed that cholesterol accumulation may lead to plaque rupture via a more direct physical pathway. Changes

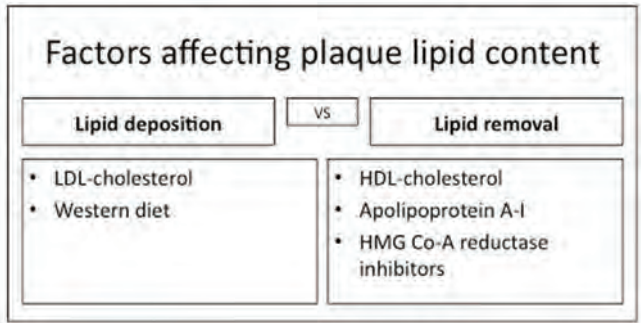

FIGURE 4.4: Factors affecting plaque lipid content

in local biological milieu such as decreased temperature or increased pH may cause in vitro precipitation of cholesterol into solid crystals. This alteration in state not only leads to a significant volume increase of up to 45%, but also leads to formation of sharp-tipped crystals that might be capable of damaging surrounding tissues and initiating plaque rupture. Using electron microscopy, Abela et al demonstrated that such crystal could be seen perforating the luminal surface of ruptured plaques from human coronary arteries.[70,71]

The cap of the plaque

The cap of the atherosclerotic plaque plays a vital role in isolating the plaque's thrombogenic core from the bloodstream. Since the thickness and collagen content of this cap are important determinants of overall plaque stability,[51] many authors now use the term 'thin-cap fibroatheroma' (TCFA) to identify those plaques most at risk of rupture. The accepted definition of TCFA is any plaque with a cap thickness of less than 65μm. Though the exact mechanisms that underlie progression from stable plaque to TCFA remain somewhat uncertain, it has been suggested that endothelial shear stress may play an important role, since TCFAs most often arise at sites of low endothelial shear stress (such as bifurcations and the concave side of arterial bends).[72]

Whatever the thickness of the fibrous cap, it is composed largely of fibrillar collagens (type I and type III[68]), though the relative proportion of collagen decreases as the cap thins. The fibrillar collagens have a lower thrombogenicity than the underlying core, but their exposure can be responsible for thrombus formation following erosion of the overlying endothelium.[73,74] This phenomenon accounts for one-third of acute coronary syndromes,[75] and the subsequent healing process of erosions can account for rapid and step-wise progression in plaque growth, leading to sudden increases in stenosis or occlusion.[76]

The most vulnerable area of the plaque is the shoulder region, where the cap is often at its thinnest.[7] Studies have shown a reduction in the collagen content of the cap around areas of plaque disruption, as well as steep transverse gradients of connective tissue constituents across ulcerated plaques.[77] This may result from a reduction in matrix production by smooth muscle cells, which exhibit diminished numbers in areas of plaque disruption,[56] or from increased degradation of matrix by proteolytic enzymes. It is most likely, of course, that a combination of excessive matrix degradation and reduced matrix production are responsible for cap thinning (Figure 4.5). A reduction in SMCs within the fibrous cap would certainly undermine its strength.[78] Recently there has been interest in the role of smooth muscle cell

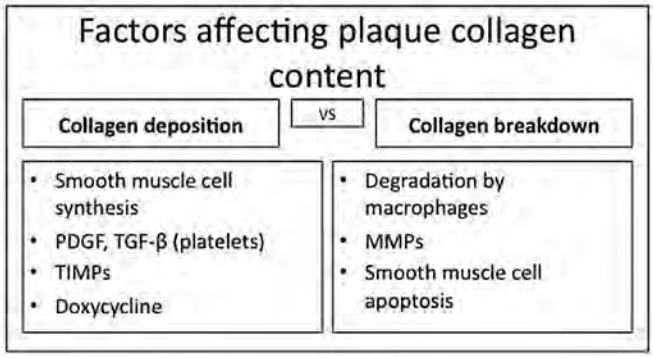

FIGURE 4.5: Factors affecting plaque collagen content

apoptosis in plaque cap weakening, caused by a combination of intrinsic and extrinsic factors, particularly macrophage and lipid derived products.[79,80]

More recently it has been suggested that plaque integrity may also be influenced by the development of minute, spherical microcalcifications within the fibrous cap. These microcalcifications are thought to represent accumulations of calcified macrophages or even post-apoptotic smooth muscle cells and result in highly focal increases in physical stress. The increased stress leads to areas of facial debonding, weakening the infrastructure of the cap and contributing to subsequent plaque disruption.[81]

Smooth muscle cells and collagen production

The SMC has a paradoxical role in plaque instability. On the one hand, SMCs are responsible for plaque matrix production and adverse arterial remodelling, while on the other, they produce collagens that give the plaque intrinsic strength. SMC inhibition therefore has potentially detrimental and beneficial effects.

In the normal arterial wall, SMCs are present in the media and express a differentiated phenotype. They are contractile and do not divide or migrate.[82] In atherosclerosis, when stimulated by the milieu of growth factors and cyokines, they 'dedifferentiate' and express a synthetic phenotype.[83] In the media, SMCs are surrounded by a basal lamina consisting of type IV collagen. Proteolytic enzymes secreted by macrophages are responsible for digestion of this supporting framework. The released SMCs are then able to migrate to the intima, where they secrete new extracellular matrix.[84] SMCs play a crucial role in stabilising atherosclerotic plaques, as they are responsible for the production of the cap fibrillar collagens.[82] In this respect, SMCs are important not only in initial formation of the fibrotic cap, but also in repair of subclinical plaque rupture. SMCs accumulate at the rupture site and secrete fibrous proteins. This restores plaque integrity, but may also lead to rapid growth of the plaque causing vessel stenosis. Certain platelet factors, including PDGF and TGF-β, are felt to be particularly important in stimulating collagen synthesis by SMCs, whereas γ-interferon (from activated T-cells) has the opposite effect.[85]

Since SMCs are the only cells producing fibrous tissue for inclusion in the atherosclerotic plaque, the balance between recruitment and degradation of these cells is clearly of great significance in plaque stability. It had previously been accepted that all SMCs involved in atherosclerosis were

derived from the local vessel media or intima. However, many groups are now examining the possibility that they may also be recruited from a circulating pool of SMC progenitor cells.[86] The prospect of manipulating the activity of these progenitor cells to increase plaque stability is an attractive therapeutic target, though further work is still required in this area.

Whatever the true origin of plaque SMCs, they play a vital role in maintaining the structure of the plaque and SMC apoptosis leads to decreased collagen production, thinning of the fibrous cap and increased volume of the necrotic core.[87-89] A recent, though small, study demonstrated that the proportion of SMCs undergoing apoptosis and the frequency of cytoplasmic remnants of apoptotic cells were significantly increased in unstable versus stable angina atherectomy specimens.[90] Apoptosis of SMCs and macrophages has been identified within plaques, but only in advanced disease with dense macrophage infiltration. Apoptotic cells are deemed to have become susceptible to a form of cell death which is distinct from necrosis and is characterised by a series of morphological changes, starting with shrinkage of the cell membrane and leading on to condensation of nuclear chromatin, cellular fragmentation and eventually engulfment of apoptotic bodies by surrounding cells.[79]

Pro-apoptotic proteins are present in advanced plaques, and it has been observed that cells derived from the plaque, but not the adjacent media, die when brought into culture.[80,91] Intimal cell apoptosis may account for the low density of smooth muscle cells in unstable plaques, and may contribute to the events leading up to plaque disruption. Though further study is still required, prevention of smooth muscle cell apoptosis may prove to be an important therapeutic target in the treatment of atherosclerotic disease.

Macrophages and collagen degradation

It is now known that inflammation plays a major role in plaque progression and especially in the period just prior to its rupture.[92] Macrophages control many of the inflammatory processes within the plaque,[93] and are responsible for the production of proteolytic enzymes capable of degrading the extracellular matrix.[94,95] The predominant proteolytic enzymes involved in plaque disruption are the matrix metalloproteinases or MMPs.[96]

The MMPs are a family of proteolytic enzymes characterised by the presence of zinc ions at their active sites. All degrade components of the extracellular matrix, and are divided into 4 main classes on the basis of their substrate specificity (Table 4.1).

MMPs are essential in normal healthy individuals, playing a key role in processes such as wound healing.[97,98] However there is growing interest in their role in disease states where ECM breakdown plays a predominant role.[99] Early interest focused on a pathological role for MMPs in the resorption of periodontal structures in periodontal disease,[100] the destruction of joints in rheumatoid arthritis,[101] and the local invasive behaviour of malignancies.[102] In vascular disease, they have been implicated in many of the stages of atherosclerosis but most particularly in acute plaque disruption.[103] The site of rupture is characterised by an intense inflammatory infiltrate consisting predominantly of macrophages,[94] that undergoes activation resulting in increased MMP expression. This shifts the delicate equilibrium towards proteolysis and away from matrix accumulation, making plaque disruption more likely (Figure 4.5).

MMP activity is tightly controlled at several levels and expression of MMPs is determined at the transcriptional level by various cytokines and growth factors.[104]

TABLE 4.1: THE MATRIX METALLOPROTEINASE FAMILY

MMP	Alternative names	Principal substrates
Collagenases		
MMP-1	Collagenase-1, Interstitial collagenase	Collagens I,II,III, gelatin, MMP-2 & 9
MMP-8	Collagenase-2, Neutrophil	Collagens I,II,III, gelatin
MMP-13	collagenase	Collagens I,II,III, gelatin, PAI-2
MMP-18	Collagenase-3	Collagen I
	Collagenase-4, Xenopus collagenase	
Gelatinases		
MMP-2	Gelatinase-A, 72 kDa gelatinase	Gelatin, collagens IV,V,VII,X,XI,XIV, elastin, fibronectin, aggrecan
MMP-9	Gelatinase-B, 92 kDa gelatinase	Gelatin, collagen types IV,V,VII,X, elastin
Stromelysins		
MMP-3	Stromelysin-1	Collagens III,IV,IX,X, gelatin, aggrecan, MMP-1,7,8,9 & 13
MMP-10	Stromelysin-2	Collagens III,IV,V, gelatin, MMP-1 & 8
MMP-11	Stromelysin-3	
Matrilysins		
MMP-7	Matrilysin-1, Pump-1	
MMP-26	Matrilysin-2, Endometase	
Membrane types		
MMP-14	MT1-MMP	Collagens I,II,III, gelatin, MMP-2 & 13
MMP-15	MT2-MMP	MMP-2, gelatin
MMP-16	MT3-MMP	MMP-2
MMP-17	MT4-MMP	
MMP-24	MT5-MMP	
MMP-25	MT6-MMP	
Others		
MMP-12	Macrophage elastase	
MMP-19	No trivial name	
MMP-20	Enamelysin	
MMP-21	XMMP (Xenopus)	
MMP-23		
MMP-27		
MMP-28	Epilysin	

In a variety of tissue types, IL-1, PDGF and TNF-α stimulate expression,[105,106] while heparin, TGF–β and corticosteroids inhibit expression.[107,108] In recent years, there has also been considerable interest in the regulatory role of extracellular matrix metalloproteinase inducer (EMMPRIN). EMMPRIN was initially identified as a tumour-derived protein that facilitated cancer cell invasion by stimulating MMP production in epithelial cells and fibroblasts.[109] However, subsequent studies have demonstrated that EMMPRIN also stimulates production of MMPs by smooth muscle cells and monocytes, making it highly relevant in atherosclerosis and plaque instability. In addition, EMMPRIN may also

lead to increased production of inflammatory cytokines which further augment MMP activity as described above.[110]

MMPs are initially secreted as latent inactive proenzymes and converted to the active state by cleavage of a propeptide domain.[111] The major physiological activator is plasmin, which in turn is regulated by PAI.[112] Thrombin has been shown to activate MMP-2 in vitro[113] and could provide a mechanism for MMP activation at sites of vascular injury. Reactive oxygen species also modulate enzyme activation.[114,115]

Metalloproteinase activity is further governed by naturally occurring MMP inhibitors. These 'tissue inhibitors of metalloproteinases' (TIMPs) provides a further level of control and overall proteolytic activity depends on the ratio of activated MMPs to TIMPs.[116]

Early studies showed that MMPs were present at increased levels in atherosclerotic arteries. Raised levels of gelatinase activity were demonstrated in the aortas of patients with occlusive disease compared to healthy controls, and zymography revealed that this was predominantly MMP-9.[117] Subsequently, quantitative studies using ELISA revealed a six-fold increase in MMP-9 levels in atherosclerotic aortas.[118] The level and expression of MMP-2 is also increased in atherosclerotic aortic tissue compared with normal aorta.[119] While expression of MMP-2 has been detected in normal arteries, it appears that most MMPs are expressed only in atherosclerotic tissue.[120] The colocalisation of MMP-1, -2, -3 and -9 to the vulnerable shoulder of the plaque provided further evidence of their potential role in acute disruption.[120]

More recent studies have demonstrated an association between MMP levels and markers of plaque instability. Increased immunostaining for MMP-9 was seen in 12 atherectomy specimens retrieved from patients with unstable angina compared to the stable form.[121] A larger study, involving 75 carotid endarterectomy specimens, demonstrated a close association between raised plaque levels of MMP-9 and a number of indicators of plaque instability, including symptomatology, cerebral embolisation and histological features of plaque rupture.[9]

Convincing evidence therefore exists of increased levels of MMP-2 and -9 in unstable plaques. However, intact type I and type III collagen molecules, which account for the load-bearing strength of the plaque cap, are not substrates for MMP-2 and -9. While it has been reported that high concentrations of MMP-2 can degrade type I collagen in an in vitro environment devoid of TIMPs,[122] it is likely that in vivo only the collagenases, MMP-1, -8 and -13, are capable of degrading fibrillar collagens.

MMP-1 and -13 levels are higher in 'atheromatous' compared to 'fibrous' plaques,[123] and MMP-8 has been demonstrated in atheroma but not normal arteries.[124] The expression of MMP-1 is increased in areas of high circumferential stress.[125] It is likely that both mechanics and proteolysis play a role in the degradation and weakening of the collagen-rich extracellular matrix, and understanding their interaction may be crucial.[126]

Evidence from our laboratories suggests that active MMP-8 is significantly raised in unstable plaques retrieved at carotid endarterectomy (Figure 4.6). The ratio of active MMP-8 to TIMP-1 and -2 (its naturally occurring inhibitors) were also significantly higher in the more unstable plaques of the 159 specimens collected in this study. This implies net proteolysis of the types of collagen found in the cap of the plaque by MMP-8. Immunohistochemistry confirmed the presence of MMP-8 protein within the plaque, which colocalised with macrophages (Figure 4.7).

Genetic variation in the genes controlling MMPs could theoretically be responsible for the susceptibility of some individuals to atherosclerotic plaque rupture. Early work has identified a number of polymorphisms that may be influential in this regard. Price et al have identified a novel genetic variation in the MMP-2 gene.[127] Ye and colleagues detected a polymorphism in the promoter region of the MMP-3 gene that may lead to increased systemic levels.[128] This polymorphism was subsequently found to be more common in patients suffering MI, compared to a control group.[129] A single nucleotide polymorphism

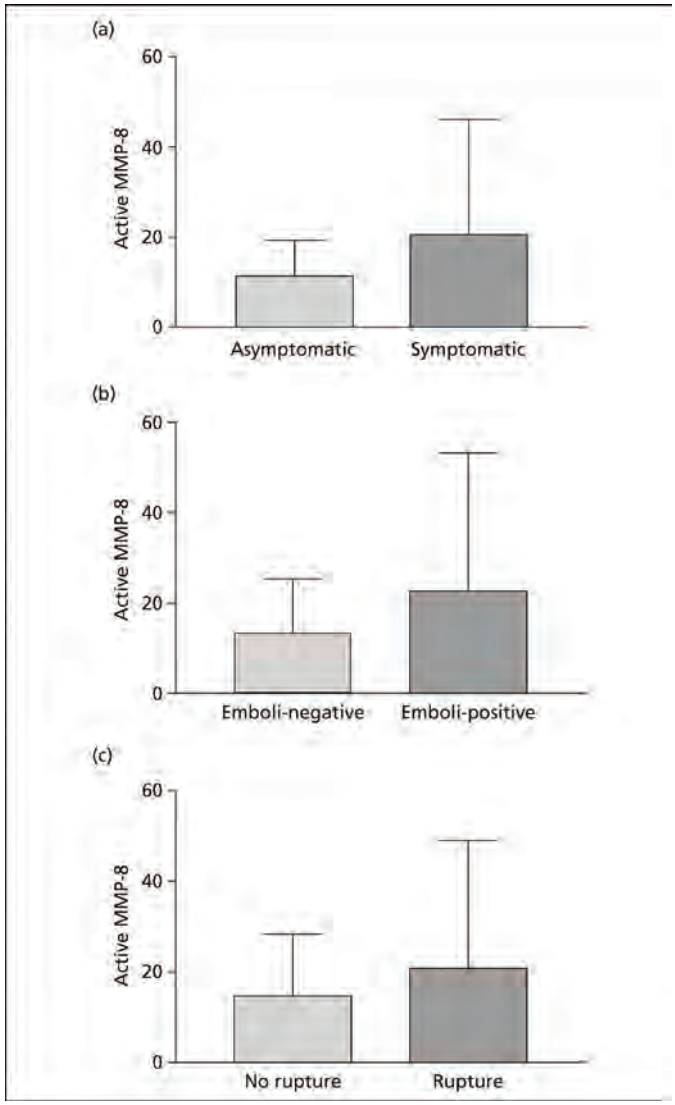

FIGURE 4.6: Plaque concentrations of active MMP-8 are significantly higher in symptomatic compared to asymptomatic carotid plaques: **(a)** from patients suffering carotid territory symptoms in the 6 months prior to surgery (p-value 0.0002), **(b)** from patients with pre-operative cerebral embolisation detected by transcranial Doppler (p-value 0.003) and **(c)** showing histological evidence of plaque rupture. Median values and interquartile ranges shown (p-value 0.003).

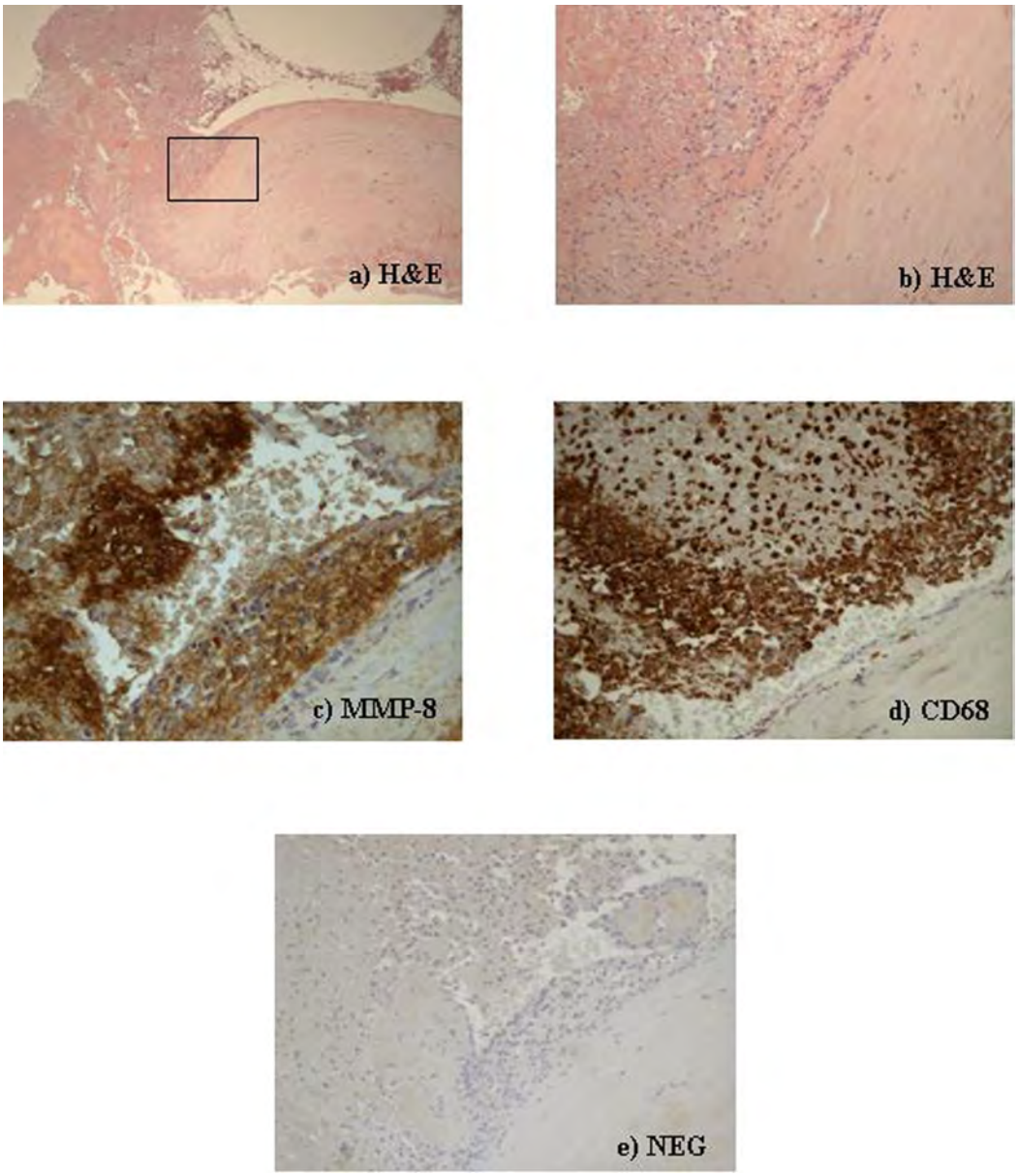

FIGURE 4.7: Histological sections taken from the shoulder region of a symptomatic carotid plaque. Some sections show disruption of the friable plaque.

(a) Low power H&E section with boxed area delineating high power view shown in **(b-e)**.
(b) High power H&E section demonstrating a cellular infiltrate.
(c) Strong reactivity for MMP-8 in cells.
(d) Positive staining for CD68 (macrophages).
(e) Negative immunohistochemistry control.

(C to T transition at position –1562) has been shown to influence MMP-9 transcription.[130] In this study by Zhang and co-workers, triple-vessel coronary artery disease was detected by angiography in 26% of patients with this polymorphism compared to 15% of those without.[130] Presenting a coherent picture of the interactions between various polymorphisms and the corresponding gene expression is difficult, and further complicated by environmental effects. However, it is clear that the potential exists to identify 'at risk' individuals in such a manner.

The vessel lumen

Disruption alone would not precipitate ischaemic syndromes without thrombus formation on the plaque surface, so plaque instability and thrombogenicity in tandem predispose to acute clinical events. Platelet adherence to the sub-endothelium after surface disruption leads to activation, with ADP and serotonin release stimulating further platelet recruitment and activation.

Once formed, thrombus can behave in three ways, dependent on the physical nature of the rupture and the balance between local fibrinolytic and coagulation processes. Firstly, the initial thrombus may progress to cause occlusion of the vessel. Secondly, the thrombus may disintegrate resulting in distal embolisation. Thirdly, the clot can undergo rapid dissolution, with the healed rupture resulting in a variable decrease in vessel lumen diameter.[76]

Tissue factor is a major regulator of haemostasis.[131] It is the most thrombogenic component of atherosclerotic plaques[132] and is expressed by numerous cell types, including endothelial cells. The level of tissue factor in coronary plaques from patients with unstable angina is more than twice the value observed in those plaques from stable angina patients.[133] Positive immunostaining for tissue factor correlates with areas of intense macrophage infiltration and SMCs, suggesting a cell-mediated increased thrombogenicity in unstable plaques. The increase in tissue factor levels seems to be linked to expression of the CD-40 receptor on the macrophage cell surface. The CD-40 ligand is expressed on activated T-lymphocytes, and other atheroma-associated cells,[134] which can therefore induce tissue factor production by macrophages via this signalling system. Expression is also regulated by cytokines and oxidised LDL.[135,136] It has been reported that a blood-borne pool of tissue factor exists,[137] though in the context of plaque disruption, macrophage production of tissue factor is predominantly responsible for plaque thrombogenicity.[133,138,139] It is interesting to note that many of the recognised cardiovascular risk factors increase the expression of tissue factor.[140,141]

THE ROLE OF ANGIOGENESIS IN PLAQUE RUPTURE

Angiogenesis is essential for normal growth and development. Neovascularisation has been observed in plaques[142] and it is postulated that it may play a role in atherosclerosis by providing growth factors and cytokines to regions of plaque development.

A study of coronary atherectomy specimens revealed the presence of neovascularisation in 50% of specimens from patients with unstable angina compared to 10% of specimens from patients with stable angina,[29] suggesting a possible role in plaque instability. Angiogenesis may contribute to plaque instability by causing intraplaque haemorrhage or extravasation of erythrocytes and inflammatory mediators into the centre of the plaque. Once red blood cells have leaked into the plaque, cholesterol from the cell membrane may become incorporated into the lipid core increasing its volume.[143]

This is supported by the finding that lipid-rich plaques have a significantly higher microvessel density than fibrous plaques.[144] The associated delivery of inflammatory cells may also lead to plaque degradation by stimulating MMP activity as described earlier in this chapter.

Perhaps more importantly, most neovascularisation occurs at the vulnerable shoulder area of the plaque. Immunostaining for inflammatory cells showed a close association between angiogenesis and inflammatory infiltration. In addition, a parallel increase in the expression of leukocyte adhesion molecules in the same vulnerable areas was demonstrated.[144]

Angiogenesis involves interactions between endothelial cells and components of the basement membrane matrix. MMP activity is required for such interactions, especially MMP-2 and MT1-MMP.[145] TIMPs have been shown to reduce angiogenesis, while up-regulation of MMP activity stimulates its increase.[146] However, whilst neovascularisation may promote and sustain inflammatory infiltration, the converse may also be true, whereby changes in the plaque associated with inflammation may themselves promote angiogenesis. Further work in this area is required.

THE ROLE OF INFECTIOUS AGENTS IN PLAQUE RUPTURE

The role of infectious agents in atherosclerosis and plaque rupture is controversial. Definitive proof of a causal relationship is lacking, although studies have reported associations between plaque development and *Chlamydia pneumoniae*,[147-149] *Helicobacter pylori*,[150] cytomegalovirus,[151,152] Herpes simplex virus types 1 and 2,[153] and hepatitis A virus.[154]

Certain infectious agents can evoke cellular and molecular changes supportive of a role in atherogenesis.[155] Work has shown that Chlamydial interaction with monocytes results in upregulation of TNF-α and IL-1β,[156,157] both of which are associated with plaque development. Chlamydial production of the HSP-60 antigen activates human vascular endothelium, and increases TNF-α and MMP expression in macrophages.[158,159] Once again, these are factors that influence plaque stability.

It has also been proposed that infective pathogens may exert their effects via direct infection of cells in the vessel wall. This establishes localised inflammation, leading to increased smooth muscle cell migration and greater uptake of oxidised low-density lipoprotein.[160]

There is some doubt about the methods employed for Chlamydia detection,[161] and also the role of potential confounding factors in epidemiological studies.[162] A large-scale prospective study of 15,000 healthy men in the United States which was controlled for age, smoking, socio-economic status and other cardiovascular risk factors, failed to show any association between Chlamydia seropositivity and the risk of MI.[163]

More recently, the STAMINA trial[164] demonstrated that eradication therapy (amoxicillin/ azithromycin, metronidazole and omeprazole) administered for 1-week after an acute coronary syndrome, significantly reduced cardiac death and acute coronary syndrome readmission rates over the following 12 months. These effects were unrelated to *Chlamydia pneumoniae* or *Helicobacter pylori* seropositivity, however, suggesting that the trial therapy prevented lesion progression by a mechanism unrelated to its antibiotic action.

Though the role of infection in atherosclerosis is still unclear, it seems that any causal relationship is likely to be highly complex and involve both direct and indirect pathways. Important factors may also include the patient's susceptibility to infection and

their innate inflammatory and immune responses.[165]

RISK PREDICTION OF PLAQUE INSTABILITY

Imaging

Angiography can demonstrate ulceration[166] but does not appear to be able to adequately distinguish between stable and unstable plaques.[167] In addition, the degree of stenosis detected by angiography does not correlate well with the future risk of events[19-21] because, as already discussed, it is often not the most stenotic plaques that are at highest risk of rupture.

Conventional ultrasound studies have shown an association between carotid plaque morphology and neurological symptoms[168] but have been unable to predict the risk of future events.[23] Intravenous ultrasound (IVUS), however, has been shown to have much greater resolution (100 μm) and provides detailed cross-sectional images of the arterial wall. It is also able to identify the increased echolucence of lipid-rich plaques and for a time it was thought that it might prove useful in detection of rupture-prone plaques.[169] Unfortunately, sensitivity and specificity were found to be low with this technique and it has largely been superseded by intravenous ultrasound virtual histology (IVUS-VH). The improved spectral analysis offered by this technology allows more detailed plaque characterization and can provide detail on lipid content, calcification and volume of the necrotic core.[170] Recent studies have shown this may be a clinically useful tool and demonstrated that IVUS-VH identified more TCFAs in patients with acute coronary syndrome than in those with stable angina pectoris.[171]

In parallel with the development of IVUS-VH, many groups have now begun to use optical coherence tomography (OCT). Also an intravenous modality, OCT is analogous to ultrasound imaging (using light rather than sound waves) and provides excellent spatial resolution (10-15μm). This allows detailed assessment of the arterial wall and can identify those plaques with a fibrous cap less than 65μm thick (i.e. TCFA) as well as areas of increased echolucency.[172] Whilst this technique has yielded very encouraging results in the identification of culprit atherosclerotic lesions, it is not without its limitations. Since blood attenuates the optical signal, the vessel under investigation must be proximally occluded for considerable periods to allow accurate imaging. An updated version of the technology has therefore been developed in recent years. This second generation of OCT is known as optical frequency domain imaging (OFDI) and involves much higher frame rates (>100 frames/sec). The higher frame rate allows rapid three-dimensional imaging of long arterial segments using high-speed pull-back of the probe. This means there is no need for proximal occlusion of the vessel and the artery can simply be purged with saline just prior to imaging[173] Further investigation will be needed to assess the true clinical utility of this technique.

Since increased inflammatory activity occurs prior to plaque rupture, attempts have been made to detect this increase, using local temperature measurements. Thermography studies have shown that temperature correlates well with macrophage cell density in human carotid plaques.[174] The temperature of coronary vessels in patients with ischaemic heart disease, in particular acute coronary syndromes, is higher than in normal controls.[175] In addition, increased local plaque temperature has been shown to be an independent predictor of adverse clinical outcome.[176]

High-resolution MRI appears to characterize the atherosclerotic plaque better than other imaging techniques.[177] It is more

accurate then angiography in measuring the degree of stenosis and, unlike angiography and IVUS, is non-invasive. However, a multicentre trial of imaging in coronary artery disease found whilst that MRI could reliably identify significant intraluminal lesions and rule out proximal or three-vessel disease, specificity was low.[178] This led to the suggestion that MRI may be more sensitive and specific if combined with intravascular enhancing agents such as gadolinium. Using such a marker improved MRI specificity and facilitated identification of carotid TCFA.[179]

MRI is still technically limited in many cases by small vessel size and movement artefact, and studies have not yet demonstrated the ability to predict risk of future cardiovascular events. Nonetheless, advances in the technique suggest a potential future role for MRI in detection of the high-risk plaque.

Just as enhancing agents may increase the accuracy of MRI, they may also prove useful in identifying atherosclerotic lesions using positron emission tomography (PET) and there has been increasing interest in the use of ^{18} fluorodeoxyglucose (^{18}FDG). Uptake of this glucose analogue is increased in metabolically active cells and early animal studies suggest it enriches in plaque macrophages and indicates areas of neovascularisation.[180] However, the clinical application of this technique has yet to be demonstrated.

Blood markers

It has long been established that adverse lipid profiles correlate with increased risk of MI and stroke though this is not a direct predictor of plaque rupture. Raised CRP levels have also been associated with increased cardiovascular risk in apparently healthy patients,[181,182] though its use as a prognostic marker of clinically significant thrombosis remains controversial.

MMP-2 and MMP-9 are raised in the peripheral blood of patients suffering from acute coronary syndromes,[183] while plasma MMP-9 is raised in patients with unstable carotid plaques.[184] A recent study of 1127 patients with coronary artery disease identified baseline plasma MMP-9 levels to be a novel predictor of cardiovascular mortality.[185]

Similarly, raised serum levels of soluble intercellular adhesion molecule-1 (slCAM-1) have been shown to be an independent predictor of future coronary event in patients with coronary heart disease.[186]

Many other molecules have also been investigated as potential prognostic markers in progression of atherosclerosis, including cytokines, lipoproteins, myeloperoxidases and placental growth factor. Though some have yielded promising results, none has yet been widely accepted as a reliable predictor of plaque rupture or clinical events.[187]

THERAPY AIMED AT PLAQUE STABILISATION

Pharmacotherapy to induce plaque stabilisation could be targeted at different aspects of the complex pathway leading up to plaque rupture, in particular:

1. the endothelium – by increasing endothelial passivation
2. the lipid core – by reducing LDL deposition/ augmenting LDL removal
3. the fibrous cap – by increasing collagen deposition/ preventing collagen degradation
4. the vessel lumen – by altering the thrombogenicity of the local environment.

Most recent interest has focussed on the role of HMG Co-A reductase inhibitors, which appear capable of influencing plaque stabilisation at all these levels.

HMG Co-A Reductase Inhibitors

HMG Co-A reductase inhibitors, or statins, are well known for their lipid-lowering action. They are the most effective group of therapeutic agents for lowering LDL and raising HDL levels. However, recent evidence suggests that they are also capable of decreasing cardiovascular events in those with normal cholesterol levels.[188,189] The Oxford Heart Protection Study[189] was a randomised controlled trial of simvastatin versus placebo in 20,536 individuals at high-risk of cardiovascular disease. Coronary death rate and other vascular events were significantly reduced in the simvastatin groups, even in patients with lipid levels below currently recommended targets (<5mmol/l total cholesterol and <3mmol/l LDL-cholesterol).

In the lipid lowering arm of the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT),[188] 10,305 individuals with total cholesterol levels <6.5mmol/l were randomised to either atorvastatin or placebo. The trial was stopped 1.7 years before the planned 5-year follow-up target was reached, as there were significantly fewer cardiovascular events in the atorvastatin group. The observed clinical benefit is probably a combination of lipid lowering below levels previously considered 'normal' and additional lipid-independent plaque stabilising actions. Several studies have reported effects other than lipid-lowering properties, including anti-proteolytic and anti-inflammatory mechanisms.[190,191]

Statins increase nitric oxide synthase activity[192] and encourage endothelial passivation (Figure 4.2). As discussed earlier, nitric oxide causes vasodilatation, inhibition of SMC proliferation and platelet aggregation and has widespread anti-inflammatory and anti-oxidant properties. Statins also reduce the expression of cell adhesion molecules,[193] interfering with the adherence of monocytes to the endothelium.

Statins may also have direct anti-inflammatory and anti-proteolytic actions, which contribute to increased plaque stability. In cell culture and animal models, statins have been shown to reduce macrophage secretion of MMP-1, -2, -3 and -9,[194] and increase the collagen content of the plaque.[195] Also, CRP levels are decreased by statins in a lipid-independent manner.[191,196]

Work from our laboratories suggests that statin therapy stabilises carotid plaques by lowering the levels of MMP-1, MMP-9 and IL-6. In an observational non-randomised study of 137 patients, we found that patients on statin therapy were significantly less likely to have suffered carotid territory symptoms within the month prior to carotid endarterectomy. The number of patients undergoing spontaneous pre-operative cerebral embolization was also significantly lower in the statin group.

HMG Co-A reductase inhibitors also have the potential to reduce thrombogenicity by decreasing tissue factor activity[197] and lowering levels of PAI-1.[198,199]

MMP Inhibition

The realisation that tissue remodelling due to increased MMP activity plays a key role in disease states has led to considerable interest in the potential for MMP inhibition. Most clinical and pre-clinical data regarding therapeutic manipulation of the extracellular matrix has been in the fields of arthritis, periodontal disease and cancer.[103] MMP inhibition aimed at plaque stabilisation aims to redress the imbalance between enzymes and inhibitors, which causes excessive tissue degradation. Potential methods of MMP inhibition include the administration of:

Tissue Inhibitors of Metalloproteinases (TIMPs)

The level of TIMPs can be increased either by the exogenous administration of recombinant TIMPs or by stimulating their local production through gene therapy. Increased TIMP-1 raised the collagen, elastin and smooth muscle content of atherosclerotic lesions in animal models,[200] while local gene transfer of TIMP-2 has been shown to decrease vascular remodelling in conjunction with lowered MMP activity (experimental models).[201]

It is difficult to extrapolate these data to potential applications in humans. The major drawback associated with TIMPs would be tissue delivery, since exogenous products would be metabolised and denatured with minimal tissue penetration at the intended site of action. Systemic stimulation of TIMPs would almost certainly have significant side effects precluding clinical use. Therefore, treatment would have to take the form of local tissue delivery or gene therapy. Clearly either system will be very expensive to develop, so more interest has concentrated on the development of synthetic MMP inhibitors.

Synthetic MMP inhibitors

Synthetic peptides work by binding to the zinc ion at the active site of the MMP, thus preventing cleavage of substrate collagen molecules.[202] Batimastat showed promise in decreasing tumour development and metastasis (animal models)[203] and limiting aneurysm expansion (experimental models),[204] but is not available in an oral form. Marimastat, which is available orally, was shown to limit intimal hyperplasia[205] and aneurysm expansion in vivo.[206] It also showed promise in early human cancer studies, but caused significant musculoskeletal side effects in 30% of patients.[207] Recent studies of MMI270, a more specific inhibitor (of MMP-2, MMP-8 and MMP-9), have shown a similar side effect profile.[208] Furthermore, recent animal studies of broad-spectrum synthetic MMP inhibitors have found them to be generally deleterious in terms of both plaque growth and plaque stability.[209]

Doxycycline

Doxycycline, a member of the tetracycline antibiotic family, is also a non-selective MMP inhibitor,[210] with a proven safety profile. Clinical trials have shown that doxycycline is capable of decreasing cartilage MMP levels when given to patients prior to hip surgery.[211] It has also been shown to limit intimal hyperplasia[212] and aneurysm expansion in vivo,[213] by reducing MMP-9 activity. Furthermore, when given to patients prior to AAA repair the expression of MMP-2 and MMP-9 was reduced in the aortic wall.[214]

A randomised clinical trial of doxycycline versus placebo in patients prior to carotid endarterectomy demonstrated decreased plaque MMP-1 levels and a potential for clinical benefit.[215] A phase II study of doxycycline administration to patients with small AAAs recently showed that it was reasonably well-tolerated (92% completed the 6-month course) and reduced plasma MMP-9 levels.[216] Further studies are on going to evaluate its effects on small aneurysm expansion.

ACE Inhibitors

ACE inhibitors (ACEI) and angiotensin II receptor antagonists decrease cardiovascular events, independently of their effects on blood pressure control. The ACEI, trandalopril, and the experimental angiotensin receptor antagonist, HR720, decrease the area

of atherosclerotic lesions in the thoracic aorta of cholesterol-fed monkeys.[217] This was achieved without alteration of mean blood pressure or cholesterol levels. The Heart Outcomes Prevention Evaluation (HOPE) study demonstrated a decrease in cardiovascular events in high-risk patients given ramipril as opposed to placebo.[218] This effect could only be partly explained by the modest decrease in mean blood pressure seen between the 2 groups (3/2mmHg).

Angiotensin II promotes endothelial activation,[219] and therefore the mechanism of action of ACEIs could be through endothelial passivation (leading to a reduction in cell adhesion molecule expression and macrophage infiltration). ACEIs may also exert their effects through bradykinin potentiation, resulting in decreased smooth muscle cell migration, decreased inflammation and decreased production of oxygen free radicals (COPPOLA 2008). Navalkar et al provided biochemical evidence to support these hypotheses by demonstrating that irbesartan (an angiotensin II receptor blocker) can decrease plasma levels of VCAM-1, TNF-α and superoxide.[220]

With ever more detailed understanding of the human genome, gene therapies have also come under investigation in the search for anti-atherosclerotic therapies. Hans et al have demonstrated that polyADP-ribose polymerase (PARP-1), a DNA-repair protein, stimulates apoptosis in the presence of local inflammation and plays an important role in plaque dynamics. They went on to show that inhibition of PARP-1 resulted in a reduction in plaque size, decreased collagen degradation and increased plaque smooth muscle content in ApoE(-/-) mice.[221] These findings suggest that PARP-1 inhibition may also represent a valuable therapeutic tool, though its applicability in humans has yet to be demonstrated.

Since the clinical significance of any plaque rupture is also governed by the intravascular environment, investigators continue to seek new therapies that may decrease the thrombogenicity of blood. Until now, this has been achieved with a combination of aspirin and another antiplatelet agent – most commonly clopidogrel – but drug resistance and side effect profiles can limit its applicability. The latest class of antiplatelet drugs is the $P2Y_{12}$ blockers, which inhibits platelet activation via blockade of the $P2Y_{12}$ ADP-receptor. Though these drugs (such as prasugrel and ticagrelor) may still have significant side effect profiles, they seem to be associated with far less unwanted bleeding and may be effective in patients who do not respond to clopidogrel.[222]

Though a number of therapeutic targets have shown promise in preventing plaque rupture, substantial work is still needed in this area since many of the potential therapeutic targets (such as smooth muscle cells and macrophages) have the ability to play both detrimental and beneficial roles in the complex process of atherosclerosis.

SUMMARY

Acute plaque disruption precedes the onset of clinical ischaemic syndromes. Exposure of the highly thrombogenic core to luminal blood results in platelet adherence and thrombosis. Inflammation is clearly involved in the process of plaque development and acute disruption, though the precise mechanism by which the inflammatory process is initiated remains unclear. The roles of angiogenesis, cellular apoptosis and infectious agents also require further clarification. Unstable plaques have a large lipid core and a thin fibrous cap with reduced collagen content. A major component of plaque destabilisation appears to be increased matrix degradation, the primary regulators of which are the MMPs and their inhibitors. There are a number

of potential therapeutic options aimed at preventing plaque disruption. In particular, MMP inhibition is an attractive target for such pharmacotherapy.

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5 • Current and Emerging Therapies in Atheroprotection

STEPHEN NICHOLLS, RISHI PURI

The Heart and Vascular Institute, Cleveland Clinic, Cleveland, Ohio

BACKGROUND

The global epidemic of cardiovascular disease (CVD) continues to rise, with atherosclerotic CVD remaining the lead cause of morbidity and mortality worldwide, comprising of 29% of all global deaths in 2003. A majority of these deaths were directly caused by coronary artery disease (CAD) or strokes.[1] It is estimated that up to 50% of all deaths and disability due to CAD and strokes could be curtailed by a number of lifestyle and therapeutic approaches that directly target major cardiovascular risk factors.

Atherosclerosis is a systemic disease process involving multiple vascular territories. The presence of established vascular disease, regardless of the territory involved, portends the greatest risk of incident cardiovascular events. The prevalence of asymptomatic coronary stenoses of greater than 50% angiographic severity in non-disabling ischaemic stroke patients has recently been estimated to be 20%,[2] and those patients afflicted with peripheral arterial disease have a probability of death due to CAD and stroke of 55% and 11% respectively.[3] Given the significant systemic plaque burden in these patients coupled with corresponding high event rates, various anti-atherosclerotic and vascular protective therapies have the potential to significantly lower absolute clinical event rates.

PATHOLOGY

Atherosclerosis is a chronic inflammatory condition, characterised by the accumulation of inflammatory cells, lipid and apoptotic material within the arterial wall. The endothelial cell layer, a single cell layer lining the lumen of the vasculature, serves to regulate permeability of the arterial wall, vascular tone and tendency for thrombus formation. Endothelial dysfunction therefore results in altered permeability of the vessel wall, increased vascular reactivity with vasoconstriction and the promotion of a number of prothrombotic substances. Such abnormalities are inherently promoted by the reduced bioavailability of nitric oxide, the principal product of the endothelium. Following transmigration across the endothelial layer, migrated monocytes transform into macrophages, which engulf extracellular lipid to become foam cells, which accumulate to form a fatty streak, considered the earliest macroscopic evidence of atherosclerosis.[4] Local smooth muscle cell migration results in collagen production, forming a fibrous cap, which separates the enlarging inflammatory and lipid milieu from the circulating blood stream. The ongoing accumulation of inflammatory cells, lipid and apoptotic material covered by a strong fibrous cap represents a mature atherosclerotic plaque.[4] Many

atherosclerotic plaques remain clinically quiescent, however some plaques may progress to an advanced stage, characterised by progressive lumen encroachment, aneurysm formation and plaque rupture. The latter two processes are mediated by the degeneration of the extracellular collagen matrix, which in turn is driven by activated matrix metalloproteinase enzymes.[5] Furthermore, plaque neovascularisation (with the adventitial proliferation of vasa-vasorum) leading to repeated intraplaque haemorrhage is now widely recognised as an important mediator of plaque progression and instability.[6]

The degradation of collagen within the fibrous cap leads to a reduction in the tensile strength, which appears more pronounced at the shoulders regions of the plaque. Higher shear stress at these shoulder region, further predispose the thinned fibrous cap to erosion or rupture. This exposes plaque components (including the highly thrombogenic tissue factor) to the circulating blood stream, culminating in the activation and aggregation of platelets and the coagulation cascade, leading to local thrombus formation. This thrombus may embolise downstream, or result in local occlusion of the arterial lumen to blood flow, with resulting acute ischaemia.[7]

Anti-atherosclerotic therapies are administered for either the primary prevention of cardiovascular events, or to prevent the recurrence of events in those patients with established vascular disease. This chapter will review the role of various strategies that have shown clinical evidence of their atheroprotective effects for the prevention of cardiovascular disease.

RISK FACTOR MODIFICATION

Population studies have established an association of the presence of a number of modifiable cardiovascular risk factors with increased risk for the development of CVD.[8,9] These risk factors include: (1) elevated plasma concentrations of various atherogenic lipoproteins, which include low-density lipoprotein cholesterol (LDL-C), lipoprotein (a) and triglycerides (TGL); (2) reduced plasma concentrations of high-density lipoprotein cholesterol (HDL-C); (3) hypertension; (4) diabetes; (5) smoking; and (6) obesity and the associated metabolic syndrome. Mechanistic studies, primary and secondary prevention clinical studies and atherosclerosis imaging studies, have demonstrated significant benefits following the reduction of pro-atherogenic lipoproteins, elevation of HDL-C and treating hypertension, whilst the aggressive glucose lowering for reducing cardiovascular events remains controversial.

TABLE 5.1: Modifiable atherogenic risk factors

- Elevated atherogenic lipoproteins:
  - *LDL-C*
  - *TGL levels*
  - *Lipoprotein (a)*
  - *Chylomicron remnant particles*
- Reduced HDL-C levels
- Hypertension
- Diabetes mellitus
- Obesity
- Smoking

Statins, LDL lowering and C-Reactive Protein

In population studies with extensive follow-up, plasma concentrations of LDL-C have been shown to be a strong and independent predictor of future cardiovascular disease.[8] Established therapies to lower LDL-C levels include hydroxymethylglutaryl Co-A reductase inhibitors (statins), bile acid sequestrants, ezetemibe, LDL-C apheresis

and ileal bypass surgery. Statins however remain the predominant means of successful LDL-C lowering with an abundance of clinical and mechanistic evidence of the favourable impact these agents have upon atherosclerosis regression, and primary and secondary prevention.

Hydroxymethylglutaryl Co-A reductase is the rate limiting enzyme in the pathway leading to the endogenous synthesis of cholesterol by the liver. Inhibition of this enzyme results in the up-regulation of hepatic LDL receptors, with the subsequent removal of LDL from the circulation. Statins are well tolerated, and result in substantial inhibition in plasma LDL-C levels, coupled with modest elevations in HDL-C levels. A minority of patients develop elevations in hepatic transaminases and myositis, while rhabdomyolysis is exceedingly rare.

Statins have resulted in relative risk reductions of 20-40% of both primary[10-12] and secondary cardiovascular events.[13-17] The West Of Scotland Coronary Prevention Study (WOSCOPS) was the first primary prevention study that highlighted a 31% and 33% reduction of fatal and non-fatal coronary events respectively in 6595 randomized middle aged males (whose total cholesterol was greater than 6.5 mmol/L) who took pravastatin for 5 years.[10] Following WOSCOPS, the AFCAPS/TexCAPS trial randomized 6605 patients with a mean total cholesterol level of 5.7 mmol/L to lovastatin or placebo. After 5 years follow-up, lovastatin reduced the combined end point of sudden death, myocardial infarction and unstable angina by 33%.[11] More recently, the JUPITER study randomized 17802 individuals without hyperlipidaemia (mean LDL-C less than 3.4 mmol/L) but with an elevated high-sensitive C-reactive protein (hsCRP) of greater than 2 mg/L to daily rosuvastatin 20 mg or placebo. The trial was halted prematurely after a median follow-up of 1.9 years due to the rosuvastatin group experiencing a 23% relative risk reduction in the combined primary endpoint of myocardial infarction, stroke, arterial revascularisation, hospitalisation for unstable angina or death from cardiovascular causes.[12] This trial therefore highlighted the primary benefit of LDL-C lowering in patients with systemic inflammation.

The Scandinavian Simvastatin Survival Study (4S) randomized 4444 patients with established coronary artery disease and fasting total cholesterol between 5.5 to 8 mmol/L to treatment with simvastatin or placebo. After 5.4 years, the primary end point of all-cause mortality was reduced by 30% with simvastatin, with similar reductions in myocardial infarction and stroke.[13] Similar magnitudes of reduction in mortality and myocardial infarction were seen in the Cholesterol and Recurrent Events (CARE) and Long-term Intervention with Pravastatin in Ischaemic Disease (LIPID) studies, which together evaluated the benefit of pravastatin versus placebo in over 13000 patients with prior myocardial infarction over a 5-year period.[14,16] Additionally, the Heart Protection Study (HPS) randomized 20536 patients with established atherosclerotic CVD (or high-risk equivalents on the basis of diabetes mellitus) and total cholesterol greater than 3.5 mmol/L to treatment with simvastatin or placebo.[15] Significant reductions in all-cause mortality (13%), cardiovascular mortality (17%), coronary events (27%) and stroke (25%) were found in the simvastatin group.

Regression of atherosclerosis within the coronary vasculature is a validated surrogate end-point of the clinical event reductions seen with statin therapies. Studies employing intravascular ultrasound (IVUS) have also highlighted the benefits of LDL-C lowering upon the coronary vessel wall. A consistent finding observed in the IVUS trials is the strong linear relationship between

mean LDL-C levels achieved on statin therapy and the median progression-regression rate of atherosclerosis. In the Reversal of Atherosclerosis with Aggressive Lipid Lowering (REVERSAL) study, halting of disease progression was achieved with aggressive LDL-C lowering to 2.0 mmol/L with atorvastatin (80 mg/day), as compared with plaque progression seen in those patients randomized to moderate LDL-C lowering with pravastatin 40 mg/d who achieved a mean LDL-C of 2.9 mmol/L.[18] The ASTEROID (A Study To Evaluate the effect of Rosuvastatin On Intravascular ultrasound –Derived coronary atheroma burden) study was the first large scale trial to show that plaque regression could be achieved by intensive LDL-C lowering to levels lower than achieved in REVERSAL. Rosuvastatin 40 mg/d lowered LDL-C levels to 1.6 mmol/L, and reductions in all volumetric measures of plaque burden was achieved.[19] More recently, a pooled analysis of more than 4000 patients who underwent serial IVUS coronary imaging in 6 trials has demonstrated a direct relationship between the baseline plaque burden, its progression and major adverse cardiovascular events.[20]

Although the clinical benefit seen in statin trials is able to be predicted from the degree of LDL-C lowering, statins are thought to exert vasculoprotective effects mediated by a variety of mechanisms other than direct LDL-C lowering. These suggested pleiotropic mechanisms include anti-thrombotic, anti-inflammatory, antioxidant, vasodilatory and anti-proliferative effects. Although such effects have been studied previously, only the anti-inflammatory CRP-lowering effects have been systematically evaluated in large scale clinical trials.

A major benefit of statin therapy is found in those patients with a high inflammatory state. Post hoc analyses of the CARE study found that those subjects with circulating biomarkers greater than the 90th percentile had the greatest risk of recurrent events, and the greatest benefit from pravastatin occurred in this group.[21] Further insights from REVERSAL revealed the superior CRP-lowering effects of atorvastatin over pravastatin. It is likely that this greater degree of anti-inflammatory effects exerted by atorvastatin contributed to a halting of atherosclerosis progression,[22] whereby the CRP reductions correlated directly with changes in atheroma volume seen with IVUS. The PROVE-IT (PRavastatin Or atorVastatin Evaluation and Infection Therapy) study complimented the imaging findings from REVERSAL using the identical regimen of statin therapy in a large cohort of patients following an acute coronary syndrome (ACS), with the atorvastatin group experiencing significantly reduced clinical event rates.[23] It is yet to be shown as to whether CRP exerts direct effects upon plaque biology, and appropriately designed clinical trials will need to be undertaken to study the clinical effects of new molecules that directly target CRP.

The pleiotropic effects of statins have been postulated to result in plaque stabilisation, which renders the plaque less likely to rupture to cause clinical events. Modulation of endothelial dysfunction and the anti-inflammatory properties of statins have been demonstrated within a few hours following statin administration. As a result, the effects of statins have been studied early in the setting of ACS or prior to elective percutaneous coronary intervention (PCI) in stable patients. Patients with ACS present a relatively high risk of recurrent adverse cardiovascular events. Hence, the early intensive use of statins may improve clinical outcomes and as such, statins have recently been included in the treatment guidelines for ACS.[24] The early benefit of statin therapy following ACS was demonstrated in The Myocardial Ischaemia Reduction with Aggressive Cholesterol Lowering (MIRACL) study.[25] This trial enrolled

3086 patients with ACS and randomized this group to atorvastatin 80 mg/d or placebo commenced within 24-96 hours following randomization. After 16 weeks, the atorvastatin group demonstrated a 14% relative risk reduction in the composite clinical endpoint of death, myocardial infarction, resuscitated cardiac arrest or admission for suspected ischaemia. Two recent meta-analyses of randomized controlled trials which evaluated for benefits of statins compared to placebo or usual care following ACS have highlighted that the real benefit of early statin therapy takes at least 4-6 months to become evident, with the significant benefits predominantly limited to reductions in unstable angina.[26]

A number of studies have also investigated the effects of high-dose statin loading upon the incidence of periprocedural myocardial infarction in the setting of PCI. A large series of patients undergoing elective PCI (n = 5052), found that patients treated with statins at the time of procedure had a significant mortality reduction at 30 days (0.8% vs 1.5% in statin naive patients; p = 0.048); with this benefit being maintained at 6 month follow-up (2.4% vs 3.6%; p = 0.046).[27] Furthermore, in a series of 1552 patients, those patients that had initiated statin therapy prior to the procedure (39.6% of the study group) had a significantly lower incidence of periprocedural myocardial infarction (5.7% vs 8.1% in statin naive; p = 0.038) with a mortality benefit seen at 1 year (3.4% vs 6.9%; p = 0.003).[28] Statin pretreatment was predictive of survival chiefly in patients within the highest CRP quartile. The Atorvastatin for Reduction of Myocardial Damage During Angioplasty (ARMYDA) trial was the first randomized prospective, placebo controlled, double-blind study that demonstrated a beneficial effect of statin pretreatment in preventing myocardial damage following PCI.[29] Patients with chronic stable angina (n = 153) were randomized to atorvastatin 40mg/d commencing 1 week pre-procedure versus placebo. Periprocedural myocardial infarction was detected in 5% of atorvastatin-treated patients compared to 18% in the placebo arm (p = 0.025). The ARMYDA ACS trial randomized 171 patients with ACS to receive placebo or atorvastatin loading (80 mg 12 hours before coronary angiography and a 40 mg dose 2 hours before intervention).[30] The primary composite endpoint of 30 day death, myocardial infarction and need for repeat target vessel revascularisation occurred in 5% of the treatment group compared to 17% of the placebo group (p = 0.04), with multivariate analysis indicating that atorvastatin pretreatment was associated with an 88% relative risk reduction of 30-day events. Furthermore, the ARMYDA RECAPTURE study showed that acute statin preloading prior to PCI in patients on chronic statin therapy had a protective effect with the combined endpoint of 30 day death, myocardial infarction and target vessel revascularisation occurring in 3.7% of patients in the re-load group vs 9.4% in the placebo group (p = 0.037); a 50% relative risk reduction on multivariate analysis.[31] Both the ARMYDA ACS and ARMYDA RECAPTURE trial 30 day combined endpoints were largely driven by a significant reduction in periprocedural myocardial infarction. Collectively, this data highlights that statin pretreatment is a low risk yet highly effective therapy of 'plaque' and 'vessel' stabilisation, mediated perhaps via a number of pleiotropic effects, in both stable and unstable patients undergoing coronary intervention, regardless of whether patients are statin naive or not.

The vasculoprotective effects of statins also extends to the perioperative period during noncardiac surgery. A retrospective case-control study of 2816 patients who underwent major non-cardiac vascular surgery was the first study to show a 4-fold

significant reduction in all-cause mortality during the perioperative period.[32] Soon after, the first prospective, placebo-controlled, blinded, randomized controlled trial evaluating the effects of 2 months of statin treatment upon perioperative cardiovascular complications after vascular surgery showed that the combined primary endpoint (cardiac death, nonfatal myocardial infarction, stroke or unstable angina) at 6-months was 3-fold higher with placebo than with atorvastatin 20 mg/d.[33] A number of retrospective studies have also evaluated the effects of statin therapy upon perioperative complications in patients undergoing noncardiac surgery. A large cohort (n = 780591) of patients undergoing noncardiac surgery found that in those 70159 statin users, there was a significant 1.4-fold reduced risk of in-hospital mortality.[34] The Statins for Risk Reduction in Surgery (STARRS) study assessed the effect of statins on cardiac complications in 1163 patients undergoing vascular surgery.[35] This study found a significantly lower perioperative complication rate in the statin group compared to statin non-users (adjusted OR 0.52, 95% CI 0.35-0.77). Furthermore, the long-term benefit of statins was observed in 510 patients undergoing successful abdominal aortic aneurysm surgery, with a significant reduction in all-cause mortality (18% vs 50%; p<0.001) seen in statin users compared to non-statin users over a median period of 4.7 years.[36] Although a risk of statin-induced myopathy exists in the surgical group of patients taking statins, this risk is considered relatively modest compared to the benefits of statins in reducing perioperative cardiac events. Also noteworthy is the evidence that suggests that statin therapy should remain uninterrupted (if possible) during the perioperative period. Due to the lack of availability of intravenous formulations, statin interruption, however, is common and usually unintended.

TABLE 5.2: LDL-C lowering summary points

- Statins are the most effective means of LDL-C lowering
- Statins inhibit the rate limiting enzyme in cholesterol synthesis
- Statins also achieve minor elevations of HDL-C, modest reductions of TGL levels
- 20%-30% reduction of events in primary prevention studies
- 30%-40% reduction of events in secondary prevention studies
- Clinical benefit proportion to degree of LDL-C lowering
- Regression of coronary atherosclerosis achievable with substantial LDL-C lowering
- Pleiotropic benefits of statins may contribute to benefit seen during ACS, peri-procedural administration during PCI, and peri-operatively for non-cardiac surgery

The complexity of HDL

Although the unequivocal benefit of LDL-C lowering has been demonstrated in a number of clinical trials, reductions in event rates by no more than 40% suggest that additional mechanisms are involved in mediating cardiovascular events, and that complementary strategies are required to achieve greater efficacy in cardiovascular risk reduction. HDL-C is emerging as an important therapeutic target for modulation of cardiovascular risk. Observational studies have suggested that the prevalence of low HDL-C levels (< 40 µg/dL, or 1.0 mmol/L) is greater in patients with established CAD, with numerous population studies also demonstrating an inverse relationship between HDL-C levels and prospective risk of CAD, regardless of the corresponding level of LDL-C.[37] HDL-C was found to be the strongest biochemical predictor of cardiovascular outcome in the Framingham study.[38] A pooled meta-analysis of four

population studies subsequently estimated that each 1 μg/dL rise in levels of HDL-C was associated with a 2% to 3% reduction in cardiovascular risk.[39]

High-density lipoprotein cholesterol possesses numerous anti-atherosclerotic properties, including the promotion of reverse cholesterol transport, protection of both LDL-C particles and endothelial cells from oxidative changes, inhibition of cytokine induced expression of endothelial cell surface adhesion molecules and the inhibition of the prothrombotic milieu associated with atheroma. Animal studies have provided support of these protective effects of HDL. Early studies showed that HDL infusions not only retard lesion formation but also promoted regression of established atherosclerotic plaque, with favourable effects also seen upon plaque composition.[40] These studies therefore provided robust pre-clinical evidence that raising HDL-C levels might be beneficial in humans.

There are a number of strategies that increase HDL-C levels. Non-pharmacological methods include weight loss, smoking cessation and mild alcohol consumption. Established pharmacological strategies include fibrates, statins and nicotinic acid, which raise HDL-C levels by 10-35%, 5-15% and 15-35% respectively. Fibrates have been shown to reduce clinical event rates in studies of primary and secondary prevention. The Helsinki Heart Study (HHS) demonstrated that an 11% increase in HDL-C independently predicted the 34% reduction in myocardial infarction and cardiovascular death with gemfibrozil. Each 1% increase in HDL-C was associated with a 3% reduction in cardiovascular risk.[41] A similar benefit with gemfibrozil was observed in patients with established CAD and low HDL-C levels in the VA-HIT (Veterans Affairs High-Density Lipoprotein Intervention Trial), whereby the 6% rise in HDL-C predicted the 22% reduction in myocardial infarction or cardiovascular death.[42]

Apart from LDL-C lowering, statins modestly raise HDL-C levels. In a pooled analysis of four clinical studies, raising HDL-C was found to be an independent predictor of the benefits of statins in slowing progression of coronary atherosclerosis, with incremental benefit regression observed within those patients whose HDL-C rose by 7.5%, despite intensive LDL-C lowering.[43]

Niacin is the most effective current method of raising HDL-C levels. Several studies have demonstrated that the use of niacin in combination with other lipid-modifying agents, had a substantial effect upon relative risk reduction and disease progression. The HATS (HDL in Atherosclerosis Study) showed that simvastatin and niacin raised HDL-C by 24%, lowered LDL-C by 42% and promoted regression of angiographically detectable CAD. Moreover, there was a profound 60% relative risk reduction in major adverse cardiac events.[44] The benefit of adding extended release niacin to statin therapy was also seen in the ARBITER-2 (Arterial Biology for the Investigation of the Treatment Effects of Reducing Cholesterol 2) study, whereby an increase in HDL-C levels of 0.21mmol/L slowed progression of carotid intimal-media thickness (CIMT) in patients with HDL-C levels below 1.2 mmol/L.[45] More recently, the addition of niacin (for HDL-C augmentation) was found to be superior to the addition of ezetemibe (for further LDL-C lowering) in a group of patients already on chronic statin therapy for CAD, whereby the niacin/statin group experienced significant regression of CIMT compared to the ezetemibe/statin group.[46] Intolerance due to flushing, which is mediated by activation of epidermal prostanoid receptors, continues to limit the widespread clinical use of effective doses of niacin. However, pharmacological strategies to inhibit these

epidermal prostanoid receptors in combination with the delivery of niacin therapy may hold promise for future trials employing niacin as an effective and well-tolerated anti-atherosclerotic therapy. Enthusiasm also exists for significantly raising HDL-C levels via the inhibition of cholesteryl ester transfer protein (CETP). In humans, CETP inhibition is capable of raising HDL-C levels by greater than 50% and reducing LDL-C levels by 20%. However the ILLUMINATE (Investigation of Lipid Level Management to Understand Its Impact in Atherosclerotic Events) trial was prematurely halted due to a higher clinical event rate seen in the patients administered Torcetrapib (CETP inhibitor).[47] These unexpected effects have been postulated to arise from activation of the renin-angiotensin system as well as a direct, unfavourable molecule-specific vasculotoxic effect of Torcetrapib itself.[48] The recent report of the safety of Anacetrapib, a next generation CETP inhibitor, has raised hopes that significant elevations in HDL-C levels via CETP inhibition will regress atherosclerosis and eventually result in reduced clinical event rates.[49]

HDL comprise a heterogenous group of particles, which vary in size, shape and content of both lipid and protein. Normal or elevated levels of HDL are not always atheroprotective in humans. The finding that greater than 40% of the clinical events observed in the Framingham cohort occurred in subjects with serum HDL-C levels greater that 1.01 mmol/L suggests that their HDL failed to possess the appropriate levels of protective properties.[50] The observation that HDL, isolated from subjects with CAD despite high HDL-C serum levels, promotes rather than inhibits monocyte chemotaxis in response to oxidised LDL-C, provided mechanistic evidence that HDL may be proatherogenic in some individuals.[51] It remains to be established whether the size and composition of HDL particles bears influence upon their functional properties. Contrasting reports from population studies have demonstrated that HDL particle size may influence prospective clinical risk. Furthermore, a potential relationship between HDL subclasses and cardiovascular risk has been reported to contribute to the relative benefit of various lipid-modifying therapies. In an exploratory analysis of VA-HIT, the generation of small HDL particles with gemfibrozil was demonstrated to independently predict protection from cardiovascular events.[52] This may explain why a relatively small rise in HDL-C levels was associated with clinical benefit. The observation of benefit by raising levels of lipid-deplete HDL particles is consistent with their ability to remove excess cellular cholesterol, prevent LDL-C oxidation, rapidly improve endothelial function, and promote plaque regression in humans.[53]

HDL therefore remains an attractive target for modification by therapies aimed to promote cardiovascular risk reduction. However, the complexity of HDL presents a major challenge in determining the optimal therapeutic approach. Although a number of groups have proposed specific HDL-C targets for treatment, there is no current evidence that treating to a particular target results in clinical benefit. At the minimum, it would seem that attempts to raise HDL-C levels above levels considered to be associated with an increase in cardiovascular risk (1.01 mmol/L in men and 1.28 mmol/L in women) would be prudent. However the emergence of the functional quality of HDL, rather than absolute HDL levels, is currently presenting a major challenge for future drug development programs. It is likely that raising the right type of HDL-C may have the greatest impact upon cardiovascular disease prevention.

TABLE 5.3: HDL-C cholesterol summary points

- Strongest independent predictor of events
- Numerous atheroprotective properties:
  - *Promotes cholesterol efflux*
  - *Anti-inflammatory actions*
  - *Reduces oxidation of LDL-C*
  - *Inhibits thrombogenecity*
- Elevated by weight loss, smoking cessation and mild alcohol consumption
- Therapeutically elevated by fibrates and nicotinic acid
- Novel therapeutic agents that elevate HDL-C levels and promote HDL-C functionality are currently being trialled in humans

The controversy of triglycerides

Despite extensive research, in the setting of clinical trials and epidemiologic studies, the exact role of serum TGL levels as an independent risk factor for CAD remains uncertain. Observational studies have produced data in support of the independent predictive value of fasting TGL levels for incident CAD. Graziano et al. investigated the interrelationships between fasting TGL and other lipid parameters, along with non-lipid risk factors, against the risk of myocardial infarction in 340 cases and 340 age-, gender- and community-matched controls. The relative risk of myocardial infarction was found to have a strong association with elevated fasting TGL levels that remained unaltered after controlling for other non-lipid risk factors; however despite remaining statistically significant, the strength of this association was attenuated somewhat after controlling for HDL-C.[54] Following this study, Jeppesen et al. examined the relationship between fasting TGL levels and ischaemic heart disease (IHD) risk in over 2,900 males in the Copenhagen Male Study, with 8-years of follow-up. Following adjustment of both lipid and non-lipid risk factors, men in the middle and highest tertile of TGL levels had relative risks of IHD of 1.5 (95% CI, 1.0-2.3; $p = 0.05$) and 2.2 (95% CI, 1.4-3.4; $p < 0.001$) respectively, when compared with men in the lowest TGL tertile.[55] When TGL levels were stratified by HDL-C levels, the risk of IHD increased with increasing TGL within each HDL-C level. Similarly, the larger Prospective Cardiovascular Munster study involving 4,849 middle-aged men who were followed for 8-years found TGL levels to be an independent risk factor for CAD irrespective of HDL- or LDL-C levels.[56]

Therefore there is no clinical trial that has been designed to specifically address the issue of TGL lowering upon incident cardiovascular events. Although patients enrolled in the HHS experienced a 35% reduction in TGL levels, it was the 11% increase in HDL-C levels that predicted the 34% relative risk reduction in the rate of myocardial infarction.[41] Limited data therefore exists from clinical trials to suggest that the specific lowering of TGL levels, independent of concomitant HDL-C raising/LDL-C lowering, will result in reduced clinical event rates within patients with normal or elevated LDL-C levels. Although current guidelines do not mandate screening for elevated TGL levels within the general population, TGL levels in patients afflicted with CAD (or CAD risk equivalent) may provide valuable prognostic information for therapeutic decision-making. At this point the presence of mild to moderate hypertriglyceridemia identifies a patient who requires more intensive management of LDL-C, with treatment guidelines advocating initiation or intensification of statin therapy as first line treatment.

Hypertension

Epidemiologic studies have established a direct relationship between both systolic and

diastolic blood pressures and cardiovascular events. The strongest relationship lies between hypertension and cerebrovascular disease. Framingham data predict the lifetime risk of developing hypertension to be about 90% by the age of 65 years.[57] The Multiple Risk Factors Intervention Trial (MRFIT) demonstrated a continuous and graded relationship between both systolic and diastolic blood pressures and death due to both CAD and stroke,[58] with the strength of this relationship greatest for systolic blood pressure. In addition, isolated systolic hypertension, the commonest form of hypertension, portends a 2.5-fold greater risk of cardiovascular disease compared with matched, normotensive patients.

Hypertension alters shear forces within the vascular lumen. Steady laminar flow stimulates endothelial cellular functions which maintain vascular tone, thrombogenecity and regulated permeability. The mechanotransduction of these altered shear-stress signals arising from the endothelial surface result in a number of changes contributing towards atherogenesis, including altered gene expression, as well as changes to vascular regulatory substances including nitric oxide, endothelin and angiotensin II.

The use of anti-hypertensive therapy for primary prevention reduces cardiovascular events. A meta-analysis of randomised controlled trials involving the reduction of systemic hypertension in primary prevention revealed a 21% risk reduction in events attributed to CAD and a 37% reduction in the incidence of stroke.[59] The Hypertension Optimization Trial (HOT) randomised patients with hypertension to therapy aimed at three groups of blood pressure targets. Analysis revealed the lowest incidence of cardiovascular events in those with a mean diastolic blood pressure of 82.6 mmHg, supporting aggressive lowering of blood pressure into the 'normal' range.[60]

The Heart Outcome Prevention Evaluation (HOPE) study supports the use of angiotensin converting enzyme (ACE) inhibition in all high risk patients. Despite the minimal blood pressure reduction seen in the ramipril arm, the use of an ACE inhibitor reduced the composite vascular end point by 22%, supporting the critical role played by the renin-angiotensin system in the promotion of atherogenesis.[61] The second Australian National Blood Pressure Study also highlighted the benefit of an ACE inhibitor-based anti-hypertensive regimen significantly lowering cardiac death in the elderly population as compared to a diuretic-based regimen.[62] The Antihypertensive and Lipid-Lowering Treatment to prevent Heart Attack Trial (ALLHAT) reported no overall differences in CAD outcome among patients treated with a diuretic-based compared to a calcium channel blocker- or an ACE inhibitor-based treatment program.[63] However, patients in the diuretic group experienced fewer episodes of heart failure than in the calcium channel blocker group. Although The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC 7) (National Heart, Lung, and Blood Institute. National Blood Pressure Education

TABLE 5.4: Hypertension summary points

- Strong independent risk factor
- Small reductions in blood pressure result in marked reduction in clinical events
- Reduction of blood pressure to the normal range results in the least number of clinical events; further reductions may be deleterious
- Blood pressure lowering per se is of utmost importance, irrespective of the choice of anti-hypertensive agent
- ACE inhibition reduces cardiovascular events in all high-risk patient groups, regardless of baseline blood pressure

Program, 2003) supports thiazide diuretics as first-step drug of choice in most hypertensive patients, what is clear from the wealth of trial data is that blood pressure lowering per se is of greater importance in reducing cardiovascular event rates rather than the choice of agent(s) used.

RISK FACTOR MODIFICATION IN THE DIABETIC PATIENT

Glycaemic Control

The incidence of diabetes continues to increase worldwide, predominantly due to the significant rise in the prevalence of type 2 diabetes mellitus, which now accounts for over 90% of all diagnosed cases. The major cause of morbidity and mortality in subjects with diabetes is attributed to atherosclerosis, particularly CAD. In subjects with type 2 diabetes, it is well established that there is a two- to four-fold increased risk of CAD, peripheral arterial disease and cerebrovascular disease than compared with matched non-diabetic controls. This persists despite accounting for other traditional cardiovascular risk factors such as hypertension, smoking and dyslipidaemia. Accordingly, treatment guidelines now consider the presence of diabetes to be an atherosclerosis equivalent, and suggest that diabetics should be treated in a similar fashion as those with established clinical cardiovascular disease in terms of risk factor control.[64]

A number of direct and indirect pathways contribute towards atherogenesis in diabetics. Indirect pathways that are promoted by hyperglycaemia include worsening of dyslipidaemia (development of atherogenic dyslipidaemia; small dense LDL-C particles, reduced HDL-C levels and elevated TGL levels), sympathetic nervous system dysfunction and the development of renal dysfunction. Direct pathways (directly pertaining to chronic hyperglycaemia) result in the overall reduction in the bioavailability of nitric oxide. The resultant endothelial dysfunction promotes vasoconstriction, pro-inflammatory and prothrombotic tendencies that contribute towards the progression of atheroma and subsequent atherothrombosis. Cholesterol and blood pressure lowering trials have demonstrated benefit in diabetic patients. Given the implicit relationship between hyperglycaemia and virtually all stages of atherogenesis, one would expect that the strict control of blood sugar levels would cause corresponding reductions in atherosclerotic cardiovascular disease. However clinical trials evaluating the impact of chronic, intensive glycaemic control upon cardiovascular disease have produced conflicting results, reflecting a complex interaction between glycaemic control and the atherosclerotic disease process.

Observational studies have tended to show a linear relationship between hyperglycaemia and cardiovascular mortality.However for levels of glucose at or below the threshold for the diagnosis of diabetes, the relationship with cardiovascular mortality is less clear-cut.[65,66] Recently, the results of large-scale intervention trials examining the relationship between intensive glucose control and cardiovascular outcomes have been published. The ACCORD (Action to Control Cardiovascular Risk in Diabetes) trial examined the effect of intensive glucose control (target HbA_{1c} under 6.0%, but achieving a mean level of 6.4%) versus standard therapy (targeting HbA_{1c} between 7.0-7.9%, achieving a mean level of 7.5%) in over 10,000 established type 2 diabetics.[67] An unexpected finding of a higher rate of cardiovascular mortality (34%, $p = 0.02$) and all-cause mortality (22%, $p = 0.04$) within the intensive glycaemic arm resulted in a premature halting of this study after a mean follow-up period of 3.5 years. Despite these

mortality rates, there was a non-significant trend towards a reduction in the primary outcome of the composite endpoint of non-fatal myocardial infarction, non-fatal stroke or cardiovascular death. Although the exact mechanisms of the resultant increase in mortality in this trial are unknown, an association between higher rates of severe hypoglycaemia within the intensive glycaemic control arm was reported, with patients with the highest baseline HbA_{1c} levels at greatest risk of hypoglycaemia compared to those with superior initial glycaemic control. In contradiction to ACCORD, the ADVANCE (Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation) trial randomised over 11,100 type 2 diabetics to either intensive glucose control (achieving a mean HbA_{1c} of 6.5%) or standard control (achieving a mean HbA_{1c} of 7.3%).[68] After a mean follow-up period of 5 years, the composite primary endpoint of combined macrovascular and microvascular events was significantly attenuated in the intensive glucose control group. However this result was primarily driven by the significant reduction in microvascular endpoints, whereas there was no significant reduction in cardiovascular events.

In the United Kingdom Prospective Diabetes Study (UKPDS), 3,867 newly diagnosed type 2 diabetics were randomised to an intensive glycaemic control compared with conventional treatment policy.[69] Over the ensuing 10-year period of the trial, the intensively treated patients achieved a mean HbA_{1c} of 7.0% compared to a mean HbA_{1c} level of 7.9% in the conventionally treated group. The intensively treated patients experienced a 16% relative risk reduction of myocardial infarction which just failed to achieve statistical significance ($p = 0.052$) compared to the conventionally treated group. However the intensively treated group did experience a significant 25% reduction in microvascular complications ($p = 0.01$). A sub-group of overweight individuals within the UKPDS study who were placed on metformin as part of their intensive glucose control regimen did experience a significant 39% reduction in myocardial infarction ($p = 0.01$) and a 36% reduction in all-cause mortality ($p = 0.01$). This data has been adopted to promote the use of metformin as a means of effectively reducing cardiovascular endpoints independently of glycaemic control. Following the publication of the UKPDS results, all surviving patients of this study entered into a post-trial monitoring program until 2007.[70] During this follow-up period, no attempts were made to continue the mode and intensity of glycaemic control according to their prior randomisation group. In the first year after entering the post-trial monitoring program, any differences in the HbA_{1c} between the previous 2 treatment groups was lost. Despite the rapid loss of glycaemic separation between the two groups in the post-trial follow-up program, a significant benefit of being previously randomised to an intensive glucose control regimen was still observed, whereby this group experienced a 15% lower rate of myocardial infarction ($p = 0.01$) as well as a 13% reduction in all-cause mortality ($p = 0.007$). Moreover, the prior reductions in microvascular endpoints as well as the benefits of metformin therapy were all maintained during this post-interventional follow-up study. These observations virtually mirror the results of the previously reported Diabetes Control and Complications Trial (DCCT); a 9-year trial highlighting the benefit intensive glycaemic control significantly delaying the development and progression of diabetes-related microvascular complications in type 1 diabetics, however failing to show any significant benefit upon cardiovascular events.[71] Following completion of the DCCT, subjects were then followed by the Epidemiology of Diabetes Interventions

and Complications (EDIC) study.[72] Again, differences in HbA_{1c} were quickly lost between the 2 treatment groups. The completion of the 11-year post trial observation period occurred in 2005, and despite the early loss in glycaemic separation between the prior 2 groups, the combined endpoint of cardiovascular events (non-fatal myocardial infarction, stroke or cardiovascular death) were reduced by 57% ($p = 0.02$) in the group of patients previously assigned to intensive glucose control.

The UKPDS follow-up study and the EDIC study suggest that the sustained, early intensive control of blood glucose levels eventually translates into a significant benefit upon the development of cardiovascular disease many years down the track. Mechanisms pertaining to this 'legacy' effect are currently unknown. At least 4 recently published meta-analyses (that have included the ACCORD and ADVANCE studies) have suggested that intensive versus standard glucose lowering reduces cardiovascular events without increasing cardiovascular or all-cause mortality. [73-75] Therefore, an HbA_{1c} target of < 7.0% still remains an acceptable target for appropriate glucose control. However under certain circumstances, especially in the setting of newly diagnosed type 2 diabetes and in young patients without significant comorbidities, aiming for a lower HbA_{1c} level may represent an appropriate strategy to lower both microvascular and macrovascular complications.

Global risk factor reduction in diabetics

The greatest benefit for diabetic patients, in terms of cardiovascular risk factor reduction, has resulted from the aggressive modification of plasma lipoproteins and blood pressure. Diabetic dyslipidaemia, characterised by elevated TGL levels, reduced HDL-C levels and the presence of small, dense LDL-C particles, represents a potent atherogenic stimulus. Weight loss and aggressive glycaemic control leads to an improvement of this lipid profile.[64] Statin trials have demonstrated greater relative risk reductions in diabetic subgroups, as have trials employing gemfibrozil.[13,42] Although the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study did not show daily fenofibrate to significantly reduce the risk of the primary outcome of coronary events (death due to CAD or non-fatal myocardial infarction) in over 4800 type 2 diabetics, there was a primary reduction in the rate of coronary and non-coronary revascularisations as well as fewer non-fatal myocardial infarctions.[76] Moreover, fenofibrate therapy resulted in the improvement in a number of microvascular parameters. Furthermore, the ACCORD study group investigated whether combination therapy with statin plus fenofibrate, as compared with statin monotherapy, would further reduce the risk of cardiovascular disease in over 5,500 type 2 diabetics at high risk for cardiovascular events. The statin/fibrate combination did not reduce the rate of fatal cardiovascular events, non-fatal myocardial infarction, or non-fatal stroke as compared with statin therapy alone.[77] A recent meta-analysis (of 18 trials, over 45,000 patients) investigating the effects of fibrates on major clinical outcomes concluded that fibrates do reduce the risk of major cardiovascular events, largely driven by a 13% relative risk reduction (7-19) in coronary events ($p<0.0001$).[78] Collectively, these trials suggest that although statin therapy should remain the cornerstone of treating dyslipidaemia in diabetics, diabetics with reduced HDL-C and elevated TGL levels may derive some additional benefit with the addition of fibrate therapy.

Similarly, lowering blood pressure is of utmost importance in diabetics for enhanced

cardiovascular risk reduction, portending a greater risk benefit in diabetics compared with non-diabetics. The blood pressure lowering sub-study of UKPDS confirmed a significant reduction in strokes (as well as microvascular endpoints) in those with aggressive blood pressure control (mean on-treatment blood pressure of 144/82 mmHg) compared with the group assigned less tight control (who achieved a mean blood pressure of 154/87 mmHg).[79] A similar observation was noted within the HOPE (Heart Outcomes Prevention Evaluation Study) study, with the benefit of ramipril seen in diabetic patients compared to those with established vascular disease.[61] However, more recent attempts to define the optimum level of blood pressure control in diabetics has highlighted the complex relationship between blood pressure levels in diabetics and cardiovascular event rates. Until recently, it was widely felt that aggressive lowering of systolic blood pressure below 135 mmHg in diabetics would be of further clinical benefit. However the results of the ACCORD study group collaborators found that in type 2 diabetics at high risk for cardiovascular events, intensive systolic blood pressure lowering to levels below 120 mmHg, as compared to less than 140 mmHg, did not reduce the rate of composite outcome of fatal and nonfatal major cardiovascular events.[80] Moreover, the patients assigned to intensive blood pressure lowering experienced significantly higher adverse event rates attributed to the antihypertensive treatment. This trial reinforced the notion that aggressive blood pressure lowering in diabetics to values below 130-135 mmHg may be problematic.

TABLE 5.5: Diabetes summary points

- Diabetes and insulin resistance are both strong risk factors for cardiovascular events
- Glycaemic control correlates with clinical outcome
- Aggressive glycaemic control reduces microvascular complications
- There is a more complex relationship between aggressive glycaemic control and reduction of macrovascular events
- Early, aggressive glycaemic control may have a longer term 'legacy' effect upon reduction in macrovascular events
- Metformin use in obese diabetics significantly lowers rate of myocardial infarction
- Statins remain the cornerstone of lipid management in diabetics
- Control of blood pressure equally important in diabetics; however aggressive lowering of systolic blood pressure to levels < 120 mmHg may be detrimental

The metabolic syndrome

The metabolic syndrome refers to the co-existence of an atherogenic lipid profile, elevated blood pressure, and elevated plasma glucose, associated with abdominal obesity and insulin resistance.[64] Although a number of definitions exist, the most recent consensus from the International Diabetes Federation defines metabolic syndrome as the presence of central obesity (defined as waist circumference with ethnic specific values, or if body mass index exceeds 30 kg/m^2, then central obesity can be assumed) and any two of the following: (1) raised TGL levels (> 1.7 mmol/L), (2) reduced HDL-C levels (< 1.03 mmol/L in males and 1.29 mmol/L in females), (3) raised blood pressure (systolic blood pressure > 130 mmHg or diastolic blood pressure > 85 mmHg), (4) raised fasting plasma glucose level (> 5.6 mmol/L).[81]

Numerous studies have investigated the cardiovascular risk associated with metabolic syndrome, with ongoing debate regarding the prognostic significance of the metabolic syndrome for cardiovascular outcomes. A recent meta-analysis was conducted of

the cardiovascular risk associated with the metabolic syndrome (as defined by the National Cholesterol Education Program), which found the presence of metabolic syndrome to be associated with a 2-fold increase in cardiovascular outcomes and a 1.5-fold increase in all-cause mortality.[82] It remains unclear, however, as to the relative contribution of the individual components of the metabolic syndrome towards the mediation of cardiovascular risk. A recent study analysing the risk of myocardial infarction conferred by the metabolic syndrome and its individual components in multiple ethnic populations was recently undertaken. It was found that the risk of metabolic syndrome upon myocardial infarction is generally comparable to that conferred by some, but not all, of its component risk factors, whereby the association with myocardial infarction being similar to that of diabetes mellitus and hypertension.[83] Accordingly, a recent analysis of over 3450 patients who participated in 7 clinical trials that monitored coronary atheroma progression with IVUS found accelerated disease progression in those patients with the metabolic syndrome. However, after adjusting for its individual components, the presence of the metabolic syndrome itself was no longer found to be an independent predictor of disease progression.[84] Infact, further analysis of the same patient cohort found that patients with diabetes mellitus had greatest coronary plaque burden, greater plaque progression and lumen constriction than those categorised as having metabolic syndrome.[85] Further studies will need to be conducted to ascertain the candidate mechanisms by which metabolic syndrome mediates cardiovascular risk. However given the strong relationship between the presence of its component risk factors, the ideal anti-atherosclerotic approach tackling the metabolic syndrome would appear multi-factorial, in addition to weight loss and life-style measures.

FUTURE TARGETS

The current generation therapeutic approach for atherosclerotic cardiovascular disease prevention is essentially aimed directly at the modulation of known cardiovascular risk factors. Although these approaches have been successful in risk reduction, a substantial residual risk remains. Given our understanding of the implicit role of oxidative and inflammatory pathways in atherogenesis, therapies directly targeting these pathologic substrates represents a potential future challenge in achieving further risk reduction in the population. As a result, various novel therapeutic agents are undergoing clinical development. These include a number of anti-oxidant and anti-inflammatory therapies. It is likely that these therapeutic approaches will find their way into clinical practice.

CONCLUSION

Atherogenesis represents the culmination of a complex series of humoral events that promote the inflammatory cascade and thrombogenicity. The optimal therapeutic approach for both primary and secondary prevention of atherosclerosis still involves the traditional approach of global risk factor modification. However emerging therapies targeting novel markers of disease will be needed to tackle the residual burden of clinical events attributable to atherosclerosis despite contemporary anti-atherosclerotic therapies. Furthermore, emerging imaging and serum markers of atherosclerosis and its inflammatory content will enhance our risk prediction capabilities for cardiovascular events, as well as the ability to monitor response to anti-atherosclerotic therapies.

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6 • Molecular Approaches to Revascularization in Peripheral Vascular Disease

GREG S. MCMAHON, MARK J. MCCARTHY

Department of Surgery and Cardiovascular Sciences, University of Leicester, Leicester, UK

INTRODUCTION

Currently, treatment options for peripheral vascular disease include angioplasty and reconstructive surgery. An attractive, less invasive alternative could involve the revascularization of ischaemic tissue by the induction of vascular growth. It would be particularly welcome for patients in whom current approaches are difficult or prone to failure, including those with conditions that make surgical intervention unsafe, patients with diffuse occlusive disease and those in whom there is significant downstream microvascular disease. Recent years have witnessed major advances in the understanding of the molecular mechanisms underlying vascular formation and remodelling, as well as the identification of key molecules controlling these processes. Most research has focused on the induction of new vessel formation by stimulating angiogenesis and this has been the goal of the clinical trials directed at peripheral vascular disease. But, whilst the stimulation of angiogenesis may relieve microvascular disease, the bypass of occluded conduit vessels requires the formation of more substantial collateral vessels by the process of arteriogenesis. This chapter will review current understanding of the mechanisms controlling angiogenesis and arteriogenesis; approaches that are, and could be pursued to induce vessel growth in peripheral vascular disease, as well as summarizing the current status of clinical trials.

MECHANISMS OF VASCULAR GROWTH

Strategies currently being developed for the therapeutic induction of vessel growth have evolved, largely, from knowledge of the physiological mechanisms of developmental vascularisation. In development, blood vessels arise initially by the process of vasculogenesis during which precursor cells, known as angioblasts, differentiate into endothelial cells and organize into primitive vessels.[1] These vessels expand by angiogenesis, which includes both sprouting growth and non-sprouting remodelling.[1]

Vasculogenesis

The angioblasts that give rise to endothelial cells in the first stages of developmental vascularisation originate in the mesoderm under the influence of fibroblast growth factor-2 (FGF-2).[2] These differentiate into endothelial cells, which proliferate and aggregate into cords, and become lumenized

to form primitive vascular plexuses.[1] Signalling through vascular endothelial growth factor receptor-2 (VEGF-R2) is crucial for angioblast survival and establishment of these first vessels.[3]

Endothelial cell formation from precursor cells, and *in situ* differentiation into vessels, was thought to be confined to developmental vascularisation. However, it has now been shown that circulating endothelial progenitor cells (EPCs) exist in the adult and that they can contribute to vessel formation.[4] These cells originate in the bone marrow and express CD34 and VEGF-R2 as well as the orphan receptor AC133.[5] EPCs can incorporate into neovessels formed in healing wounds, ischaemic tissue and tumours.[6] The mechanisms controlling the incorporation of EPCs into neovessels are at present poorly defined although a number of growth factors, including VEGF, FGF2, granulocyte-macrophage colony stimulating factor (GMCSF), angiopoietins and cytokines have all been shown to increase the mobilization of EPCs from bone marrow.[7] Crude cell fractions that include EPCs can be expanded *ex vivo* and transplanted into ischaemic tissue in animal models where they have been reported to incorporate into neovessels.[4] Importantly, in some situations neovessels comprising only EPC-derived cells were observed. Several studies have highlighted that the increase in the number of circulating progenitors, induced by cell transfusion or enhanced mobilization, can also enhance restoration and integrity of the endothelial lining, suppress neointimal formation, and increase blood flow to ischaemic sites.[8] Whilst it is possible that EPCs, or EPC-derived cells, can form vessels *in situ*, by a process similar to vasculogenesis, current thinking suggests that the principal role of vascular stem and progenitor cells is paracrine, that is, these cells promote proliferation and migration of existing endothelial cells, as well as produce additional cytokines and chemokines to continue stem and progenitor mobilization, trafficking and adhesion.[9]

Angiogenesis

Establishment of the vascular network in development, as well as new vessel formation in the adult, requires angiogenesis. The two processes of sprouting and non-sprouting angiogenesis are responsible for remodelling of the primitive vascular plexus into a complex functional network. In sprouting angiogenesis, endothelial cells are activated by growth factors to undergo migration, proliferation and morphogenesis into new vessels, and VEGF is the major physiological activator.

Ischaemia is the primary initiator of sprouting angiogenic growth. Low oxygen tension activates expression of a wide range of angiogenic factors including VEGF, VEGF receptors, angiopoietin-2 and platelet-derived growth factor (PDGF). Genes for these factors contain hypoxia responsive elements in their promoters and some, like VEGF, have been shown to be direct targets of the transcriptional regulator hypoxia inducible factor (HIF). HIF-1 is a heterodimer composed of HIFα and HIFβ subunits.[10] Under normoxic conditions HIF-1α is held at low intracellular concentrations by proteosomal degradation. With decreased oxygen tension HIF-1α becomes hydroxylated preventing its association with the von Hippel-Lindau ubiquitin ligase complex that is responsible for directing HIF-1α for degradation.[11] Thus, HIF-1α accumulates in the cell allowing it to activate transcription of hypoxia-inducible genes via the HIFα:β dimer.

VEGF expressed in response to HIF is secreted by ischaemic cells and acts on endothelial cells in adjacent microvessels. In these previously quiescent microvessels, endothelial activation, proliferation and migration are

normally suppressed by signals from abluminal perivascular support cells; pericytes. This suppression needs to be relieved before angiogenesis can proceed.[12] The close interaction between endothelial cells and pericytes is promoted by the ligand angiopoietin-1, which is produced by the pericyte and acts on the endothelial receptor Tie2.[13] Disruption of this signalling interaction is likely to involve angiopoietin-2, another hypoxia-responsive molecule.[14] Angiopoietin-2 can act to inhibit angiopoietin-1-induced activation of Tie2.[15] Once released from the pro-stabilizing effects of pericytes, the endothelial cells are free to invade the perivascular space, aided by proteases that degrade extracellular matrix. Many metalloproteinases have been implicated in angiogenic sprouting, including matrix metalloproteinases 2, 3, 7 and 9 as well as other proteolytic enzymes such as urokinase-type plasminogen activator.[16] Migration of the activated endothelial cells is aided by plasma proteins that extravasate from the activated microvessels in response to the vasodilatory and pro-permeability effects of VEGF. This growth factor is a potent chemoattractant and mitogen for endothelial cells, and directs their migration and proliferation. Interestingly, *in vivo* endothelial cells in the developing vascular sprouts respond differentially to VEGF, with the cells at the tip migrating and those behind the tip proliferating.[17] Migration and proliferation give rise to endothelial cords that become lumenized, a process which is poorly understood, though is known to be enhanced by angiopoietin-1.[18]

Neovessel maturation

Whether by vasculogenesis or angiogenesis, newly formed blood vessels are highly unstable, and are prone to haemorrhage, thrombosis and spontaneous regression in the absence of elevated growth factors.[19] Such vessels are characteristically found in pathological vascularisation and their phenotype can directly contribute to the disease process. Newly formed primitive vascular channels are maintained by local high concentrations of VEGF, withdrawal of which leads to endothelial apoptosis and neovessel regression.[20] Transformation to a functional vessel requires interaction of endothelial cells in the nascent vessel with pericytes, which originate as mesenchymal cells that are recruited to the developing vessel and differentiate into pericytes on contact with the endothelium.[21] Proliferation and migration of partially or fully differentiated pericytes along established microvessels also contributes to mural cell acquisition by sprouting neovessels, and sprouts can themselves recruit mesenchymal cells.[22] Migration and proliferation of pericytes is regulated mainly by platelet-derived growth factor-β (PDGFβ) secreted by endothelial cells. Interaction between mesenchymal cells and endothelial cells in a developing vessel produces phenotypic changes in both cell types. The mesenchymal cell is directed toward a pericyte or smooth muscle phenotype and the endothelial cell adopts the phenotype required for formation of a stabilized microvessel.[1] Pericytes supply anti-apoptotic ligands, including angiopoietin-1,[23] to underlying endothelial cells allowing neovessels to survive the decrease in VEGF concentrations that occur as the ischaemia is relieved by increased perfusion. In addition, pericytes suppress endothelial proliferation and migration, and increase deposition of the perivascular basement membrane, all of which contribute to switching the fragile nascent vessel into a quiescent functional microvessel.[24] Transforming growth factor-β, produced by proteolytic cleavage from a precursor form as a result of endothelial:pericyte interaction,[25] has a central role in these effects.

Microvascular network maturation

Maturation of vascular channels into functional vessels is accompanied by maturation of the neovessel network. This involves optimization of the new vessel configuration, density, branching pattern and vessel hierarchy. Spatial distributions of angiogenic initiators, like VEGF, have a major influence on the direction of the initial branches. There are six members of the VEGF family and VEGF-A is expressed as a number of alternatively spliced variants; in humans these are mainly forms with 121, 165, 189 and 206 amino acids.[26] VEGF165, 189 and 206 possess heparin-binding domains that allow these forms to interact with the extracellular matrix. The ability of VEGF isoforms to be retained by the matrix is important in regulating the spatial organization of vessel branching.[27]

Patterning also occurs through selective loss of certain vessels by regression. Another major determinant of network maturation is the branching and 'splitting' of vessels by non-sprouting angiogenesis. This occurs by the process of intussusception in which vessel lumens are internally divided by insertion, and subsequent growth and stabilization, of transcapillary tissue pillars.[28] The mechanism of intussusception and factors that regulate it are poorly understood, though the Tie receptors are known to have a role.[29]

Arteriogenesis

Further expansion and muscularization of vessels occurs by the process of arteriogenesis; the development of large calibre collateral arteries from a pre-existing network, in response to occlusive disease. This involves recruitment of additional mural cells and their proliferation, as well as expansion of the abluminal extracellular matrix. Whilst angiogenesis is driven by ischaemia, the initiator of arteriogenesis is increased fluid shear stress, which acts directly upon gene expression involved in the endothelial cell cycle.[30] PDGFβ with its effects on smooth muscle cell recruitment and proliferation, and TGFβ, a known regulator of vascular extracellular matrix synthesis, are likely to be key regulators.

Arteriogenesis of collateral vessels has been demonstrated in a number of animal models following the occlusion of major vessels.[31] In humans, well-developed collateral vessels that bypass occluded arteries have been found frequently and those patients with the best developed collaterals often have minimal symptoms.[32] Arteriogenesis of collaterals in response to the occlusion of primary supply vessels occurs in two phases. An initial increase in the lumen size occurs within a few days and this is followed by a slower remodelling of smooth muscle cell and extracellular matrix cover.[31] The early phase of this adaptive arteriogenesis is associated with inflammation. There is monocyte attachment to endothelium secondary to release of monocyte chemoattractant protein-1 (MCP-1), GMCSF and stromal-cell-derived factor-1 (SDF-1), which recruit CD14+ monocytes to the activated endothelial cell surface as well as extravasation and accumulation in the adventitia and perivascular space, with mast cells and T lymphocytes.[33] Monocytes have a critical role in adaptive arteriogenesis as experimental suppression of monocyte numbers decreases arteriogenesis.[34] These cells provide growth factors to stimulate vessel enlargement and proteases that can act on the extracellular matrix to accommodate increased vessel size. Changes to the wall of the vessel involve remodelling of the media, with increased turnover of medial smooth muscle cells and a shift towards a synthetic phenotype, assuming a contractile phenotype after enlargement of arterial diameter.[33]

A new internal elastic lamina is established and vessel wall thickening results from increased extracellular matrix deposition.

THERAPEUTIC INDUCTION OF VASCULAR GROWTH

Most studies on the therapeutic induction of vessel growth at a pre-clinical level, and all clinical trials, have focused on angiogenesis. Clinical trials aimed at relieving peripheral vascular disease by therapeutic angiogenesis have had limited success. This is perhaps not surprising given the very limited ability of angiogenic growth to compensate for the loss of conductance vessels. It is now being generally recognised that revascularisation in peripheral vascular disease, as in coronary heart disease, would be best achieved by the therapeutic activation of collateral arteriogenesis. Nevertheless, the ability to activate angiogenesis therapeutically would be valuable where significant microvascular disease exists. In addition, the early work on therapeutic angiogenesis has provided data and approaches that may be valuable in future studies aimed at the activation of arteriogenesis.

Delivery of molecular activators of vascular growth

Induction of angiogenesis in the appropriate ischaemic areas and in future local arteriogenesis at suitable sites for collateral development, requires activating agents to be delivered in a manner that ensures controllable local activity and minimizes systemic side effects. Growth factors are readily degraded and if administered systemically would have to be used at high concentrations in order to ensure enough active growth factor reached the appropriate site. There are risks associated with non-local delivery, for example, systemically delivered VEGF produces severe hypotension in animal models.[35] In addition to localization, activators must be present for sufficient time to induce optimal vascular growth.

The two principal methods used to deliver angiogenic activators in pre-clinical studies as well as clinical trials have been as recombinant proteins or as the genes that encode these proteins. Activators can be delivered locally via intramuscular catheters, direct injection into the muscle or use of coated stents. An important consideration in the use of peptide growth factors is ensuring sufficient longevity of the molecules at the desired site. Where recombinant proteins are injected this would necessitate multiple injections during the course of treatment. An alternative strategy is the use of local reservoirs of recombinant protein, such as biodegradable microspheres.[36] A major limitation to the use of recombinant protein, however, is the expense and difficulty in obtaining large enough quantities of appropriate purity, especially when cocktails of growth factors are required.

Delivery of angiogenic factors by gene transfer has significant advantages over the administration of recombinant protein. It is relatively easy to produce high purity DNA in large quantities and the transfected genes remain active over a period of several days to several weeks. In contrast to gene therapy aimed at correcting genetic diseases, gene transfer as a means of providing short term local expression of therapeutic proteins has been successful. Surprisingly, small amounts of DNA plasmid vectors can be taken up by muscle cells *in vivo* and are reported to result in significant gene expression in humans.[37] Improvements in transfer efficiency have been sought by the use of liposomal carriers and viral vectors. Adenovirus is the most common viral vector used for the delivery of angiogenic genes, and since genes transferred in this way do not integrate into

chromosomes of transduced cells, transient expression is provided.

There have been reports of an inflammatory reaction to adenoviral vectors in human trials, but no long term safety problems at doses appropriate for angiogenic therapy. Second generation adenoviral vectors with deletions of E1 and E4 regions have better transfection efficiency and elicit a decreased inflammatory response[38] and further improved adenoviral vectors can be expected.[39] The adeno-associated viruses (AAV) offer an alternative viral means for gene delivery. AAV efficiently transduce skeletal muscle and vasculature.[40] However, along with retroviruses and lentiviruses, AAV integrate into the recipient genome requiring the development of regulatory systems if they are to provide controllable expression of vascular growth genes. As with recombinant protein, local delivery of genes can be accomplished by direct intramuscular injection, implantation of coated stents or catheters. It may also be possible to utilize tissue-specific endothelial surface molecules for targeting vectors to particular vascular beds. Implantation of cells transfected *ex vivo* offers an additional route of local delivery.

Angiogenic activators

Perhaps the simplest approach to activating angiogenesis is the administration of a soluble angiogenic activator. VEGF is relatively specific for endothelial cells and physiologically relevant. Although VEGF-A, -B, -C, -D and –E, as well as the VEGF-R1 ligand placental growth factor, have all been shown to activate angiogenesis when administered in animal models, most studies have concentrated on VEGF-A. Administration of VEGF alone results in a high percentage of malformed capillaries in animal models.[41] This growth factor also induces vessel permeability resulting in local hypotension and oedema.[35] Neovessels induced by VEGF are transient, with regression occurring on growth factor withdrawal.[42] These data indicate formation of a sustained microvessel network may require relatively long term exposure to the angiogenic initiator.

The fibroblast growth factors have also been examined as potential therapeutic agents to induce angiogenesis in clinical trials. There are 23 members of the FGF family and FGF-2 and FGF-4 have been used in clinical trials. FGF-1, -2, -4 and -9 are highly mitogenic for endothelial cells, although these growth factors are also active on non-endothelial cells.[43] Another growth factor that induces angiogenesis and is in early trials is hepatocyte growth factor; again its effects are not confined to endothelial cells.[44]

With the recognition that physiological angiogenesis requires a spatially and temporally co-ordinated repertoire of signals, attempts have been made to improve capillary formation by providing cocktails of growth factors. Indeed combination of VEGF with the pro-stabilizing Tie2 agonist angiopoietin-1 in a mouse model does produce microvessels with increased lumen size, less thrombosis and increased perfusion compared with VEGF alone.[18] These vessels are also less permeable than those formed in response to VEGF alone.[45] Another approach aimed at providing a more physiological range of angiogenic factors is the targeting of HIF. Expression of a form of HIF-1α that is resistant to oxygen-induced degradation in mouse skin led to up-regulation of HIF-sensitive angiogenic genes and the stimulation of microvessel formation.[46] Again the vessels produced were not associated with oedema. The cell permeable peptide, PR39, inhibits proteosomal degradation and can stabilize HIF-1α.[47] PR39 stimulates angiogenesis in mouse heart, although further studies are required to determine its specificity.[47]

Pre-clinical and early clinical studies have shown that angiogenesis can be induced *in vivo* by a variety of approaches. The challenge is to devise a means to stimulate the conversion of these neovessels into optimally organized, persistent and functional microvascular networks.

Arteriogenic activators

Little is known about the molecular mechanisms of arteriogenesis. In contrast to the ischaemic tissue microenvironment in which angiogenesis occurs, collateral arteriogenesis in the limb takes place in normoxic conditions.[48] In adaptive arteriogenesis studied in animal models, the biochemical effects of the increased flow that the collaterals experience as a result of occlusion of conductance vessels plays a major role. These effects include increased wall shear stress as well as tangential and axial stresses. Increased shear can up-regulate vascular cell adhesion molecule-1 and intracellular adhesion molecule-1, as well as MCP-1 which contributes to monocyte recruitment.[49] Growth factors are undoubtedly involved in adaptive arteriogenesis, but again more work is required to identify the key factors and their roles. In animal models FGF-1 and FGF-2 were found to be unchanged during adaptive arteriogenesis, although there was a transient increase in expression of FGF-receptor-1.[50]

TGFβ is increased during collateral development and can enhance arteriogenesis in animal models. Several factors have been found to enhance arteriogenesis when administered to animals, including FGF-2, VEGF, placental growth factor, angiopoietin-1 and MCP-1, although the exact mechanisms of action remain undefined.[31] Many appear to have indirect actions, for example, VEGF and placental growth factor infusions are likely to enhance arteriogenesis via their monocyte chemoattractive activity. Given the great potential of therapeutic induction of collateral arteriogenesis for the treatment of peripheral vascular disease, it is important that a better understanding of the molecular mechanisms be gained. Identification of key regulators that can induce or enhance arteriogenesis of collaterals is a priority.

Clinical trials for angiogenic therapy of peripheral vascular disease

There have been a number of phase 2 and 3 clinical trials aimed at relieving peripheral vascular disease by angiogenic therapy. The Therapeutic Angiogenesis with Recombinant Fibroblast Growth Factor-2 for Intermittent Claudication (TRAFFIC) study used single or repeated doses of recombinant FGF-2 delivered by arterial puncture and cross-over catheter in patients with intermittent claudication. Patients receiving a single FGF-2 dose showed a significant improvement in peak walking time at 90 days.[51] In the Regional Angiogenesis with Vascular Endothelial Growth Factor (RAVE) study, VEGF121 gene transfer by adenovirus was utilized but failed to produce a significant improvement in peak walking time at 12 weeks.[52] In contrast, in a different study adenoviral delivered VEGF165 given during angioplasty did produce an increased angiographically-assessed vascularity at 3 months.[53] The Vascular Endothelial Growth Factor in Ischemia for Vascular Angiogenesis (VIVA) trial failed to show a difference between the treatment and placebo groups for the primary endpoint of walking time.[54] The TALISMAN collaborators evaluated a plasmid-based angiogenic gene delivery system for the local expression of FGF-1 in patients with non-healing ulcers who were not suitable for invasive re-vascularisation. Whilst there was no difference observed in the primary endpoint of complete ulcer healing, there was a reduced risk of major amputation.[55] Initial

reports from the ongoing Bone Marrow Outcome Trial in Critical Limb Ischemia (BONMOT-CLI), show promise for the intramuscular injection of autologous bone marrow stem cells into ischaemic limbs, with significant improvements in Rutherford categories and a trend against major limb amputation.[56]

A meta-analysis of 5 randomised controlled trials investigating the role of gene therapy as an option for the treatment of peripheral vascular disease concluded that the current literature does not demonstrate a clinical benefit for patients with peripheral vascular disease.[57] Although these trials have met with limited success, together with phase 1 studies and earlier small trials, they demonstrate the feasibility and safety of molecular approaches to therapeutic modulation of vascular growth. The trials have also been valuable in aiding development of techniques for delivering therapeutic agents, as well as helping clinicians refine aspects of trial design for future clinical work on arteriogenesis and angiogenesis.

The realization that therapeutic induction of collateralization by arteriogenesis would be most appropriate for occlusive disease, whilst angiogenic therapy would benefit patients with microvascular defects, should help improve selection of the most appropriate populations for use in future trials. Clear clinical end-points are required in such work. Where angiogenesis is the aim, establishment of optimal treatment modalities will depend on further pre-clinical work focused on determining ways to establish mature, correctly patterned vascular networks. This may involve defined cocktails of stimulators, or activation of transcriptional factors triggering coordinated expression of stimulators. In both cases distinct spatial and temporal expression patterns are likely to be required.

CONCLUSIONS

The prospects for a molecular approach to stimulate vascular growth as a means of relieving tissue ischaemia in peripheral vascular disease are promising. Early clinical work, together with a better understanding of vascular growth mechanisms, has allowed identification of the key areas in which progress is required in order to bring therapeutic vascular growth to the clinic. Yet Isner predicted in therapeutic angiogenesis 'a new frontier for vascular therapy' in 1996.[58] That today the underlying cellular mechanisms and processes continue to be elucidated is testament to the complexity of this area of vascular medicine. Bypassing the occluded conductance vessels is now recognised to require collateral growth by arteriogenesis, rather than angiogenesis. In comparison to angiogenesis our understanding of arteriogenesis remains rudimentary. Significant work is needed therefore to understand the mechanisms regulating physiological arteriogenesis as well as adaptive arteriogenesis, and to identify key molecular regulators. Activation of angiogenic growth will be valuable where microvascular disease is prevalent. Indeed, situations in which activation of arteriogenesis to restore conductance level flow together with activation of angiogenesis to relieve microvascular defects can be envisaged. It is clear that optimum microvascular growth will require correctly patterned, functional and persistent mature microvessel networks. Further work on the basic biology of angiogenesis is needed to determine the best means of inducing this therapeutically.

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7 • Biology of Restenosis and Targets for Intervention

RICHARD D. KENAGY

Centre for Cardiovascular Biology, Department of Surgery, University of Washington, Seattle, WA, USA

INTRODUCTION

Restenosis is usually defined as a re-narrowing of the arterial lumen occurring after a vascular intervention intended to treat ischemic lesions. This loss of lumen area results from the injury caused by all forms of vascular intervention, including direct repair (patch angioplasty, endarterectomy) and intraluminal approaches (balloon angioplasty, atherectomy, stent angioplasty). While there are clear differences between arteries and veins (review in[1]), this review will also include discussion of stenosis after vein bypass grafting or creation of arteriovenous fistula dialysis access because stenosis in these cases also results from injury.

Lumen enlargement after angioplasty or stenting is the result of a combination of plaque reduction (compression and embolization), plaque redistribution within or outside the lesion area, and vessel expansion (see review[2]). Failure occurs at early times because of technical problems and thrombosis (e.g. small diameter vein graft, limited outflow, or hypercoagulability) and later times (1-18 months) primarily because of injury-induced scarring. At much later times (> 18 months), failure primarily results from the ongoing atherosclerotic process. Although restenosis occurs within the context of atherosclerosis, the clinical features and genetic control of atherosclerosis and restenosis are different.[3] Atherosclerosis develops slowly over decades,[4] while restenosis occurs within months to years.[5]

The costs of restenosis are considerable, since greater than 20% of all interventions fail because of restenosis. In the U.S. alone, it is estimated that as many as 200,000 cases of drug eluting stent (DES) restenosis occur every year (see review[6]). The goal of research in this area is to modify vascular healing so that injury is repaired without lumenal narrowing. The objectives of this chapter are to review mechanisms of restenosis and current and potential molecular targets to prevent restenotic lesions.

MECHANISMS OF RESTENOSIS

Restenosis results from a combination of elastic recoil, thrombosis, remodelling and intimal hyperplasia. Three decades ago the prevailing view was that restenosis was primarily a problem of intimal hyperplasia caused by migration of SMCs from the media to the intima followed by excessive growth. Two decades ago a role for remodelling was confirmed and more recently a possible role for progenitor/stem cells has gained ground. Because recoil is not an issue after stenting

and is a mechanical property of elastic layers of the artery, we will focus on the roles of thrombosis, remodelling and intimal hyperplasia, which contribute to varying degrees depending on the intervention employed (e.g. angioplasty vs. stenting).

Thrombosis

Thrombosis may occur after vascular intervention because of damage to the endothelium and possible intimal and medial dissection. Mechanisms of this process are presented more fully in Chapter 10 on the haemostatic system. Briefly, exposure of underlying tissue factor to blood causes thrombin and fibrin generation, which along with platelets may lead to thrombotic occlusion.[7] Adherence of platelets is mediated by receptors such as the integrin, IIb/IIIa. Aggregation of platelets causes the release of numerous factors, including thromboxane A2, ADP, serotonin, and matrix metalloproteinases 2 and 9, that further stimulate platelet adherence and/or aggregation.[5,8] Platelets also release a variety of growth and chemotactic factors.[8] Thrombus can act as a scaffold through which SMCs migrate and both synthesize and degrade extracellular matrix components, thus reorganizing the thrombus. While anti-platelet therapy largely prevents acute thrombosis after vascular intervention, late thrombosis in DES remains of concern prompting prolonged anti-platelet therapy.[6] Even in the absence of thrombotic occlusion, there is considerable evidence of a relationship between the early thrombotic response and the later development of restenosis.[9] Anti-platelet therapy, particularly inhibitors of IIb/IIIa in both animals and humans has demonstrated the importance of platelets to the restenotic process.[8,10-13] For example, knockout of P2Y12, the ADP receptor on platelets, or its blockade using clopidogrel inhibits neointimal formation after arterial injury.[14] Fibrin deposition on stent struts and decreased heparin cofactor II, a thrombin inhibitor, are both associated with in-stent restenosis[15,16] (Table 7.1). Larger platelets, which contain more prothrombotic material per unit volume, are also associated with restenosis.[17] However, the clinical relevance of these findings has not been fully evaluated. For example, low platelet responsiveness to clopidogrel, a predictor of thrombotic complications, is not a predictor of DES restenosis.[18]

Remodelling

Remodelling refers to a change in the total area of the vessel (generally measured as a loss of the area within the external elastic lamina) that affects lumenal dimensions of the blood vessel not attributable to vasospasm, vasodilation, or changes in wall area. Remodelling can be favorable (outward, positive, compensatory, or adaptive) or unfavorable (inward, negative, or maladaptive). Vascular remodelling occurs normally in response to changes in blood flow, wall mass, or wall tension (as during normal development, atherosclerotic lesion development, or hypertension) as an adaptation to maintain normal blood flow (see review[19]). Post-angioplasty, arteries show further gains in lumen area between 1 day and 1 month after angioplasty, but then lose some vessel area thereafter with restenotic arteries showing a greater loss than non-restenotic arteries. Negative remodelling contributes more than intimal hyperplasia to restenosis after coronary and peripheral artery angioplasty[5,96] and in vein graft stenosis,[1,97,98] but in rigid artificial grafts and stented arteries intimal hyperplasia is the primary mechanism of restenosis.[5]

The regulation of arterial remodelling is poorly understood, but since wall tension and shear stress are normally regulated

TABLE 7.1: Recent Studies of Biologically Relevant, Non-technical Factors Implicated in Restenosis

Factor (+ if positively, - if negatively associated)	Independent Predictor of restenosis after:	Not an Independent Predictor of restenosis after:	Patients, N	Citation
Patient Characteristics				
End stage renal disease **(+)**	Fem-pop BMS		511	[20]
	Coronary DES (recurrent restenosis)		990	[21]
	Coronary BMS		34	[22]
Diabetes **(+)**	Carotid endarterectomy		243	[23]
		Carotid endarterectomy	308	[24]
	Lower extremity angioplasty		40	[25]
	Angioplasty of AV fistula		140	[26]
		Renal artery stent/ angioplasty	91	[27]
	Coronary SES or PES		545	[28]
	Coronary SES		1312	[29]
	Coronary stent		3104	[30]
	SES for coronary BMS restenosis		244	[31]
		Coronary BMS	345	[32]
		Coronary BMS and DES	274	[33]
		Coronary angioplasty	92	[34]
		Coronary BMS	109	[35]
Vascular Characteristics				
Echolucent femoral (non-target) lesion **(+)**	Carotid endarterectomy		321	[36]
Echolucent plaque **(+)**	Carotid endarterectomy		308	[24]
	Coronary angioplasty		92	[34]
Collagen content **(+)**	Femoral endarterectomy		217	[37]
Positively remodeled lesion **(+)**	Coronary BMS		85	[38]
	Coronary BMS		113	[39]

Factor (+ if positively, - if negatively associated)	Independent Predictor of restenosis after:	Not an Independent Predictor of restenosis after:	Patients, N	Citation
Decreased macrophages, lipid core in lesion **(+)**	Carotid endarterectomy		500	[40]
Atherosclerotic burden – Gensini score **(+)**	Coronary angioplasty		345	[32]
Brachial intima/media thickness	Iliac/femoral angioplasty; BMS		128	[41]
Impaired forearm reactive hyperemia **(+)**	Coronary BMS		47	[42]
	Coronary BMS DES		136	[43]
		Iliac/femoral angioplasty; BMS	128	[41]
High collateral function **(+)**	Coronary BMS		95	[44]
	Coronary angioplasty		64	[45]
	Coronary angioplasty		91	[46]
		Coronary BMS	58	[47]
SNPs and soluble factors *Growth Inhibitors and Stimulants*				
eNOS (Glu298Asp) **(+ TT)**	Coronary BMS		106	[48]
eNOS (Glu298Asp) **(+ TT)**	Coronary BMS		226	[49]
eNOS (Glu298Asp)		Coronary BMS	3104	[30]
Heme Oxygenase-1 promoter $(GT)_n$ length polymorphism **(+)**	Coronary BMS		323	[50]
		Coronary BMS	1807	[51]
p27 -838C>A **(+ CC)**	Coronary BMS		715	[52]
Nurr1 haplotype **(-)**	Coronary BMS		601	[53]
Pre-procedure Adiponectin **(-)**	Coronary BMS in end stage renal disease		71	[54]
Pre-procedure HMW adiponectin **(-)**	Infrainguinal saphenous vein graft		225	[55]

Factor (+ if positively, - if negatively associated)	**Independent Predictor of restenosis after:**	**Not an Independent Predictor of restenosis after:**	**Patients, N**	**Citation**
Change in adiponectin **(-)**	Coronary BMS and DES		32	[56]
Pre-procedure Resistin **(+)**	Infrainguinal saphenous vein graft		225	[55]
	Coronary BMS		70	[57]
Inflammation				
Pre-procedure CRP **(+)**	Angioplasty of femoral, popliteal arteries		172	[58]
		Carotid endarterectomy	64	[59]
	Coronary DES		167	[60]
		Coronary DES	134	[61]
	Coronary angioplasty and BMS		850	[62]
		Coronary angioplasty	345	[32]
		Coronary angioplasty	162	[63]
		Coronary angioplasty	168	[64]
		Coronary angioplasty	345	[65]
		Coronary angioplasty	216	[66]
	Meta-analysis of coronary angioplasty (includes [64] [66])		1062	[67]
IL-*1B–511* SNP*(T/C)* **(+ TT)**	Coronary stent (type unclear)		165	[68]
		Coronary angioplasty	171	[69]
IL-1R antagonist*2 **(-)**	Coronary angioplasty		171	[69]
Pre-procedure IL-3 **(+)**	Coronary stent (type unclear)		205	[70]
IL-6 SNP (-174 G/C) **(+ CC)**	Fem-pop angioplasty		281	[71]
IL-6 SNP (-174 G/C and -572 G/C)		Coronary BMS	3104	[30]
Pre-procedure soluble CD40 ligand **(+)**	Coronary angioplasty		70	[72]
		Coronary angioplasty	162	[63]

Factor (+ if positively, - if negatively associated)	**Independent Predictor of restenosis after:**	**Not an Independent Predictor of restenosis after:**	**Patients, N**	**Citation**
CD11b level and activation on leukocytes **(+)**	Coronary BMS		62	[73]
CD18 SNP (1323 C/T) **(+ TT)**	Coronary stent		1207	[74]
CD14 SNP (-260C/T) **(+ CC)**	Coronary BMS		3104	[30]
Change in MCP-1 blood levels **(+)**	Coronary DES BMS		32	[75]
TNFα –238G-1031T haplotype **(+)**	Coronary angioplasty		3104	[76]
TNFα release during procedure **(+)**	BMS into stenotic saphenous coronary bypass graft		18	[77]
CCL11 (Eotaxin) SNP (-1328G/A) **(+ GG)**	Coronary BMS		3104	[30]
Colony Stimulating Factor 2 SNP (Ile117Thr) **(+ Ile)**	Coronary BMS		3104	[30]
Colony Stimulating Factor 3 at 24 hours **(+)**	Coronary BMS		40	[78]
Oxidized LDL change **(+)**	Coronary BMS after acute infarction		109	[35]
Pre-procedure monocyte VEGF expression **(+)**	Coronary BMS and SES		41	[79]
$CD34^+$ cells on day 7 **(+)**	Coronary BMS		40	[78]
$CD34^+$ cells **(+)**	Coronary BMS		17	[80]
Complement/Lectin				
Mannose Binding Lectin (MBL) 2, A/A alleles **(+)**	Carotid endarterectomy		123	[81]
Pre-procedure Complement C3 **(+)**	Carotid endarterectomy		64	[82]

Factor (+ if positively, - if negatively associated)	**Independent Predictor of restenosis after:**	**Not an Independent Predictor of restenosis after:**	**Patients, N**	**Citation**
Complement C3 SNP (Arg102Gly)		Coronary BMS	3104	[30]
Pre-procedure C1-Inhibitor **(+)**	Carotid endarterectomy		64	[59]
Change in VEGF and PDGF-AB in patients with MBL2 A/A alleles **(+)**	Carotid endarterectomy		53	[83]
Vasoactivity				
Beta2 adrenergic receptor SNP (Arg16Gly) **(+ Gly)**	Coronary BMS		3104	[30]
Asymmetric dimethyl arginine **(+)**	Angioplasty of failed AV fistula in ESRD		100	[84]
Pre-procedure N-terminal Brain Natriuretic Protein **(+)**	Coronary angioplasty		345	[32]
Post-procedure N-terminal Brain Natriuretic Protein **(+)**	Coronary BMS and DES		249	[85]
Haemostatic/Fibrinolytic system				
Mean platelet volume **(+)**	Meta-analysis coronary angioplasty and stent		430	[17]
Heparin Cofactor II **(-)**	BMS in restenotic fem-pop post angioplasty		63	[16]
	Coronary BMS		134	[86]
Urokinase **(+)**	Coronary BMS		49	[87]
Other				
Pre-procedure LDL particle size **(+)**	Coronary BMS and DES		274	[33]
Lipoprotein(a) **(+)**	Coronary BMS		109	[88]
Active MMP9 **(+)**	Coronary BMS		286	[89]
MMP9 **(+)**	Coronary stent		40	[90]

Factor (+ if positively, - if negatively associated)	Independent Predictor of restenosis after:	Not an Independent Predictor of restenosis after:	Patients, N	Citation
Active MMP3 or MMP9	Coronary BMS		152	[91]
MMP2 and MMP9 **(+)**	Coronary DES		85	[92]
5A/6A MMP3 SNP **(6A+)**	Coronary BMS		344	[93]
		Coronary BMS	198	[94]
	Coronary angioplasty		287	[94]
		Coronary angioplasty	48	[95]
Pre-procedure Pregnancy-associated plasma protein A **(+)**	Coronary angioplasty		162	[63]

This table is not an inclusive list of all studies of risk factors for restenosis. Studies showing non-technical factors as independent risk factors for restenosis (i.e. angiography, duplex US) by multivariate analysis were included as were studies showing a lack of independence of these same factors.

within a narrow range in mammals (see review[19]), it must involve the transduction of the forces of shear stress and wall tension into biochemical signals leading to the breaking and reforming of ECM attachments, both matrix to matrix and cell to matrix. For example, the wall thickening that occurs in vein grafts and hypertensive arteries normalizes wall tension. A mechanoregulation model of remodelling proposed by Grinnell and colleagues, in which strain-regulated cell migration and collagen translocation can produce large scale tissue movement,[99] is relevant to both vascular and cutaneous injury, which share several features. For example, skin wounds contract as do negatively remodelling arteries, ECM changes are similar in injured skin and artery (increased biglycan and versican and less decorin), and smooth muscle actin-positive cells appear in the skin wound (myofibroblasts) and in the injured arterial adventitia. Of interest, there is a possible association between abnormal skin wound healing and restenosis.[100] Support for a mechanoregulatory model comes from observations after vascular and cutaneous injury. Releasing tension by external wrapping of arteries and by external pressure or skin flapping to skin wounds causes tissue regression through apoptosis and loss of ECM.[101-103] Detaching collagen gels embedded with SMCs so that they float releases tension and causes SMC death and inhibits ECM production.[104] Finally, increasing tension by stretching arteries ex vivo and by external skin splinting causes cell proliferation and increased tissue mass.[105-107] The tension in the cell-ECM has been shown to regulate which signaling pathways are utilized,[99] and several signaling mediators are implicated in

the regulation of remodelling. One example is NO, but while NO formed by iNOS and nNOS inhibits negative remodelling, that formed by eNOS does not influence remodelling. Whether the particular isoform of NOS or the particular cell expressing NOS is critical is not known. The P2X4 ion channel, which is needed for NO production, is also necessary for flow-mediated remodelling, as is the cytoskeletal filament, vimentin, which is a key intracellular protein in the transmission of contractile forces.[19]

The roles of the adventitial, medial, and intimal layers of the artery in remodelling are not clear though each provide significant mechanical strength to the human artery.[108] Some data suggest that adventitial fibrosis and collagen accumulation contribute to negative remodelling.[109] SMCs synthesize collagen fibrils in a manner dependent on fibronectin fiber assembly, $\alpha 2\beta 1$ integrin, and RhoA activity, as well as an intact actin cytoskeleton, which is required for tension development.[110] Of interest, blockade of fibronectin assembly or Rho kinase prevents positive arterial remodelling.[111,112] Fibronectin regulates signaling by the transcription factor NFκB,[113] which mediates $\alpha 2\beta 1$ integrin-directed SMC collagen gel contraction[114] and remodelling of vessels caused by circumferential and axial stress.[106,107,115,116]

Finally, inhibition of ECM protein cross linking by inhibition of lysyl oxidase or transglutaminase inhibits negative remodelling after angioplasty and blood flow reduction, respectively.[117,118] Thus, arterial remodelling may involve the interaction of mechanical stress with fibronectin/collagen fibrillogenesis mediated by integrin/NFκB signaling as well as ECM cross-linking. The possible roles in remodelling of MMPs and other ECM components will be discussed below (sections on matrix metalloproteinases and extracellular matrix/receptors). Finally, ongoing studies are focused on defining genetic determinants of murine flow-induced remodelling.[19]

Intimal hyperplasia

Intimal hyperplasia results from the net increase in cell number and ECM, which are dependent on rates of cellular migration, proliferation, and death and on rates of ECM synthesis and degradation, respectively. Our understanding of the underlying cellular mechanisms of intimal hyperplasia comes from animal models of vascular injury, since the ability to study the human response at the cellular and biochemical level has been limited. A variety of methods of arterial injury has been employed including a partially inflated Fogarty embolectomy catheter, loose-fitting external cuffs, endolumenal wires or nylon loops, adventitial electrical injury, complete or partial arterial ligation, and stents. Some of these methods share features of clinically used interventions (e.g. stents) while others do not (ligation; see review[119]). Despite their limitations, animal models of vascular injury have provided a general understanding of the sequence of events after injury.[120]

Sequence of Events after Injury

The sequence of events after arterial injury is illustrated in Figure 7.1. Depending on the type of injury, endothelial cells are either injured or completely removed resulting in the loss of the quiescent endothelial cell layer, which inhibits SMC proliferation and intimal hyperplasia.[121,122] Within 30 minutes of balloon injury up to 70% of medial SMCs die via apoptosis. Placement of vein grafts and stents into the arterial circulation, as well as arterial ligation, also cause apoptosis (review in[2]). Some data suggest that cell death increases intimal hyperplasia,[123-125] but SMC death by itself does not appear to cause intimal hyperplasia.[126]

Prior to the start of proliferation, SMCs

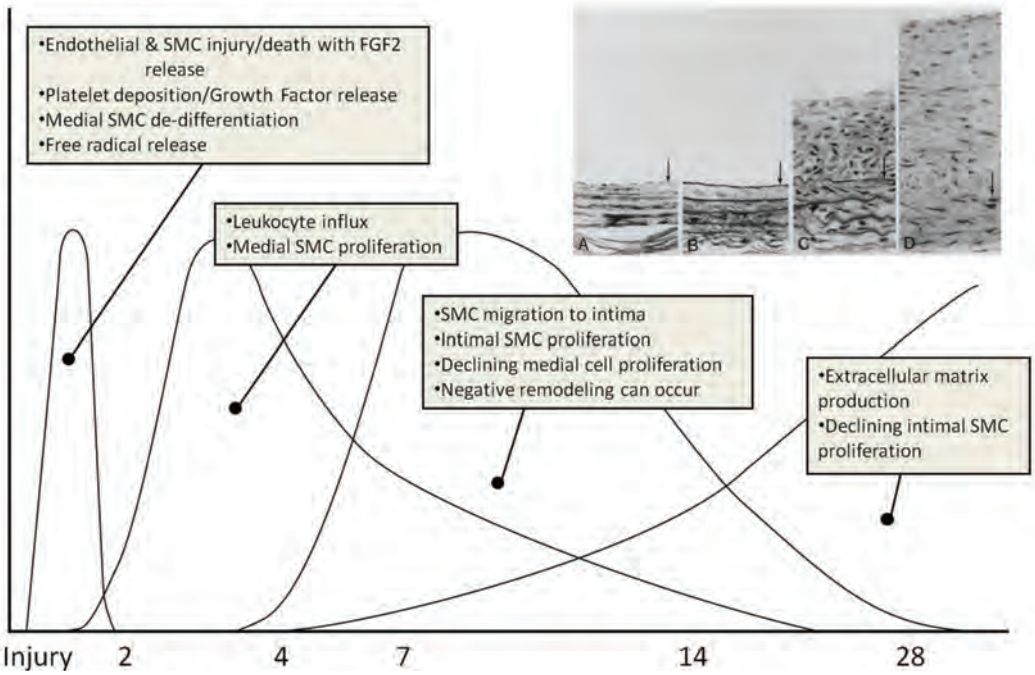

FIGURE 7.1: Sequence of events leading to intimal hyperplasia after arterial injury including histological cross-sections of injured rat carotid arteries: (a) normal vessel. Note the single layer of endothelium in the intima; (b) denuded vessel at two days. Note the loss of endothelium; (c) denuded vessel at two weeks. Intima is now markedly thickened due to smooth muscle migration and proliferation; and (d) denuded vessel at 12 weeks. Further intimal thickening has occurred. Internal elastic lamina indicated by an arrow.

in the media express high levels of SMC-specific contractile proteins, such as smooth muscle alpha actin (SMA), and are quiescent with no significant ECM production. After injury, SMCs change to a de-differentiated phenotype with SMCs expressing decreased levels of SMC-specific contractile proteins[127] and increased levels of migration, proliferation, and ECM synthesis. The regulation of this transition has been studied at the transcriptional level for markers of SMC differentiation[128] and for many years it has been assumed that expression of these genes might inhibit proliferation. Recent data supports this for SMA in that specific mutations in SMA cause SMCs to proliferate faster in vitro and cause coronary artery disease.[129]

The medial SMC proliferation rate during the first two to four days jumps from 0.06% before injury to 10-40% in the injured arteries of rats, mice, rabbits, and non-human primates (review in[2]). Adventitial proliferation begins earlier and is maintained along with medial proliferation.[130] Animal and clinical studies with stents indicate that intimal hyperplasia is generally correlated with the degree of injury.[2,15] By four weeks cell growth in the media and adventitia returns to baseline. By eight weeks, when intimal growth is maximal, growth returns to baseline in endothelialized intima, but SMCs at the luminal surface in deendothelialized areas continue to proliferate at a low rate.[131]

Migration of medial SMCs into the rat and mouse neointima occurs as early as 4 days after injury.[132,133] The role of SMC migration in human vessels remains an unknown,

because the presence of intimal SMCs in normal arteries as well as in arterial lesions makes the measurement of migration impossible by currently available methods. In addition, the long term significance of medial SMC migration is uncertain, since pharmacological inhibition of migration causes only transient inhibition of intimal hyperplasia (e.g. MMP inhibitors and heparin; see review[2]). Thus, especially when there are pre-existing intimal SMCs, the impact of medial SMC migration is not clear.

There are very little data on the rate of growth of lesions in humans as most studies report baseline and final lesion size and serial angiography after angioplasty does not differentiate between negative remodelling and intimal hyperplasia. Available data indicates that intimal growth in stents is greatest from 0-6 months with a small increase (in DES) or decrease (in BMS) between 6–24 months.[134,135] Maximal intimal hyperplasia is achieved by 2 months after arterial injury in rats, rabbits and baboons[131,136,137] and after stenting in rats.[138] Differences in the thrombotic response[139] as well as differences in rates of recovery of the endothelium[140] may explain some of this variability. Genetic differences can explain some but not all of these differences in animals as well as humans.[3,141-143]

A considerable number of risk factors for restenosis are associated with intimal hyperplasia (Table 7.1). Regarding renal failure and type 2 diabetes, while there are few studies of arterial injury in animal models of type 2 diabetes (in contrast to type 1)[144] or renal failure, increased intimal hyperplasia is observed in arterio-venous fistulas in a mouse model of renal failure and in vein grafts in a mouse model of type 2 diabetes.[145,146] In addition, SMCs obtained from type 2 diabetic patients display increased proliferative and migratory capacity in vitro compared to SMCs from non-diabetic patients.[147] A SNP of p27, which is an inhibitor of cyclin-dependent kinases and proliferation,[148] increases basal promoter activity and is associated with less restenosis.[52] Certain haplotypes of Nurr1, a transcription factor that inhibits SMC proliferation, are associated with restenosis.[53] eNOS SNPs associated with restenosis may decrease levels of NO, which is a SMC growth inhibitor as well as vasodilator,[149] Adiponectin, which inhibits SMC growth,[150] is negatively associated with restenosis. In contrast, resistin, which is positively associated with restenosis, stimulates SMC growth.[151] Finally, a role for cell proliferation in vein graft stenosis is supported by the increased proliferative capacity of cells cultured from veins of patients that develop stenosis.[152]

Several predictive factors for restenosis may be associated with decreased blood flow through the lesion (Table 7.1), which is known to increase intimal hyperplasia in animal models.[153] These factors include impaired forearm reactive hyperemia, which may indicate poor dilation in the lesion area, and high collateral function, which may divert blood from the lesion. Other factors influence eNOS, which synthesizes the vasodilator NO. These are an eNOS SNP associated with restenosis, which may decrease eNOS function, and asymmetric dimethyl arginine, which inhibits eNOS and which is increased in the blood of restenotic patients.[84] Finally, a beta2 adrenergic receptor SNP may decrease the vasodilatory function of this receptor[30] (Table 7.1).

Origin of intimal cells

Early experiments on the arterial response to injury indicated that medial SMCs were the source of intimal cells after injury (see [2]), but more recent studies of arterial injury in chimeric mice and rats suggested that a significant number of intimal SMCs are of bone marrow origin(review in;[154,155] see

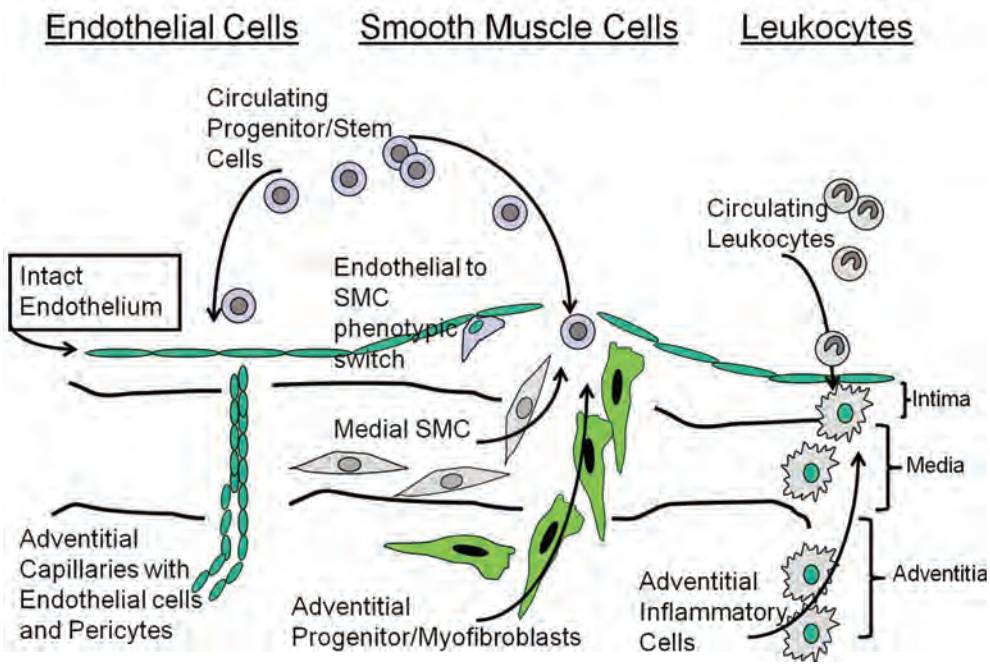

FIGURE 7.2: Possible sources of intimal cells after arterial injury.

figure 7.2 for possible sources of the major cells of the intima). However, these data have been called into question by investigators using more robust microscopic techniques for detecting double labeled cells.[156-159] In addition, while some studies have shown a correlation between the risk of human coronary BMS restenosis and the increase in circulating $CD34^+$ cells and their ability to differentiate into SMCs in vitro,[78,80] the significance of these data is not clear in the absence of data showing a contribution of these cells to intima formation in humans. Another possible source of intimal cells is suggested by studies in rats, pigs, and rabbits that indicate that adventitial cells migrate into the intima (review in[160]). However, other investigators did not find significant adventitial involvement in porcine coronary intima formation with[161,162] or without complete interruption of the media (see review[154]). While recent reports demonstrate the presence of adventitial cells in rat and human arteries and veins that can differentiate into SMCs, the contribution of adventitial cells to intimal hyperplasia remains uncertain.[158,163,164] In addition, evidence from studies of embryonic development and with cultured endothelial cells demonstrate that endothelial cells can undergo a phenotypic transition to SMCs making these another potential source of intimal cells.[165] Overall, the data suggest a medial and possibly adventitial origin of intimal SMCs.

Inflammation

There is a strong correlation between inflammation and restenosis after angioplasty or stent placement[15,166] and between macrophages in the primary lesion and the occurrence of restenosis after angioplasty[167] (reviewed in[168]). While many individual studies have not demonstrated an independent association between pre-procedural blood levels of the acute phase protein, CRP, and coronary restenosis, a meta-analysis of

coronary angioplasty studies showed CRP as an independent predictor (Table 7.1). In addition, a study of peripheral angioplasty showed a much stronger relationship between restenosis and 48 hour post-angioplasty CRP levels than with pre-procedural levels.[58] Whether CRP is related to vein graft stenosis is not clear.[169]

Data from models of injury that both do and do not denude the vessel of endothelium indicate that inflammation promotes intimal hyperplasia. Leukocyte recruitment to the injured vascular wall occurs via binding to adherent platelets[170] and to cell adhesion molecules that are up-regulated by injury, such as ICAM-1 and VCAM-1.[171] Knockout or blockade of VCAM-1 or of the inflammatory cell integrin, Mac1, inhibits lesion formation after injury because of decreased leukocyte recruitment.[10,172,173] Inhibition of monocyte recruitment using a dominant-negative mutant of MCP-1, a major monocyte chemoattractant, also inhibits intimal hyperplasia after angioplasty in rats, non-human primates,[174] and hypercholesterolemic rabbits.[175] Finally, simultaneous myocardial infarction increases intimal hyperplasia after femoral artery injury possibly via increased levels of circulating IL-6 and TNFα.[176]

A number of inflammatory factors are predictors of restenosis. The number of inflammatory cells in stented lesions,[15] activation status of Mac1 on leukocytes,[73] a CD18 (a Mac1 subunit) SNP,[74] and the change in blood levels of MCP-1 are associated with restenosis[75,177] (Table 7.1). Of note, rapamycin is anti-inflammatory.[178] In addition, prednisone, another anti-inflammatory drug, decreases late lumen loss in coronary BMS and the release of TNFα from the patients' monocytes. This reduction in TNFα release correlates with late lumen loss[179] (Table 7.1). However, there are conflicting results with the -174 G/C and -511 polymorphisms of IL-6 and IL-1, respectively, in studies of the coronary and peripheral circulation.[66,69,180] Despite this there is a clear association of inflammation with restenosis.

Role of ECM production

Both cell proliferation and ECM production contribute to intimal hyperplasia[131], and intimal area more than doubles between two and eight weeks after injury because of ECM accumulation. The stable neointima is about 20% SMCs and about 80% ECM.[131] Rates of SMC replication are extremely low in restenotic tissue,[5,181] leading Glover and colleagues to suggest that changes in ECM are the major factor in restenosis several months to years after stent placement. Of interest, re-injury to the rat carotid artery one month after a prior balloon injury increases intimal lesion size entirely as a result of increased ECM.[182] In this regard, it should be noted that rapamycin inhibits induction of major ECM proteins such as collagen and hyaluronan.[183,184] The lack of evidence for substantial SMC proliferation in either angioplasty or stent stenosis suggests that therapies may be better aimed at altering the synthetic phenotype of SMCs, which would control ECM synthesis as well as proliferative capacity.

THE CONTRIBUTION OF SPECIFIC FACTORS TO RESTENOSIS

Growth factors/cytokines

Activated platelets, leukocytes, endothelial cells, macrophages, and SMCs can release a great number of growth factors and cytokines after arterial injury, including PDGF, FGF2, TGFβ, TGFα, vascular endothelial cell growth factor, macrophage colony stimulating factor, granulocyte macrophage colony stimulating factor, platelet-derived endothelial cell growth factor, IL-1, IL-4, IL-6, IL-8, IL-18, MCP, and TNF.[185] Many

of these factors have been shown to play roles in intimal hyperplasia and remodelling after arterial injury,[2] but we will focus on only three of these factors.

PDGF is a family of four gene products (A, B, C, and D chains) that dimerize into five functional growth factors (AA, AB, BB, CC, and DD), which bind differentially to homo- or hetero-dimers of two receptor subunits, α and β (see review[186]). PDGF plays a major role in the migration of SMCs after injury, while playing a minor role in SMC proliferation.[186] The β receptor subunit mediates intimal hyperplasia in non-human primates, which suggests a role for the PDGF isoforms B and D, and possibly C. However, there is also evidence for a role of PDGF-AA and, therefore, PDGF receptor α in intimal hyperplasia in the rat.[186] Of interest, concomitant treatment of non-human primates with blocking antibodies to both PDGF receptor isoforms causes intimal regression in polytetrafluroethylene grafts, while treatment with either alone does not.[187] A single intravenous infusion of a humanized version of this PDGFRβ antibody did not alter BMS restenosis,[188] but plasma concentrations considered effective were maintained for only two weeks.

FGF2 stimulates medial SMC proliferation and migration to the intima in the injured rat carotid, and blockade of both FGF2 and PDGF with antibodies results in an additive inhibition of intimal hyperplasia. However, FGF2 plays no role in the proliferation of intimal SMCs, and the intimal hyperplastic response in FGF2 knockout mice is normal (see review[2]). However, these mice display decreased SMC contractility,[189] which is consistent with the observation that a blocking antibody to FGF2 inhibits negative remodelling in the mouse carotid tie-off model.[190]

Although infusion of TGFβ1 stimulates medial SMC proliferation and antibody blockade slightly inhibits intimal hyperplasia, the more striking effect of blocking the action of TGFβ is on remodelling and ECM production (see review[191]). Blockade of TGFβ with a soluble receptor blocks negative remodelling, the transition of adventitial fibroblasts to myofibroblasts, and the deposition of collagen and versican. In stents, blockade of the TGF receptor does not alter intimal thickening but alters inflammatory cell number and extracellular matrix composition.[192]

Interactions among growth factors after injury are a significant though largely unexplored aspect of restenosis. For example, TGFβ can synergistically augment the mitogenic action of PDGF and FGF2.[191] In addition, IL-1β augments the proliferative effect of PDGF-BB on SMCs by inhibiting expression of p21 and p27, but inhibits PDGF-BB mediated SMC migration (see review[2]). Such interactions may augment hyperplasia in areas of inflammation (such as near stent struts) by slowing SMC movement and increasing proliferation.

Inhibitors

Numerous factors are inhibitors of intimal hyperplasia. For example, NO can inhibit SMC migration and proliferation after arterial injury[149] and prostacyclin inhibits intimal hyperplasia via its receptor IP.[193] Heparin and the heparan sulfate proteoglycans, perlecan and syndecan-1, inhibit SMC proliferation and migration, thus inhibiting injury mediated intimal hyperplasia.[194-196] In addition, overexpression of heparanase, the enzyme responsible for degrading the heparan sulfate glycosaminoglycans, causes increased intimal hyperplasia after stent-induced injury.[197] The normal adventitia inhibits medial SMC migration and proliferation.[121] Similarly, normal periadventitial adipose tissue inhibits intimal hyperplasia after injury at least partially through the action of adiponectin.[198] Other factors known to inhibit intimal hyperplasia

include interferon γ, hepatocyte growth factor, interleukin 10, adrenomedulin, somatostatin, and endothelin.[2]

In recent years, it has become clear that the effect of a single ligand is often dictated by the relative expression of receptor isotypes, which can have opposing roles. For example, in the vascular system S1P binds to the GPCRs, S1PR1, 2, and 3. Data indicate that S1PR1 and S1PR3 are stimulators of intimal hyperplasia after injury, while S1PR2 is an inhibitor in this regard.[199] Another GPCR ligand, LPA, binds to LPA1 through 5. LPA1 is inhibitory towards SMC migration, while LPA3 appears to be stimulatory.[200] Prostaglandin E_2 also binds to GPCRs, EP1 through EP4. Receptors EP_1 and EP_3 induce vasoconstriction, whereas EP_2 and EP_4 induce vasodilatation.[201] Activation of EP4 increases ductus intimal cushion formation[202] suggesting the possibility that other EP receptors may mediate the growth inhibitory effects often described for PGE in vitro.

Coagulation and fibrinolytic factors

Arterial injury in pigs and primates is associated with thrombosis, which is usually not occlusive but does release mediators of SMC growth and migration. Rodent models of arterial injury are usually not associated with thrombus formation,[203] although it is possible that intimal hyperplasia is being driven by short-lived microthrombi or by thrombogenic factors such as TF[7], thrombin,[204] and factor Xa[205] at levels too low to generate thrombus formation. While TF drives intimal hyperplasia after injury because of increased SMC migration[7], after double injury TF mediates negative remodelling,[206]

Balloon injury also increases expression of the plasminogen activators, urokinase and tissue-type plasminogen activator, in SMCs (review [207]). The plasminogen activators, in turn, proteolytically activate plasmin, which has a major role in fibrinolysis. Several lines of evidence indicate that urokinase is also required for SMC migration and proliferation.[207,208] In this regard plasma urokinase is a predictor of restenosis.[87] Also of interest, the urokinase receptor interacts with PDGF receptor β which in turn mediates the effects of urokinase on migration and proliferation in a PDGF-independent manner.[209] Finally, in contrast to urokinase, tissue plasminogen activator has been shown to inhibit SMC accumulation after injury and to cause positive arterial remodelling.[207]

Matrix metalloproteinases

MMPs are involved in the regulation of both intimal hyperplasia and remodelling. Arterial injury in numerous species induces the production of a number of MMPs, including MMPs 2, 3, and 9,[208,210] which promote intimal hyperplasia and are associated with BMS restenosis (Table 7.1).[89-92] For example, MMP9 is increased in coronary sinus blood after stent placement and is associated with increased levels of $CD34^+$ progenitor cells,[211] which are predictors of restenosis (Table 7.1). High blood flow-mediated positive remodelling is mediated by MMP9.[212-213] Blockade of MMPs with synthetic drugs has mixed results on intimal hyperplasia and remodelling[2] probably because of the lack of specificity of small molecule inhibitors. In addition, hydroximate-based MMP inhibitors can inhibit MAP kinase signaling and collagen synthesis, which itself can inhibit SMC migration.[2] Finally, there are active site independent effects of MMPs as demonstrated by the inhibitory effect of MMP9 on SMC-mediated collagen gel contraction.[214]

Extracellular matrix/receptors

At late times after arterial injury as SMC proliferation decreases, intimal hyperplasia

continues as the result of ECM accumulation. Restenotic tissue from humans demonstrates lower cell density and substantial amounts of an ECM that differs from primary atherosclerotic lesions from which restenotic lesions arise.[5,215] ECM molecules induced by angioplasty[216] and stenting[217] include type I collagen, elastin, and hyaluronan as well as the proteoglycans versican, perlecan, biglycan, and decorin. The ECM of restenotic lesions has more biglycan and hyaluronan[181,216,218,219] and no decorin, unlike primary plaques.[5] Consistent with lesion formation during restenosis, both biglycan and hyaluronan increase SMC proliferation, while decorin inhibits ECM accumulation after injury.[220-222]

Cellular receptors for ECM components by which SMCs might act to remodel the artery includes the integrins, discoidin domain receptors, TLRs, and CD44, all of which are induced after vascular injury.[223-226] The family of integrins provides the classic example of a binding partner, which is able to mediate subcellular signaling.[227] Blockade of $\alpha v\beta 3$ integrin inhibits intimal hyperplasia without an effect on arterial remodelling.[228] The stimulatory effect of CCN1, an ECM protein upregulated by injury, on intimal hyperplasia may be via interaction with integrins.[229] Discoidin domain receptor 1, first described as a signaling receptor of collagens, is required for SMC migration and MMP production.[230] CD44 binds hyaluronan, collagens, and other ECM molecules and mediates SMC proliferation, migration, and SMC-mediated collagen gel contraction.[231,232] TRL2 and TLR4, which are important components of the innate immune system, recognize not only microbial components but also endogenous molecules such as versican, biglycan, heparan sulfate, and hyaluronan.[233-235] Both TLR2 and TLR4 appear to be required for cuff-mediated intimal hyperplasia[226,236] and TLR4 is also required for high blood flow-mediated outward remodelling.[237] MyD88, which is a major intracellular mediator of TLR signaling, is also required for flow-mediated remodelling.[238]

TARGETS FOR INTERVENTION

Intracellular signaling molecules

mTOR and microtubules

Current interventions that significantly prevent restenosis utilize DES for the local application of either rapamycin or taxol related drugs.[6] The molecular targets of rapamycin and taxol are mTOR and microtubules, respectively (see review[239]). Rapamycin is known to function as an antiproliferative drug through the inhibitory effect of a rapamycin/FKBP12/mTOR complex. This complex inhibits pro-proliferative molecules such as $p70^{s6k}$ and inhibits anti-proliferative molecules such as p27.[239] mTOR has two isoforms, mTORc1 and mTORc2. Inhibition of the latter isoform appears to mediate endothelial cell toxicity that can lead to thrombosis.[240] Newer rapamycin analogs, such as everolimus and zotolorimus, show promise of decreasing problems with late thrombosis.[6] The other primary type of drug currently used in DES is paclitaxel, which binds to the β subunit of tubulin thereby stabilizing microtubules and preventing mitotic spindle formation during cell division. However, this drug also has cell-cycle independent effects on cell spreading and migration.[239]

Transcription factors

One transcription factor that has been targeted is E2F, a family of transcription factors required for DNA synthesis and cell cycle progression. Two large clinical trials of an inhibitor of E2F were based on animal studies in which an E2F decoy (a short

double-stranded oligodeoxynucleotide that binds E2F) blocked intimal hyperplasia. Saphenous vein grafts were treated with the E2F decoy before implantation for coronary (PREVENT IV) or peripheral (PREVENT III) bypass. However, neither trial of 3400 and 1600 patients, respectively, showed an effect on graft failure.[241,242] Unfortunately, the use of a non-selective E2F decoy that inhibits all E2F family members may have cancelled out an effect, since a more recent study indicates that E2F3 stimulates and other E2F family members inhibit SMC proliferation and intimal hyperplasia.[243] Despite these negative results vein grafts still provide an exceptional opportunity for intervention ex vivo before implantation. In preclinical studies, other transcription factor targets using the decoy technology have included Egr-1, which has been implicated in many cardiovascular disorders.[244]

miRNA

It is likely that future targets will include miRNAs, since these small RNA molecules regulate multiple gene products and it is unlikely that a single gene will regulate all pathways of a complex pathology like restenosis. Possible miRNA targets include Mir-21, Mir26a, Mir143/145, and MiR-221, which have been shown to regulate SMC phenotype, growth, death, and migration as well as intimal hyperplasia.[245-248]

Inflammation targets

One anti-inflammatory drug that has been tested is pimecrolimus. This drug binds to the cytosolic receptor FK506 binding protein, which inhibits the calcium-dependent phosphatase calcineurin and the translocation of the transcription factor, nuclear factor of activated T-cells, to the nucleus preventing induction of inflammatory cytokines in T cells and mast cells. Based on animal models this was expected to reduce arterial inflammation and, therefore, neointimal hyperplasia and restenosis.[249] Instead, patients treated with pimecrolimus-eluting stents were reported to fare worse than patients treated with stents that delivered a combination of pimecrolimus and paclitaxel, or paclitaxel alone.[250] However, other drugs are being tested. An anti-inflammatory drug with a long history, salicylic acid, is being tested as a component of the biodegradable backbone of a stent that also has a coat of sirolimus.[251] Finally, liposomal alendronate, a bisphosphanate compound that depletes monocytes and inhibits restenosis in rat and rabbit models of injury, is in phase II trials testing its effects on BMS restenosis (http://clinicaltrials.gov/ct2/show/NCT00739466). Overall it appears that targeting inflammation alone may be inadequate to inhibit restenosis, although it may be effective as an adjunctive target.[249]

Brachytherapy

Brachytherapy (radiation treatment) has shown success as an adjunctive therapy for BMS restenosis after successful balloon angioplasty.[6] While brachytherapy inhibits both negative remodelling and intimal hyperplasia, one limitation found initially was hyperplasia at the ends of the stents or the angioplasty zone when radiation was not complete.[5,252] In addition, prior brachytherapy is a risk factor for stent thrombosis.[6] Brachytherapy is less common now because of procedural logistics, concern of long-term thrombosis and delayed restenosis, but more importantly the availability of DES.[6]

Extracellular targets and cell-based therapies

Angiotensin pathway

While angiotensin II type 1 receptors mediate vascular SMC migration, proliferation, and extracellular matrix production after arterial

injury and angiotensin-converting enzyme inhibitors or specific receptor antagonists reduced intimal hyperplasia in several animal models, large scale trials of the angiotensin-converting enzyme inhibitor, cilazapril, for balloon angioplasty or BMS failed to show benefit.[120] However, more recent studies show that neointimal hyperplasia is inhibited by the angiotensin-converting enzyme inhibitor, quinapril, in patients with the D/D and I/D genotypes of angiotensin-converting enzyme.[253,254] In addition, use of the angiotensin II type 1 receptor antagonist, valsartin, decreases the incidence of stent restenosis[255] and a valsartin-eluting stent is equivalent to a rapamycin-eluting stent.[256] These data suggest that angiotensin II type 1 receptor antagonists may be useful in preventing restenosis.

Cell-based therapies

Use of engineered allogeneic endothelial cells applied to the adventitial surface to prevent restenosis in peripheral interventions is in Phase I/II trials at this time (http://clinicaltrials.gov/ct2/show/NCT01099215). This trial is based on work in a porcine stent model[257] and utilizes the same concept as a trial aimed at inhibiting stenosis of arterio-venous fistula bypass grafts.[258] A Phase IV study involving endothelial cells utilizes a coronary stent with immobilised anti-CD34 antibody with which to capture circulating endothelial progenitor cells (http://clinicaltrials.gov/ct2/show/NCT00494247).[259] Finally, the possibility of bioengineered bypass grafts developed from induced pluripotent stem cells or other autologous progenitors holds promise as these may also be engineered to express or repress targets of choice.[260]

Differential effects on endothelium and SMCs

A differential effect on endothelium compared to SMCs is a helpful attribute for any target for restenosis. The lack of selectivity is a problem for rapamycin for which effects on endothelium mediate its thrombotic side effects. A20 is a zinc finger protein that prevents intimal hyperplasia in the injured rat carotid artery[261]. Expression of A20 in medial SMCs prevents neointima formation by shutting down inflammation and proliferation via inhibition of NF-κB and by increased expression of the cell cycle dependent kinase inhibitors $p21^{waf1}$ and $p27^{kip1}$. However, A20 is anti-inflammatory in endothelial cells by inhibiting NFκB, but this is antiapoptotic via inhibition of the activation of caspase 8. Therefore, A20 has protective effects for endothelial cells and anti-proliferative effects on SMCs making it a good candidate for inhibiting SMC accumulation while minimizing endothelial damage. Another differentially effective target may be Nogo. Nogo-B is expressed by both endothelial cells and SMCs, but it increases endothelial cell migration and inhibits SMC migration. Nogo-B is down-regulated after injury and intimal hyperplasia is greatly increased in the Nogo-B null mouse. Transfection of the murine arterial adventitia or porcine vein graft adventitia with an adenoviral vector of Nogo-B greatly inhibited intimal hyperplasia.[262]

Delivery devices

Garg et al have recently reviewed the use of DES and future design technology for the coronary arteries.[6,251] The issue of drug-eluting polymers is an area of advancement with next generation polymers, including biodegradable polymers, avoiding many of the past problems with allergic and inflammatory reactions. In addition, biodegradable stents with and without anti-proliferative drugs are under development. These would avoid long term issues of inflammation from a foreign body. Stents with multiple therapeutic targets are under

development. One example is a DES that targets SMC proliferation and platelets with sirolimus and cilostazol, respectively. Bi-directional drug delivery from stents may allow functional separation of effects on SMCs and endothelial cells. Finally, as more is learned about the effects of co-morbitities on restenosis, such as diabetes, specific DES or drug-eluting balloons may be developed to use in subsets of patients.

A common treatment of in-stent restenosis is the use of DES. However, it is unclear if another stent adds to the risk of stent thrombosis or reoccurrence of restenosis. Drug-eluting balloons are being studied as an alternative for this situation. In addition, DES have not shown benefit in femoropopliteal arteries, which are often significantly occluded throughout their length and are subject to forces not experienced by coronary arteries (e.g. compression and bending) that may cause strut fracture.[263] Drug-eluting balloons have shown promise on peripheral artery lesions.[264] One advantage of drug-eluting balloons is illustrated by a study of the use of nanoparticles, since the nanoparticles move further into the vascular wall before eluting their drug cargo and thus avoid endothelial cell toxicity.[125] There are many differences between DES and drug-eluting balloons including the issue of drug delivery kinetics. These as well as current and past trials with drug-eluting balloons are reviewed by Gray.[265]

Prevention vs. reversal of restenosis

Because current strategies to treat restenosis are intended to prevent intimal hyperplasia, all patients must be treated, even though less than one-third of all vascular interventions fail. Other options are to develop the ability to screen for those patients at high risk or to develop a treatment that would reverse the restenotic process.[261] Observations of various SNPs and soluble factors associated with restenosis (Table 7.1) suggest that screening may be possible in the future. The possibility of reversing the restenotic process comes from the observation that intima formation in stents increases for about 3 months, but starts regressing spontaneously after 6 months[261], as well as effects of anti-hypertensive drugs and of A20, which induce vascular regression via SMC apoptosis.[261,266] In addition, when global gene expression is compared in two distinct models of vascular regression in non-human primates, only 7 genes are regulated in the same manner in *both* models of atrophy (Kenagy et al, in press). Six of these atrophy-associated genes are also induced in vitro by the ligand for the death receptor Fas, suggesting that these genes are an important part of the cell death program active during atrophy. Also, a third of the up-regulated genes (ADAMTS4, tissue plasminogen activator, and hyaluronidase2) degrade components of the ECM, loss of which is a major feature of vascular atrophy. These commonly regulated genes may play a fundamental role in vascular atrophy and may lead to drug candidates useful for reversing restenosis in those situations where intimal hyperplasia is the primary cause.

CONCLUSIONS

Restenosis is primarily a problem of excessive intimal hyperplasia and negative remodelling. Human risk factors for restenosis include diabetes, renal failure, factors associated with decreased blood flow, factors leading to decreased growth inhibition, factors leading to increased growth stimulation, as well as increased inflammation (Table 7.1). These data are consistent with data from animal models of intimal hyperplasia and support further research into how these factors impact restenosis. While animal models have been useful for understanding basic mechanisms

of restenosis, the predictive power of animal models for clinical efficacy is still unclear, particularly concerning peripheral vessels. Correlating animal models and clinical application is an active area of research.[267] In addition, most clinical studies are of the coronary circulation. The degree to which results in the coronary circulation match the peripheral circulation is not known, but there are differences. Coronary and peripheral vessels are embryologically distinct,[268] and injury of the coronary artery leads to greater intima formation than does injury of peripheral arteries in pigs and dogs.[269-271] In addition, the femoropopliteal arteries, but not the coronary arteries, are subject to the forces of compression, bending, twisting, and axial changes caused by joint flexion and external impact, which may have a significant effect on restenosis.

Our greatest success at preventing restenosis, DES, comes as much as a result of engineering than of drug development. As endovascular techniques continue to evolve, the efficacy of drug targets should keep pace. Unfortunately, the early promise of the Human Genome Project for new therapeutics aimed at human disease such as restenosis has not been forthcoming. Genome-wide association studies, designed to reveal markers or potential causal factors, have revealed that cardiovascular pathologies have a complex genetic structure. While >20,000 genes and more regulatory factors of the RNA world have been identified, how these factors are related remains largely unknown. However, the field of systems biology is poised to have a significant impact on vascular biology over the coming decade. New types of analysis have revealed networks that can explain up to 50% of the variance of a complex clinical phenotype.[272-273] It is hoped that a systems biology or similar approach will soon reveal key networks and regulatory hub molecules that control restenosis. Based on research to date it is likely that networks regulating cell differentiation, growth, inflammation, and ECM production, major aspects of intimal hyperplasia, will be featured and that hub molecules that link these networks will be identified as promising therapeutic targets.

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8 • Vascular Arterial Haemodynamics

MICHAEL MD LAWRENCE-BROWN[1], KURT LIFFMAN[1,2,3]
JAMES B SEMMENS[1], ILIJA D SUTALO[1,2]

[1]Curtin Health Innovation Research Institute, Curtin University, Perth, Western Australia, Australia

[2] Materials Science and Engineering, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Highett, Victoria, Australia

[3] School of Mathematical Sciences, Monash University, Victoria, Australia

INTRODUCTION

Vascular interventions have developed rapidly since the first aortic replacement with Dacron by Dubois in 1952. Understanding vascular haemodynamics and the biological response to implanted materials is essential for vascular surgeons and scientists developing new interventional technologies.[1,2]

This chapter will summarise and discuss the following laws, equations and phenomena to give a basic understanding of the haemodynamic principles of the conduits and fluids with which we work:

- Laplace's law of wall tension
- Newtonian Fluid
- Non-Newtonian fluid
- Poiseuille Flow
- Bernoulli's equation
- Young's modulus and pulsatile flow
- Mass conversation
- Reynolds' number: laminar and turbulent flow
- Shear stress and pressure
- Forces on graft systems
- Computational modelling

For those who understand electrical circuit theory, there is much similarity with haemodynamics. Understanding the physiology and physics of blood flow is aided by the use of that recognition. When considering fluid dynamics instead of:

$$V = IR, \tag{1}$$

where V is the voltage, I is the current and R is the electrical resistance. This formula maybe substituted by:

$$P = QR, \tag{2}$$

with P the pressure, Q the volume flow rate and R the flow resistance.

Resistors in series and parallel govern degrees of ischaemia and the behaviour of blood flow and contribution of collaterals, and hence degree of ischaemia of limbs and organs.

The great vessels, like the aorta, are without muscle and their walls are composed of collagen and elastin fibres. This allows them

to behave as capacitors and store some of the energy in systole to be released to power flow in diastole, that is so important *into* vessels such as the coronary artery. The elastic arteries stiffen with age and explain the flow changes that occur with ageing and for progressive arterial disease due to this most important of all the risk factors.

LAPLACE'S LAW OF WALL TENSION

Laplace's law relates the tension in an arterial or venous wall with the pressure that the elastic tube can apply to material inside the tube. To assist in understanding this law we consider Figure 8.1. In this figure, w, represents the thickness of the arterial wall, r is the inner radius of the artery, P the inward pressure force due to the elastic nature of the artery and T is tensional stress within the wall of the vessel, where the tensional stress points in a direction that is tangential to the vessel wall. Due to mass conservation the wall thins as the vessel expands.

The formula for Laplace's law is given by the Eq.:

$$P = \frac{w}{r} T, \tag{3}$$

where it is usually assumed that the wall thickness, w is small relative to r. This law tells us that the inward pressure that is exerted by the vessel wall on the blood is directly proportional to the tensional stress in the wall and inversely proportional to the radius of the wall. Thus the smaller the vessel the larger the pressure it can apply on the blood.

Large thin-walled vessels are low pressure vessels. Increasing the pressure distends the vessel and increases the vessel volume which is a characteristic property of veins. For arteries to maintain pressure, the width of the wall must obviously be greater, so large veins are thin-walled and arteries are thick-walled.

One consequence of this behaviour is that, to a certain extent, an artery acts like a long cylindrical party balloon. When one attempts to blow up such a balloon, it is quite difficult to do at the first blow, however once the balloon reaches a particular radius, it usually becomes much easier to expand the balloon. That is you require less pressure to increase the size of the balloon. This phenomenon is known as instability. If this happens to an artery, then we are dealing with an aneurysm and the relatively constant blood pressure will keep on increasing the size of the aneurysm.

The radius of the artery at which this instability occurs is difficult to compute accurately, but some fairly general arguments suggest that the following formula is a good guide: where r_c is the critical radius for the onset

$$r_c \sim 2r_0, \tag{4}$$

of the instability and r_0 is the initial radius of the artery. The median diameter of the aorta is 23 mm and the thus aortic rupture is very rare when less than 50 mm in diameter, which is consistent with recent clinical

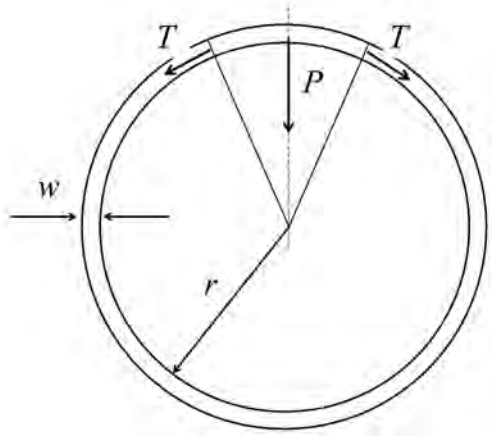

FIGURE 8.1: Cross section of an artery showing the various physical components that make up Laplace's law.

data.[3,4] This guide also directs us to consider that the ratio of the diameters is probably more important than the absolute diameter and this should be taken into account when assessing aneurysms in the smaller diameter vessels of women. How arterial wall instability arises is illustrated in Figure 8.2, where in Figure 8.2(a) we show the stress structure within a small artery. Here the tensile stresses have a component in the radial direction, where the letter T labels this component. In Figure 8.2(b) the aneurysm/balloon has become very large, such that over a small segment of the wall the artery has hardly any curvature. This is an extreme case, but it does show that there is now no radial component to the tensile stresses. In such a case, the aneurysm can expand freely for just about any internal arterial pressure.

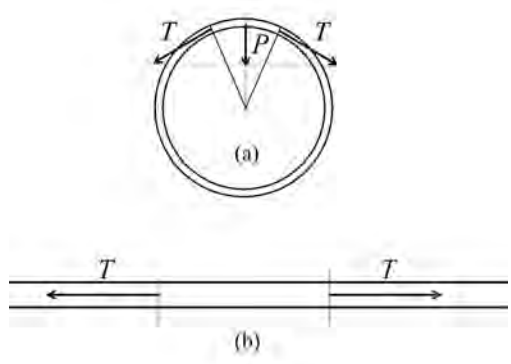

FIGURE 8.2: Cross sections of a small artery (a) and a very large artery (b) showing the stress distribution within the artery.

NEWTONIAN FLUID

When we wish to describe the behaviour of a fluid it is necessary to know something about the frictional properties of the fluid. Consider the schematic depiction of a fluid shown in Figure 8.3. In this figure, fluid is flowing from left to right along the x direction. For purposes of illustration, we assume that the speed of the fluid, u, is increasing with increasing height (*i.e.*, increasing y). This means that elements of fluid are sliding past each other and so generate some frictional stress τ. In a Newtonian fluid, the frictional stress is proportional to the rate at which the speed changes as a function of distance, where μ is the viscosity (Eq (5)). The du/dy in equation (5) corresponds to the shear rate. To a reasonable approximation, one can assume that blood is a Newtonian fluid, at least for flow along the major arteries.

$$\tau = \mu \frac{du}{dy}, \tag{5}$$

NON-NEWTONIAN FLUID

Non-Newtonian fluids have a viscosity that depends on the strain rate. A shear thinning fluid is a fluid that changes from "thick" to "thin" when force is applied to the fluid. Examples of such fluids are shampoos and paints. This behaviour usually occurs, because, at rest, a shear thinning fluid typically has a tangled molecular structure, which makes the fluid relatively viscous. When force is applied, the molecules become ordered, the fluid viscosity decreases and the fluid begins to flow more easily. In Figure 8.4 we show the experimentally determined shear thinning behaviour of blood, where the hematocrit value for the blood is 45%. These data show that for high shear rates, which may occur in the large arteries of the body, the viscosity of blood is about four times that of water (where the viscosity of water is approximately one centipoise (cP)). However for lower shear rates, the viscosity of blood can be over one hundred times that of water.

This change in viscosity is mostly due to the collective behaviour of red blood cells. At low shear rates, red blood cells form aggregates where they stack one upon another, somewhat like a cylindrical pile of coins. These "stacks" of red blood cells are known

as "rouleaux" (Figure 8.5). When the shear rate increases, these aggregates of blood cells are broken down and the blood viscosity decreases. For high shear rates, the blood cells tend to become elongated and line up with the flow of the liquid. This also tends to decrease the viscosity of the blood.

Given that the viscosity of blood increases with decreasing shear, one would think that the viscosity of blood within the body should increase as blood travels from the arteries through the arterioles and into the capillaries. This is because the shear rate and velocity of the blood decreases as the blood travels from the arteries through to the capillaries. The viscosity of blood, however, may be approximately constant throughout much of the body. This

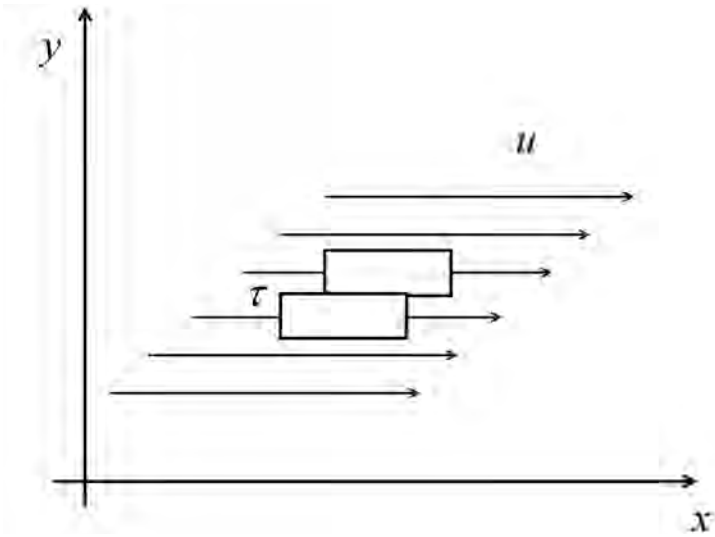

FIGURE 8.3: Elements of fluid slide past each other and generate a frictional shear stress.

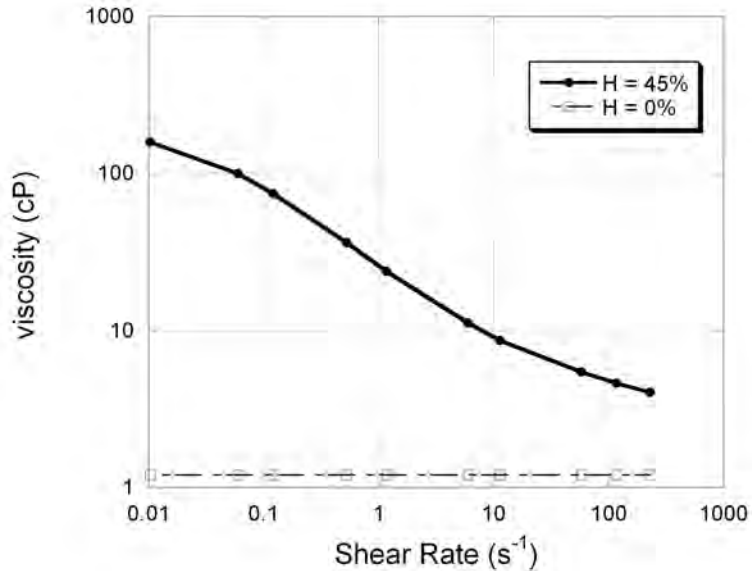

FIGURE 8.4: Blood viscosity as a function of shear rate for 0% and 45% hematocrit.[5]

effect arises due to separate physical flow phenomena:

First, the viscosity of blood is dependent on the hematocrit. If the hematocrit decreases then the blood viscosity decreases. For example in Figure 8.4 we show the viscosity of blood as a function of shear rate for 45% and 0% hematocrit. For 0% hematocrit line the viscosity of the blood is constant and has a value of approximately 1.6 cP.

Second, the hematocrit level is dependent on the diameter of the blood vessel. As the blood vessel decreases in diameter, the hematocrit level also decreases (Figure 8.6). This effect occurs because the blood cells tend to move away from the vessel walls and travel where the flow velocity is a maximum. This behaviour is known as the Fahraeus Effect and it has been shown to occur in tubes with a diameter as small as 29 μm.[6,7] Given that a blood cell has a diameter of around 8 μm it is possible that the Fahraeus Effect may occur in tubes with diameters less than 29 μm.

The combination of these two effects implies that the viscosity of blood is approximately constant throughout the body. Understanding these properties affects the thinking of shear stress between blood and

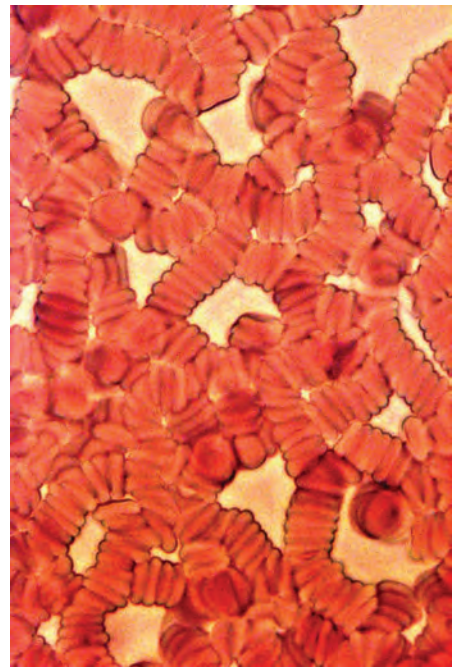

FIGURE 8.5: Rouleaux blood cell network.

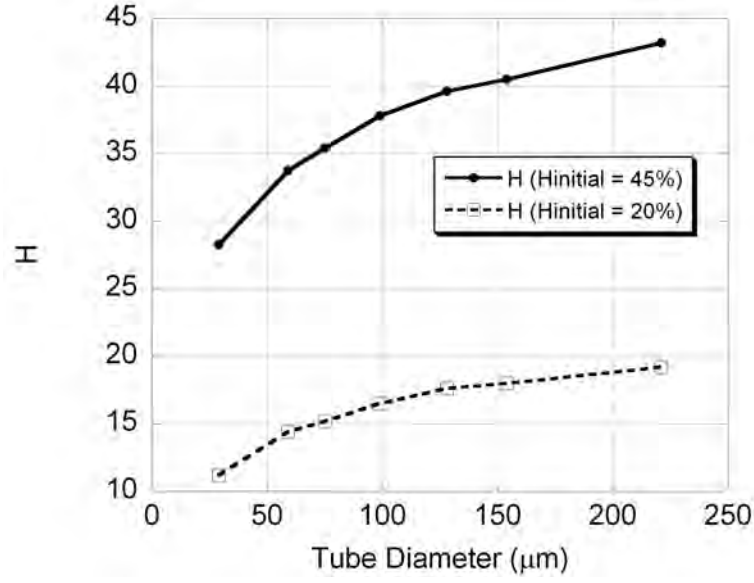

FIGURE 8.6: Hematocrit as a function of tube diameter. The initial hematocrit value for each line is shown in the inset box[8].

vessel walls – or more relevantly between blood and atheroma.

POISEUILLE FLOW

Suppose that you have a Newtonian fluid flowing, in a steady, non-pulsatile manner, down a cylindrical, non-elastic pipe of length L and radius a. If the pipe is long enough, the flow will develop a parabolic velocity profile, which is generally called a Poiseuille flow profile (Figure 8.7). The flow takes its name from Jean Louis Poiseuille, a physician with training in physics and mathematics, who first described the flow structure in 1846.

The volumetric flow rate (q) for Poiseuille flow, *i.e.*, the volume of fluid flowing along the tube per unit time is given by the formula

$$q = \frac{(p_1 - p_2)\pi a^4}{8\mu L}, \qquad (6)$$

where $p_1 - p_2$ is the pressure difference between the two ends of the tube and μ is the viscosity of the fluid.

The physics of the flow is nicely described by this equation. That is, flow is driven by the pressure gradient in the tube or conversely, when there is flow in a tube then you must have a pressure gradient to drive the flow.

Prostheses are subject to the intermittent forces of pulsation and flow. The large elastic vessels are capacitors and provide on-flow in diastole and the muscular peripheral vessels maintain pressure by altering resistance mediated via physiological feedback. Current prostheses are not able to do this and have to withstand the forces.

Note also the parameter of length. Flow is therefore also related to length. Patency, such as in femoro-popliteal synthetic conduits, maybe as much, if not more, related to length of conduit as it is to angulation across bend points depending on the haematological factors depositing thrombus. This may also partly explain better patency in shorter bypass grafts.

BERNOULLI'S EQUATION

Johann Bernoulli (1667–1748) was a professor in Basel and taught physics, anatomy and physiology and his understanding lies at the heart of vascular physics and relates pressure to motion and energy. For a fluid that has no viscosity, one can write

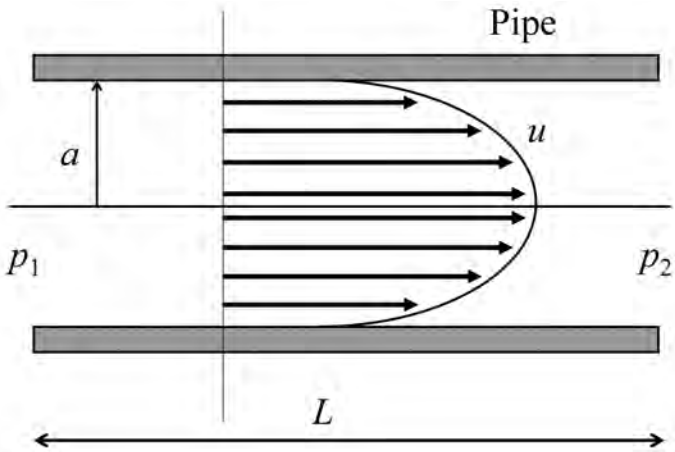

FIGURE 8.7: Parabolic velocity profile for fully developed Poiseuille flow.

$$p + \rho\frac{u^2}{2} + \rho gy = \text{constant of the flow,} \qquad (7)$$

where p is the pressure, ρ the mass density of the liquid, u the speed of the fluid, g the gravitational acceleration, and y the height. In other words, the Bernoulli equation states that the pressure plus the kinetic energy per unit volume, $\rho\frac{u^2}{2}$, plus the potential energy per unit volume, ρgy, is a constant at any point along the blood vessel. So for a constant height, an increase in flow speed implies a decrease in pressure, while for constant flow speed, an increase in height implies a decrease in pressure.

It should be understood that Eq. (7) is an approximation, as it ignores the loss of energy due to shearing friction between the flowing blood and the walls of the artery. Even so, it does provide us with an intuitive understanding of the physics of the arterial/venous system. For example, suppose we wish to measure the blood pressure of a person. Typically one places a sleeve or an external cuff around the upper arm. The upper arm is chosen because it is at approximately the same level of the heart and so the pressure will not be affected by any difference in height. To measure the systolic pressure, the cuff pressure is increased until all blood flow ceases from Eq. (7) we know that this "cut-off" pressure is the maximum pressure in the artery. The pressure in the external cuff is then decreased until the flow is a maximum. We then know that the pressure will be a minimum and this is the diastolic pressure in the artery.

In practice, the arterial system has two sources of potential energy to drive the blood forward. The first is blood pressure and this is transformed into kinetic energy of flow during the period between systole and diastole, and the second is stored energy in the wall of the artery – its capacitance. Consider what might happen when the kinetic energy meets a resistive obstacle – some energy is dissipated as heat as with circuit theory and some is stored for use in diastole for onward flow in the period of heart filling by the elasticity of the great vessels acting as a capacitor. However, some energy is used up as a water hammer. The repetitive alterations in forward pressure and resistive back-pressure with pulsatile flow in a physiologically responding, pressurized system sets up the potential for the water hammer. The injury and healing cycle effect of these water hammers on atherogenesis and aneurysm behaviour at stress points has yet to be fully determined.

YOUNG'S MODULUS AND PULSATILE FLOW

Blood flows through the arteries in a pulsatile fashion. Arteries are semi-elastic tubes and the arteries expand and contract as the pulse of blood flows along the artery. The speed, c, at which blood flows along an artery is determined by the speed that a pulse of fluid can travel along an elastic tube. This speed is given, approximately, by the Moen-Korteweg formula:

$$c \approx \sqrt{\frac{Eh}{\rho d}}, \qquad (8)$$

where E is Young's modulus for the wall of the artery, h is the thickness of the artery, d is the inner diameter of the artery and ρ is the density of blood. A schematic depiction of how a pulsatile wave propagates along an artery is given in Figure 8.8.

As can be seen from Eq. (8), the speed at which blood travels along an artery is partially dependent on the Young's Modulus of the arterial wall. To illustrate the definition of Young's Modulus it is useful to consider Figure 8.9, where a block of material is being stretched due to an applied force on one end of the block.

The block has a natural length denoted by L, when a force F is applied to one side of the block then the length of the block increases by ΔL. This change in length is known as a *strain*, ε, and it is defined by the equation

$$\varepsilon = \frac{\Delta L}{L}. \tag{9}$$

The *stress* that the force applies to the block of material has the definition

$$\sigma = \frac{F}{A}. \tag{10}$$

Young's Modulus is defined as the stress over the strain, *i.e.*,

$$E = \frac{\sigma}{\varepsilon}. \tag{11}$$

Young's modulus is a measure of how easy it is to stretch and compress a material. Thomas Young (1773 – 1829) was a medical physician who made significant contributions to fields of Physics (through his experiments which demonstrated the wave-like nature of light), linguistics (via his identification of the Rosetta Stone), medicine (with his studies of blood flow), and structural mechanics (*e.g.*, Young's Modulus). He was well aware of

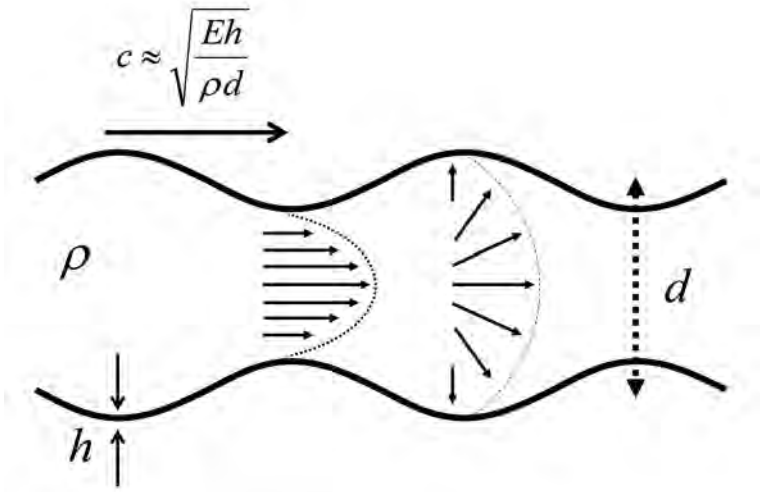

FIGURE 8.8: An exaggerated, schematic view of blood flow in an artery.

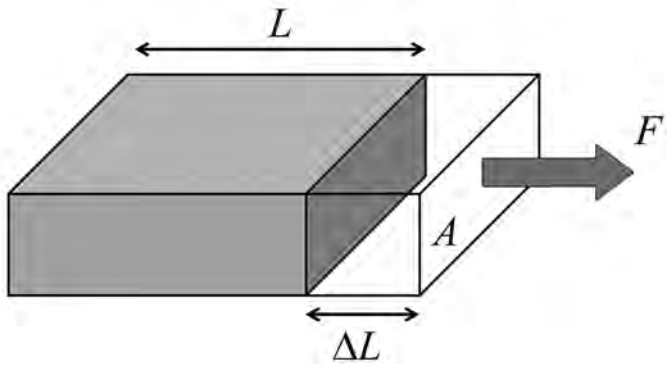

FIGURE 8.9: A block of material with a length, L, and side area A is subject to a force F. The applied force stretches the block a distance ΔL.

the elastic nature of arteries, but, somewhat ironically, does not appear to have used Young's Modulus to describe their properties.

One consequence of aging is increasing stiffness in the arteries. This means that the Young's modulus increases and this, as a consequence of Eq. (8), increases the speed of pulsatile flow within the arterial system.

MASS CONVERSION

In Figure 8.10 we view a schematic depiction of an artery that is changing in shape as one travels along the artery. The blood flows in at one end with a speed u_1. The area at the inlet of the artery is given by A_1. In its simplest form, the mass conservation equation provides us with the relationship between the quantities at the proximal and distal ends of the artery:

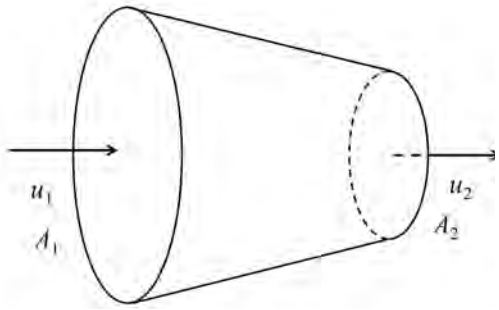

FIGURE 8.10: A change in the diameter of an artery leads to a change in the blood flow speed.

$$u_1 A_1 = u_2 A_2, \tag{12}$$

here u_2 and A_2 are the outlet flow speed and area, respectively. In plain English, Eq. (12) is another way of saying "what goes in must come out".

We can see from Eq. (12) that if an artery becomes narrower, i.e., A_2 becomes smaller, then the flow speed, u_2, increases. This occurs because the mass flow is conserved and so if the tube becomes narrower then the flow rate has to increase.

Some diseased blood vessels develop a constriction or stenosis (Figure 8.11). This narrowing of the blood vessel wall may be caused by atherosclerosis or neo-intimal hyperplasia after an intervention. If we assume steady-state, Newtonian blood flow and ignore gravity then the pressure in a compromised blood vessel with a stenosis can be calculated by combining Bernoulli's equation (equation 7) and the mass conservation (equation 12) to obtain

$$p_1 + \frac{\rho v_1^2}{2} = p_2 + \frac{\rho}{2}\left(\frac{v_1 A_1}{A_2}\right)^2, \tag{13}$$

$$p_2 = p_1 + \frac{\rho v_1^2}{2}\left(1 - \left(\frac{A_1}{A_2}\right)^2\right). \tag{14}$$

Since $A_2 < A_1$ the energy last term of equation 14 becomes negative so then the blood

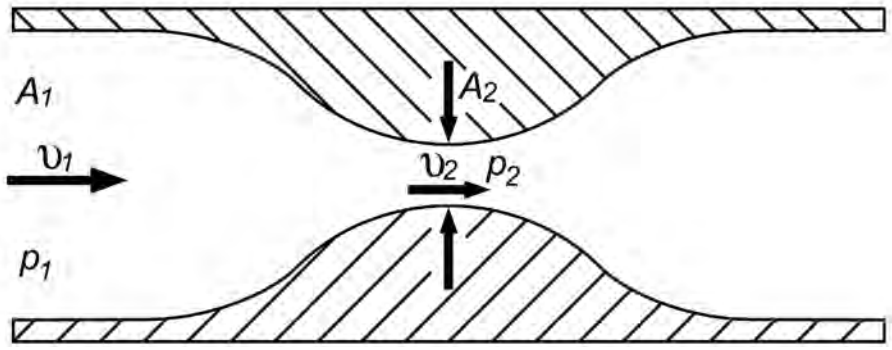

FIGURE 8.11: Blood vessel with a stenosis.

pressure is lower at the stenosed section of the blood vessel (the constriction) to ensure that the sum of the pressure and energy at each point along the blood vessel remains equal. The lower pressure at the stenosis makes the blood vessel with a stenosis more prone to collapse if an external pressure were applied to the blood vessel. Stents or drug -eluting stents may be inserted in an artery that has a stenosis to keep the artery open after the blockage has been cleared using angioplasty. Mass conservation shows that the velocity is higher at the stenosis due to a smaller area at the stenosis.

REYNOLD'S NUMBER

The Reynolds' number (Re) is a dimensionless number, which provides an indication of how blood is flowing in an artery. The Reynolds' number is given by:

$$\mathrm{Re} = \frac{UD\rho}{\mu}, \tag{15}$$

where U is the speed of the flow, D is the diameter of the blood vessel, ρ the blood density and μ the blood viscosity. For an artery, the flow tends to change from laminar to turbulent at a Reynolds' number of approximately 2000. This number should be treated as only a representative value, since the transition from laminar to turbulent flow may occur at higher Reynolds' numbers.

To see a representative peak value of Reynolds' number, we consider an abdominal aorta of diameter, D = 2.5 cm = 0.025 m, peak blood flow speed U = 60 cm/s = 0.6 m/s, blood density ρ = 1 gram/cc = 1000 kg/m^3 and blood viscosity μ = 0.0036 Pa s. These values give Re ≈ 4,200. So, in principle, it is possible for turbulent flow to occur in the aorta during the systolic phase.

Fluid flowing in a laminar fashion is dominated by the viscosity and at a high Reynolds' number by its inertia. A bruit is audible chaotic flow at high velocity with energy transformed to noise – inefficient flow that maybe disruptive as in a carotid stenosis – and blood needs to be able to flow fast in order to deliver its load at a cardiac output of up to 30L/min in an athlete.

Turbulent flow is less efficient relative to laminar flow. This means that more energy or a greater pressure drop is required to drive turbulent flow compared to laminar flow. A quantitative way of measuring this inefficiency is given by the formula for energy or "head" loss for flow along a pipe

$$h_L = f\,\frac{L}{D}\frac{U^2}{2g}, \tag{16}$$

where f is the loss coefficient, L the length of the artery or appropriate subsection of an artery and g the acceleration due to gravity. For laminar flow,

$$f_{lam} = \frac{64}{\mathrm{Re}}, \tag{17}$$

while for turbulent flow

$$f_{lturb} \sim \frac{0.316}{\mathrm{Re}^{1/4}}. \tag{18}$$

One can show that $f_{\mathrm{turb}} > f_{\mathrm{lam}}$ when Re > 1200, which implies that turbulence consumes more energy relative to laminar flow. This result is represented schematically in Figure 8.12, where we have plotted the ratio $f_{\mathrm{turb}}/f_{\mathrm{lam}}$ as a function of Re. Here we see that at a Reynolds' number of around 2000, turbulent flow loses 1.5 times more energy relative to laminar flow. As Re approaches 5000 turbulent flow tends to lose 3 times as much energy as laminar flow.

It is interesting to speculate that the particulate nature of blood and plasma composition may act to discourage the formation of turbulent flow. Each red cell, being bi-concave, could change the local interactions between the cells and the blood plasma so that the flow tends to remain laminar. The shape of the red cell then may enhance the efficiency of blood flow, in addi-

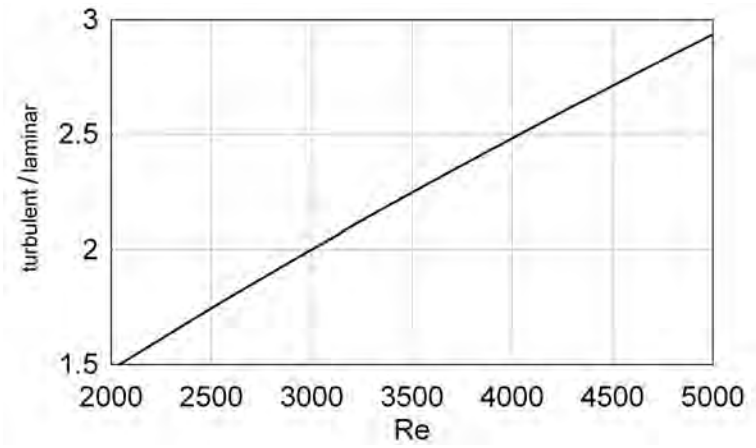

FIGURE 8.12: Plot of the ratio of turbulent to laminar energy loss coefficients.

tion to increasing surface area for oxygen delivery.

ARTERIAL DISSECTION, COLLATERAL CIRCULATION AND COMPETING FLOWS

To this point we have essentially discussed flow in series. Much of the normal flow and some pathological flow occurs in parallel. For example, the collateral circulation in each segment of the body; the profunda system in the thigh, the geniculate system around the knee and the tibial systems in the leg. Another good example is the carotid and vertebral systems combining to form the cerebral circulation. In parallel circulation, the pressure at the separation of the two systems is theoretically the same for each, and the pressure at the re-union is also the same for each. The proportion of ongoing flow from the two systems is determined by the resistance of each system. These two therefore compete for the proportion of on-flow. This works well to direct or redirect the flow to the target tissues. The body may select priorities for flow, for example, the brain and heart in shock or the muscles during exercise. The branches of the great vessels and arteries to the tissues are resistance vessels and they have muscular walls for this purpose. The formula for resistors in parallel circuits is

$$\frac{1}{R_{total}} = \frac{1}{R_1} + \frac{1}{R_2} + \ldots + \frac{1}{R_n}, \qquad (19)$$

where n is the number of parallel circuits.

These circuits also provide alternative channels should the dynamics change due to injury or disease. Not all parallel circuits are beneficial. Detrimental competing flows may occur with artificially created channels, for example, aorto-bifemoral bypass, when one iliac system is normal and the other occluded. The competing flows on the normal side predispose for either that limb of the graft or part of the iliac system on that side to occlude. Similarly, with femoro-popliteal bypass after long-standing superficial femoral artery occlusion when the profunda collateral flow has been well developed.

In aortic dissection, the outflow from the false lumen is met with greater resistance than the outflow from the true lumen. The flows compete where the intima has been torn off the origin of a branch vessel which therefore comes off the false lumen and leaves a hole in the membrane at that point. The pressure

is higher in the false lumen at any time in the cardiac cycle other than peak systole.

Figure 8.13 shows the trace from true and false lumens of a dissected aorta. Note the systolic pressure is same in each lumen at 138 mmHg. The diastolic is higher in the false lumen at 93 mmHg compared to the diastolic in the true lumen of 82 mmHg. The area under the curve is the same and so the pulse wave in the false lumen is wider. The mean pressure in the false lumen is higher at 109 mmHg than the true lumen where the mean is 91 mmHg.

This means that the false lumen is almost always the larger of the two and is more likely to dilate. Flow of contrast injected into the true lumen is not seen to flow out to the false lumen through the holes in the membrane unless the pressure of the injection and the pressure of the lumen together exceed the pressure of the false lumen. The membrane that is the remnant of the intima oscillates as the pressure ratio between the true and false lumen changes during the cardiac cycle. This dynamic also applies for a Type 1 endoleak into the residual sac of an aortic aneurysm treated by an endovascular graft.

SHEAR STRESS AND PRESSURE

All vascular clinicians are familiar with the ultimate shearing force injury of high velocity impact when the mobile arch of the aorta and heart continue to move forward while the descending aorta, held by the intercostals and posterior mediastinum, is held to the vertebral bodies. What of subtle persistent long-term shear stresses and the relationship with the greatest risk factor for arterial disease – age? There are known common sites for occlusive atheromatous plaques e.g. the carotid bifurcation, aortic bifurcation, origins of branches of the aorta and coronary arteries and shear stress points such as the adductor canal.

Atheroma is an arterial lesion. Occlusive and dilating diseases of the arteries progressively occur with ageing, and obviously age is the greatest risk factor. It is not seen in children and only seen in veins subject to long term pulsatile pressure when they are said to be "arterialised". For example, when a vein is used for an arterial bypass or for a dialysis fistula. Pressure and pulsatility are the forces involved. Persistent raised blood pressure above the norm causes progressive

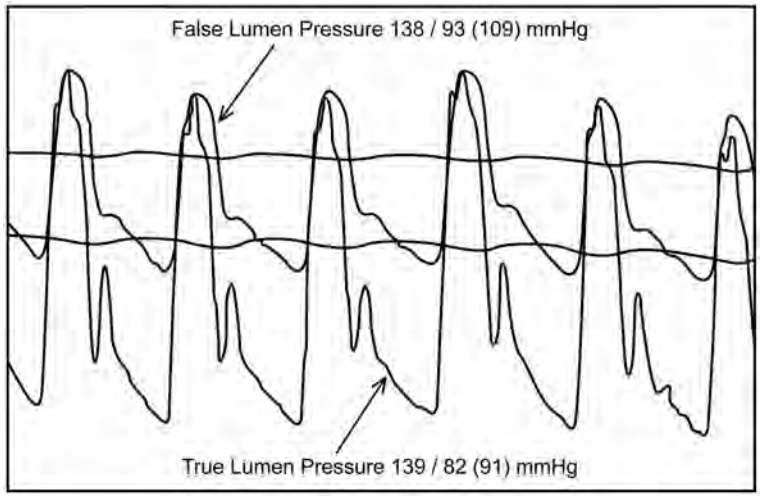

FIGURE 8.13: Pressure readings from the true and false lumens of a dissected abdominal aorta (courtesy of Dr John Anderson).

wall damage. With age there is degeneration of the wall of the artery and loss of compliance. Pulse pressure and peak systolic pressures rise because of the loss of compliance. Peaks of pressure occur with exertion and acute damage may occur at such times. Age will eventually affect all, but some are more genetically predisposed to arterial lesions and other risk factors such as poor diet and smoking accelerate any genetic predisposition.

Shear stress on an arterial wall, τ_w, due to Poiseuille fluid flow is given by the formula

$$\tau_w = \frac{4\mu q}{\pi a^3}, \qquad (20)$$

where a is the radius of the artery and q is the volume flow rate of blood through the artery. From this formula it can be seen that shear stress increases with the increase of blood flow through the artery and tends to increase as the artery becomes smaller in diameter – provided that the volume flow rate and the viscosity are approximately constant.

Atherosclerotic lesions form at specific areas where low and oscillatory endothelial shear stress occur. High risk plaques have a large lipid core, thin and inflamed fibrous cap and excessive expansive remodelling[9]. Wall shear stress may rupture the established plaque. Plaque rupture and intraplaque haemorrhage are recognized causes of cardiac events. Computational modelling of carotid bifurcations with atherosclerotic plaques that had patient-specific geometries obtained from Magnetic Resonance Imaging (MRI) scans and modelled the fluid-structure interactions have shown that stresses in the fibrous cap and around the plaque shoulders affect plaque rupture risk, with higher stress and plaque rupture risk for thinner caps.[10-13]

FORCES ON GRAFT SYSTEMS

The performance of endoluminal grafts (ELG) was found to be different to open repair with a sewn replacement of the artery because of unsuspected influences, as mentioned above, that relate to sustained physical forces.[1] The openly-sewn prosthesis binds the wall of the artery to the prosthesis with a transmural suture. The artery may expand above or below the prosthesis. However, at the point of attachment the artery wall is held to the fixed diameter by the through-wall suture for as long as the suture holds. ELG's to date do not bind the adventitia to the prosthesis – they merely attach. The ELG must continue to act to bridge the gap between normal artery above and below until, if ever, the aneurysm's cavity shrinks right down. In open surgery, the suture is binding and the tissues around supportive. The diameters of the grafts used for the same abdominal aortic aneurysm (AAA) differ markedly between the open and ELG methods. The common diameters used for tube replacement surgically of infrarenal AAA is 18 or 20 mm. The commonest diameter for an endoluminal graft is 26 or 28 mm and 30+ mm is not uncommon. Why such a discrepancy when the surgeon judges the diameter to suitable fit? This discrepancy is due to the different types of attachment of an open graft and an endoluminal graft. With the former, there are sutures through the graft and the full thickness of the aortic wall. This means that the aortic diameter at that point is permanently fixed to the diameter of the graft in its pressurised state. The diameter of a crimped vascular graft is, by definition, the minimum internal distance between the crimps in the non-pressurised state. It is increased by approximately 10% when pressurized. With the ELG, a residual radial force is required for seal and the attachment may or may not be enhanced by latching barbs. The oversize allowance must accommodate elasticity and compliance while maintaining the seal between pulsations for the whole of the length of the sealing zone.

These latching barbs sometimes cross the renal arteries but have been shown to have a minor effect of 1 % on the renal artery flow rate for 3 mm diameter artery.[14]

With an endoluminal graft the device must bridge a gap for an indeterminate time before the body reabsorbs the contents of the aneurysm and encases the graft in foreign body fibrous tissue support. Therefore the long term function and durability demands are different and more demanding.[1] Understanding the forces involved is basic to design and use of new technology and the weaknesses that lead to aneurysmal disease provides a challenge.[1,15]

A mistaken clinical impression is that the forces on a thoracic ELG should be greater than those on an abdominal ELG. The flow and diameter of the thoracic aorta are greater and the haemodynamic forces potentially much larger. However, because the diameter of the graft changes little, if at all, the downward displacement force in the thoracic ELG is small as the resistance in the graft is low – except on the aortic arch. The resistance of any graft that extends into the iliac vessels is much greater because of the significant change in diameter and high resistance within the graft acting like a windsock or sea anchor.[15] An aorto uni-iliac device affords greater resistance than a bifurcated graft and detachment at the neck and migration is a common problem due to high displacement forces. In contrast with the thoracic aorta there is little drag because there is little or no change in diameter. In contrast, there is little drag on a graft in the thoracic aorta because there is little or no change in diameter along the graft. The force applied to the graft is on the curve and centrifugal forces apply. Since every action has an equal and opposite reaction (Newton's third law), one must ask where is the reaction. The reaction is to pull the graft out from the top and the bottom almost equally. When endoluminal grafts were first used in the thorax, unexpected upward migration of the distal end emerged as the problem, especially when there was a significant curve on the graft. For the same reason, this 'lift out' may also be seen from the iliacs when the graft fixation is weak because of ectasia and/or short length of distal attachment. Type 1B endoleak can be more dangerous than Type 1A if this factor is ignored.

An important issue in vascular intervention is the durability of endoluminal grafts. Such grafts are often used to protect aneurysms from the effects of arterial pressure. Unfortunately, hemodynamic forces can displace a graft and thereby, potentially, interrupt the seal between the graft and the neck of the aneurysm. It is important, therefore to have an understanding of the possible forces that may be exerted on a graft.

To illustrate the steps used in determining the forces on a graft system, via analytic equations, we consider the steady flow of blood through a bent pipe (Figure 8.14). In this figure, the proximal inlet entrance is labelled by 1 and the distal exit by 2. D_1, A_1 and D_2, A_2 are the diameters and cross-sectional areas, respectively, of the graft at the points 1 and 2. The vector normals of the cross-sectional areas are, respectively, at angles of θ_1 and θ_2 to the vertical. Similarly p and $\boldsymbol{v}$ refer to the pressures and velocities at these points. R_x and R_y are the x and y components of the restoring force. The external pressure on the graft system is denoted by p_{ex}.

In our analysis, we assume steady-state, *i.e.*, non-pulsatile, flow. We do this as it gives us a basic idea of how the system is behaving.

The first equation is the steady-state **mass conservation** equation, which we rewrite in the form

$$v_1 A_1 = v_2 A_2. \tag{21}$$

One should note that v_1 and v_2 are average flow speeds, where the average is taken

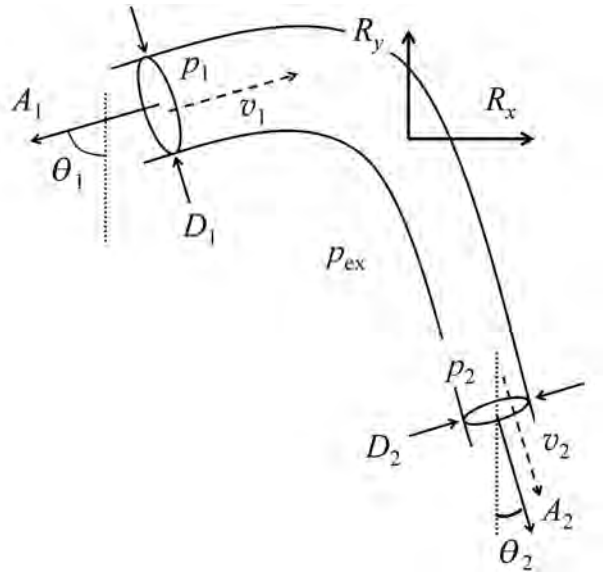

FIGURE 8.14: The characteristic velocity, pressure, area and force vectors required to compute the restraining forces on a bent, single-tube graft system.

over the areas of A_1 and A_2 respectively.

The next analysis tool at our disposal is the **momentum conservation** equation, which can be expressed in the form

$$R_x = \begin{matrix}(p_2 - p_{ex})A_2\sin\theta_2 - (p_1 - p_{ex})A_1\sin\theta_1 \\ + \rho v_2^2 A_2\sin\theta_2 - \rho v_1^2 A_1\sin\theta_1\end{matrix} \qquad (22)$$

and

$$R_y = \begin{matrix}-(p_1 - p_{ex})A_1\cos\theta_1 - (p_2 - p_{ex})A_2\cos\theta_2 \\ - \rho v_1^2 A_1\cos\theta_2 - \rho v_2^2 A_2\cos\theta_2\end{matrix} \qquad (23)$$

where in these formulae, we have ignored the weight of the graft and the weight of blood in the graft. These terms are easily included into the equations, if required.

Energy is the final conserved quantity that we can use in our analysis. The **energy conservation equation** has the form:

$$\frac{p_1}{\gamma} + \frac{\alpha_1 v_2}{2g} + z_1 = \frac{p_2}{\gamma} + \frac{\alpha_2 v_2^2}{2g} + z_2 + h_L, \qquad (24)$$

where g is the gravitational acceleration, $\gamma = \rho g$ is the weight density of blood, z_1 and z_2 are the vertical heights of the proximal and distal ends of the graft, respectively, and h_L is the 'head loss' in the pipe, *i.e.*, the amount of pressure or energy that is lost due to frictional viscous effects as the fluid travels through the pipe. Head loss is usually given by the equation

$$h_L = K_L \frac{v_2^2}{2g}, \qquad (25)$$

where K_L is a constant, the value of which is usually dependent on the shape, length and diameter of the pipe. The coefficients α_1 and α_2 are kinetic energy correction factors that have different values depending on the type of flow. For example, for uniform flow $\alpha = 1$, turbulent flow has $\alpha \approx 1$, and laminar flow gives $\alpha = 2$.

By combining Eqs (21), (24) and (25), one obtains

$$p_2 = p_1 + \frac{\gamma v_1^2}{2g}\left(\alpha_1 - (\alpha_2 + K_L)\left(\frac{A_1}{A_2}\right)^2\right) + \gamma(z_1 - z_2). \qquad (26)$$

So, by using Eqs (26) and (21), we can express p_2 and v_2 in terms of quantities at the entrance of the graft. This then allows us to compute the restraining forces on the graft system by then using Eqs (22) and (23).

Case 1 – The cylindrical graft

For this case, the inlet and the outlet areas are the same, so, by Eq. (21), the inlet and outlet flow speeds are also equal. The angles θ_1 and θ_2 are equal and have a value of 90°. The inlet and outlet pressures are not equal due to the frictional, shear interaction between the blood and the graft (*i.e.*, the head loss as given by Eq. (25)). This frictional interaction causes the outlet pressure, p_2, to be less than the inlet pressure, p_1. This is called a pressure drop.

From all of this information, one can write down the restraint forces on the graft. So, from Eqs (22) and (23):

$$R_y = 0, \tag{27}$$

i.e., there are no vertical forces generated by blood flowing through a horizontal graft and

$$R_x = (p_2 - p_1)A_1, \tag{28}$$

where we have set the external pressure to zero. In this case, the horizontal force on the graft is quite small, because p_1 will only be a little larger than p_2. One can conclude from this analysis that straight, cylindrical grafts only feel a relatively small drag force in the direction of the flow.

Case 2 – The windsock graft

Suppose now we consider a graft in the shape of a windsock, such as in Figure 8.16.

For this case, the inlet area is now larger than and the outlet area, so, by Eq. (21), the outlet flow speed is greater than the inlet flow speed as given by

$$v_2 = \left(\frac{A_1}{A_2}\right)v_1 \tag{29}$$

As in the previous case, the angles θ_1 and θ_2 are equal and have a value of 90° and the inlet and outlet pressures are not equal due to the frictional, shear interaction between the blood and the graft.

The restraint forces on the graft are from Eqs (22) and (23):

$$R_x = p_2A_2 - p_1A_1 + \rho v_2^2A_2 - \rho v_1^2A_1, \tag{30}$$

and

$$R_y = 0\ . \tag{31}$$

When you put in the appropriate numbers into Eq. (30), it is found that the dominant term in this equation is the p_1A_1 term. Many endoluminal grafts have this 'wind sock' shape with a distal exit area, which is smaller than the proximal, inlet area. This shape has a much larger drag force than for a cylindrical graft.

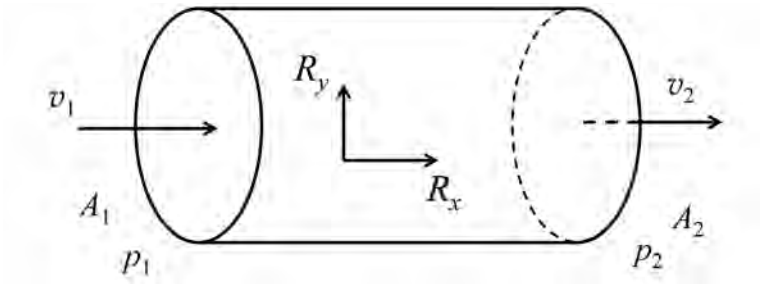

FIGURE 8.15: Cylindrical graft.

Case 3 – The curved graft

As with the cylindrical graft, the inlet and the outlet areas are the same, so, by Eq. (21), the inlet and outlet flow speeds are also equal. Due to the symmetry of the situation, the vertical restraint force is zero, the horizontal restraint force is given by

$$R_x = -p_2A_2 - p_1A_1 - \rho v_2^2A_2 - \rho v_1^2A_1 . \qquad (32)$$

So, now both the pressure and velocity components add together to produce a greater total force on the graft. This result suggests that a curved graft may be subject to greater forces than a wind-sock shaped graft.

Case 4 – The symmetric bifurcated graft

Suppose that we consider a symmetric bifurcated graft, such as shown in Figure 8.18, where the two outlet distal legs of the graft are at an angle α to the horizontal, the two distal ends are equal and gravity is ignored. The proximal end of the graft is labelled by the number 1, the symmetric distal ends by 2 and 3. By satisfying momentum conversation the horizontal restraint force is given by:

$$R_x = -p_1A_1 - 2p_2A_2\cos\alpha + \rho v_1^2A_1 - 2\rho v_2^2A_2\cos\alpha . \qquad (33)$$

The more general, non-symmetric case with gravity is described elsewhere.[15]

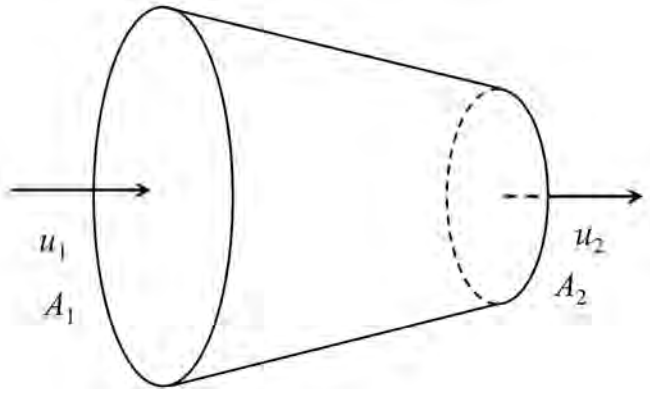

FIGURE 8.16: An endoluminal graft in the shape of a wind-sock.

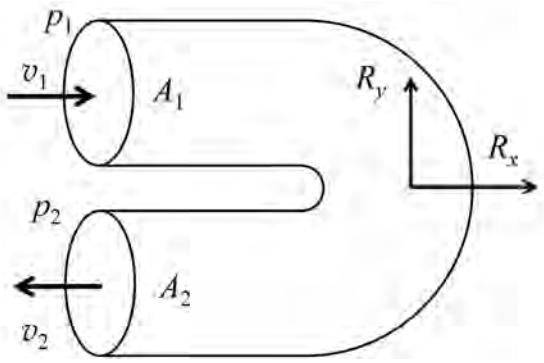

FIGURE 8.17: Curved graft.

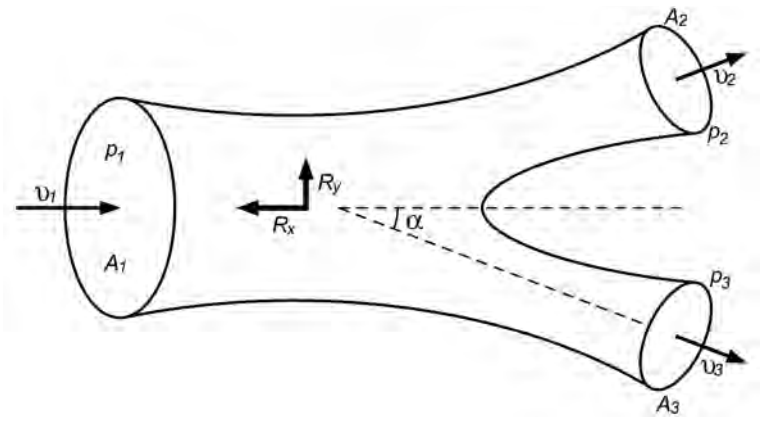

FIGURE 8.18: Symmetric bifurcated graft.

We also know to satisfy mass conversation that the flow in has to equal the sum of the outflows

$$v_1 A_1 = v_2 A_2 + v_3 A_3, \tag{34}$$

and since it is a symmetric bifurcated graft then

$$v_1 A_1 = 2 v_2 A_2. \tag{35}$$

By applying Bernoulli's equation 7 and mass conversation equation 35 then p_2 and v_2 can be eliminated from equation 33.

This equation shows that the horizontal restraint force is strongly dependent on inlet area, pressure and on the bifurcation angle (especially > 15°). But the blood inlet velocity or flow rate has negligible effect on the horizontal restraint force.[16-18] Naturally, a steady-state assumption is questionable, since pulsatile flow occurs in the human body. However, it was shown experimentally that a steady-state analytical model can be used, with variable pressure and flow rate inputs, to predict forces on a symmetric, bifurcated graft in pulsatile flow with reasonable approximation within design limits.[17-18]

$$R_x = p_1 A_1 - \rho \frac{A_1^2}{2A_2^2} v_1^2 \cos\alpha + \rho v_1^2 A_1 - 2A_2 \left[p_1 + \frac{\rho}{2^2} v_1^2 \left(1 - \frac{A_1^2}{2A_2^2} \right) \right] \cos\alpha \,. \tag{36}$$

This steady-state analytical force model is now used in the design of grafts.

COMPUTATIONAL MODELLING

Computational fluid dynamics (CFD) and finite element modeling can assist in our understanding of vascular haemodynamics. CFD uses numerical methods to discretize and mesh the geometry and algorithms to solve the equations of motion (for example, the Navier-Stokes equation) and other relevant equations. In the last several years with advances in computing the computational modeling capability has greatly improved. It is now possible to incorporate patient-specific geometry from MRI, CT or magnetic resonance angiography (MRA) data. The computational models also now include fluid-structure interactions (fluid flow and wall deformation interaction), pulsatile flow and non-Newtonian flow. Some computational modelling of vascular haemodynamic systems include AAA grafts,[19-22] fluid-structure interactions with cerebral aneurysms,[23-25] patient-specific cerebral aneurysms with coils,[26-28] and patient-specific circle of

Willis.[29-33] For example, Li and Kleinstreuer[19] modelled blood flow and structure interactions in a AAA with and without a graft where they incorporated fluid-structure interactions, flexible walls, pulsatile flow and non-Newtonian blood flow. They confirmed that the force on the graft is highly dependent on the diameter, blood pressure and bifurcation angle. They also showed significant reduction in AAA stress, displacement and pressure after graft placement as shown in Figure 8.19.

Endoleaks which are blood flow between graft and the AAA wall can also cause problems in AAA such as elevated sac pressure and high stresses which may lead to rupture. Li and Kleinstreuer[34] modelling analysis indicated the sac pressure caused by type II endoleaks (leakage via collateral arteries) depends on the inlet branch pressure; thus, type II endoleaks may increase sac pressure to near the systemic pressure levels, which could cause more clinical concern. Other studies have shown that intrasac pressure measurements and haemodynamic analysis of the graft-aortic wall interactions can be used to detect type II endoleaks.[35,36]

RECENT DEVELOPMENTS AND FUTURE DIRECTIONS

Mathematics, principles of physics and computational modelling of vascular haemodynamics have been useful in verifying or modifying intuitive engineering of endovascular stent grafts; and towards better understanding of failure modes of the cardiovascular system and its prostheses. For example, based on vascular haemodynamics analysis relating a vascular geometric ratio to the likelihood of aneurysm rupture, plaque rupture and stent graft migration.

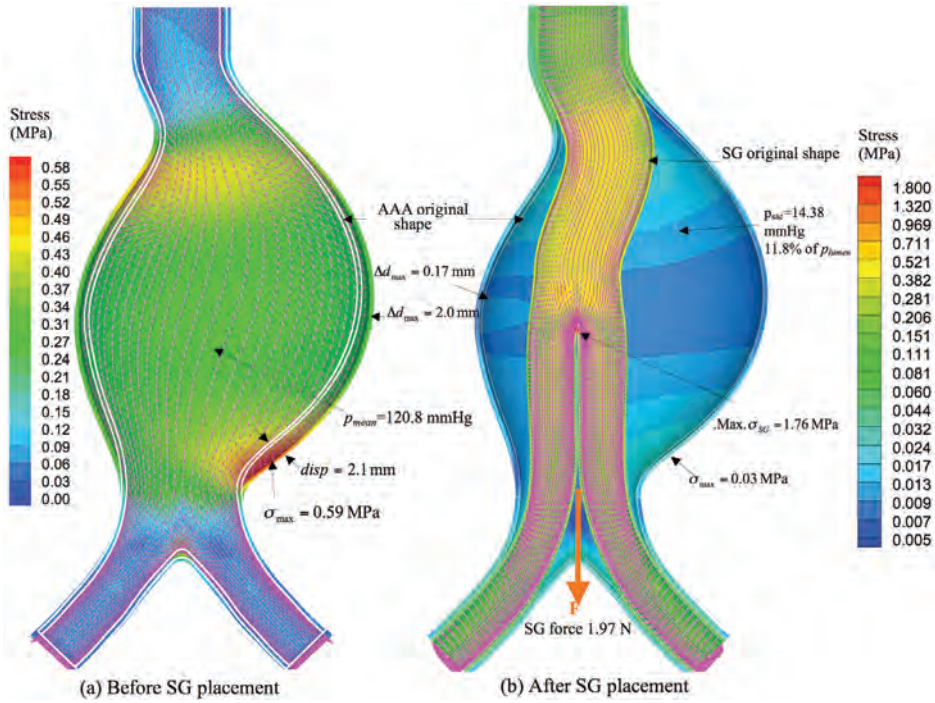

FIGURE 8.19: Effect of graft placement on blood flow and AAA wall at peak systole pressure level (courtesy of Professor Clement Kleinstreuer).[19]

CFD modelling of vascular haemodynamics is currently being used in the design and evaluation of implanted medical devices such as grafts. It is still a complex process to create patient-specific computational models and difficult for clinicians to interpret the results. In the future, computational modelling of vascular haemodynamics may be used as a tool for patient-specific blood flow quantification relevant to clinical practice to assist intervention planning, decision-making and optimization.

However, for computational modelling to be more easily translated to clinical relevance there needs to be more extensive comparisons of *in vitro* and *in vivo* clinical studies to validate the codes, with the uncertainties quantified, so they can be used with confidence. Patient-specific geometries and measured flow distribution boundary conditions should be included. There needs to be better models of arterial mechanical properties during each stage of a disease state (e.g. aneurysm formation), more accurate imaging techniques that can provide better information of the wall thicknesses, details on perivascular environment and much better coupling with vascular biology, mass transport and cellular biophysics. With grafts now being used in high curvature areas it is more important to understand the forces required to keep the grafts in place to mitigate their migration.

The intersect of clinical arterial pathology, feedback systems in physiology and computational fluid dynamics leads us to potentially the most exciting time in advances for arterial disease ever. The vascular system is dynamic in its function, its response to demand, its injury and repair cycle and its aging. The arterial system is intricately designed so that each arterial division is specific for its function and the demands placed upon it for up to one hundred years. To the empirical, statistical, biochemical, genetic and molecular biology knowledge of the cardiovascular system must be added the central role of haemodynamic physics and the pathology that results from the relentless forces of blood pressure and pulse wave. Modelling of arteries opens the door to much better understanding of why atheroma occurs at the known predictable sites such as the carotid bifurcation, the origins of branch vessels of the aorta and sites of stress for example the adductor canal.

CONCLUSION

Understanding the physics of the vascular system in health and disease will influence vascular management. This is a rich field for further research. Further clues to atherogenesis may lie in the differences of the fluid dynamics and stresses applied to the arterial systems. Computational modelling will be of increasing importance, as the science evolves, to our understanding of vascular haemodynamics.

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9 • Physiological Haemostasis

SIMON MCRAE

Royal Adelaide Hospital & The Queen Elizabeth Hospital, Adelaide, South Australia.

INTRODUCTION

Physiological haemostasis involves complex interactions between endothelial cells, platelets and coagulation proteins, that result in a prompt platelet plug and then localised thrombus formation at the site of a break in vascular integrity. Numerous regulatory processes prevent widespread activation of coagulation, ensuring that blood remains fluid in the absence of vascular injury or other pathology. All components of the haemostatic process can be disturbed resulting in either a pro-thrombotic or bleeding tendency, and drugs that modify the haemostatic process are commonly used, particularly in patients with vascular disease. An understanding of normal haemostasis is therefore important for all clinicians that deal with this patient group.

PRIMARY HAEMOSTASIS

Primary haemostasis is the initial response of the body to vascular injury, and involves interaction between platelets, adhesive proteins located in the subendothelial matrix (including collagen and von Willebrand factor), and circulating fibrinogen.[1] The end result of primary haemostasis is the formation of a stable platelet plug around which a fibrin network can then be built. This same process is responsible for the pathogenic thrombus formation in patients with arterial disease. Disorders of primary haemostasis tend to manifest in the main as mucosal bleeding, including epistaxis, oral bleeding and menorrhagia, and often immediate difficulty with haemostasis in the post-operative setting.

Platelets

Platelets are small fragments of megakaryocyte cytoplasm that in the resting state are small discoid structures. The normal range for circulating platelet count in adults is between 150 to 400 $\times$ 10^9/L. Although anucleate, platelets are metabolically active, and interact with the local environment through the binding of surface glycoprotein receptors to specific ligands. Platelets go through a predictable cycle of response to vessel wall injury that involves initial platelet adhesion to the sub-endothelium, subsequent intracellular signalling that triggers platelet shape change and activation with granule release, and finally aggregation (Figure 9.1).[2]

Platelet adhesion

Endothelial injury results in the exposure of circulating blood to the subendothelial

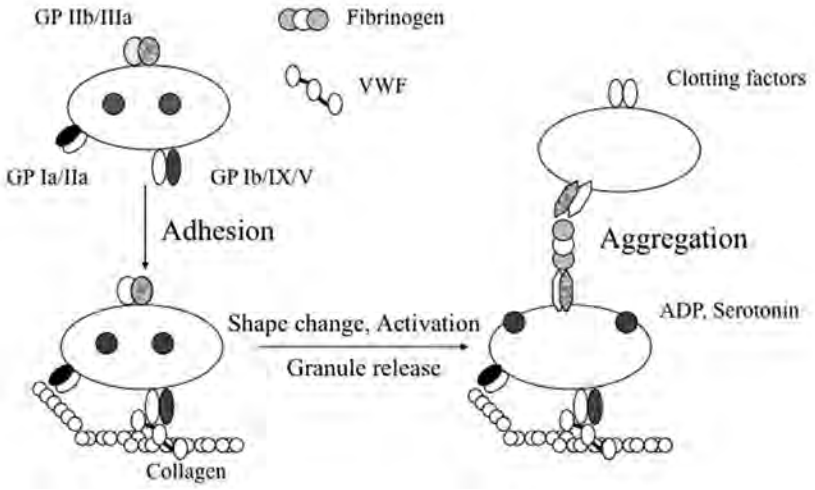

FIGURE 9.1: Mechanism of platelet aggregation

matrix that is rich in a number of adhesive proteins. von Willebrand factor (vWF) is a large adhesive glycoprotein produced by endothelial cells and megakaryocytes that is central in initial platelet adhesion.[3] The mature vWF molecule consists of disulphide-linked multimers of high molecular weight of up to 20,000,000 daltons.[4] When secreted into the plasma, these high molecular weight (HMW) vWF multimers are digested into smaller forms by the metalloprotease ADAMTS13 (a disintegrin and metalloprotease with a thrombospondin type 1 motif, member 13). These smaller soluble forms bind less readily to platelet receptors, reducing the chance of spontaneous platelet aggregation. However vWF secreted into the subendothelial space binds to other molecules such as collagen, resulting in a conformational change that exposes the binding site for platelet glycoprotein (GP) receptor Ib.[4] Subendothelial vWF is therefore 'primed' to interact with circulating platelets in the event of endothelial injury. Other important adhesive proteins include collagen type 1 and type 4, fibronectin, thrombospondin, laminin and vitronectin.

Initial platelet adhesion, particularly in high shear conditions, involves interaction between vWF and the GPIb/IX/V complex located on the platelet surface. This complex consists of four trans-membrane subunits GPIba, GPIbb, GPIX and GPV, with the N-terminal globular domain of GPIba responsible for the interaction with the A1-domain of vWF.[1] Binding of vWF to GP Ib is often reversible, and in animal models platelets can be seen to initially slide or translocate along the subendothelial surface due to cyclical attachment and then dissociation of the GP Ib/IX/V complex to vWF.[2] However, finally through further platelet receptor ligand interactions the platelet is stabilized on the subendothelial surface. The platelet glycoprotein Ia/IIa receptor (integrin $\alpha_2\beta_1$) binds collagen, an interaction that appears to be more important in low-shear conditions.[5] Glycoprotein VI, a platelet surface receptor that belongs to the immunoglobin superfamily, also directly binds collagen and further activates the GPIa/IIa receptor via intracellular signaling.[6] Other β_1 integrins also bind their respective subendothelial ligands ($\alpha_6\beta_1$ – laminin; $\alpha_5\beta_1$ – fibronectin),

and there is increasing evidence that early binding of vWF to the glycoprotein IIb/IIIa ($\alpha_{IIb}\beta_3$) receptor contributes to the initial adhesion process.[2] Finally there is evidence that formation of platelet membrane tethers, that consist of smooth cylinders of lipid membrane pulled from the platelet surface under the influence of hemodynamic drag forces, contribute to platelet adhesion in high shear conditions.[7]

Platelet activation and shape change

Following platelet adhesion, multiple pathways lead to platelet activation that results in platelet shape change, platelet granule release, and conformational change in the GP IIb/IIIa receptor that allows binding to fibrinogen and vWF, leading to platelet aggregation. Binding of vWF to the GP Ib receptor and collagen to the GP VI during the adhesion process triggers intracellular signaling via a pathway that involves activation of Src family kinases (Src), Syk and PI 3-kinase (PI3K). These events lead to the activation of phospholipase C-b (PLC), which hydrolyses membrane phospholipids to generate inositol (1,4,5) trisphosphate (IP3).[8] The binding of IP3 to its receptors (IP3R) on the dense tubular system (DTS) then results in mobilisation of intra-platelet calcium stores, which has a number of consequences including;

1. *Thromboxane A2 (TXA2) generation* – the increase in intracellular calcium stimulates the production of arachadonic acid by PLC and phospholipase A2. Arachadonic acid is converted into TxA2 via the actions of the enzymes cyclooxygenase 1 (COX-1) and Tx synthase. TxA2 is released from the platelet and binds platelet receptors TPα and TPβ. Its effects in platelets are mediated primarily through TPα. Binding of TxA2 to this G-protein coupled receptor results in further PLC activation, leading to further intracellular calcium increase further reinforcing platelet activation.[9] Local diffusion of TxA2 also contributes to the recruitment to the site of injury and activation of further platelets. Aspirin or acetyl salicylic acid exerts its antiplatelet effect by blocking TXA2 synthesis, due to the irreversible acetylation of Ser-529 in COX-1. Because platelets are anucleate, no new COX can be generated, explaining why aspirin has a persistent functional effect that lasts the lifespan of the platelet (approximately 7 days).
2. *Granule release* – intracellular calcium mobilization also results in the release from the platelet of both the dense and alpha-granules. The dense granules contain high concentrations of the small molecules adenosine diphosphate (ADP) and serotonin, which further act to reinforce local platelet activation by binding to specific platelet surface membrane receptors upon release. ADP is a central player in sustained platelet activation. The receptors for ADP, the $P2Y_1$ and $P2Y_{12}$ are seven transmembrane receptors that are coupled via heterotrimeric G-proteins to numerous intracellular effector molecules. $P2Y_1$ links to the G-protein Gq resulting in further activation of PLC and also protein kinase C activation. $P2Y_{12}$ is linked to the G-protein Gi that has an inhibitory effect on adenylate cyclase. ADP induced activation of the $P2Y_1$ receptor induces platelet shape change and rapid transient aggregation,[10] whereas activation of the $P2Y_{12}$ receptor results in sustained irreversible aggregation.[11] The thienopyridine class of antiplatelet agents, ticlopidine, clopidogrel and prasugrel exert their antiplatelet effect by blocking the $P2Y_{12}$ receptor. The active metabolites of all agents have a free thiol moiety that forms a disulfide bridge

with the extracellular cysteine residues Cys17 and Cys270.[12] Released serotonin also binds to a G-protein coupled platelet surface receptor, the 5-HT_{2A} receptor. Binding is also associated with Gq-dependent activation of PLC, resulting in amplification of platelet activation, platelet shape change, and weak reversible platelet aggregation.[13]

3. *Activation of the GP IIb/IIIa receptor* – in its resting state the GP IIb/IIIa receptor is unable to bind its ligands, namely fibrinogen and vWF. The above platelet signaling events through the activation of the small GTPase Rap1b and its interaction with a Rap1-GTP interacting adapter molecule (RIAM), lead to the binding of the proteins talin and kindlin to β3 tail of GP IIb/IIIa receptor.[14] This leads to activation of the receptor and the resulting change in conformation allows the surface portion of the receptor to bind readily to fibrinogen and vWF. The binding of talin to the receptor tail also links it to the underlying actin cytoskeleton of the platelet, enhancing adhesive strength and platelet cohesion.[15]
4. *Platelet shape change* – the normally discoid-shaped platelet with a smooth surface membrane undergoes dramatic shape change with stimulation, including extension of filopodia, and flattening or spreading on the subendothelial surface. The platelet cytoskeleton is primarily responsible for regulating the platelet's shape. Platelet activation leads to the rapid reorganization and polymerization of actin into filaments, resulting in the above conformational change.[16]

Along with ADP, the serine protease thrombin appears to play an important role in sustaining platelet activation leading to irreversible platelet aggregation. Thrombin specific receptors, the protease-activated receptors (PARs), are located on the platelet surface. Two main PARs, PAR1 a high affinity receptor and PAR4, a low affinity receptor, are involved in thrombin mediated platelet activation.[17] Thrombin activates PARs by cleaving the N-terminal of the receptor, unmasking a hidden receptor-linked ligand. This ligand then interacts with the remainder of the receptor leading to G-protein coupled signaling that results in further platelet activation.

Finally platelet activation also results in the surface expression of a number of adhesion molecules, such as the glycoprotein P-selectin which is involved in interaction with both endothelial cells and also the recruitment of inflammatory cells to the area of injury, via binding of P-selectin to P-selectin glycoprotein ligand 1 (PSGL-1) located on the surface of leucocytes.[18] Platelets also secrete chemokines such as RANTES/CCL5 and platelet factor 4 that also increases the local recruitment of inflammatory cells such as monocytes. This contributes to and can exacerbate the local inflammatory response that often presents in atherosclerotic plaque.[19]

Platelet aggregation

As the final part of the primary haemostatic response, platelets recruited to the site of vascular injury and activated by the above soluble agonists then undergo irreversible aggregation. This is mediated via the concurrent binding of either fibrinogen or vWF to the activated GP IIb/IIIa receptors on separate platelets, leading to their cross-linking and the formation of a platelet aggregate. In low flow vascular beds binding of fibrinogen to the GP IIb/IIIa receptor appears to be the main process involved in platelet aggregation, whereas the interaction between GP IIb/IIIa and vWF is more important for aggregation in high

shear vascular beds and pathological arterial thrombosis.[7]

INTERACTIONS BETWEEN PRIMARY AND SECONDARY HAEMOSTASIS

While the primary and secondary haemostatic processes are often considered separately, they are intrinsically linked. As described above, the coagulation protease thrombin plays a central role in the activation of platelets. The activated platelet in turn provides the surface upon which the reaction complexes of the coagulation cascade form. In addition, as part of platelet activation the content of the negatively charged phospholipid phosphatidylserine on the outer surface of the platelet membrane increases from almost 0% up to 12%, providing a binding site for the proteins of the coagulation cascade.[20] Release of clotting factors, such as factor V, from platelet alpha granules, and the expression of other as yet still poorly defined platelet receptors for coagulation factors on the platelet surface provide additional methods in which activation of the coagulation cascade is localised to the site of platelet activation and vascular injury.[21]

SECONDARY HAEMOSTASIS

Secondary haemostasis describes the process whereby exposure of tissue factor to the bloodstream leads to a series of enzymatic reactions that result in a sufficient burst of thrombin production to convert soluble fibrinogen into a stable network. A repetitive theme in this process is the formation of a series of reaction complexes consisting of an active enzyme and a co-factor, in which the presence of the latter results in a order of magnitude increase in the efficiency of the enzyme to bind to and convert its target substrate, itself a pro-enzyme or zymogen, to its active form. Defects of secondary haemostasis, as typified by factor VIII deficiency or haemophilia A, result in muscle, joint and soft tissue bleeding, and delayed bleeding post surgical or traumatic haemostatic challenge.

The coagulation factors involved in secondary haemostasis belong to the class of proteins known as serine proteases, so called because they have a serine residue which, along with histidine and aspartic acid, forms a catalytic triad at the centre of the active site of the enzyme.[21] Most of the reactions of secondary haemostasis take place on a phospholipid membrane surface, which is normally the surface of an activated platelet. Binding of the coagulation proteins to the phospholipid membrane surface requires the presence of calcium, and agents that chelate calcium such as EDTA or citrate can therefore be utilised to prevent activation of the coagulation cascade after blood collection.

The coagulation factors have a modular structure, and different factors share similar structural features. The coagulation factors II, VII, with IX and X along with the natural inhibitors of coagulation, protein C and protein S, all undergo post-translational gamma-carboxylation of glutamate residues located at the amino-terminus. This modification is necessary for the efficient binding of these proteins to the phospholipid surface. The carboxylation process is dependant on the presence of vitamin K, which is a co-factor for this process. Vitamin K deficiency or Vitamin K antagonists, such as warfarin that prevent the conversion of vitamin K to its reduced form by blocking the activity of the enzyme vitamin K epoxide-reductase, leads to a reduction in the activity of the coagulation factors resulting in an anticoagulant effect.

THE COAGULATION CASCADE

Early observations noted that clot formation in plasma would occur after the addition of exogenous biological material such as macerated brain extract, but that exposure of blood or plasma to surfaces such as glass would also precipitate clot formation without the addition of further material. This led to the concept of 'extrinsic' and 'intrinsic' pathways of coagulation, and over time the coagulation factors involved in these separate pathways were identified (Figure 9.2).[21,22] Tissue factor was identified as the 'active' factor in the added tissue extract, and was demonstrated to activate factor VII in the first part of the extrinsic pathway. The intrinsic pathway, sometimes also called the contact activation pathway, was found to involve serial activation of the coagulation factors XII, XI and IX, with factor VIII acting as a co-factor for the latter. Both extrinsic and intrinsic pathways were found to then converge on the 'common pathway' involving factor X, prothrombin (factor II), and finally the conversion of fibrinogen to fibrin. The concept of the two separate pathways was reinforced by the fact that the most widely utilised laboratory assays of coagulation evaluated the extrinsic (the prothrombin time or PT assay) and intrinsic pathway (the activated partial thromboplastin time or aPTT) separately, with both assays affected by common pathway defects.

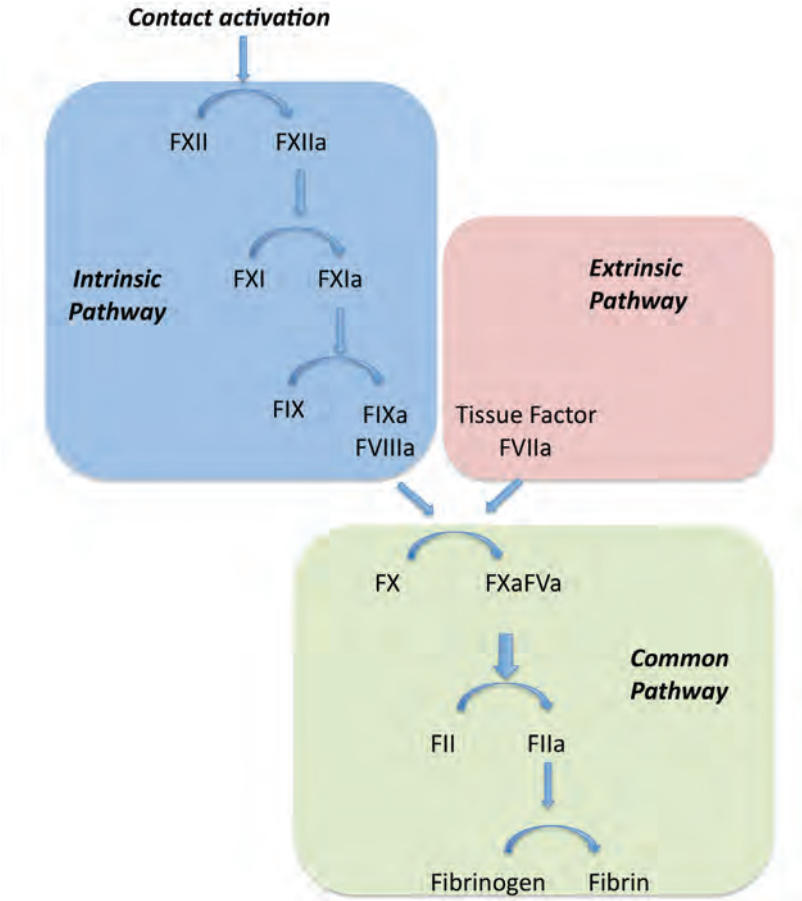

FIGURE 9.2: The extrinsic and intrinsic pathways of coagulation

It however became clear with time that the above model was unlikely to reflect physiological coagulation. The observation that inherited factor XII deficiency was not associated with a bleeding tendency raised questions regarding the physiological role of the intrinsic pathway.[23] It was also demonstrated that activated factor VII, or factor VIIa, had the ability to activate factor IX as well as factor X, and therefore that cross-talk between the pathways was likely.[24] With increasing knowledge of the role of the cell surface proteins in the coagulation process, and in particular the role of platelets, a cell-based model of haemostasis then emerged.[25] This model divides the coagulation cascade into the separate steps of initiation, amplification, and then propagation (Figure 9.3).

Initiation

Exposure of cells expressing the transmembrane protein tissue factor to circulating blood is the physiological trigger of the coagulation cascade. Tissue factor (TF) is a transmembrane protein that is constitutively expressed on the surface of most non-vascular cells, including those located in the subendothelium. There is also some evidence that tissue factor expression can be induced in the setting of inflammation on the surface of monocytes and that microparticles derived from monocytes may also express TF in pathological states.[26] Upon exposure to circulating blood TF binds to factor VII, converting it to its active form factor VIIa. The resulting enzymatic structure is known as the extrinsic tenase complex, with TF then acting as a co-factor for VIIa and greatly potentiating conversion of factor X to factor Xa, and, to a lesser degree, factor IX to factor IXa. The activated factor Xa formed then binds to the surface of the tissue factor-expressing cell, and converts a small amount of prothrombin (factor II) to thrombin, while the small amount of factor IXa produced diffuses away with the potential to bind locally to the surface of activated platelets.[27]

Amplification

The small amount of thrombin formed during the initiation stage, while insufficient

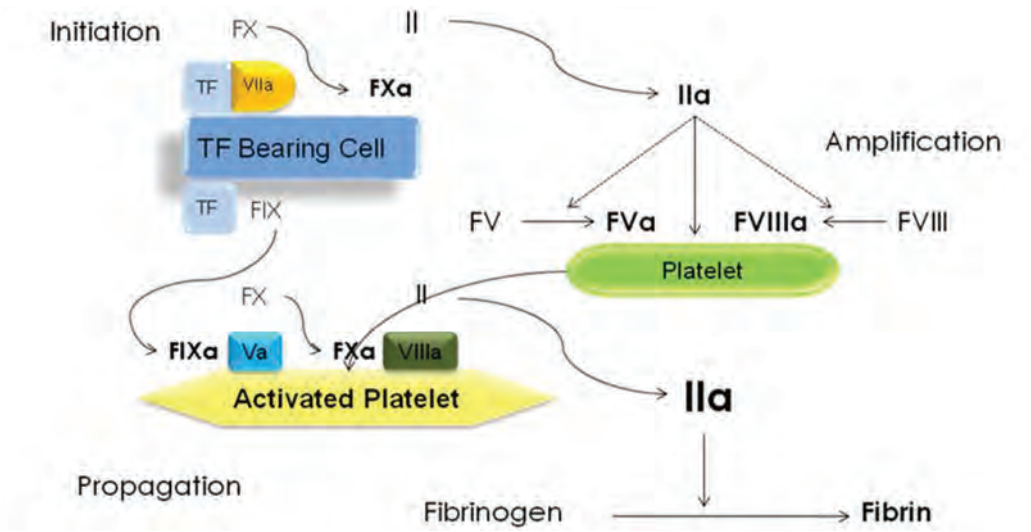

FIGURE 9.3: Cell based model of haemostasis

to convert adequate amounts of fibrinogen to fibrin, is none-the-less enough to be responsible for the subsequent amplification of the coagulation cascade. The thrombin produced results in 1) further local activation of platelets resulting in the phospholipid surface on which the reactions of the coagulation cascade can proceed; 2) activation of the co-factors factor V and factor VIII that then localize on the nearby surface of activated platelets; and 3) activation of factor XI that also binds locally to the platelet surface.[28]

Propagation

Following the activation of the co-factors and their localization on the platelet surface, the stage is set for the formation of highly efficient enzymatic complexes that are responsible for the burst of thrombin generation that leads to clot formation. Factor IXa formed during the initiation step, binds to factor VIIIa on the platelet surface to form the intrinsic tenase complex. This then efficiently converts factor X to factor Xa, with the latter then binding to its co-factor, factor Va, to form the prothrombinase complex responsible for the effective conversion of prothrombin to thrombin. Factor XIa produced during amplification activates further factor IX, further reinforcing or enhancing the whole process from above.[25]

The burst of thrombin generated during propagation then cleaves the fibrinopeptides a and b from soluble fibrinogen to form insoluble fibrin monomers. The transglutaminase Factor XIII, itself activated by thrombin, then forms bonds between separate fibrin monomers to form a firm network of cross-linked fibrin that is a requirement for stable thrombus formation.[29]

Natural inhibitors of coagulation

Normal coagulation is kept in check by several regulatory processes that cause thrombin production to plateau and then diminish, preventing appropriate localized activation of coagulation from becoming an inappropriate widespread activation of the clotting cascade. The initiation phase of coagulation is regulated by tissue factor pathway inhibitor (TFPI), a protein produced by endothelial cells.[30] After a sufficient local concentration of FXa is generated in the initiation step of coagulation, TFPI is able to form an inhibitory quaternary complex with FXa, FVIIa, and tissue factor, preventing continued activation of the cascade from above.

Central to regulation of the propagation phase of the coagulation cascade is the protein C anticoagulant pathway that involves protein C and protein S, both vitamin K dependent plasma glycoproteins synthesized in the liver.[31,32] Thrombin itself initiates this inhibitory pathway after binding to thrombomodulin, a transmembrane protein located on the intact endothelial cell surface in all vascular beds particularly in the microcirculation. Binding of thrombin to thrombomodulin results in a change in substrate specificity that favours cleavage of the vitamin K dependent protein C to its activated form activated protein C (APC).[33] Binding of thrombin to thrombomodulin therefore results in its net enzymatic effect being switched from pro-coagulant to anticoagulant. Another endothelial transmembrane protein, the endothelial protein C receptor (EPCR) binds protein C, helping to localize the protein at the endothelial surface potentiating activation by thrombomodulin bound thrombin. Once activated APC diffuses away from EPCR, and binds to the extrinsic tenase and prothrombinase complexes where it acts to inactivate factor VIIIa

and factor Va respectively. Protein S acts as a co-factor for protein C in these reactions, as well as having some direct anticoagulant activity.[34] In plasma, PS circulates both free (40%) and bound to the C4b-binding protein (60%). It is the free form of PS that has cofactor activity.[32]

Finally antithrombin (AT) is a single chain plasma glycoprotein that belongs to the serine protease inhibitor superfamily (serpins). It plays a central role in the inactivation of circulating activated clotting factors, forming a 1:1 complex that is cleared by the liver. It is the main physiological inhibitor of thrombin and also binds to factors Xa, IXa, XIa, and XIIa.[35] Thrombin inhibition by AT is potentiated more than 1000-fold by heparin, due to conformational change of the AT molecule upon heparin binding, and it is this mechanism that results in heparin's activity as an anticoagulant agent.[36]

Inherited deficiency states of the main inhibitory proteins of coagulation, namely protein C, protein S and antithrombin, have all been described, and result in a prothrombotic tendency. Such deficiency states are relatively rare accounting, when combined, for less than 5% of individuals with venous thrombosis in a Caucasian population.

Fibrinolysis

The fibrinolytic system is responsible for the dissolution of thrombus composed of cross-linked fibrin, and plays a major role in helping maintain a patent vascular system.[37] It is composed of a number of enzymes, most of which are serine proteases, that act in concert to convert insoluble fibrin to soluble fibrin degradation products (FDPs). The central protein of the fibrinolytic system is plasminogen, a single-chain glycoprotein consisting of 791 amino acids, which is converted to its active form plasmin by the cleavage of a single Arg561–Val562 peptide bond. Tissue-type plasminogen activator (tPA) is the physiological activator primarily involved in the dissolution of fibrin from the circulation. Activation of plasminogen to plasmin is potentiated in the presence of fibrin due to the fact that both plasminogen and tPA bind to lysine residues on the surface of fibrin, and are as a result brought into close proximity to each other. Both tPA and another plasminogen activator, urokinase-type plassminogen activator, play a role in the activation of plasminogen that is bound to the endothelial cell surface. Once activated, plasmin cleaves fibrin into soluble fibrin degradation products, of which D-dimer is one. D-dimer consists of two cross-linked fibrin D-domains, and is not normally present in the absence of recent plasmin activity. It is therefore used as a laboratory marker of active thrombosis, and is a sensitive test that can be used to rule out recent venous thromboembolism.

Like the coagulation cascade, the fibrinolytic system also has a number of inhibitory proteins that in normal circumstances prevent widespread activation of fibrinolysis. Plasminogen activator inhibitor-1 (PAI-1) is a 52-kd, single-chain glycoprotein that belongs to the serpin family, that is the main inhibitor of both tPA and uPA, doing so by forming a 1:1 complex that is cleared by the liver.[39] Circulating plasmin is quickly mopped up by α_2-plasmin that is present in the circulation at a high concentration. The most recently described inhibitor of fibrinolysis is thrombin-activatable fibrinolysis inhibitor (TAFI), a carboxypeptidase.[40] TAFI is activated by thrombin, a process that is markedly accelerated if thrombin is bound to thrombomodulin. The antifibrinolytic activity of TAFI is due the fact that it cleaves C-terminal lysine and arginine residues from fibrin. This significantly reduces the binding of plasminogen to fibrin, therefore decreasing

the activation of plasminogen by tPA on the surface of the fibrin clot.

The fibrinolytic system is manipulated therapeutically by administration of either naturally occurring (streptokinase) or recombinant protein (r-tPA) that exert the same effect as endogenous tPA, leading to activation of plasmin and resulting thrombus lysis.

CONCLUSIONS

Primary and secondary haemostasis both involve carefully balanced systems that if disturbed can lead to issues with either bleeding or pathological thrombosis. An improved understanding of the molecular processes involved has lead to the development of more targeted therapeutic options, such as the direct thrombin inhibitors and direct factor Xa inhibitors, with the aim of increasing the benefit and reducing the risks associated with anticoagulation. Continued advances in our understanding of the relationship between the structure and function of the proteins and receptors involved in haemostasis, along with improved technology, is likely to lead to further therapeutic advances in coming decades.

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10 • Hypercoagulable States

SIMON McRAE

Royal Adelaide Hospital & The Queen Elizabeth Hospital, Adelaide, South Australia.

INTRODUCTION

Abnormal thrombus formation is central to the acute pathophysiology of both arterial and venous disease. Formation of thrombus superimposed upon the surface of ruptured atherosclerotic plaque, producing vessel occlusion and resulting tissue ischemia, is the common mechanism leading to acute symptoms and presentation in patients with arterial disease. Likewise deep vein thrombosis and pulmonary embolism, important community causes of morbidity and mortality, both result from abnormal thrombus formation in the venous circulation. An understanding of conditions that may predispose to abnormal thrombus formation, including a knowledge of how the presence of these conditions may or may not impact on patient management, is important for all clinicians involved in the management of vascular disease.

First used in 1937,[1] and then also in the first description of inherited antithrombin deficiency, the term 'thrombophilia' can be defined as an increased tendency to develop thrombosis, which may be either acquired or inherited.[3] Thrombophilic conditions vary both in prevalence and in the magnitude of the associated increase in risk of thrombosis. The discovery during the 1990's of the high prevalence factor V Leiden and prothrombin gene point mutations that predispose to thrombosis,[4,5] meant that an underlying thrombophilic condition could be found in approximately 50% of unselected patients with venous thrombosis.[6] This fact, along with the belief that the presence of such a condition may influence prognosis and may therefore help guide patient management, has led to a significant increase in laboratory testing for inherited thrombophilia.[7] Recent data has however suggested that testing for thrombophilia, particularly the more common inherited conditions, is unlikely to influence the management of the majority of patients in whom it is performed,[8] and guidelines as a result have recommended against widespread testing in unselected patients.[9]

This chapter will first describe individual inherited and acquired conditions that predipose to an increased risk of thrombosis. The potential clinical rationale will then be outlined, and finally current evidence and recommendations regarding the clinical utility of laboratory testing in specific clinical scenarios will be discussed.

CLASSIFICATION OF THROMBOPHILIA

Thrombophilic conditions can be broadly classified as being either inherited or acquired and will be described in these two broad categories.

Inherited Thrombophilia

In 2003 Crowther and colleagues proposed a further sub-classification of inherited thrombophilia into either type 1 conditions that involve a deficiency of one of the naturally occurring inhibitors of coagulation, and type 2 conditions that result in a gain of function or an increase in the level of one of the procoagulant proteins.[10] The distinction is of clinical relevance as the majority of patients with a type 1 condition will develop a symptomatic episode of venous thrombosis during their lifetime, whereas the majority of individuals with a type 2 condition will not. Similarly the presence of a type 1 thrombophilia clearly increases the risk of recurrent venous thrombosis and therefore influences decision making regarding the duration of anticoagulation, where again type 2 conditions in isolation do not strongly influence recurrence risk and their absence or presence should not be used in isolation to determine duration of treatment.[12]

Type 1 Conditions

Antithrombin Deficiency

Antithrombin (AT) is a single chain plasma glycoprotein belonging to the serine protease inhibitor superfamily (serpins).[3] It is a physiological inhibitor of thrombin and other activated coagulation factors (factors Xa, IXa, XIa). Heparin exerts is anticoagulant effect by binding to AT, resulting in a conformational change that increases the affinity of AT for thrombi more than 1000-fold. Familial AT deficiency, described in 1965, was the first identified inherited thrombophilia.[2,3] Individuals with AT deficiency typically have AT levels ranging between 40-80%, and estimates of the prevalence of the condition range from 0.02% to 0.15% of the general population.[13] Approximately 0.5-2% of unselected individuals with venous thromboembolism (VTE) will have AT deficiency.[14] Estimates of the increase in risk of VTE associated with AT deficiency vary from 5 to 20-fold.

Protein C and Protein S Deficiency

Protein C (PC) and protein S (PS) are both vitamin K dependent plasma glycoproteins synthesized in the liver.[3] When activated by thrombin, a process potentiated by the binding of thrombin to thrombomodulin, PC is converted to the active serine protease, activated protein C (APC).[15] In combination with its cofactor, PS, APC inactivates both factor Va and VIIIa, and plays a central role in controlling the procoagulant pathway. In plasma, PS circulates both free (40%) and bound to the C4b-binding protein (60%). It is the free form of PS that has cofactor activity.

Inherited PC deficiency was first described as a cause of venous thrombosis in 1981,[16] whereas PS deficiency was initially described as a cause of venous thrombosis in 1984.[17] PC and PS deficiency both have type I (quantitative deficiency) and type II (qualitative deficiency) subgroups, and in addition a type III PS deficiency state with normal total circulating but reduced free levels can occur. The estimated prevalence of heterozygous PC deficiency in the general population is between 0.2 and 0.4%,[18] and many of these individuals have no history of thrombosis. The community prevalence of PS deficiency is estimated at approximately 0.2%.[19] PC deficiency is found in 1–3%, and PS deficiency in 1–7% of unselected patients diagnosed with VTE. Estimates

from case-control and family cohort studies of the increase in risk of VTE associated with PC deficiency range from 5.0 to 10-fold, and 8.5 to 30-fold for PS deficiency.[8]

Type 2 Conditions

Factor V Leiden

In 1993, Dahlback and colleagues noted that plasma taken from a family with a strong history of venous thrombosis was resistant to the anticoagulant effect of APC.[20] This phenotype became known as APC Resistance. A point mutation in the factor V gene (G1691A) resulting in an amino acid change (Arg^{506} to Gly) at the cleavage site involved in the inactivation of factor Va by activated protein C was identified as the cause in more than 90% of individuals and became known as factor V Leiden (FVL).[4,21] The FVL mutation has a high community prevalence with 3 to 7% of Caucasians being heterozygous for the mutation, although a lower incidence is found in other ethnic groups.[22] It is the most commonly identified cause of inherited thrombophilia, being present in 12 to 20% of unselected patients with VTE,[23] and up to 50% of individuals from thrombophilic families presenting with venous thrombosis. The heterozygous state is a relatively low risk thrombophilia being associated with a 3 to 7 fold increase in risk of VTE,[8] with one study finding greater than 90% of individuals remaining event free by the age of 65.[24] Unlike individuals homozygous for natural anticoagulant deficiency states, homozygosity for FVL does not result in a catastrophic thrombotic state early in life, and it is estimated that 0.1% of the population are FVL homozygotes.[8] The risk of VTE, however, in homozygotes for FVL is greater than that in heterozygotes, with estimates of the magnitude of risk ranging form 25 to 80-fold that of the healthy controls.

The Prothrombin (G20210A) Gene Mutation

In 1996, Poort and colleagues described a common mutation (G20210) of the prothrombin gene, which has become known as the prothrombin gene mutation (PGM).[5] Located in the 3' untranslated region of the gene, the mutation is associated with increased mean plasma prothrombin levels due to increased efficiency of 3' end processing of the gene resulting in accumulation of the encoded mRNA.[26] The prevalence of the mutation in Caucasian populations is approximately 2%, and it is rare in Asian and African populations.[27] In unselected patients with venous thrombosis the mutation has been found in between 4.0 to 7.1% of individuals,[8] and 18% of individuals with a strong family history of VTE. The PGM is a relatively weak risk factor for VTE, being associated with a 2 to 5 fold increase in risk.[8]

FVL/PGM compound heterozygotes

Given the high community prevalence of both the FVL and PGM mutations it is not uncommon for individuals to be heterozygous for both conditions, with an expected prevalence of 1 per 1000 in Caucasian populations.[28] In a pooled analysis of case control studies, double heterozygotes were estimated to have a 20-fold increase in risk of VTE in comparison to healthy controls.[28]

Other inherited conditions

Homozygosity for the C667T mutation in the methylenetetrahydrofolate reductase (MTHFR), producing a thermolabile gene product with reduced function, is the commonest inherited cause of raised plasma homocysteine levels.[29] In prospective studies a 5µmol/L (micromolar) increase in total plasma homocysteine levels has been shown to be associated with an approximate 1.3-fold

increase in the risk of venous thrombosis,[30] and patients with peripheral vascular disease have been shown to have a slight elevation of homocysteine levels in comparison to controls.[31] Conversely homozygosity for the C667T MTHFR mutation has been shown to have no association with venous thrombosis in folate replete societies,[29] and to have only a weak association with arterial disease (OR 1.2, 95% CI 1.0-1.4).[32] Performing testing for this mutation is therefore not recommended outside the research setting.

Elevated levels of the coagulation factors VIII, IX, XI and prothrombin (factor II) have all been shown to be associated with increased VTE risk. In the case of factor VIII, familial clustering of individuals with elevation of this factor has been demonstrated suggesting an underlying inherited cause, although a specific genetic defect is yet to be identified.[8] Other common mutations within coagulation proteins that have been documented to increase the risk of venous thrombosis include the Plasminogen Activator Inhibitor 4G/5G mutation (OR 1.62) and the alpha-fibrinogen Thr312Ala point mutation (OR 1.4). However there is no clear evidence that the presence of these mutations should alter patient management at present. [33]

Acquired Thrombophilia

There are a number of important acquired conditions that predispose to venous or arterial thrombosis that can be defined by laboratory testing. External or environmental acquired risk factors such as recent surgery or hospitalization, while often playing a central role in the causation particularly of venous thrombosis, will not be discussed further.

Antiphospholipid antibodies

The term antiphospholipid antibody syndrome (APLAS) was first used in the 1980's to describe a non-inflammatory autoimmune condition characterized by the presence of antibodies targeting a variety of phospholipid membrane associated proteins, and a history of either arterial or venous thrombosis or adverse pregnancy outcomes.[34] Laboratory confirmation of the presence of antiphospholipid antibodies requires the demonstration of the presence of a lupus anticoagulant, characterized by prolongation of phospholipid dependant coagulation assays such as the APTT, or a positive immunoassay for anti-cardiolipin or anti-beta2-glycoprotein1 antibodies. To classify a patient has having APLAS, antibody testing should be positive on at least two occasions 12 weeks apart.[35] The risk of an initial thrombotic event in patients with a positive test for antiphospholipid antibodies varies from no increase in blood donors in whom the often transient antibodies are an incidental finding, to annual risk of thrombosis of 2 to 4% in patients with SLE who are antibody positive.[34] As will be discussed, patients with the APLAS, particularly those with a positive test for a lupus anticoagulant, are at increased risk of recurrent thrombosis and therefore they will usually receive long-term anticoagulation after an initial event.

Heparin Induced Thrombocytopenia

Heparin induced thrombocytopenia (HIT) is immune-mediated adverse drug reaction to heparin. It results from the formation of antibodies, in the majority of patients, directed against a complex of heparin and the positively charged molecule platelet factor 4 (PF4).[36] These antibodies then bind to the heparin-PF4 complex bound to the platelet surface, leading to platelet activation most likely due to signalling via the platelet Fc receptors. Platelet and probable concurrent endothelial activation result in activation of the coagulation cascade and

increased thrombin generation, manifesting clinically as increased risk of venous and arterial thrombosis. Without institution of alternative anticoagulation, patients with confirmed HIT have a daily incidence of new thrombotic complications of up to 6%, with the historical risk of death or amputation due to venous gangrene approaching 50%. Early recognition of HIT is therefore important and monitoring of platelet counts between day 2 and 14 of exposure should be performed in all patients receiving heparin. A fall in platelet count to less than 150×10^9/L or a fall in total platelet count by greater than 50% should prompt laboratory investigation for HIT antibodies. Patients testing positive for HIT antibodies should be started on a non-heparin alternative anticoagulant such as lepirudin or danaparoid.[36]

Myeloproliferative Disorders

The primary bone marrow disorders polycythaemia rubra vera (PRV), myelofibrosis and essential thrombocytosis (ET) make up the bcr-abl negative myeloproliferative disorders. In almost all patients with PRV, and a significant proportion with ET, a somatic acquired mutation known as the JAK2 V617F mutation will be detected.[37] Patients with PRV and ET in particular have been shown to be at an increased risk of both venous and arterial thrombosis. The annual incidence of thrombosis in patients with essential thrombocytosis has been shown to be 12 per 1000 per year, of which approximately 50% will be arterial and 50% venous.[37] This compares with a background incidence in the general population of approximately 1 per 1000. Full blood examination is therefore recommended in all patients with venous thrombosis. It has also been recently observed that a significant proportion of patients with unprovoked portal and mesenteric vein thrombosis will be found to have the JAK2 V617F mutation present, often without clear evidence of a myeloproliferative disease on the peripheral blood examination.[38] While the therapeutic implications of this finding are still being evaluated, testing for this mutation should be considered in this patient group.

Potential Reasons for Performing Thrombophilia Testing

Clinical utility is an important concept when considering laboratory investigations for any condition. The clinical utility of any investigation can be defined as the degree to which the clinical outcome of an individual patient is improved by the performance of that test. Potential ways in which testing for an underlying thrombophilic condition may improve patient outcome are discussed below.

Patients With Venous Thrombosis and Their Relatives

a) Providing an understanding of the aetiology of a thrombotic event

As discussed above, a number of conditions have been shown to be clearly associated with an increased risk of a first episode of venous thrombosis (Table 10.1).[8,9] Patients with venous thrombosis are often keen to have an understanding as to why an event occurred, and therefore thrombophilia testing may help provide some explanation as to the aetiology of an event. It however should be emphasized that venous thrombosis is a multifactorial disease with many risk factors present at the time of an event, and therefore care should be taken in attributing an event entirely to an underlying thrombophilic condition.

The cost-effectiveness of performing thrombophilia testing solely to understand the aetiology is questionable. As discussed below, it is also important that both the patient and clinician understand that testing for the common genetic mutations, the FVL

TABLE 10.1: Increase in risk of initial and recurrent venous thrombosis with inherited thrombophilia.

	AT Deficiency	Protein C Deficiency	Protein S Deficiency	FVL mutation*	PGM mutation*
Increase in risk of first episode VTE	5 to 20-fold	5 to 10-fold	5 to 30-fold	3 to 7-fold	2 to 3-fold
Increase in risk of recurrent VTE	2.0-fold (pooled data)			1.2 to 1.6 fold	1.4 fold

*Refers to heterozygote state

and PGM mutations, is unlikely to change management, and that the results of a positive test for these conditions are not over-interpreted. Finally the potentially negative impact of testing including implications for insurance should be taken into account before testing is performed.

Determining risk of recurrence and therefore optimal duration of anticoagulation

Patients with venous thrombosis are at risk of recurrent events, with approximately 30% of affected individuals subsequently experiencing a recurrent event within 5 years of ceasing anticoagulation.[38] A potential role for thrombophilia testing is therefore to identify those patients at greatest risk of recurrent thrombosis, in whom exposure to the increased risk of haemorrhage with long-term anticoagulation may be justified. This is most relevant in patients with unprovoked venous thrombosis who have a substantially increased risk of recurrent thrombosis in comparison to patients in whom the event was associated with a definite provoking risk factor.

The high incidence inherited thrombophilic conditions, the FVL and PGM mutations, do not significantly increase the risk of recurrent thrombosis. A recent meta-analysis found that patients heterozygous for the FVL mutation compared to patients without the mutation had an approximate 1.6-fold increase in the risk of recurrent thrombosis.[39] When this analysis was restricted to patients with an unprovoked event this decreased to a 1.2-fold increase in risk that was no longer statistically increased. The same analysis found a borderline significant 1.4-fold increase in risk of recurrent venous thrombosis in patients heterozygous for the prothrombin gene mutation. This data suggests heterozygosity for the FVL or PGM should not be used by itself to determine duration of anticoagulation.

There is less data regarding the impact of antithrombin, protein C and protein S deficiency on the risk of recurrent venous thrombosis, and due to their lower incidence data tends to be pooled for all three conditions. Data from prospective cohort studies of unselected patients with venous thrombosis has suggested an approximate 2-fold increase in the risk of recurrence in patients with deficiencies of these proteins in comparison to patients with normal levels.[8] A retrospective study of thrombophilic families found that individuals with AT, PC and PS deficiency had a cumulative incidence of recurrent thrombosis of 55% by 10 years after ceasing anticoagulation, in comparison to a figure of 25% in patients with FVL, PGM or elevated FVIII levels.[40] These data suggest that patients with confirmed AT, PC or PS deficiency may benefit from long-term

anticoagulation. It is important to stress that the levels of these proteins may be spuriously low, for example in the case of recent extensive thrombosis, and, for protein C and S due to warfarin therapy. Therefore repeat testing in the absence of confounding factors should be performed to confirm the diagnosis prior to therapeutic decisions being made. While data is lacking on clinical factors that can be used to reliably identify patients with venous thrombosis that will have a deficiency of one of the natural inhibitors of coagulation, it would appear reasonable to focus testing on patients with unprovoked events, younger age (<50 yrs of age), unusual site of thrombosis, or a strong family history (>1 first degree relative) of venous thrombosis.

As previously mentioned, patients with antiphospholipid antibody syndrome have been demonstrated to have an increased risk of recurrent thrombosis, with estimates of risk ranging from 10 to 60% per annum.[34] In addition, patients with antiphospholipid antibody syndrome have been demonstrated to have an increased risk of death after ceasing anticoagulation, contributed to by the fact that this patient group is at increased risk of not only recurrent venous thrombosis but also arterial complications.[41] Therefore long-term anticoagulation is generally recommended for patients who meet the diagnostic criteria for this condition.

Determining the need for primary prophylaxis in asymptomatic family members

Another possible role for thrombophilia testing is determined if the baseline risk of venous thrombosis is sufficient to warrant primary prophylaxis with anticoagulation. Given the lack of evidence supporting a role for anti-platelet therapy in preventing venous thromboembolism, at present this would require a sufficiently high risk to justify exposure to the 2 to 3% annual risk of major haemorrhage associated with vitamin K antagonist therapy of which approximately 20% will be fatal.

As shown in Table 10.2, the annual risk of venous thrombosis in previously asymptomatic patients with venous thrombosis varies from approximately 0.3% with the PGM to up to 2% in patients with AT or protein S deficiency.[8,9,40] This is against a background rate of approximately 0.1% per annum in the general population, with incidence increasing with age. It is generally accepted that given the risk associated with oral anticoagulation, that primary prophylaxis is therefore not justified in patients with any of the known inherited thrombophilias. It has been shown that between 50 to 60% of episodes of venous thrombosis in previously asymptomatic family members with thrombophilia will occur in

TABLE 10.2: Risk of venous thrombosis in asymptomatic family members with inherited thrombophilia.

	AT Deficiency	Protein C Deficiency	Protein S Deficiency	FVL mutation*	PGM mutation*
Overall risk (risk / year)	1.5–2.0%	1.0–1.5%	1.5–2.0%	0.5%	0.3–0.4%
Oral Contraception (risk / yr exposure)	4 to 5% (pooled data)			0.3–0.5%	0.2%
Pregnancy (risk / pregnancy)	~ 4.0% (pooled data)			~2.0%	~2.0%

*Refers to heterozygote state

the context of an additional environmental risk factor such as surgery. While not clearly demonstrated in clinical trials, it is possible that more aggressive thromboprophylaxis may be justified particularly in patients with type 1 thrombophilic conditions.[9] Again, if testing is performed for this indication, care must be taken to avoid over-interpretation of the test result by both patient and other clinicians.

Making decisions regarding the use of the oral contraceptive pill

Knowledge of whether a previously asymptomatic individual is a carrier of a known inherited thrombophilia may influence decision-making regarding exposure to the pro-thrombotic effects of oral contraception. Estimates of the annual risk of thrombosis with use of a combined oestrogen/progesterone oral contraceptive (OCP) are shown in Table 10.2.[8] Generally women of child bearing age have a low annual risk of thrombosis of approximately 1 to 2 per 10000 per year. Therefore despite the combination of oral contraceptive use and being heterozygous for the FVL mutation producing an approximate 30-fold increase in risk, the absolute risk per year is still relatively low at no greater than 0.5% per annum.

Most clinicians would accept that the degree of risk associated with OCP use in patients with type 1 thrombophilic conditions justifies avoidance and use of other contraceptive measures, including progesterone only pills or intrauterine devices that do not increase the risk of thrombosis. The decision regarding OCP use in women heterozygous for FVL and PGM is less black and white, and will be influenced by patient perception of the benefit obtained from OCP use, and the presence of other risk factors for venous thrombosis such as obesity.

Determining the need for thromboprophylaxis during pregnancy

The risk of venous thrombosis during pregnancy in women with no prior history of thrombosis associated with the presence of common inherited thrombophilic conditions is shown in Table 10.2.[8,9] Two-thirds of pregnancy related episodes of venous thrombosis will occur during the post-partum period. Again the case for prophylactic anticoagulation during pregnancy can be made most strongly for women with type 1 conditions, particularly for antithrombin deficiency that in some studies is associated with a risk of ante-partum events of up to 10%. As a minimum, post-partum prophylaxis should be administered for 6 to 8 weeks. In FVL and PGM heterozygotes ante-partum prophylaxis is generally not recommended in women with no prior history of events. Post-partum prophylaxis should be considered, particularly in women with additional risk factors.

Patients with arterial thrombosis

The association between inherited thrombophilic conditions and arterial disease has not been clearly demonstrated. Case reports and small studies have linked antithrombin, protein C and protein S deficiency to arterial disease, however the data are inconclusive.[8] Larger studies have evaluated the link between the FVL and PGM mutations with both coronary artery disease, myocardial infarction and stroke. Generally the findings have been of either no link or a weak association with odds ratios of < 1.5,[8,9] with some data suggesting a stronger association with myocardial infarction in younger patients with the additional risk factor of smoking. There is also no conclusive evidence supporting an association of thrombophilia with peripheral arterial disease. Based on the lack of a clear association of inherited

thrombophilia with arterial disease, and no data supporting a change in management based on the knowledge that the presence of a thrombophilic condition improves patients outcome, is has been strongly recommended that testing for inherited thrombophilia should not be performed in patients with arterial disease.

As stated above, the association of antiphospholipid antibodies with an increased risk of arterial disease is more definitive. It is generally recommended that patients with APLAS and arterial disease should be treated with warfarin rather than antiplatelet agents, although the evidence supporting this approach remains minimal.[34]

The clinical utility of measuring homocysteine levels in patients with arterial disease at present remains unclear. While a number of trials have shown benefit of B-vitamin supplementation on surrogate end-points of arterial disease, a recent meta-analysis found no reduction in clinical end-points in patients with either cardiovascular disease or stroke with supplementation therapy.[42]

POTENTIAL DETRIMENTAL EFFECTS OF THROMBOPHILIA TESTING

A small number of studies have examined the potential psychological impact on patients of performing thrombophilia testing.[43] While the general conclusion was that the impact was low, it was clear that many patients were unclear that they had been tested, and the knowledge of having a thrombophilia did cause significant distress in some individuals. Other potential drawbacks to testing for inherited thrombophilia may include difficulty with obtaining or changes to the cost of life-insurance, and questionable cost-effectiveness.[8]

CONCLUSION

It can be concluded that despite the ability to detect an underlying thrombophilia in up to 50% of patients with venous thrombosis, it is doubtful that performing laboratory testing for thrombophilias has a positive effect on patient outcome in the majority of patients. The strongest case for testing for inherited thrombophilia can be made for type 1 conditions, although these conditions will be detected in only approximately 5% of patients. The evidence that testing for FVL and the PGM abnormalities improves patient outcome is limited. Widespread testing in unselected patients is recommended against, and a stronger case can be made for patients with female first-degree relatives of child-bearing age. Prior to any testing being performed, the clinician involved in test-ordering should counsel the patient regarding the implications of both a positive and negative test result, and how this will change patient management. If it is unclear how the test result will change treatment for the individual or relatives, then testing should not be performed.

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11 • Platelets in the Pathogenesis of Vascular Disease and their Role as a Therapeutic Target

SANDEEP PRABHU[1], RAHUL SHARMA[1], KARLHEINZ PETER[1,2]

[1.] Alfred Hospital, Melbourne, Australia
[2.] Baker IDI Heart and Diabetes Institute, Melbourne, Victoria, Australia

INTRODUCTION

Platelets are key blood components with a physiological role in the initiation of endogenous haemostasis and effective endothelial repair following vascular injury. Platelets are responsible for the initiation of a series of complex interactions culminating in platelet aggregation and thrombus formation. As such, key platelet functions, such as adherence, activation, aggregation and interaction with coagulation factors, operate in the context of a complex and balanced interplay of receptors and mediators that ensure this process is controlled and specifically targeted to areas of vascular injury. However, in disease states, such as atherosclerosis, the abnormal initiation of platelet functions also contributes to the pathogenesis and propagation of vascular disease. Consequently, targeted therapeutic inhibition of platelets has demonstrated an important clinical role in situations of both pathological and iatrogenic vascular injury, such as atherosclerosis and angioplasty. This chapter will firstly outline the relevant platelet receptors, their agonists and other important structural platelet components and their role in platelet function. Secondly, it will outline the role of these functions in the pathogenesis and propagation of vascular disease. Finally, the mechanism of therapeutic anti-platelet agents will be reviewed along with a description of currently used methods to assess platelet function.

PLATELET FUNCTION – ADHESION AND ACTIVATION

Platelets are enucleated cytoplasmic fragments of bone marrow megakaryocytes with a limited capacity for protein synthesis. Although lacking DNA, platelets do contain megakarocyte mRNA along with components necessary for protein synthesis,[1] and are capable of performing nuclear functions such as pre-RNA splicing.[2] Once in the bloodstream, platelets have a lifespan of 7–10 days. The primary function of platelets is to stop haemorrhage from sites of vascular injury. This is accomplished through the key platelet functional processes of adhesion, activation, cross-linking or aggregation, with the involvement of several important pro-activation mediators.

Platelet adhesion

Platelet adhesion is initiated by tethering of circulating platelets to an area of vascular injury. Usually, the intact endothelium prevents unwanted platelet activation by acting as a physical barrier to underlying thrombogenic substances (such as collagen, tissue factor and von Willebrand factor) and by releasing mediators that inhibit platelet activation (Figure 11.1). This involves three separate pathways, (1) the arachadonic acid-prostacyclin pathway, (2) the L-arginine-nitric oxide pathway and (3) the endothelial ecto-adenosine diphosphatase (ecto-ADPase) pathway.[3] Endothelial cyclooxygenase 1 & 2 (COX-1 & 2) convert arachadonic acid to prostacyclin metabolites (such as prostaglandin I2 (PGI2)) which elevate platelet intracellular cAMP levels and inhibit platelet activation in a process thought to be mediated by protein kinase A.[4,5] Nitric oxide, produced by endothelial cells, passively diffuses into platelets causing an increase in cytosolic cyclic guanine monophosphate (cGMP) levels and activation of cGMP dependant protein kinases with a consequent reduction in intracellular calcium.[6] Ecto-ADPase is a protein constituent of the endothelial cell surface, which upon activation, limits the recruitment phase of platelet reactivity by reducing plasma concentrations of nucleotides, particularly ADP.[7]

Endothelial cells with impairment of the above processes are termed dysfunctional, and express an 'atherogenic' profile of receptors such as P-selectin, E-selectin, ICAM-1 and VCAM-1 (as seen in Figure 11.1), as do endothelial cells which have been activated by exposure to various mediators (such as thrombin, TNF-α and LPS), sepsis, trauma, rapid temperature variations, shear stress and minor alterations to the local micro-environment.[8,9] These features usually also accompany acute vessel injury. However, in the absence of acute injury, an activated or dysfunctional endothelium may result from prolonged exposure to high blood pressure, shear stresses and dyslipidaemia, and consequently result in pathological platelet activation and inflammatory cell recruitment.[10]

Platelet adhesion begins by the exposure of circulating platelets to an activated or dysfunctional endothelium, or to exposed sub-endothelial matrix proteins such as collagen, fibrinogen and von Willebrand factor following endothelial injury.[11] These ligands are capable of binding to receptors on inactivated platelets at high shear rates and tethering them to the site of vascular injury. Collagen binds to glycoprotein VI (GPVI), whilst von Willebrand factor binds to the platelet receptor GPIb-IX-V.[12] In addition, collagen also binds von Willebrand factor, which is a mechanism of facilitating the adhesion of other inactivated platlelets. P selectin on the surface of activated endothelial cells, also binds to GP1bα and PSGL-1 on the platelet surface, facilitating tethering and rolling. Platelet adhesion triggers the process of platelet activation, culminating in the activation of the GPIIb/IIIa receptor, enabling it to bind soluble fibrinogen and von Willebrand factor allowing firm adhesion of the platelet to the endothelium[13] via fibrinogen bound to receptors on the endothelial surface (αvβ3 and ICAM-1). Figure 11.1 illustrates how a tethered platelet becomes activated and firmly adherent via fibrinogen bound surface receptors.

GPIIb/IIIa (αIIbβ3, CD41/CD61) is a member of the superfamily of 'integrin' type receptors, which are transmembrane proteins comprising of various combinations of non-covalently bonded subtypes of α and β subunits. Integrins are involved in intracellular and extracellular signal transduction (in both directions) as well as the mechanical coupling of cytoskeleton proteins to either the extracellular matrix or surface receptors on other cells.[14]

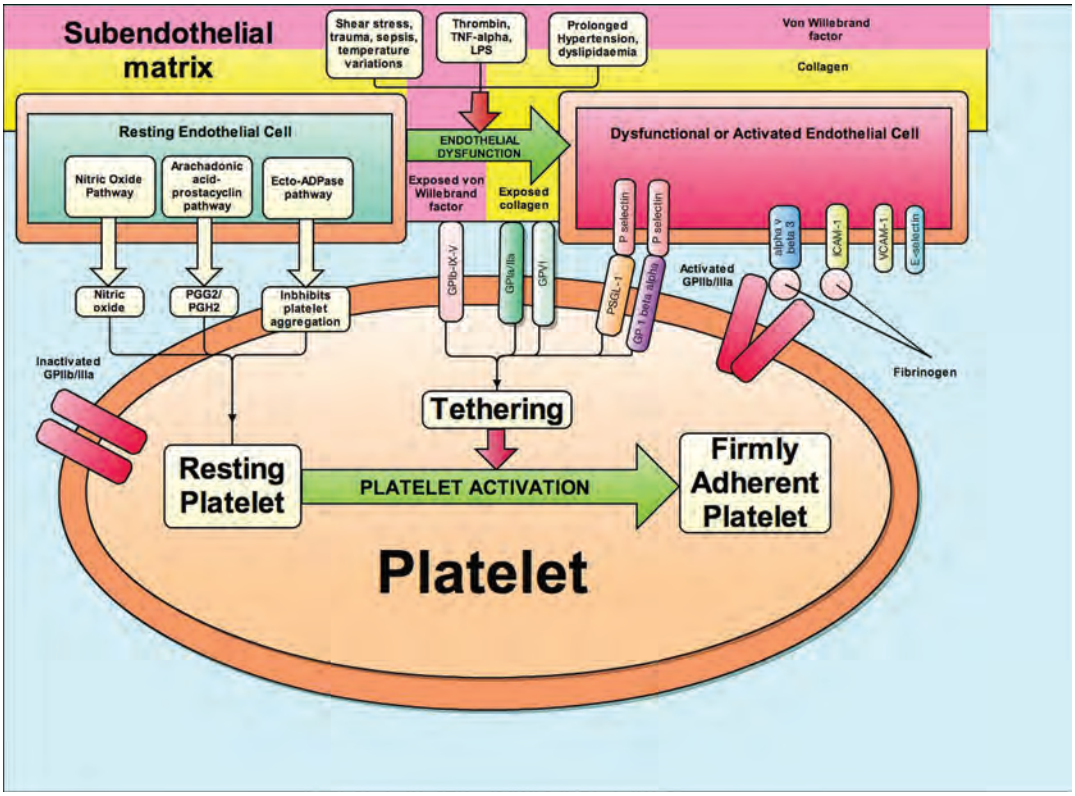

FIGURE 11.1: Mechanisms of platelet adhesion to dysfunctional endothelium. Normal endothelial cells inhibit platelet activation by three primary pathways as shown. Endothelial cells activated by injury, sepsis or inflammation, or dysfunctional (for example, after exposure to prolonged hypertension), express an array of receptors that facilitate tethering of platelet to the vascular wall, removing it from the blood stream. These interactions, particularly with potent platelet stimulators such as collagen, promote activation of the platelet; enabling activated GPIIb/IIIa receptors to bind fibrinogen bound on the surface of endothelial cells by receptors such as $\alpha v\beta 3$ and ICAM-1. Platelet-adhesion is also mediated by interactions between platelet integrin receptors and exposed sub-endothelial matrix proteins such as collagen and von Willebrand factor. These mechanisms result in firm adhesion of the activated platelet to the vessel wall.

Platelet activation

In contrast to the relatively passive process of platelet adhesion, platelet activation is a metabolically active process involving several important and generally irreversible, biochemical and physical alterations to the platelet. These processes include the release of preformed mediators, alteration to the surface receptor profile and cytoskeletal changes resulting in a dramatic physical change to the platelet structure. These changes serve to facilitate the incorporation of the activated platelet into a developing platelet thrombus, activate neighbouring platelets via positive feedback mechanisms whilst also playing a key a role in the recruitment of inflammatory cells and the propagation of a broader inflammatory response.

Mediators of platelet activation and 'outside-in' signaling

Various non-chemical stimuli can activate platelets. These include hypothermia, trauma and alterations to acid-base balance. Nonetheless, endogenous molecules mediate the vast majority of platelet activation in both

the physiological and pathological setting, acting via both autocrine and paracrine mechanisms. The most important of these are collagen, thrombin, adenosine diphosphate, adrenaline and thromboxane A2.

'Outside-in signaling' refers to the process of mediators binding to specific receptors on the platelet surface and initiating a secondary messenger response inside the platelet. These processes are mediated via several secondary messenger pathways namely: the phospholipase C and PI-3 kinase pathway, the eicosanoid and arachidonate pathway, protein kinase C and the cAMP and cGMP pathways.[15] 'Inside out signaling' refers to intracellular pathways mediating functional changes to surface receptors, such as the GPIIb/IIIa receptor (as discussed below).

Thrombin and collagen

Collagen and thrombin are the most potent platelet stimulators. Collagen binds directly to GPVI and the integrin $\alpha_2\beta_1$ (also known as GPIa/IIa) and is crucial in the initial tethering of platelets to sites of vascular injury. Other integrin receptors bind collagen bound to von Willebrand factor. Collagen types I, III and VI are the most common type of collagen in the blood vessel subendothelial matrix and they bind directly to GPVI (see Figures 11.1 and 11.3). After binding, a series of intracellular signaling processes result in protein phosphorylation and consequently platelet activation.[16]

Thrombin is generated at sites of vascular injury from its precursor prothrombin, by virtue of the intrinsic, tissue factor-driven pathway of the coagulation cascade. Thrombin binds to G-protein linked protease activated receptors (known as PARs) on the platelet surface. Human platelets express two distinct PAR receptors, PAR1 and PAR4. PAR1 is coupled to Gα12/13, Gαq and Gαi proteins, which mediate cytoskeletal responses, increased intracellular calcium and reduced cAMP respectively, each of which are crucial steps in platelet activation (Figure 11.2). PAR1 appears to be a more potent activator of platelets than PAR4 and has been proposed as a potential target for therapeutic inhibition (atopaxar – see Figure 11.3). Effective inhibition of PAR1 and PAR4 leads to near complete inhibition of platelet activation, even in the presence of high concentrations of thrombin.[17]

Adenosine diphosphate (ADP)

ADP is generated and stored in platelets and red blood cells in dense granules. Platelet activation results in the release of stored ADP granules and activation of nearby platelets – a key amplifying process (Figures 11.2 & 11.3). Several receptors, known as P2 receptors interact with ADP resulting in platelet activation. Present on platelets are the P2X2, P2Y1 and P2Y12 receptors. P2X2 is an intrinsic ion channel, which upon ligand binding allows calcium influx into the platelet, promoting activation.[18] P2Y1 is a G-protein coupled seven transmembrane domain receptor which activates protein phospholipase C causing release of stored intracellular calcium and facilitating conformational change of the platelet. P2Y12 is similarly a G-protein coupled receptor that, upon activation, mediates a reduction in intracellular cAMP (Figure 11.2). Of these receptors, P2Y12 plays the more significant role in amplifying and sustaining the platelet activation process. Platelet function studies have demonstrated that simultaneous activation of both P2Y1 and P2Y12 receptors is needed for the full platelet response to ADP. Both these features have made inhibition of P2Y12 with clopidogrel a very effective therapeutic target.[19]

Thromboxane A2 (TXA2)

Thromboxane A_2 is synthesized from arachadonic acid in activated platelets via

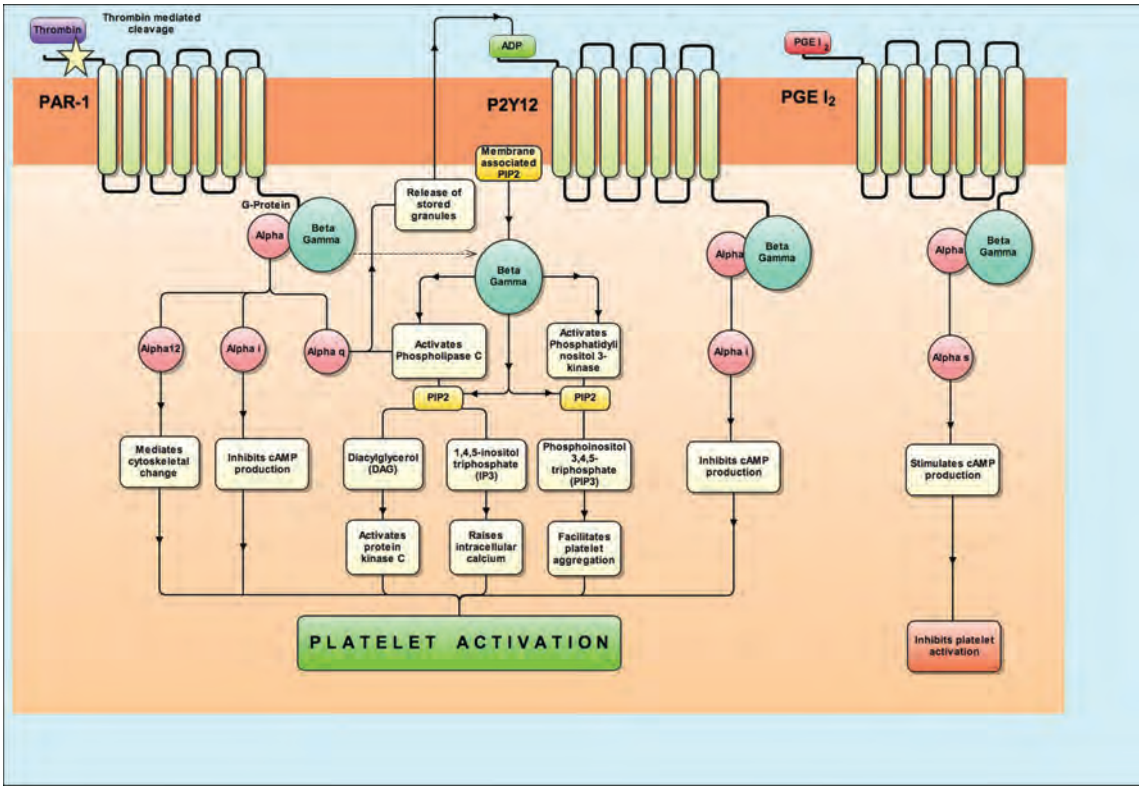

FIGURE 11.2: Intracellular signaling mechanisms involved in platelet activation. The signaling pathways of three platelet receptors are depicted as examples: PAR-1 (protease-activated receptor-1; the main ADP receptor, P2Y12; PGE I2). Each of these receptors is a seven transmembrane domain G-protein coupled receptor. Each G-protein consists of an α and $\beta\gamma$ subunit. The $\beta\gamma$ subunit is involved in the formation of DAG, IP3 and PIP3, which play key roles in platelet activation. The Gα subunit exists in several isoforms. Gα12 mediates platelet shape change by activating cytoskeletal proteins. Gαq plays a pivotal role by activating phospholipase C, and facilitating the formation DAG and IP3, which promote platelet activation by activating protein kinase C and raising cytosolic calcium. In addition, Gαq also promotes release of pre-formed granules containing pro-activating and pro-inflammatory mediators, such as ADP. Gαi and Gαs inhibit and stimulate cAMP production respectively with lower intracellular cAMP concentration favoring activation. Gαs is primarily made available in response to prostaglandin I_2 action, primarily from release of endothelial cells, inhibiting platelet activation. (Adapted from Abrams CS et al 'Platelet Biology' – *UptoDate article* Jan 2010 and Bhatt DL et al 'Scientific and therapeutic advances in antiplatelet therapy' (2003) *Nature Reviews Drug Discovery* 2: 15-18). cAMP = cyclic adenosine diphosphate, PGE I_2 = prostaglandin I_2.

the cyclooxygenase pathway. It freely diffuses across the platelet membrane to activate neighbouring platelets. Thromboxane A_2 binds to Tα or Tβ receptors, which are in turn coupled to G-proteins Gαq, Gα12 or Gα13 – each of which activate phospholipase C – an enzyme which degrades membrane phosphoinositides creating key second messengers inositol triphosphate (IP3) and diacylglycerol (DAG) which facilitate protein kinase C mediated intracellular protein phosphorylation and raised intracellular calcium respectively (Figures 11.2 and 11.3). The G-protein linked receptors aid in amplification of the response to ligand binding as a single receptor may interact with multiple G-proteins.[20]

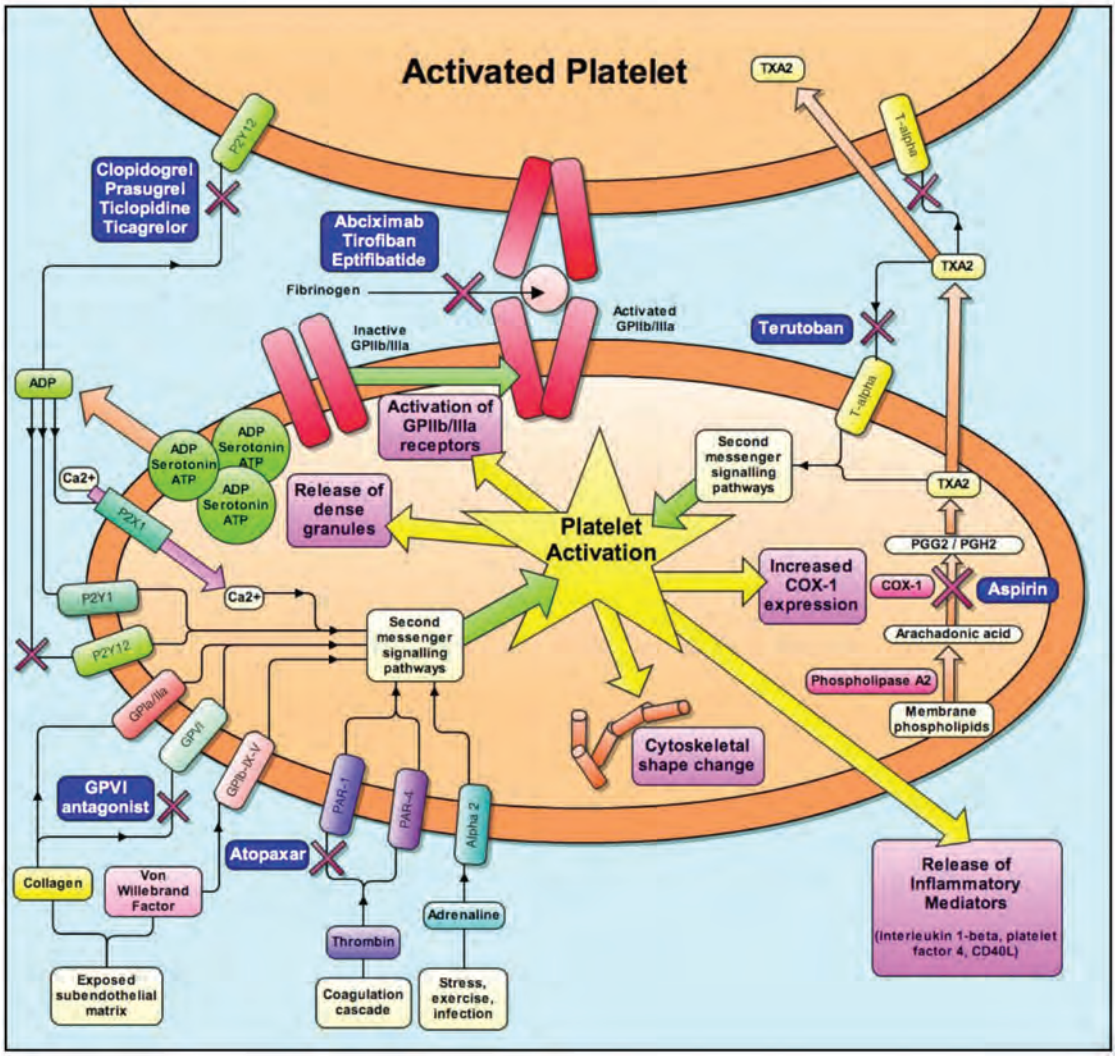

FIGURE 11.3: Mechanisms of platelet activation and platelet inhibition. A wide array of mediators can trigger platelet activation. Thromboxane A2 is produced from membrane derived arachadonic acid in a process inhibited by aspirin. TXA_2 can then freely diffuse across the platelet membrane and activate both its own and neighboring platelets. A similar mechanism of amplification is provided by the release of ADP and the stimulation of P2Y12 receptors. Other mediators act via specific receptors that promote platelet activation by raising intracellular calcium (eg. thrombin, TXA2) or reducing intracellular cAMP concentrations (ADP). These receptors provide useful therapeutic targets for platelet inhibition (shown in blue). Once activated, platelets can interact with neighboring activated platelets via fibrinogen bound to activated GPIIb/IIIa receptors, allowing cross linking and the formation of a platelet thrombus. (Adapted from Hankey GJ et al 'Antiplatelet Drugs' (2003) *MJA* 178(11): 577-8).

Adrenaline

Adrenaline is the least potent physiological stimulator of platelet activation. Surface α2 adrenergic receptors are G-protein linked receptors which are potent inhibitors of cAMP formation, although studies suggest supra-physiological doses are required for activation of platelets by adrenaline alone.[21] Nonetheless, circulating adrenaline release by a stress response, may serve to reduce

overall platelet activation threshold, making platelets more susceptible to lower doses of other platelet activating mediators.

Second messenger systems

Platelet-activating ligands bind to G-protein coupled receptors inducing intracellular second messenger pathways, which then mediate the biochemical and structural changes associated with platelet activation. G-proteins usually consist of α and $\beta\gamma$ subunits. The α subunit exists in several isoforms, each mediating a specific intracellular function.[22] The α_q and $\beta\gamma$ subunits activate phospholipase C (both) and PI3K ($\beta\gamma$) in a manner discussed further below. The α_s and α_i subunits promote or inhibit intracellular cyclic AMP activity respectively – although the exact mechanism involved is as yet unclear. Increased intracellular cAMP activity is associated with inhibition of platelet activation, a process utilized by the anti-platelet agent dypridimole. The α_{12} subunit is involved in mediation of shape change by promoting cytoskeletal reorganization (as discussed further below).[23] Figure 11.2 provides a broad outline of the key intracellular second messenger systems involved in platelet activation by several key receptors.

There are two central intracellular pathways involved in platelet activation – the phosphoinositide hydrolysis pathway and the eicosanoid synthesis pathway. The former is a consequence of the activation of phospholipase C beta (via G alpha-q subunit of G-protein linked receptor proteins) and the activation of phosphoinositol 3-kinase gamma (via G beta-gamma). Once activated phospholipase C hydrolyzes PI-4,5-P(2) (PIP2) to DAG and IP(3). DAG in turn binds to and activates protein kinase C, causing phosphorylation of key enzymes known as protein kinase C iso-enzymes. IP(3) binds to receptors within the intracellular tubular system, resulting the release of sequestered intracellular calcium.[15]

The eicosanoid pathway results in the formation of thromboxane A_2. Platelet activation results in the release of arachidonate from membrane phospholipids by the action phospholipase A_2, which is stimulated by raised intracellular calcium. Arachidonate is then metabolized to thromboxane A_2 by the action of cyclooxygenase-1 (COX-1), by a process inhibited by aspirin.[24]

Physiological consequences of platelet activation

Platelet activation involves four primary features: (1) An important conformational change of the GPIIb/IIIa integrin receptor, allowing it to bind fibrinogen and von Willebrand factor, promoting platelet aggregation and the formation of a platelet thrombus. (2) The release of preformed intracellular granules of ADP and thromboxane A_2 promotes further platelet activation and a local positive feedback of the activation process. (3) Activation results in a conformational change in the platelet itself, by rearrangement of the internal cytoskeletal ultra-structure. (4) It is increasingly recognized that platelet activation augments an inflammatory response by the surface expression of receptors, the recruitment of inflammatory cells, and the release of pro-inflammatory mediators. These functions have an important physiological role in the engagement of repair processes following injury. Nonetheless, inappropriate activation of these processes are crucial elements in pathogenesis of atherosclerosis.

The GP IIb/IIIa receptor and 'inside-out' signaling

The GPIIb/IIIa receptor is from the β_3 subgroup of the integrin receptor super-family.[25]

The receptor consists of two proteins each with a transmembrane domain. GPIIb consists of a heavy (105kD) and light (25kD) chain linked via a disulfide bond. GPIIIa consist of a single (95kD) chain.[26] Once activated, the receptor recognizes RGD (arginine-glycine-aspartic acid) and KQAGDV (glycine-glutamine-alanine-glycine-aspartic acid-valine) peptide sequences present on fibrinogen (both sequences), von Willenbrand factor and fibronectin (RGD sequence) making each of these proteins ligands for the activated receptor – with fibrinogen being the most potent.[27] As shown in Figure 11.3, one fibrinogen molecule can serve to crosslink platelets and augment platelet aggregation.[25]

GPIIb/IIIa receptors are found exclusively on platelets (with the exception of the platelet precursor, the megakaryocyte), with approximately 60-80,000 receptors per platelet comprising 2% of all protein present in the platelet. They have been utilized in the clinical setting as potent anti-platelet targets as discussed further below.

Granule exocytosis

Granule exocytosis is a key consequence of platelet activation, allowing platelet activation to be exponentially amplified and a local inflammatory response to ensue. Microscopically, platelets contain dense, alpha and lysosomal granules. Dense granules contain platelet agonists, which promote platelet activation such as ADP, ATP and serotonin. Alpha granules contain adhesion-promoting proteins including fibrinogen, fibronectin, vitronectin and von Willebrand factor. These mediate platelet aggregation. Lysosomal granules contain glycosidase and proteases, the role of which is unclear. SNARE complex proteins (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) are thought to be the primary mechanism regulating the vesicle-membrane interactions. Granule exocytosis is thought to be modified by the presence of aspirin, explaining part of its anti-platelet action.[28]

Activation-induced conformational change of platelets

Once activated, platelets undergo a dramatic physical shape change, losing their discoid shape and developing elongated projections of cytoplasm, called filopods, mediated by reorganization of the cytoskeletal ultra structure. This reorganization involves alterations to three primary components of the platelet cytoskeleton: the cytoplasmic actin network, the cytoskeletal rim and the marginal band. The membrane associated cytoplasmic actin network consists of both filamentous polymers and monomeric globular forms of actin, a 42kDa abundant cytoskeletal protein. Platelet activation results in an increase in the proportion of filamentous or F-actin, and reorganization of the actin network into longer actin filaments promoting conformational change. This process is mediated by phosphatidylinositol produced during platelet activation. F-actin filaments are anchored to the plasma membrane, via actin binding protein via the GPIb/IX complex.[29] The cytoskeletal rim contains multiple components including actin, filamin, talin, vinculin, spectrin (also seen in red blood cells), and alpha actin along with multiple membrane glycoproteins. The interaction of filamin with actin is the key factor in preserving the discoid shape of the resting platelet. Disruption of this interaction occurs in the presence of rising cytosolic calcium concentration, resulting in a loss of tethering of the GPIb to the cytoskeletal ring, promoting conformational change. Shape change is also facilitated by contraction of the tubulin polymers of the marginal band, however the exact biomechanical significance of this is still yet to be determined.[30] The net result of the above processes is the

physical transformation of the platelet from a discoid to a flat, broadened and star-shaped conformation known as spreading – allowing efficient incorporation of the platelet into an evolving platelet thrombus.

PLATELETS AND ATHEROSCLEROSIS

There is increasing evidence that platelets play a crucial role in all stages of the pathogenesis of vascular disease – particularly atherosclerosis. Research over the last 10–15 years has demonstrated that atherosclerosis involves an active inflammatory process rather than the benign accumulation of intra-luminal lipids.[31] Platelets play key roles in the development and progression of atherosclerotic plaques, by the action of released mediators and facilitating interactions with other inflammatory cells. For the subset of atherosclerotic plaques that are unstable or prone to rupture, localized platelet activation and aggregation result in an occlusive platelet thrombus interrupting blood flow and causing distal ischemic injury. This mechanism underpins myocardial infarction and acute coronary syndromes, and explains to some degree the effectiveness of anti-platelet agents in the treatment and prevention of such conditions.[32]

Role of platelets in the initiation of atherosclerosis

Platelets are the first cell to arrive at the developing atherosclerotic lesion. Studies demonstrate that platelets adhere to carotid endothelium of ApoE deficient mice.[33] P-selectin (CD62P) and E-selectin are expressed on the surface of activated endothelial cells (and platelets) which interact with GP1βα, PSGL-1 and the von Willebrand receptor complex receptors on the platelet surface in a loose manner which is insufficient for stable adherence, but instead facilitates the rolling process[34] (Figure 11.1). In addition, soluble von Willebrand factor is secreted by endothelium in response to inflammatory stimuli. Mice deficient in von Willebrand factor demonstrate a reduced propensity towards atherosclerosis.[35]

As platelets roll along the surface of activated endothelium, they become activated, and firm adhesion can occur via the interaction between β_3 integrins present on endothelial cells and the fibrinogen bound GPIIb/IIIa receptor on platelets (Figure 11.1). Once activated, platelets become firmly adherent and are able to recruit other platelets to the area of endothelial injury.[36] Inhibition of platelet activation, such as suppression of COX-1 dependant thromboxane A2 production or activity, has been demonstrated to slow the formation of atherosclerosis in murine models.[37]

Role of platelets in the progression of atherosclerosis

Activated platelets also express P-selectin on their surface, which not only mediates platelet-endothelial interactions, but also stimulates neighboring monocytes and macrophages to release pro-inflammatory mediators. For example, P-selectin mediated signaling between aggregated platelet and monocytes promotes up regulation of COX-2 mRNA and the production of interleukin-1β, which promotes inflammation and further platelet activation.[38]

Firmly attached platelets have also been shown to recruit monocytes from the blood stream to the site of vascular injury in a process described as 'tethering'. Such platelets interact with circulating monocytes via PSGL-1 (P-selectin glycoprotein ligand-1 – on monocytes) and P-selectin expressed by platelets, multiple platelet receptors including

the fibrinogen bound activated GPIIb/IIIa receptor and MAC-1 (on monocytes) and the lymphocyte function associate antigen (LFA-1), which binds to ICAM-2 on platelets. These interactions result in monocyte recruitment to the injured endothelium.[39]

These platelets release an array of pro-inflammatory mediators such as interleukin-1β, platelet factor 4, RANTES (regulated upon activation, normal T cell expressed and secreted) and CD40 ligand.[25] These mediators promote localized inflammation and atherosclerotic development by activating the vascular endothelium to facilitate the chemoattraction, chemotaxis and transmigration of monocytes.

Role of platelets in vulnerable plaques and plaque rupture

Platelets have a well-established role in the development of a thrombus after the rupture of the thin fibrous cap present in vulnerable plaques. Disruption of the thin fibrous cap, usually in the adjoining shoulder region, exposes the highly thrombogenic lipid core to the bloodstream, triggering a cascade of platelet activation and thrombosis.

However, the extent to which platelets interact with an established vulnerable plaque before it undergoes a clinically significant rupture is uncertain. It is circumstantially suggested by the success of antiplatelet therapies in reducing ischemic events.[40] Subclinical plaque rupture is a frequent event with 9% of autopsies on patients not dying from myocardial infarction demonstrating ruptured fibrous caps (22% in patients with cardiovascular risk factors). This suggests that rather than every plaque rupture precipitating an ischemic event, it is likely that the thrombotic response to plaque disruption is dynamic with thrombosis and thrombolysis occurring simultaneously in patients with acute coronary syndrome.[41,42] Consequently, a rupture prone plaque may suffer periodic disruptions in its fibrous cap resulting in ongoing interactions with activated platelets.[43] Thus, in addition to their role in acute plaque rupture, at any given time, activated platelets may be associated with unstable plaques presumably in a number and frequency proportional to the degree of plaque instability. The detection of such activated platelets potentially may allow identification of unstable plaques prior to rupture.[44]

CURRENT AND FUTURE ANTI-PLATELET AGENTS

Given the pivotal role of platelets in atherosclerosis, platelet inhibition provides major benefits in the treatment of atherosclerotic disease, both in the acute and preventative settings. Several targets have proven suitable therapeutic targets. Figure 11.3 schematically illustrates the mechanism of action of these agents.

Aspirin (Salicylic acid)

Aspirin, or salicylic acid, was the earliest known anti-platelet agent, used as early as 500BC. Aspirin irreversibly inhibits COX-1 enzymes stored in platelets, preventing the conversion of arachadonic acid to PGG_2 and PGH_2, which are substrates for formation of thromboxane A_2 – one of the platelet's primary positive feedback system mediating amplification of both intra and inter-platelet activation (Figure 11.3). Aspirin acetylates a key serine residue at the COX-1 catalytic centre, irreversibly corrupting its enzymatic function. Platelets lack the machinery to resynthesize COX-1, thus aspirin leads to irreversible platelet inhibition for the life of the platelet (7-10 days), despite its relatively short plasma half-life of 15 minutes.[45]

Aspirin has a well-established clinical benefit in vascular disease, particularly myocardial infarction and stroke, in both the acute and chronic setting. In acute myocardial infarction, aspirin demonstrated a 23% reduction of mortality at 5 weeks, in addition to thrombolysis – with benefits still measurable at 10 years.[46] Aspirin also reduces death and myocardial infarction in unstable angina, and is associated with reduced acute vessel closure following coronary angioplasty. With regards to secondary prevention, recent metanalyses of patients with previously diagnosed vascular disease (coronary artery disease, TIA, stroke or peripheral vascular disease) demonstrated a 25% reduction in vascular death, myocardial infarction or stroke. In addition, patients with stable angina, intermittent claudication and atrial fibrillation (who cannot be fully anticoagulated) also derive similar benefit from aspirin.[47] However, the benefits are less well established in the primary prevention population as the benefits of a reduced rate of myocardial infarction are tempered by an increased rate of hemorrhagic stroke. A net benefit is likely to be limited only to those patients with known cardiovascular risk factors such as diabetes.[48]

The optimal dose of aspirin had been established as 75-150mg daily, in order to achieve its full platelet inhibitory effect. Higher doses (up to 325mg daily) demonstrate increased gastrointestinal side effects with no additional anti-platelet effect.[48]

Aspirin resistance is a newly recognized phenomenon where aspirin is unable to exert its full antiplatelet action and confer its cardiovascular protection, in certain patients. Aspirin resistance is multifactorial. Pharmacological reasons include: (1) competitive inhibition by co-administration with NSAIDs (reversible COX-1&2 inhibitors); (2) the action of possible inhibitory proteins, such as vitamin D binding protein which may act directly or indirectly; and (3) genetic polymorphisms in cyclooxygenase making the active site less susceptible to the action of aspirin. Non-pharmacological reasons include: (1) an increased reactivity of platelets to other activating factors such as collagen, ADP, von Willebrand factor and adrenaline; (2) an increased rate of platelet formation, associated with myocardial infarction and coronary artery bypass graft surgery, resulting in an increased proportion of uninhibited platelet COX-1; (3) the presence of alternative pathways to thromboxane A2 generation which bypass COX-1. These may include COX-2 (present in the setting of inflammation), which requires a higher dose of aspirin to be effectively inhibited. Furthermore thromboxane A_2 precursors may be acquired from monocytes and endothelial cells, despite inhibition of COX-1. Some forms of aspirin resistance are amenable to an increased dose of aspirin, whilst other forms require a reliance upon other agents to effectively inhibit platelets.[49]

Thienopyridines

Thienopyridines are pro-drugs which, when metabolized to their active form, cause irreversible inhibition of the P2Y12 receptor and inhibit the action of ADP. The three in clinical use are ticlopidine, clopidogrel and prasugrel, of which cloidogrel is currently the most widely used.

Clopidogrel

Once in the bloodstream, clopidogrel undergoes enzymatic alteration via a two-step pathway to produce the short lived active metabolite R-130964. These steps involve hepatic enzymes of the cytochrome P450 family, particularly CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP3A4/5.[50,51] Binding of the active metabolite to the

P2Y12 receptor results in irreversible conformational change, which inhibits ADP binding for the life of the platelet.[52]

As an antiplatelet agent, clopidogrel reduces platelet aggregation, platelet-leukocyte interactions and expression of cellular adhesion molecules such as P-selectin.[53] In addition, clopidogrel's active metabolite has been shown to improve endothelial function possibly through blocking P2Y12 receptor and/or by direct effects independent of its antiplatelet activity.[54] Clopidogrel also limits ADP's role in amplifying platelet activation through other agonists such as thrombin and collagen.[55]

The CAPRIE trial demonstrated that clopidogrel had moderately improved cardiovascular secondary protection in patients with atherosclerotic disease (myocardial infarction, ischemic stroke, peripheral vascular disease), compared to aspirin with similar tolerability. The combination of aspirin and clopidogrel, known as dual antiplatelet therapy, significantly reduces the risk of subacute in-stent restenosis post angioplasty within the first month (bare metal stents) or twelve months (drug-eluting stents), and has an evolving clinical role in the long term management of refractory unstable angina.[56]

Clopidogrel is dosed using a loading and maintenance regime. The loading dose is aimed at obtaining rapid antagonism of the P2Y12 receptor. Greater platelet inhibition is achieved more promptly with a 600mg loading dose (maximum inhibition of 40-45% 2–3 hours after loading dose) compared to a 300mg dose. However a 900mg dose does not lead to further improvements in platelet inhibition or earlier onset of action.[57] The standard maintenance dose is 75mg daily, however recent studies suggest that a 150mg daily dose has a small additional benefit in reducing the risk of stroke, MI, cardiac death and in-stent thrombosis in the subset of patients undergoing PCI for acute coronary syndromes – however the optimal duration of such therapy is yet to be determined.[58,59]

Like aspirin, a proportion of the population displays a degree of resistance to the therapeutic effects of clopidogrel. This is though to be due to polymorphisms in the hepatic enzyme responsible for the conversion of the pro-drug to its active form. The most prevalent is the 681G>A polymorphism (also known as *2 allele) in the CYP2C19 enzyme, which is responsible for first stage of clopidogrel metabolism. This allele is present in roughly 30% of the population with higher rates in the Asian population (up to 50%). Studies have suggested the presence of this polymorphism is associated with a higher rate of in-stent thrombosis, and is associated with increased risk of ischemic stroke, MI and vascular death in patients with the mutation receiving clopidogrel for secondary prevention.[60] Such patients may require an increased dose of clopidogrel, or the use of alternative agents. Genotyping prior to commencing therapy, although available, is not currently routine practice, although studies are ongoing.[58]

Given its reliance upon liver metabolism, there exists the potential for drug interactions to limit the effectiveness of clopidogrel by reducing its bioavailability. In particular, an interaction with proton-pump inhibitors such as omeprazole has been proposed. Although retrospective and observational analyses have suggested an increased rate of death or hospitalization for ACS,[61,62] and in vitro platelet aggregometry studies have suggested an impaired platelet response to clopidogrel in the presence of omeprazole, several randomized clinical trials have failed to identify adverse clinical endpoints attributable to clopidogrel and PPI interaction at this stage.[63,64,65] Such studies may have been limited by power, and further studies are currently in progress.

Prasugrel

Prasugrel is the newest thienopyridine. It has several advantages over clopidogrel. Firstly, its onset of action is significantly quicker than clopidogrel (30 minutes versus 2 hours).[66] Secondly, unlike clopidogrel, prasugrel requires only a single hepatic enzyme step for conversion to its active metabolite. Consequently, this significantly improves its bioavailability compared to clopidogrel.[67] The intermediate form of prasugrel is primarily formed through hydrolysis by the intestinal enzyme hcE2. Subsequently, intestinal (CYP3A, CYP2C9, CYP2C19) and hepatic (CYP3A, CYP2B6, CYP2C9, CYP2C19) cytochromes are involved in conversion from intermediate form to active metabolite.[68]

The decreased reliance on hepatic enzymes for active metabolite formation and its increased bioavailability explain the stronger and more consistent (amongst individuals) inhibition of P2Y12 induced platelet activation compared to clopidogrel.[69] The recent TRITON trial demonstrated a reduction in ischemic events in patients with acute coronary syndromes managed with PCI, and 50% reduction in acute and sub-acute in-stent restenosis in patients on prasugrel compared to clopidogrel. However, this benefit came at the expense of an increased rate of major bleeding.[70]

Ticlopidine

Ticlopidine was the first available thienopyridine. Like clopidogrel, it requires hepatic activation in a two-stage process. It has several disadvantages compared to clopidogrel that limit its clinical usefulness. Firstly, its onset of action is significantly slower than clopidogrel. Secondly, it requires twice daily dosing, which noticeably reduces patient compliance. Importantly, it also has several noticeable side effects including skin rashes, gastrointestinal upset and life threatening blood dyscrasias such as neutropaenia.[71]

Ticagrelor

Ticagrelor is a new non-thienopyridine competitive P2Y12 antagonist, which has recently undergone Phase III clinical trials.[72] Unlike the thienopyridines, ticagrelor is not a pro-drug, but a direct P2Y12 receptor antagonist – requiring no hepatic or intestinal enzymatic activation. It has several advantages over clopidogrel: a more reliable pharmacokinetic profile, faster onset of action, and a lack of susceptibility to genetic based resistance as with thienopyridines. In addition, reversible inhibition allows its antiplatelet effect to cease rapidly after stopping therapy, unlike thienopyridines, which require 7–10 days for a return to normal platelet function. Furthermore, a recent randomized controlled clinical trial comparing ticagrelor with clopidogrel in patients with acute coronary syndromes, found a significantly reduced rate of death from vascular causes, stroke and myocardial infarction.[72] Unlike prasugrel, this benefit was not realized at the expense of increased major bleeding. There are, however, some notable issues: firstly, ticagrelor was associated with a higher rate of procedure related bleeding compared to clopidogrel. Secondly, ticagrelor was associated with idiosyncratic side effects of symptomatic dyspnoea and transient increase in ventricular pauses. Lastly, ticagrelor requires twice daily dosing and would likely reduce patient adherence.[71] Nonetheless, ticagrelor remains an exciting agent, which may address some of the shortcomings of the thienopyridines.

GPIIb/IIIa Antagonists

The GPIIb/IIIa receptor is a useful therapeutic target given its prominent role in platelet

aggregation and thrombus development. Diverse arrays of drugs have been developed to target the GPIIb/IIIa receptor including monoclonal antibodies, cyclic peptides and chemical compounds.

Abciximab is a fully humanised monoclonal antibody specifically targeted to inhibit the GPIIb/IIIa receptor. It consists of the murine generated variable domains linked to human IgG antibody structure which limits the immunogenicity of abciximab.[73] Eptifibatide was developed from a template peptide extracted from the venom of the south-eastern pygmy rattlesnake. It consists of a cyclic heptapeptide with a KGD sequence, which confers particular specificity for the GPIIb/IIIa receptor (as opposed to the RGD sequence in other endogenous ligands).[74] Tirofiban is a small molecular weight non-peptide compound based, again on a snake venom template, which inhibits GPIIb/IIIa receptor.[75]

Of these agents, abciximab demonstrates the highest affinity for the receptor, followed by eptifibatide and tirofiban. Taking into account other pharmacokinetic properties, abciximab has a duration of action of several days, compared to 2-4 hours for eptifibatide and tirofiban. The shorter acting agents are thus preferred in patients likely to undergo cardiac surgery following catheterisation. Profound thrombocytopaenia is well-described complication of GPIIb/IIIa blocker therapy. It is likely mediated by a host immunological response towards neoepitopes exposed after the binding of the GPIIb/IIIa blockers to the receptor.[76]

GPIIb/IIIa antagonists are potent inhibitors of platelet aggregation that improve mortality in patients presenting with acute coronary syndromes, particularly those undergoing percutaneous coronary intervention (PCI).[1,2] Abciximab, has shown a 10-35% reduction in mortality. Similar results, albeit of a smaller magnitude, have been seen with the small-molecule antagonist's tirofiban and eptifibatide, with a 16% to 35% reduction in ischemic events in patients undergoing PCI. A greater benefit was seen in higher acuity patients (patients with elevated troponin levels and/or diabetes).[77] In patients presenting with an ST elevation myocardial infarction undergoing primary angioplasty, GPIIb/IIIa blockade reduced death, subsequent infarction and need for revascularization within 30 days by 46%.[78] The addition of GPIIb/IIIa blockage to thrombolysis therapy has thus far not shown to be beneficial due to an increased bleeding risk.[79]

Interestingly, instead of reducing major ischemic events, long-term oral GPIIb/IIIa inhibitor therapy has uniformly increased the mortality rate. As a potential reason for this, it is postulated that exposure of GPIIb/IIIa to antagonists, which typically mimic the ligand fibrinogen, induce 'outside in signaling', as would be expected for ligand binding to an integrin receptor.[80,83] This can lead to paradoxical platelet activation and aggregation, implying that although GPIIb/IIIa is a good target for platelet inhibition, the ligand-mimetic strategy of receptor blockade is not an ideal pharmacological strategy.[81,82] Allosteric inhibition or selective inhibition of activated GPIIb/IIIa receptors have been recently employed as novel drug developments.[83,84]

Other anti-platelet agents and promising new developments

Dipyridamole is well known antiplatelet agent currently in clinical use for the secondary prevention of stroke. Dipyridamole increases intracellular cyclic AMP by inhibiting enzymes responsible for adenosine breakdown. Raised cAMP suppresses platelet activation, promotes vasodilatation, and stimulates prostacyclin release and coronary artery vasodilation.[85] In combination with

low dose aspirin, it has been shown in one trial to offer additional stroke protection than aspirin alone.[86] It has no demonstrated role in preventing cardiovascular disease.

Cilostazol is a Type III inhibitor of phosphodiesterase in platelets, promoting increased cAMP, and is currently approved for use in peripheral vascular disease. The KAMIR and DECREASE trials have suggested cilostazol confers a modest benefit in patients with coronary artery disease undergoing PCI in addition to standard dual antiplatelet therapy with respect to cardiac death, stroke, MI and instent-thrombosis at 8-12 months.[87,88] However, the outcome of a double-blinded, randomised controlled trial is required before it can be recommended for routine use.

Other new agents to mediate platelet inhibition are currently in development. One such is an oral protease receptor antagonist-1 (PAR-1) antagonist, currently known as atopaxar. PAR-1 is the primary platelet receptor for thrombin and is a potent activating agent. Phase III studies are underway to determine its clinical usefulness, with early Phase II data suggesting it may offer improved clinical outcomes with respect to death, stroke and MI, when added to other antiplatelet agents.[89] Similarly, animal studies have demonstrated that inhibition of the platelet collagen receptor, GPVI, with monoclonal antibodies and Fab fragments, can inhibit collagen induced platelet aggregation in rats. Phase II studies are currently in progress with a view to commence human trials in the near future.[90] Terutoban is an orally active inhibitor of the thromboxane A2 receptor, currently undergoing phase III trials.[91]

A major limitation in the development of new anti-platelet agents is the recurring observation that with increasingly effective platelet inhibition comes an inevitable increased bleeding risk, either tempering or negating the cardiovascular benefit. Novel classes of therapeutic drugs, currently under development, seek to circumvent this problem by selectively targeting activated platelets, for example activation specific GPIIb/IIIa antagonists[84] and PI3 kinase inhibitors.[92] These agents demonstrate potent antiplatelet activity without prolonging bleeding time in animal models. Clinical performance of these agents is still yet to be evaluated, however they remain promising.

PLATELET FUNCTION TESTING

Platelet function testing refers to in vitro measurements of platelets' response to activation in an attempt to quantify the degree of platelet aggregation. It has evolved significantly over the last decade from a laborious laboratory based process to rapid point of care commercially available kits. Despite this, there still remains a surprising lack of standardisation of platelet function testing. Table 11.1 summarises some of the currently available methods, along with their incumbent advantages and limitations.

Light transmission aggregometry

Light transmittance aggregometry (LTA) is currently the gold standard for assessing platelet activation as it is able to measure the functional ability of platelets to aggregate in response to known agonists such as ADP. Platelet aggregation uses the principle that the amount of light transmitted through the sample increases proportional to an increase in platelet aggregation. Lack of standardization, poor reproducibility and spontaneous platelet activation through sample preparation are a few limitations of standard light transmittance aggregometry.[93]

TABLE 11.1: A list of commonly used platelet function tests, their advantages and limitations.

Test	Method	Advantages and Limitations
Classical (or turbidometric) platelet aggregometry	Blood is centrifuged at low force to isolate platelet rich plasma, which is then stirred in a curvette at 37°C and placed between a light source and measuring photocell. Platelet agonists (such as ADP, collagen, adrenaline or ristocetin) are added. As individual platelets aggregate, turbidity of the PRP is reduced, and the increasing light transmission is detected by the photocell.	• Original 'gold standard' technique • Limited to specialised laboratories as requires specialised expertise for accurate performance and interpretation • Labour intensive • Limited sensitivity in detecting small (<100 platelets) aggregates, or preformed aggregates. Thus, limited sensitivity to detect early aggregates in platelet hyperfunction. • Limited ability to detect duration and efficacy of antiplatelet therapy, especially GPIIb/IIIa blockade
Whole blood aggregometry	Whole blood is stirred at 37°C. Platinum electrodes at a fixed distance are added to the blood. Once electrically active, platelet aggregates amass upon the electrodes resulting in a measurable increase in electrical resistance in a manner proportional to the degree of platelet aggregation.	• Comparative accuracy with classical aggregometry • Insensitive to small platelet aggregates • Requires significant technical expertise and expense
The VerifyNow® Assay (Accumetrics Inc, San Diego, California, USA) – previously known as the Ultegra Rapid Platelet Function Assay (RPFA)	A commercially available 'point-of-care' assay. GPIIb/IIIa kit: Whole blood is added to disposable cartridge containing fibrinogen beads and an activating Thrombin Receptor Activating Peptide (TRAP). Platelets activated by TRAP, bind to fibrinogen in a degree proportional to the amount of available receptors. Thus the degree of GPIIb/IIIa blockade can be quantified. Aspirin and Clopidogrel kits: These utilise the same principle using arachadonic acid and ADP as agonists in order to assess the activity of aspirin and clopidogrel respectively.	• Fast and easy to use with results available in 2–3 minutes • Limited to assessing the effectiveness of antiplatelet therapy and no role in assessing platelet activity in disease states • Comparable sensitivity to classical platelet aggregometry • Ideal cut-off points for diagnosing antiplatelet resistance are still a matter of conjecture.

Test	Method	Advantages and Limitations
Flow Cytometry	Flow cytometry uses the principle of light scattering and fluorescence to accurately quantify platelets, blood cells, platelet aggregates and platelet leukocyte aggregates as well as the functional state of platelets.	• Most versatile and best quantitative platelet function measurements • Utilising gating technology, the expression of activation specific markers on platelets can be quantified. This provides very high sensitivity for detecting and quantifying platelet activation. • Can identify platelet/leukocyte aggregates in addition to platelet aggregates • Utilising fluorescence labelled ligands of antibodies such as fibrinogen-FITC or anti-P-selectin monoclonal antibody, direct quantification of receptor blockade can be achieved (for example GPIIb/IIIa blockade) • Requires considerable expertise and specialised laboratory • Time consuming and expensive
Vasodilator Stimulated Phosphoprotein (VASP) Assay	Using flow cytometry, the VASP Assay measures the degree of phosphorylation of VASP which correlates with the degree of activity of the ADP receptor. Phosphorylated VASP correlates with P2Y12 receptor inhibition. The degree of phosphorylation in the presence of ADP, suggests effective antiplatelet activity.	• Best available test for identifying patients with possible clopidogrel resistance • Requires considerable expertise and specialised laboratory • Time consuming and expensive

Whole blood aggregometry

Impendence aggregometry, a newer technique that measures electrical resistance due to aggregation of stimulated platelets on two platinum electrodes, has been shown to have improved reproducibility and sensitivity. The increase in resistance between electrodes is used to determine the amount of platelet aggregation after stimulation by an agonist. Impedance aggregometry correlates well with LTA.[94]

VerifyNow® assay

Verify Now® is a cartridge based rapid point of care test that measures aggregation of platelets via GIIb/IIIa receptors in response to ADP. The extent of platelet aggregation measured by a technique based on light transmittance aggregometry, is expressed as platelet reactivity units (PRU), which is specific to the action of the P2Y12 platelet receptor. Simplicity of technique, reliability of results, lack of reliance on lab equipment

and good correlation with established tests of platelet function are advantages of this test. All point of care tests are limited by low to moderate sensitivity and specificity. However, VerifyNow® P2Y12 assay has been shown to predict clinical adverse outcomes.[95]

Flow cytometry

Flow cytometry enables identification of expression of substances on platelets and leukocytes, particularly formation of platelet-leukocyte aggregates. Fluorophores (substances that fluoresce when stimulated with energy) attached to antibodies which bind to substances of interest; emit light when excited by energy from lasers. These lasers transmit light at a specific frequency aimed at particles flowing past the laser in single file. The intensity of light emitted by the fluorophores allows quantification of the substance targeted by conjugated antibodies. However, accuracy and reproducibility of results vary from one protocol to the next. The ability to assess multiple markers relating to antiplatelet activity at the receptor is the main advantage of flow cytometry. The main disadvantages of this technique is the need for experienced operators, time consuming process for analysis with narrow sample preparation windows, lack of standardized lab protocols and access to lab facilities.

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12 • Pathogenesis of Aortic Aneurysms

JONATHAN GOLLEDGE[1], GUO-PING SHI[2], PAUL NORMAN[3]

[1]Vascular Biology Unit, School of Medicine and Dentistry, James Cook University, Townsville, Qld, Australia.
[2]Department of Cardiovascular Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, USA.
[3]School of Surgery, University of Western Australia, Fremantle Hospital, Fremantle, WA, Australia.

INTRODUCTION

Over the last decade aortic aneurysm has gained increased focus for a number of reasons. Firstly, the widespread use of imaging and screening in some countries has resulted in greater detection of the condition. Secondly since the condition is much more common in the elderly the number of patients with the problem is projected to increase progressively with the rising average age of the population. Surgical and endovascular treatments have not been shown to reduce mortality for patients with small abdominal aortic aneurysms (AAAs) and thus other therapies are required to deal effectively with the large group of patients being identified with small AAAs.[1,2] There is also a need to provide better prognostic information for patients with AAA by risk stratification in terms of the likelihood of complications, including AAA progression to a stage where surgery is required and also other cardiovascular complications.[3] As a result of these current management deficiencies, there has been an explosion in studies directed at improved understanding of AAA pathogenesis. These studies have mainly consisted of investigations in animal models or assessments of human DNA, tissue or blood samples.[3] Mice models have been particularly popular due to the ability to perform elegant interventions and assess their influence on AAA. Data from such studies has thus had a major impact on pathogenesis theories and treatment targets. However, animal models have limitations and cannot be expected to be completely comparable to human disease. Human AAAs develop over many decades in patients with multiple risk factors and with numerous mechanisms leading to the final common result of an AAA. In contrast rodent models usually involve techniques to induce aortic aneurysms over a few weeks or result due to single genetic deficiencies. These models have allowed considerable insight not possible with human association studies alone but their exact importance will only become evident when treatment strategies they have suggested are examined by interventional trials in patients with small AAAs.

DIFFERENCES BETWEEN THORACIC AND ABDOMINAL AORTIC ANEURYSMS

Numerous factors contribute to differences between the thoracic and abdominal aorta. In adults, the thickness and number of elastic lamellae gradually decreases along the aorta resulting in the wall thickness falling from about 1.5mm at the arch to less than 1mm in the distal abdominal aorta.[4] The fall in elastin is particularly notable in the aneurysm-prone infra-renal aorta.[5] As a result, the abdominal aorta has a smaller cross-sectional area and a stiffer wall. Compared with the thoracic aorta, the infra-renal aorta is exposed to reflected pressure waves from the iliac bifurcation, higher pulse pressures and increased wall shear stress due to its position proximal to a major bifurcation.[6] These adverse haemodynamic conditions make the abdominal aorta particularly prone to both atherosclerotic and aneurysmal disease.[7]

The propensity for aneurysm formation in the infrarenal aorta may also relate to differences in aortic smooth muscle cell lineage: cells forming the arch of the aorta are derived from neural crest; of the thoracic aorta from somite-derived cells; and of the abdominal aorta from splanchnic mesoderm.[8] Localised intrinsic aortic wall characteristics may influence a range of molecular pathways that are important in aneurysm formation – notably angiotensin II and transforming growth factor beta (TGFβ) signaling.[9] Divergent inflammatory responses may also contribute to differences between thoracic and abdominal aortic aneurysms. The more florid inflammation seen in AAAs may reflect greater vasa vasorum density and differential immune responsiveness.[10] In addition, the expression of pathogen-sensing Toll-like receptors by resident dendritic cells (resulting in differential T cell response) varies considerably, with each artery having a distinct profile.[11]

There are clear differences in the genetic factors associated with thoracic and abdominal aortic aneurysms. The importance of single gene mutations as causes of aneurysms decreases from the proximal to the distal aorta.[9] The role of common susceptibility genes has yet to be clarified. Given that aneurysms of the abdominal aorta are ~5 times more common than in the thoracic aorta, this chapter will focus primarily on AAA.

SUMMARY OF CURRENT THEORIES AND STAGES OF AAA EVOLUTION

The natural history of an AAA can be divided into initiation, progression and rupture. This concept is useful since there is evidence that the factors promoting each stage may be different.[12] Whilst understanding AAA initiation may ultimately lead to the goal of primary prevention, an understanding of AAA progression can guide the development of therapy for patients with small AAAs in an attempt to reduce expansion and rupture rates. An attempt has been made to link putative mechanisms to different stages of AAA in Table 12.1. In the subsequent sections the evidence implicating these mechanisms in AAA pathogenesis will be discussed.

ATHEROSCLEROSIS AND AAA

Patients with AAAs frequently have generalized atherosclerosis, and numerous studies show the association of coronary and peripheral atherosclerosis with AAA.[3] Whether this association between AAA and aortic atherosclerosis is causal or simply due to common risk factors is unknown. The most compelling argument for a causative role of

TABLE 12.1: Mechanisms implicated in AAA pathogenesis

Mechanism	AAA stage
Atherosclerosis	Initiation
Innate immunity	Initiation and progression
Extracellular matrix dysfunction	Initiation
Infection	Initiation
Biomechanical disturbance	All stages
Angiogenesis	Progression
Intra-luminal thrombus	Progression
Extracellular matrix destruction	All stages

atherosclerosis in AAA has been centered on arterial remodeling.[13] A large body of *in vitro*, animal, and histology data suggests that when an arterial luminal stenosis develops, compensatory changes occur in the media in response to shear stress alterations. The extracellular matrix remodeling promotes expansion of the artery in an attempt to normalize lumen diameter and shear stresses. Excessive remodeling could explain the medial thinning typically seen in AAAs. Elastin breaks stimulated by medial proteolysis and the diffusion of pro-inflammatory cytokines from inflammatory cells present within atheroma or associated thrombosis could also provide the stimulation for the chronic inflammation seen in AAAs. On the basis that atherosclerosis stimulates AAA development, all patients with AAA would necessarily have significant atherosclerosis and thus should be considered for indicated medical therapy, as currently advised by American Heart Association guidelines in which AAA is considered an atherosclerotic equivalent.[14]

An alternative theory suggests that the development of AAA and atherosclerosis are independent. Shared environmental and genetic risk factors may promote the development of both atherosclerosis and AAA in some patients, but the mechanisms involved are distinct. A third possibility is that either aortic atherosclerosis or AAA can develop first and both can subsequently stimulate the development of the other (Figure 12.1). Currently, evidence to support one of these theories over the other is largely limited to documenting similarities and differences in risk factors and findings within rodent models for atherosclerosis and AAA (Table 12.2).[3,15-19]

Further insight into the importance of atherosclerosis in AAA is of therapeutic relevance. Recent human association studies have shown conflicting results on whether drugs that are effective for atherosclerosis, such as statins or angiotensin converting enzyme inhibitors, inhibit AAA progression.[20-23]

IMMUNE MECHANISMS IN AAA

Relatively few studies have been carried out to establish the role of immune mechanisms in AAA by comparison to athero-thrombosis. Examination of biopsies removed from large AAAs demonstrate a marked inflammatory infiltrate, particularly within the adventitia. Assessment of the relative numbers of different cell types within AAA biopsies have been performed using a variety of techniques including histology, flow cytometry and genomic techniques.[24,25] These studies suggest that in end-stage human AAA, the predominant inflammatory cell types are T and B lymphocytes.[24] Other inflammatory cells are also identified, such as macrophages, dendritic cells and mast cells.[24] Current evidence implicates both innate and adaptive immunities in AAA pathogenesis.

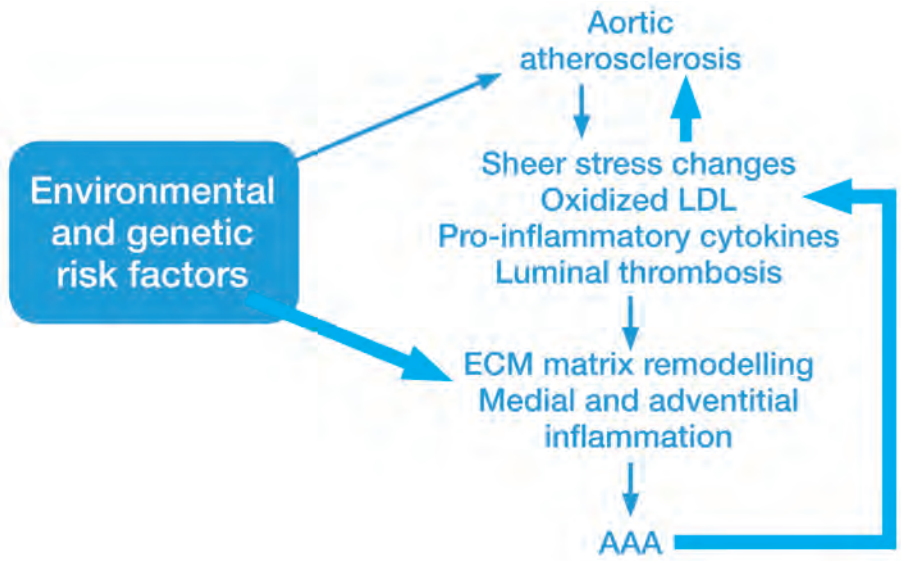

FIGURE 12.1: Theories regarding relationship between atherosclerosis and AAA. According to theory 1 (thin line + arrow), environmental and genetic risk factors lead to development of aortic atherosclerosis. Resultant positive remodeling, intimal thrombosis, and release of proinflammatory cytokines stimulate secondary matrix degradation and adventitial inflammation which promotes AAA development. According to theory 2 (thick line + arrow), environmental and genetic risk factors directly stimulate aortic medial degradation and adventitial inflammation, leading to AAA formation, which secondarily stimulates intimal atherosclerosis. More likely, both pathways act to some extent, with the relative proportion varying from patient to patient depending on the risk profile. ECM indicates extracellular matrix; LDL, low-density lipoprotein.

T cells, including both $CD4^+$ and $CD8^+$ cells, are probably the best-studied inflammatory cells in human AAAs. T cells may be demonstrated in both the adventitia and media of human AAA biopsies (Figure 12.2). The high numbers of T cells in human AAA may reflect both increased influx and also reduced clearance of these cells. T cells isolated from human AAAs are more resistant to apoptosis than those from healthy donors or patients with aortic occlusion disease (AOD).[26] Different subsets of T cells may play varying roles in AAA. For example $CD4^+CD31^-$ cells have been implicated in AAA progression by enhancing $CD8^+$ cell-mediated vascular smooth muscle cell (VSMC) cytolysis and macrophage-derived matrix metalloproteinase (MMP)-2 and -9 production.[27] Compared with healthy aortic tissues, AAAs also contain higher numbers of $CD69^+DR^+$ active T cells with lower expression of adhesion molecule CD62L,[28] although the potential antigens that activate T cells remain undiscovered.

The role of T helper (Th) cells in human AAA is controversial. According to some reports a significant percentage of freshly isolated T cells express markers of a Th1 immune response.[29] Furthermore cytokines associated with Th1 immune responses are elevated in the blood and aortic tissue of patients with AAA.[30] In the $CaCl_2$ AAA mouse model, absence of CD4 or Th1 cytokine IFN-γ suppressed AAA formation.[31] In contrast, several groups report different observations. These include high concentrations of Th2 cytokines IL4, IL5, and IL10, but negligible Th1 cytokines IL2 and IL15 in AAA biopsies.[32] In mice major histocompatibility complex

TABLE 12.2: Some similarities and differences between atherosclerosis and AAA.

Characteristic	Similarities	Differences
Clinical risk factors	Smoking, hypertension, and obesity are common risk factors for AAA and aortic atherosclerosis.[3]	Diabetes is a negative or neutral risk factor for AAA but important risk factor for atherosclerosis.[3] Male gender and smoking are much more dominant risk factors for AAA than atherosclerosis.[3]
Circulating risk factors	AAA and atherosclerosis have many similar biomarkers, e.g. fibrinogen, CRP and HDL (negative).[15]	There are a number of disparate markers for AAA and atherosclerosis, e.g. LDL has no clear association with AAA but is an important risk factor for atherosclerosis.[16]
Genetic risk factors	Family history is an important risk factor for both AAA and atherosclerosis.[3] A locus on chromosome 9p21 is associated with CHD, stroke and AAA.[17]	Some recognised genetic determinants of atherosclerosis have no consistent association with AAA, e.g. Apolipoprotein E single nucleotide polymorphisms.[18]
Histology	Intimal atheroma and thrombosis are usually present in both AAA and atherosclerosis.[3]	Marked elastin fragmentation and adventitial chronic inflammation is mainly restricted to AAA.[3]
Rodent models	Some mice (e.g. Apolipoprotein E deficient) prone to atherosclerosis are also more sensitive to AAA induction.[3] Interventions protective from AAA frequently also reduce atherosclerosis.[3]	There are examples of differential effects of interventions on AAA and atherosclerosis progression, e.g. TNF and MMP-12 deficiency.[19]

mismatched aortic transplants develop an immune response dominated by IL4. These transplanted aortas develop severe inflammation, elastin degradation, marked MMP-9 and MMP-12 expression, and AAA. These AAAs can be prevented by anti-IL4 antibody or by concomitant IL4 mutation.[33] Overall it is possible from current data that both Th1 and Th2 responses are involved in AAA pathogenesis.

Macrophages, neutrophils, and mast cells have also been implicated in AAA formation. Macrophages are demonstrated within the adventitia and media of human AAA biopsies (Figure 12.2). Human AAA biopsies contain significantly higher levels of total neutrophil elastases, localized in the adventitia and thrombus than aortic tissue from patients with AOD or without significant disease.[34] Neutrophil recruitment to AAAs may be mediated by complement components C3a and C5a or neutrophil-derived IL8 and leukotriene B4.[35,36] Antagonism of C3a and C5a blocked AAA formation in a mouse model.[35] Antibody depletion of neutrophil protected mice from elastase induced AAA development.[37] Diminished neutrophil recruitment to the elastase-injured aortic wall due to dipeptidyl peptidase I-deficiency impaired AAA formation. This protection was lost when wild type normally functioning neutrophils were restored in these mice.[38] All these data support a role for neutrophil in AAA formation and progression in mice models.

Small numbers of mast cells are found within the outer media and adventitia of human AAA (Figure 12.2) and the cell

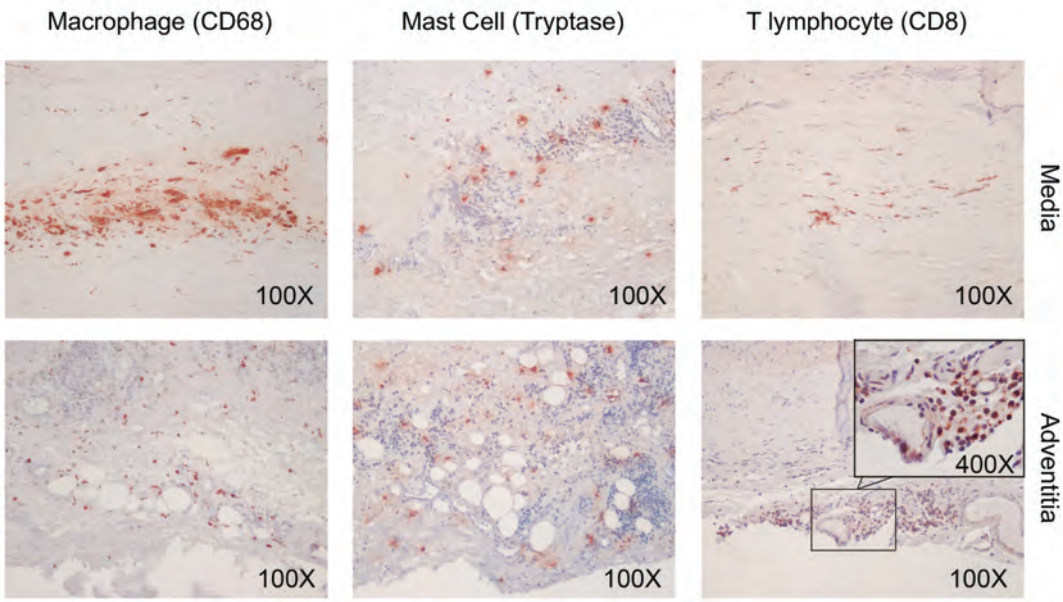

FIGURE 12.2: Example of assessment of inflammatory infiltrate in a human AAA biopsy. Macrophages, mast cells and CD8+ T cells within the media and adventitia of human AAA biopsies. Rabbit anti-human CD8 monoclonal (1:75, Abcam, Cambridge, MA), mouse anti-human CD68 monoclonal (1:60, Dako, Carpinteria, CA), and mouse anti-human tryptase (1:1500, Chemicon International, Inc., Temecula, CA) antibodies were used to detect $CD8^+$ T cells, macrophages, and mast cells in paraffin sections.

number has been correlated with AAA diameter.[39] Neovessel area within biopsies of human AAAs is also correlated with mast cell number. In rats, peri-aortic $CaCl_2$ injury induces AAA with associated increase in aortic mast cell content. Mast cell-deficient rats (*Ws/Ws*) or those that received mast cell inhibitor tranilast were protected from AAA formation. This effect was associated with reduced inflammatory cell infiltration, MMP-9 activity, angiogenesis and elastin degradation.[39] Similar observations were demonstrated in mast cell-deficient ($Kit^{W\text{-}sh/W\text{-}sh}$) mice.[40] In an aortic elastase perfusion-induced AAA model, absence of mast cells protected mice from AAA, whilst pharmacological mast cell stabilization with an anti-allergy medicine cromolyn significantly inhibited AAA expansion.[40] The findings suggest the intriguing possibility that mast cell stabilizing agents may be able to slow AAA progression.

EXTRACELLULAR MATRIX DYSFUNCTION

Examination of biopsies of large human AAAs indicates marked medial thinning and deficiency of VSMCs as consistent features.[3] In human AAA lesions, the best-studied extracellular matrix (ECM) proteins include elastin and collagen. Loss of functional elastin is an important feature of human AAA and may be a more generalized feature of arteries of patients with aneurysms.[41] Disruption of elastin has been particularly implicated in AAA development and progression, while degradation of collagen is thought to be more important in AAA rupture.[42] The availability of knock-out mice models has allowed the dissection of some of the mechanisms by which the extracellular matrix might influence AAA development. These studies suggest that extracellular matrix elements play more than just a structural role. Studies

in fibrillin-1 deficient mice suggested that the loss of this extracellular protein led to alteration in the bioavailability of TGFβ. The marfan-like phenotype stimulated by fibrillin-1 deficiency could be inhibited by using a neutralizing antibody to TGFβ.[43] Deficiency of other extracellular matrix proteins such as fibulin-4 has also been suggested to alter TGFβ signaling.[44] The mechanism by which alterations of TGFβ promotes AAA in these pre-clinical studies is currently controversial since the effects of blocking this cytokine varies in different models.[43,45] The most detailed study to date suggests that at least in one model TGFβ protects against aortic aneurysm formation by inhibiting monocyte-macrophage based tissue destruction via MMP-12 production.[45]

INFECTION

The development of aneurysms in which infection is a primary pathogen is well documented albeit rare. It has also been suggested that infection could play a more general role in aneurysm formation and progression. *Chlamydia pneumonia* in particular has been investigated for a role in AAA.[46] Evidence of previous *C. pneumonia* infection has been demonstrated in some patients with AAAs although the frequency of this varies due to the different methods of detection used. Conclusive evidence of *C. pneumonia* infection in a large series of patients with AAA compared to controls is currently lacking. Serological evidence of *C. pneumonia* infection has been related to more rapid AAA expansion. These findings stimulated two small trials involving a total of 124 patients to examine the influence of short course antibiotic therapy on AAA progression.[46] These studies both suggested a reduction in AAA growth over a short follow-up period but do not appear to have generated enthusiasm for larger studies directed at *C. pneumonia* eradication.[46] There are however two large on-going trials of doxycycline therapy in patients with AAA although the rationale for this therapy is based on MMP inhibition rather than antibiotic properties.

BIOMECHANICAL FORCES

One of the suggested reasons for the focal nature of AAA formation has been the variation in haemodynamic forces throughout the aorta.[7] Hypertension is a risk factor for AAA in human population studies and in rodent models it promotes AAA formation.[3,47] Focal increases in shear stress and wall strain have been demonstrated to inhibit AAA development in rodents due to reduction in macrophage induced oxidative stress.[48] These findings have in part been the stimulus for a current randomized trial examining the effect of supervised exercise in patients with small AAAs. Results from this trial are expected in 2012. Biomechanical forces may also have a role to play in the development of aortic rupture.

ANGIOGENESIS

New vessel formation has been demonstrated within the adventitia of human AAA biopsies and implicated in promoting influx of inflammatory cells.[49] In the angiotensin II induced mouse model, vascular endothelial growth factor promoted, and an angiogenesis inhibitor attenuated, AAA formation.[50,51] Of possible relevance to human therapy, simvastatin has been shown to inhibit angiogenesis in the same AAA pre-clinical model.[52]

INTRA-LUMINAL THROMBUS

Intra-luminal thrombus is a usual finding in large AAAs but rare in aneurysms situated at

more proximal sites in the aorta. The volume of thrombus is closely correlated to the size of the AAA suggesting that the thrombus could simply be a result of the changes in flow pattern due to aortic dilatation.[53] The thrombus is the likely source of a range of biomarkers which have been associated with AAA, such as fibrinogen degradation products including D-dimer.[54] These thrombus products maybe of diagnostic and prognostic value for AAA.[54] The thrombus has been demonstrated to contain large numbers of neutrophils, MMPs and cytokines.[55,56] Furthermore in a rodent model of AAA, inhibition of platelet activation has been shown to slow AAA progression.[55] In one cohort of patients with small AAAs aspirin prescription has been associated with reduced AAA progression.[57] Trials of the efficacy of anti-thrombotic therapies in reducing AAA progression and perhaps reducing other complications of AAA may follow in the near future.

EXTRACELLULAR MATRIX PROTEOLYSIS

The ECM in the healthy aortic wall is balanced by controlled biosynthesis and destruction. Either impaired production or enhanced degradation of ECM may promote AAA formation. Seeding of syngeneic rat VSMC stabilizes experimental AAAs[58] and this effect is enhanced by adenoviral expression of TGF-β.[59] Biosynthesis of collagen and elastin is regulated at both expression and post-translational modification. The latter is catalysed by poly-4-hydroxylase, procollagen lysyl hydroxylase (PLOD) and lysyl oxidase (LOX). Disruption of the LOX gene in mice leads to AAA formation and rupture.[60] LOX expression is reduced in AAA prone mice and in experimental AAA.[61] A PLOD mutation in humans predisposes to arterial rupture.[62] Adenoviral expression of exogenous LOX inhibits AAA in circumstances where endogenous LOX activity is suppressed.[63] In a rat $CaCl_2$ model, peri-adventitia delivery of an aortic elastin binding polyphenol and stabilizer pentagalloyl glucose inhibited elastin degradation and attenuated AAA expansion, without modifying inflammation, calcification, and high MMP activity. The polyphenol protected elastin from proteases induced degradation.[64] Excessive ECM accumulation may also play a detrimental role in AAA. This theory was supported by studies in Apolipoprotein E deficient (*Apoe*$^{-/-}$) mice which express collagenase-resistant mutant collagen. These mice develop increased interstitial collagen accumulation, aortic wall stiffness, susceptibility to mechanical failure and increased AAA development after angiotensin II infusion.[65]

ECM proteolysis in human AAA may be mediated by virtually all classes of proteases – MMPs, cysteine proteases cathepsins, and serine proteases that are derived from inflammatory or vascular cells after stimulation with cytokines (Figure 12.3). MMPs have been most studied. MMP-2, 3, 7, 12 have elastinolytic activity and MMP-2, 3, 7, 8, 9, 13, 14 demonstrate collagenolytic activity.[66] Studies of human AAAs biopsies suggest that a range of MMPs are upregulated, while concentrations of tissue inhibitors of metalloproteinase (TIMPs) are reduced.[67,68] It is postulated that inflammatory cells secrete MMPs and stimulate vascular cells to express MMPs in addition (Figure 12.3). In experimental AAA there is an inverse correlation between the number of inflammatory cells, neovessels and aortic wall collagen and elastin.[69] Mice in which MMP deficiency is introduced are protected from AAA induction.[70-72] Similarly seeding of VSMC which express high levels of the MMP inhibitor TIMP-1 into aortas of an experimental model of AAA prevented aortic rupture.[73] These findings implicate MMPs in

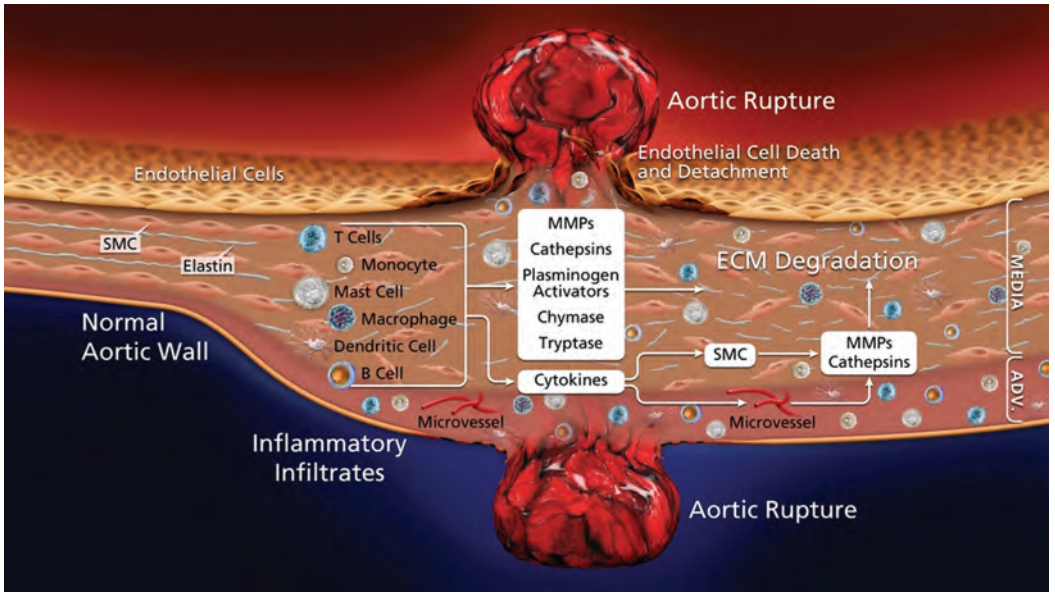

FIGURE 12.3: Schematic diagram summarizing the involvement of inflammatory cell infiltration and proteases in human AAAs. MMPs, cathepsins, plasminogen activators, chymases and tryptases are the best-studied proteases in AAA. Inflammatory infiltrates are the main sources of these proteases. Furthermore, these inflammatory cells also produce cytokines to stimulate VSMC and endothelial cells to release MMPs and cathepsins.

the development, progression and rupture of AAAs.

Increased expression of the cysteinyl cathepsins S, L, and K and decreased concentrations of their endogenous inhibitor cystatin C has previously been reported in human AAA biopsies.[74,75] Recent quantitative analysis suggested that cathepsins B, H, L, and S activities were increased by 376%, 191%, 223%, and 20% in biopsies of human AAA compared to AOD.[76] The effect of this excess cathepsin activity is currently controversial. In the elastase perfusion model, deficiency of cathepsin C, also called dipeptidyl peptidase I, reduced neutrophil infiltration and AAA expansion.[38] In contrast, *Apoe*$^{-/-}$ mice lacking cystatin C developed significantly enlarged AAAs compared with control mice.[77]

The serine proteases urokinase and tissue-type plasminogen activator (uPA and tPA) have received considerable interest in AAA research. Plasma tissue plasminogen activator has been compared in subjects with and without AAA in 5 studies.[15] Only one of the studies reported significantly higher concentrations in patients with AAA. A local role for serine protease in AAA may be more plausible than a systemic one. Absence of uPA in *Apoe*$^{-/-}$ protected mice from angiotensin II induced AAA.[78] Intra-adventitial introduction of an adenovirus over-expressing plasminogen activator inhibitor-1 (PAI-1) completely prevented AAA formation in *Apoe*$^{-/-}$ mice infused with angiotensin II. Local delivery of this virus two weeks after angiotensin II infusion prevented further expansion of small AAAs, but had no significant effect on larger AAAs.[79]

Mast cell chymase has also recently been implicated in AAA formation. In human AAA, increased chymase expression within the media and adventitia has been reported.[80] Mice with deficiency of chymase are protected from elastase induced AAAs.

In mice, chymase enhances the expression and activities of cysteinyl cathepsins, and promotes aortic elastin degradation, angiogenesis, and VSMC apoptosis.[80] Oral administration of the chymase inhibitor NK3201 (30mg/kg/day) significantly suppressed the severity and expansion of AAAs in the angiotensin II induced aneurysm model.[81] Similar observations have been reported in dogs where the chymase inhibitor NK3201 (1mg/kg/day, p.o.) inhibited AAA.[82]

Pharmacological inhibition of proteases has been widely investigated in animal models. Most of these studies focus on inhibition of MMP activity. Both MMP inhibitors BB-94[83] and doxycycline[84-86] are effective in preventing animal AAA formation. Oral treatment (100mg/kg/day) or peri-aortic perfusion of doxycycline (0.75 to 1mg/kg/day) inhibited elastase induced AAA in mice. Overall doxycycline has been shown to inhibit AAA in experimental models in >10 studies supporting the potential of this approach in human AAA.[3] In a recent trial 60 patients awaiting open AAA repair were randomized to a control group or low, median or high doses of doxycycline (50, 100, 300mg/day) for two weeks. Overall AAA biopsies from patients receiving doxycycline had reduced aortic wall neutrophil and $CD8^+$ T cell infiltration; reduced pro-inflammatory cytokine and MMP concentrations; and increased TIMP-1 and cystatin C concentrations.[87,88] Doxycycline appeared to be well tolerated with no major adverse events reported. However, a previous double blinded trial of low dose doxycycline (100mg/d) given one month prior to open AAA surgery reported no effect of the medication on MMP or TIMP expression.[89] Two randomized trials are underway examining the effect of oral doxycyline on small AAA progression.

GENETICS

Family history is a risk factor for all types of aortic aneurysms. Up to 20% of thoracic aortic aneurysms are in individuals with a familial preponderance and the understanding of the genetics of aneurysms at this site is advancing rapidly. Approximately 5% of thoracic aortic aneurysms occur in patients with well defined monogenetic connective tissue diseases, such as Marfan syndrome (mainly due to mutation in fibrillin-1).[90] Recently a range of further mutations have been identified in genes associated with thoracic aortic aneurysm, including TGFβ receptor type I and II, myosin-11 and alpha 2 actin, and aortic smooth muscle.[90] It appears likely that genetic inheritance plays a more significant role in aneurysms which develop in the ascending aorta by comparison to the abdominal aorta. Recent evidence for example suggests that the role of TGFβ receptor mutations in the development of AAAs appears to be less clear cut than described for the ascending aortic aneurysms.[91,92] It is possible that genes predisposing to AAA may have more comparability with those associated with atherosclerosis. A site on chromosome 9 originally identified in a whole genome association study for coronary heart disease has now been repeatedly associated with AAA.[17,93] Current evidence suggests that multiple genetic loci have a small effect in increasing the risk of AAA, depending on the exposure of patients to environmental or other complex risk factors, such as smoking, diet and other health behaviours. Meta-analysis of current small studies suggest that contributing genes include angiotensin converting enzyme, angiotensin type 1 receptor, methyltetrahydofolate reductase and MMP-3.[12,94]

AAA RUPTURE

Recent elucidation of the biological processes causing aneurysm development and expansion has led to translational research investigating the use of novel pharmacotherapeutic agents aimed at retarding aneurysm growth. In contrast to the expansion of AAA, the biological processes initiating aortic aneurysm rupture have received little attention. AAAs were traditionally considered to be a simple biomechanical problem, resulting from irreversible structural damage to the aortic wall that results in weakening, dilatation and eventual rupture when wall stress from the circulation exceeds the tensile strength of the wall. Focusing on aortic wall stress as the cause of rupture has led to a simplistic view that the natural history of AAAs is regulated solely by biomechanical factors. Just as the complexity of the atherosclerotic plaque has become apparent, it is now recognised that rupture of an AAA is a multifaceted biological process involving biochemical, cellular, haemodynamic and proteolytic influences.[95]

Biomechanical factors in aneurysm rupture

In a search for a more specific clinical parameter than diameter alone, biomechanical investigations have tried to predict aneurysm rupture as a function of wall stress. Several investigators revealed that ruptured or symptomatic AAAs had a significantly higher peak wall stress compared to asymptomatic AAAs, independent of blood pressure or AAA diameter. In addition, these in vivo measurements of peak wall stress using finite element analysis (FEA) predicted rupture risk more accurately than the Law of Laplace.

There are several limitations to the finite element analysis reported in these studies that assume the homogenicity of structure and thickness of the aortic wall and do not account for intraluminal thrombus. In a recent review McGloughlin and Doyle[96] suggest that aortic rupture cannot be predicted by wall stress alone and that wall strength is equally important. These authors suggest that biomechanical analysis has an increasing role to play in aortic research but that a numerical biomechanical risk index is some way off.

The role of enzymes in AAA rupture

Ex vivo mechanical testing of healthy and aneurysmal abdominal aortic wall specimens revealed that the failure strength of a typical AAA wall was lower than that of non aneurysmal aorta. The mechanism for aneurysmal wall weakening is unknown but it seems likely that the increased local production of enzymes capable of degrading elastin and interstitial collagen alters the structural integrity and predisposes the aortic wall to weakening. The earliest experimental work on AAA rupture was by Dobrin *et al* in 1984 who investigated the proteolytic effects of purified collagenase and elastase. Treatment with collagenase caused the blood vessels to dilate, become more compliant and rupture. In contrast, treatment with elastase caused the vessels to dilate markedly and become stiffer (probably due to recruitment of previously unstretched collagen fibres) but was not related to rupture. These findings have fostered the notion that elastin degradation is a key step in the development of aneurysmal dilatation but that collagen degradation is ultimately required for aneurysm rupture.

The pathological processes associated with the natural history of aneurysms to dilate and rupture are not well documented in clinical studies. Wilson *et al* found no significant differences in MMP levels in the AAA sac of large (>6.5cm) and medium (5-6.5cm) sized

aneurysms, or ruptured and non-ruptured AAA sac. When the same group analysed paired samples of aortic sac obtained from the anterior sac and the site of rupture in MMPs-8 and -9 were significantly higher at the site of rupture than in the anterior sac.[97] Aortic rupture is therefore likely caused by localised elevations in proteolytic enzymes and focal wall weakening. This concept of localised 'hot spots' of MMP hyperactivity was supported by Vallabhaneni *et al*, who demonstrated marked heterogeneity of tensile strength and MMP activity in the aneurysmal aortic wall.[98]

Role of intraluminal thrombus in aneurysm rupture

Intraluminal thrombus (ILT) is found in about 75% of all AAAs. Some authors have suggested that rupture is associated with growth of thrombus in the aneurysm, whilst there is evidence that larger AAA thrombus load is associated with a higher growth rate. Acute hemorrhage seen in the mural thrombus of patients with ruptured AAAs has led others to suggest that blood entering thrombus may have a role in rupture. There is some suggestion that AAAs with thick ILT also had increased cytokine concentrations, greater inflammation, and lower tensile strength. It was postulated that ILT, by creating a hypoxic environment may lead to compensatory inflammatory response, increase in local proteolytic activity of the wall, local wall weakening and subsequent rupture.

Investigations have demonstrated that the thrombus lining an aneurysm is an active and complex biological entity, containing many inflammatory cells, including macrophages and neutrophils. The ILT is a site of proteolytic enzyme release and activation, and it may be that mural thrombus acts as a source of proteolytic enzymes by aggregating platelets, trapping circulating cells and adsorbing plasma components.

In the future it is hoped that research into the mechanism of aortic rupture will integrate biomechanical and basic science research pathways. There is considerable evidence from isolated systems that shear and wall stress can influence the behaviour of biological processes. Understanding the interaction of these processes in the large aneurysm is key to unraveling the mechanisms of aortic rupture.

FUTURE RESEARCH

The understanding of mechanisms important in AAA is expanding rapidly within pre-clinical models. This however has not currently been matched by large trials to examine the role of therapies targeting these pathways in patients. Such trials are urgently needed given the paucity of aneurysm specific medications currently available. It is hoped that over the next decade a number of agents efficacious in slowing AAA progression and reducing other AAA specific complications, such as the high rate of cardiovascular events in these patients will be identified.

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13 • Pharmacological Treatment of Aneurysms

MATTHEW THOMPSON, JANET T POWELL

St George's Hospital, London, UK.

BACKGROUND

Abdominal aortic aneurysms (AAAs) are present in 5 to 10% of men over the age of 65, and elective surgical intervention has long been the mainstay of treatment. There is widespread consensus that operative repair is the treatment of choice in larger AAAs, where the risk of rupture increases with the size of the aneurysm. However, even elective operations carry a significant mortality risk, and the UK small aneurysm trial has shown that for smaller aneurysms (between 4 and 5.5cm) there is no difference in outcome between operation and no intervention. Currently such patients are treated with best medical therapy, but there has been considerable research into finding a pharmacological treatment to prevent aneurysm expansion and rupture.

SCREENING PROGRAMMES

A major obstacle to the prevention of mortality and morbidity associated with aneurysms has been the fact that the majority are asymptomatic, and therefore often remain undetected. Abdominal aortic aneurysms have tended to present either as emergencies or as a result of their increasing size, and it has been shown that larger aneurysms grow more rapidly than their smaller counterparts and are at greater risk of rupture.[3] These patients would therefore benefit most from operative repair rather than medical intervention. In order for a medical treatment to be of benefit, it needs to be targeted at aneurysms that are small and asymptomatic. The most obvious way of doing this would be the initiation of a mass screening programme, and indeed, the Multicentre Aneurysm Screening Study (MASS)[1] has shown that as many as 88% of screen-detected aneurysms are below the threshold for surgery.

The introduction of screening programmes will identify a large population of patients with small aortic aneurysms that are at present untreated. The concept of pharmacotherapy for AAAs has evolved over the past decade, to encompass a medical treatment for aneurysms. Pharmacotherapy aims to reduce the expansion and rupture rate of aortic aneurysms by modifying aortic wall biology. A pharmacotherapeutic approach might be used to reduce the expansion rate of small, screen detected, abdominal aneurysms, and therefore reduce the proportion of patients requiring surgery. Alternatively, effective drug treatment might be able to reduce rupture rates in patients with large aneurysms unsuitable for aneurysm repair. Applications

to endovascular aneurysm repair have yet to be explored.2

PATHOPHYSIOLOGY

Abdominal aortic aneurysms have long been known to be associated with increasing age, male gender, cigarette smoking, chronic lung disease, hypertension and genetic factors. Despite the fact that most of these risk factors are shared with patients with atherosclerosis, aneurysmal disease appears to be a separate entity. Diabetes is a strong risk factor for developing atherosclerosis but not for AAAs. The genetic component of aneurysm aetiology is not fully defined at present, but may be due to inborn errors of the connective tissue matrix, such as mutation of the COL3A1 gene coding for the A chain of type 3 collagen. Information from gene wide association studies is awaited to further inform the genetic basis of AAA.

The detailed pathophysiology of the developing and expanding aneurysm has been covered in Chapter 12. Abdominal aortic aneurysms are characterised by several inter-related processes; degradation of the extracellular matrix, excessive proteolysis, apoptosis, oxidative stress, angiogenesis and widespread inflammation.

An approach to developing a suitable pharmacotherapy may be considered from one of two perspectives. Firstly, the drug may be targeted to one of the specific processes that have been shown to influence aneurysm development. The second approach hinges on newer theories about the nature of arterial disease. Increasingly it has been recognised that arterial disease is neither a matter of simple deranged lipid metabolism or of isolated local mechanical effects. The current belief is that arterial disease represents a low-grade systemic inflammation, which can therefore manifest itself at any point – coronary, carotid, aneurysmal or peripheral vascular disease. The Oxford Heart Protection Study has shown that generalised treatment of arteriopathic patients with statin therapy can reduce their chance of undergoing major adverse events including AAA rupture, regardless of their initial cholesterol level.

The majority of agents proposed to alter aneurysm expansion have been tested in animal models of aneurysm disease, and consequently there has been the typical disconnect between findings in the experimental situation and application to clinical practice. It must be remembered that humans show a great redundancy of biological processes that suggests that any effective pharmacotherapeutic agent must have pleiotropic actions. The next section of this chapter will concentrate on agents that have been tested in – albeit small scale – clinical trials. The final section will examine novel therapeutic approaches that have not yet been evaluated clinically.

THERAPEUTIC STRATEGIES

Beta blockade

Hypertension has been associated with AAAs, and investigations have shown that hypertension increases the development of aneurysms in the Anidjar/Dobrin rat model.

Since beta-blockers have been used successfully in the treatment of hypertension, it was not unreasonable to investigate the effect of beta-blockade on aneurysm expansion, as these agents have been shown to slow aneurysm progression in both experimental models and retrospective studies of patients with AAAs. Initially this was thought to be purely due to the drugs' effects on blood pressure, but there is considerable evidence to support the theory that beta-blockers may exert any beneficial effects on AAAs through enhancing

the cross linkage of elastin molecules, making them less prone to degradation.

A randomised clinical trial was instigated by the Propanolol Aneurysm Trial Investigators and reported in January 2002.3 548 patients with asymptomatic aneurysms between 3 and 5cm in diameter were randomised to receive either placebo (n = 272) or propanolol (n = 276) and were followed for a mean of 2.5 years. The primary criterion was the mean growth rate of the aneurysm. There was no significant difference in the growth rates of the two groups, although there was a trend towards more elective operations in the placebo group. There was no difference in death rates, but patients in the treatment arm of the study reported a poorer quality of life, and more of this group stopped taking their medication.

In this robust study it was clear that propanolol has little, if any, effect on the growth rate of AAAs. Subsequent studies of other beta-blockers have suggested that beta-blockade has little effect on the growth rate of AAA.4

Modification of the inflammatory response

With considerable evidence to support the theory that aneurysm expansion and rupture are both mediated by the immune system, it is unsurprising that there has been interest in modifying this response as a means of attenuating growth.

In the rat elastase perfusion model of AAA, the effect of treating experimental aneurysms with powerful immunosupression was tested. At nine days post infusion, a significant difference in the diameters of the aortas was observed, with the control group having expanded to approximately three times their pre-infusion size, but the treatment groups only grew to around twice their original size. These findings indicate that, at least in this experimental model, preventing the infiltration of inflammatory cells could halt the main spurt of aneurysm growth. Similar results were seen by Ricci et al when using a monoclonal antibody against the macrophage adhesion molecule CD-18.5

The one constant factor in these experiments on immune-modification has been that the compounds used have been unacceptable as clinical treatment strategies due to their wide range of action and many side effects.

Non steroidal anti-inflammatories

Indomethacin is a powerful anti-inflammatory drug that has been investigated both in the rodent elastase model and in human aortic aneurysmal tissue. Indomethacin reduced both aneurysm growth and MMP-9 activity in the rat and the levels of prostaglandin E2 (PGE-2), interleukin 1 beta (IL-1β and interleukin 6 (IL-6) in human tissue.

In a retrospective analysis of the large group of patients in the UK small aneurysm trial, indomethacin was also shown to inhibit aneurysm growth *in vivo*.6 The trial was not designed for this purpose and was the result of sub-group analysis, so further trials would be required. There is preliminary evidence that non-selective COX inhibition by indomethacin prevents aneurysm growth, but the side effects of this treatment on the gastrointestinal, renal and hepatic systems are well known.

Matrix metalloproteinase (MMP) inhibition

Many observers have noted an imbalance between MMPs and their naturally occurring inhibitors (TIMPs) in aortic disease, and one of the modes of action of indomethacin is to reduce the activity of matrix metalloproteinases. Many other compounds

have also been investigated for their anti-MMP properties and most have been effective in the experimental setting.

Tetracyclines have long been known to prevent connective tissue breakdown by their inhibitory effect on MMPs and several experimental studies have suggested that doxycycline reduced the growth of degenerative aneurysms and suppressed MMP-9 production in the rat elastase model. In a clinical trial, preoperative treatment with doxycycline caused a reduction in both the expression of macrophage MMP-9 mRNA and the activity of MMP-2 in aneurysm tissue.7 Also, a small double-blinded, randomised and placebo controlled pilot study from Finland has shown that treatment with doxycycline for a three month period significantly reduced the rate of aneurysm growth in a cohort of patients as measured by serial ultrasound scans.8 At six months, there was also a significant reduction in the serum C-reactive protein levels of the treatment group. Although the sample size was small and preoperative confounding effects are not taken into consideration, this trial has provided evidence to support further research into this area. In recent years a clinical trial has demonstrated that administration of doxycycline can produce a profound but selective effect on vascular inflammation that reduces aortic wall neutrophil and cytotoxic T-cell content.9 Additionally a clinical trial of doxycycline after endovascular aneurysm repair demonstrated that patients treated with doxycycline exhibited greater decreases in maximum aortic diameter and significantly reduced aortic neck dilatation at 6 months.10 Large-scale clinical trials of this agent appear warranted.

Anti-chlamydial therapy

The hypothesis that atherosclerosis may have an infective aetiology is not new, and it is clear that AAAs and atherosclerosis share some of the same risk factors. These associations were the rationale for clinical trials of anti-chlamydial therapy for AAA.

One RCT examined the effect of roxithromycin on aneurysm growth.11 Patients with small aneurysms were given either roxithromycin or placebo for four weeks, and subsequently followed up for a mean of 1.5 years. Once adjustments had been made for smoking, blood pressure and IgA, there was a significant difference in aneurysm growth between treatment and placebo groups.

DRUGS ACTING ON THE RENIN/ANGIOTENSIN AXIS

In 1998 a French group reported the effects of angiotensin converting enzyme (ACE) inhibitors and angiotensin II antagonists in a strain of rat prone to rupture of the internal elastic lamina of the aorta. To ensure any beneficial effects were not due to the antihypertensive properties of the drugs, they were compared to hydralazine and two calcium channel antagonists. Both ACE inhibitor and angiotensin II antagonists prevented rupture of the internal elastic lamina, suggesting this was due to the effect on angiotensin II and not on another part of the renin/angiotensin system.

In recent years there has been some intriguing, and contrasting clinical evidence regarding ACE inhibition in AAA. A Canadian group using epidemiological methodology reported that patients who received ACE inhibitors before admission to hospital were significantly less likely to present with ruptured aneurysm (odds ratio 0.82) than those who did not receive ACE inhibitors.12

Similar associations were not observed for beta blockers, calcium channel blockers or angiotensin receptor blockers. Conversely, analysis of patients taking ACE inhibitors in the UK small aneurysm trial demonstrated that these patients had enhanced aneurysm growth.13 A clinical trial is underway in the UK to define the action of ACE inhibitors in AAA expansion.

HMG CO-A REDUCTASE INHIBITORS

The HMG Co-A reductase inhibitors (statins) are a group of drugs in which there has been considerable interest recently. The statins have been used successfully for their lipid-lowering properties for some time now, but have also exhibited beneficial effects in cardiovascular disease unrelated to this. In laboratory experiments, statins have been proven to reduce MMP-9 expression by human macrophages, and their anti-inflammatory effects are well documented. Their pleiotropic actions are well suited to target aneurysm expansion and experimental effects are encouraging in reducing aortic inflammation and proteolysis.[14,15]

A small scale clinical trial of statin therapy in AAA demonstrated a reduced expansion rate in the treatment group16 and a recent systematic review and meta-analysis suggested that there is evidence to suggest that statins reduce aneurysm growth.4 However, aside from aneurysm expansion, there is now overwhelming evidence that patients with aneurysms and peripheral vascular disease derive benefit from statins with regard to cardiovascular death and outcome following surgery.17 In this regard all patients with aortic aneurysms should be on statin therapy if tolerated.

THE FUTURE – DATA FROM RECENT EXPERIMENTAL STUDIES

Recent experimental data have suggested some possible significant advances in pharmacotherapy for AAA. A recent study by Satoh *et al*[18] demonstrated the significant role that oxidative stress plays in the development of experimental aneurysms. This study revealed that angiotensin II, through induction of reactive oxygen species, induces cyclophilin A in smooth muscle cells which then stimulates recruitment of inflammatory cells, activation of MMPs and production of reactive oxygen species. These factors then initiate the biological events responsible for aneurysm formation. This study and subsequent commentaries have illustrated the importance of oxidative stress in aneurysm formation and suggest a target for pharmacotherapy.

Perhaps the most significant new studies in aneurysm biology over the last few years have been those investigating the effect of inhibiting signalling pathways. As stated previously, aneurysms form by multiple pathways in the milieu of extensive biological redundancy. Two recent reports using inhibition of signalling pathways (JNK and NFκB) have reported that inhibition of crucial signalling mechanisms can actually result in regression of established experimental aneurysms.19,20 These results clearly represent a paradigm shift in aneurysm pharmacotherapy and offer the potential goal of aneurysm regression. The inhibitors used would not be safe in clinical use but recently, drugs with the potential to inhibit signalling pathways have been investigated experimentally. Rosiglitazone – a drug used extensively in diabetic control – has the ability to inhibit JNK and MAPK and has been effective in reducing aneurysm formation in the experimental setting.21

Clearly the goal over the next few years will be to discover drugs with the potential ability to regress established aneurysms.

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14 • Pathophysiology of Aortic Dissection and Connective Tissue Disorders

MARK HAMILTON

Discipline of Surgery, The University of Adelaide,
The Queen Elizabeth Hospital,
Woodville South, South Australia, Australia

INTRODUCTION

Thoracic aortic dissection (TAD) is the most common aortic catastrophe, occurring in approximately 5 to 30 cases per 1 million persons per year.[1] It carries a significant morbidity and mortality risk, with 21% of patients dying prior to hospital admission.[2] Recent improvements in the understanding of both the molecular biology and genetics of vascular disease has led to greater clarity of the pathogenesis of acute TAD and a number of associated diseases of the thoracic aorta. Since the initiation of the International Registry of Aortic Dissection (IRAD)[3] in 1996 there has been an evolution of terminology in relation to TAD, and the more encompassing term acute aortic syndrome (AAS) is now utilised to include TAD and a number of other pathologies including intramural haematoma (IMH) and penetrating aortic ulcer (PAU).

In broad terms this classification reflects recent advances in understanding in relation to the pathology and natural history of TAD, and the recognition that TAD is part of a spectrum of thoracic aortic pathology. These individual processes will be discussed separately along with some of the underlying pathologic phenomena that lead to AAS/TAD.

Embryology of thoracic aorta and arch vessels

The formation of blood vessels occurs between the third and eighth week of embryological development. The ventral aortas fuse into the endocardial tube and circulate blood by the end of the third week. During this process a series of mesenchymal clefts lined by what will become endothelium fuse and form two pairs of longitudinal channels – one medial and one lateral. The medial channels form the primitive aortas which with elongation and folding of the embryo become paired into ventral and dorsal arrangements, joining the cephalad end of the primitive heart tubes (formed by the ventral components). Five further pairs of arterial arches pass around the developing pharynx connecting the cephalad end of the heart to the remaining unfused dorsal aortas. These branchial arch arteries in pairs form a number of components of the definitive circulation as outlined below. The paired aortas fuse over much of their length around the end of the fourth week and give a number

of intersegmental branches in dorsal, lateral and ventral patterns. During weeks 5–7 there is a significant evolution in pattern or, in particular the cephalad arches regressing and new caudad arches forming.

The aortic arches are part of this cephalad to caudal progression, with six initial sets of arches regressing in a step-wise fashion starting around weeks four to five. The first, second, and fifth arches regress and are largely gone by the start of week five having contributed to the formation of parts of the maxillary and external carotid circulation (1st), the stapedial arteries (2nd) and a pair of early regressing rudimentary vessels from the 5th. Above the level of the 3rd arch the dorsal aortas remain fused and communicate with the 3rd arch to form the definitive internal carotid artery. The common carotid artery is formed by the proximal parts of the 3rd arch (hence it is also referred to as the carotid arch). The remaining proximal external carotid artery arises as new growths of artery from the aortic sac and are not part of the arches per se, but migrate up the third arch to their final position.

Of particular relevance to the thoracic aorta, the 4th arches both persist, the left as the aortic arch and the right as the root of the right subclavian artery. The subclavian arteries initially arise as outgrowths of the terminal paired aortas just proximal to their union and subsequently the resorption of the right aorta between the origin of the subclavian and the fused trunk causes the right subclavian to be isolated.

The sixth arches fuse with the developing pulmonary arteries, the right partly regressing and partly forming the pulmonary artery with the left becoming the ductus arteriosus.

There is also a concurrent change in cardiac anatomy at this time with the heart separating into its left and right sides (aortic and pulmonary).

There is an excellent demonstration of these embryological changes in Valentine and Wind[4] and a discussion of the most prevalent anomalies in a paper by Kau *et al*.[5] Conceptually this may give some understanding of why different segments of the aorta behave in different ways, and what the influences of the embryological derivation may be on the likelihood of differing pathologies in each segment. The embryonic origin of the vascular smooth muscle cells (VSMC) at differing levels of the aorta varies, with the predominant origin being neurectoderm in the thoracic aorta, versus mesoderm in the abdominal aorta.[6]

This difference in origin influences the response of VSMC's to a number of mediators such as Transforming Growth Factor Beta 1 (TGFβ1), an important modulator of the extracellular matrix (ECM) in the thoracic aorta. Neurectodermal VSMC growth is potentiated by TGFβ1, as is Collagen I production. This is in comparison to mesodermal VSMC's where TGFβ1 inhibits growth and has no influence on collagen deposition. Given that VSMC's are influential in aortic strength, it would be expected that varying concentration of VSMC's and differential response to TGFβ1 and haemodynamic strain in the aorta would influence the sites of ECM degradation and hence likelihood of aortic pathology such as dissection and/or aneurysm.[7] There is some evidence that VSMC's in the abdominal aorta under cyclical haemodynamic stress secrete TGFβ1, leading to an increase in aortic wall mass (however not in the thoracic aorta).[6]

There is also a differential pattern of elastic lamellar units (the functional elastic unit in the aorta – combining elastin lamellae and VSMC's) through the aorta, with higher levels of elastic lamella and VSMC's in the thoracic aorta than in the abdominal. Similarly there is a decrease in the elastin to

collagen ratio in the abdominal aorta compared to the thoracic aorta.[6] It is interesting to note that acardiac foetesus do not develop differential structure throughout the length of the aorta, suggesting that there is a significant influence of haemodynamic cyclical strain on the secretion of mediators such as TGFβ1 and hence architecture.

Haemodynamics of Thoracic Compared to Abdominal Aorta

There is convincing evidence to suggest that dissection flaps occur at the points in the aorta subject to the greatest fluctuations in pressure over time. Due to the torsional manner in which the heart contracts, and the physical effects of cardiac motion on the arch of the aorta, the areas subject to the greatest changes in pressure are the ascending aorta and the proximal descending aorta. This was demonstrated elegantly in a model created by Qiao et al based on a thoracic aortic aneurysm.[8] This model demonstrated differential shear and flow at varying points in the thoracic aorta, particularly the outer curves of the ascending and proximal descending aorta. There were also areas of increased transit/contact time on the concavity of the arch and proximal descending aorta (along with branch vessel origins in the arch) which as an aside may be the reason for the preponderance of atherosclerotic change at these points.

The alterations in elastic recoil ability and collagen concentrations and function in the aorta that are present in a number of aortic pathologies, combined with the magnitude of the force involved in blood flow (related to absolute blood pressure, pulse pressure and dP/dT) results in the most likely sites of dissection being where the physical forces on the aorta are greatest and the diminution in aortic strength is maximal. There is reasonable evidence that suggests that VSMC apoptosis (which is influenced by TGFβ1) is greatest at the convexities of the ascending and descending aorta – particularly in patients with bicuspid aortic valves, and that this may alter aortic strength at these sites,[7] predisposing to dissection or aneurysm at these sites.

Sizes of Normal Aorta

There is an excellent outline of both the normal sizes of the thoracic aorta at differing ages, allowing a basis for sizing in different pathologies, in the European Society of Cardiology Task Force document.[9]

CLASSIFICATION OF AORTIC SYNDROMES

The acute aortic syndromes can be classified in a number of ways, included chronicity, and anatomy and on the basis of the underlying pathology and complications.

Acute/Chronic

Acute dissections are those present for less than 14 days and chronic are those present for longer.[10]

DeBakey Classification of Class 1 Dissection – Type 1, 2 and 3

The DeBakey classification system separates TAD into three types, with subtypes of Type 3. Initially described by De Bakey and colleagues in 1965,[11] this classification is based on both the anatomy of the entry tear and the extent of the dissection. It is an anatomical classification and has been simplified on the basis of outcome measures and prognosis into the Stanford Classification.

TABLE 14.1: DeBakey Classification of Class I Dissection

Type	Tear	Extent
I	Ascending Aorta	Propagating up ascending aorta, across arch and through descending aorta
II	Ascending Aorta	Confined to ascending aorta/intrapericardial aorta
III	Distal to the Left Subclavian Artery	Descending aorta +/- retrograde across arch
Subtype IIIa		Confined to descending aorta above diaphragm
Subtype IIIb		Extends through diaphragm into visceral or abdominal aorta

TABLE 14.2: Relationship between Stanford and DeBakey classification of class I dissection

Stanford Type	De Bakey Equivalent	Site of Involvement
A	Type I and II	Ascending aorta +/- Arch
B	Type III	Descending thoracic aorta distal to left subclavian artery
Subtype a and b		Above or below diaphragm, similar to DeBakey

Stanford Classification

The Stanford classification arose from the recognition that prognosis was largely dependant on the involvement or not of the ascending aorta in the dissection process and was published by Dailey and colleagues in 1970.[12] The De Bakey Classification was thus simplified into two subclasses, Type A and B depending on involvement of the ascending aorta. Although the Stanford classification has allowed stratification into surgical treatment or conservative management groups, it fails to take into account the variations of thoracic aortic pathology that are now recognised to make up what is referred to as the Acute Aortic Syndrome. A review of recent literature proposed a more complex but inclusive classification which has been adopted by the European Task Force on Aortic Dissection.[9]

European Task Force

In 1999 Svensson et al[13] published a classification of thoracic aortic pathology that included not only classical TAD but also a number of newly recognised subtype pathologies that were felt to make up part of the continuum of aortic dissection. This system should be considered a subclassification to the Stanford and/or Debakey classifications. This classification is outlined in Figure 14.2.

PATHOGENESIS OF THORACIC AORTIC DISSECTION

Hypertension is recognised as one of the most significant risk factors for thoracic aortic dissection, and the treatment regimes for acute dissection syndromes utilise anti-hypertensive therapy as their mainstay.

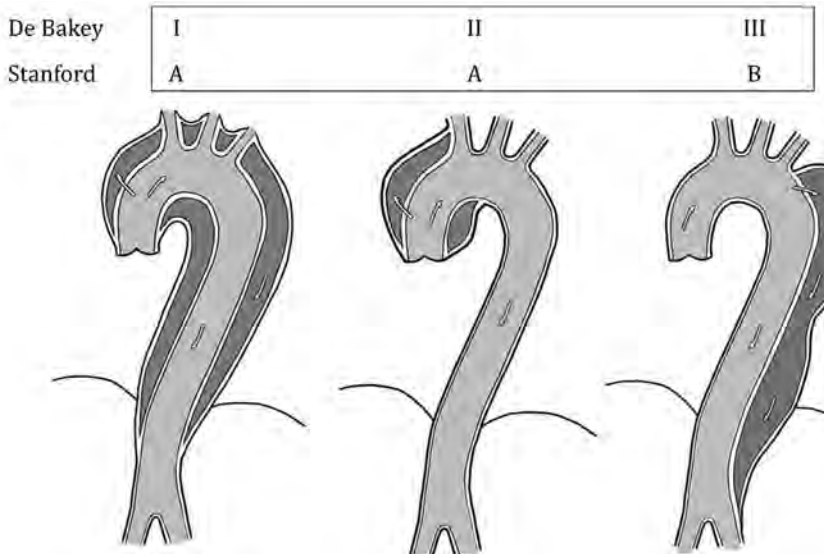

FIGURE 14.1: Diagramatic representation of aortic dissection class 1 divided into De Bakey and Stanford Classifications. Based on figure 4 from Erbel *et al*. 2001.

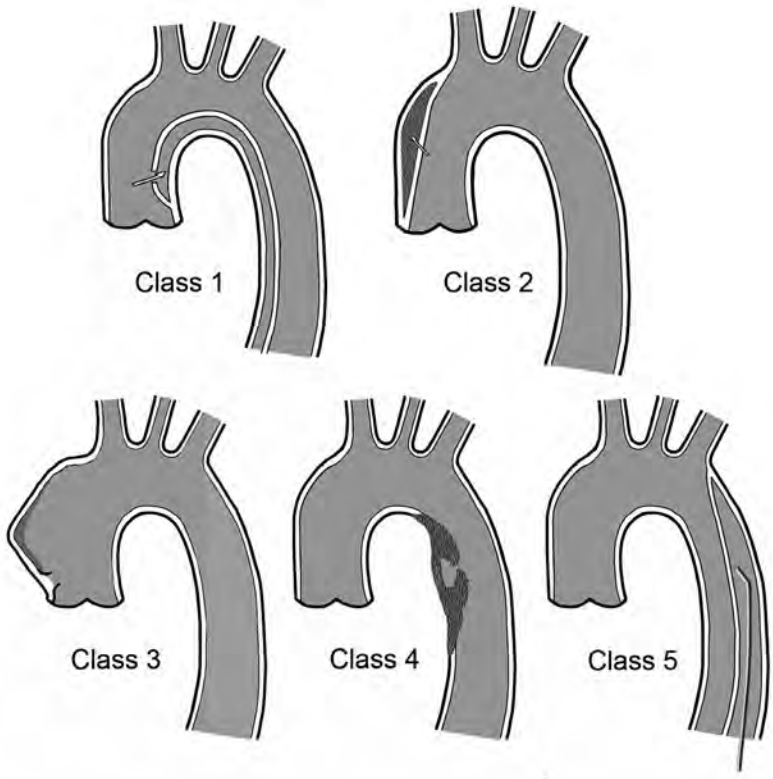

FIGURE 14.2: Classes of aortic dissection. Class 1- Classical Aortic Dissection (Intimal flap between true and false lumen); Class 2 – Intramural haematoma (Medial disruption with formation of IMH); Class 3 – Discrete/ Subtle dissection without haematoma. Eccentric bulge at tear site; Class 4 – Penetrating Aortic Ulcer (Plaque rupture leading to aortic ulceration, or a classical penetrating aortic ulcer with surrounding haematoma. Usually sub-adventitial); Class 5 – Iatrogenic and Traumatic Dissection. Based on figure 5 from Erbel *et al*. 2001[9] and Svensson *et al*. 1999.[13]

TABLE 14.3: Summary of aortic dissection classification systems

Stanford Classification	
Type A	Dissection of the ascending and descending aorta
Type B	Dissection of the descending aorta
De Bakey Classification	
Type 1	Dissection of the entire aorta
Type 2	Dissection of the ascending aorta
Type 3	Dissection of the descending aorta
New Classification	
Class 1	Classical aortic dissection with an intimal flap between true and false lumen
Class 2	Medial disruption with formation of intramural haematoma/haemorrhage
Class 3	Discrete/subtle dissection without haematoma, eccentric bulge at tear site
Class 4	Plaque rupture leading to aortic ulceration, penetrating aortic atherosclerotic ulcer with surrounding haematoma, usually subadventitial
Class 5	Iatrogenic and traumatic dissection
Class 1–5 Represent a subdivision to the Stanford or De Bakey classification	

There is good evidence that suggests that aggressive blood pressure management reduces mortality, particularly the use of beta blockers. There is also evolving evidence that a number of the newer antihypertensives such as losartan, which exerts some effect on mediators such as TGFβ1, may have extra effects on the turnover of thoracic aortic ECM, and may reduce the risk of both dissection and aneurysmal degeneration in some of the connective tissue disorders.

Smoking and hypercholesterolaemia have deleterious effects on the thoracic aorta and the catecholamine drive that is present in chronic tobacco use may exacerbate dissections and increase the risk of aneurysm disease.

Cocaine use is recognised as a risk factor for the development of the AAS, in particular in young african american males. Amphetamines similarly have a linkage with development of TAD, presumably for similar reasons to cocaine, with surges in catecholamines and concomitant acute rises in dP/dT and blood pressure in the aorta.[15,16]

Classical Thoracic Aortic Dissection (Class 1 Dissection)

The pathognomonic lesion in aortic dissection is a tear in the intima which allows pulsatile surging of blood into the intimomedial plane of the aorta. Typically the entry site is transverse but not involving the whole

circumference of the aorta. It usually extends down the left posterolateral plane of the aorta, in a spiral fashion.[10] These dissections may have communication between the false and true lumen, with intimal flap tears being present in >70% of cases at autopsy.[9] In a series of sudden deaths however, fenestrations were absent in 67% of cases.

Flow in the false lumen is usually antegrade but occurs retrograde in a small number of cases. Differences in the elasticity of the dissection flap and the aortic adventitia, and the increase in pressure in the false lumen predispose to collapse of the true lumen, with higher frequency of true lumen compression in non-fenestrated aortic dissection.

In 65% of cases of dissection, intimal tears occur in the ascending aorta, 20% in the descending and 10% in the arch, with 5% in the abdominal aorta.[10] There is a male:female ratio of 5:1 with a peak incidence of 50–60 years for proximal dissections, and 60–70yrs for distal dissection.[10]

There has been no particular success in defining the anatomical features of particular aortas that make them prone to dissection and long-term sequelae, despite analysis of multiple factors including tear depth and angle, local wall stress and the status of the vasa vasorum.[17]

Intramural Haematoma (Class 2 Aortic Dissection)

Intramural haematoma (IMH) otherwise known as Class 2 aortic dissection is a less common precursor or variant form of TAD. It comprises approximately 6–10% of all acute aortic syndromes. Approximately 10–30% of patients presenting with symptoms consistent with TAD may have IMH on imaging.[9] Occurring in greater prevalence in Asian populations, it makes up 30–40% of AAS in Asian series. Defined as a bleed into the outer layers of aortic media that lacks a discrete or detectable entry tear, it is usually seen on CT as a crescent shaped or concentric thickening of aortic wall. It may involve a longer segment of aorta than classical dissection. It appears as a 'dissection without intimal tear', and was previously described as such – however it is now recognised that there may be small atherosclerotic plaque ruptures in the wall of the vessel that are related to the proximal extent of the IMH.[18] There has been some suggestion that there may be focal rupture of vasa vasorum in the aortic wall which causes IMH, however with the advent of newer multidetector CT arrays, previously invisible intimal defects are now being recognised. This has lead to the proposition that a ruptured vasa vasorum leads to increased focal transmural pressure and consequent 'retrograde' rupture of a pre-existing aortic plaque and intimal disruption.[19]

Two subtypes of IMH/Class 2 dissection are recognized.[9] The features of the two subtypes of IMH are summarized in Table 14.4.

IMH seems to be associated with a lower risk of malperfusion syndromes than classical TAD, although complications are common. Between 28–47% of cases of IMH progress to overt false luminal dissection. Early aneurysm formation or contained rupture develops in 20–45% of patients.[14] There is considerable crossover between these groups Spontaneous regression is seen in approximately 10% of patients.[9]

Predictors of progression to TAD include recurrent/persisting pain, and presence of PAU. Some IMH may improve spontaneously with medical management only (particularly in Asian series). Younger ages, smaller aortic diameter (<4-4.5cm) and thinner haematoma (<1cm) confer better prognosis[9,20] and may allow conservative/non operative treatment with close observation. In one series, a 30-fold increase in

TABLE 14.4: Subtypes of IMH

	IMH Type 1	IMH Type II
Wall thickness	≤0.5cm	0.6 – 4.0cm (median >1.3cm)
Vessel diameter	<3.5cm	>3.5cm
Mean length in IMH	<11cm	>11cm
Presence of flow in IMH on echocardiography	Less common	Common
Association with calcified plaque	Not usually associated	Commonly associated
Intimal appearance	Smooth	Rough / Atherosclerotic
Echocardiographic appearances	Echo free zones present <30%	Echo free zones present >70%

progression to rupture was demonstrated if the aortic diameter was greater than 40mm. Wall thickness >1cm was associated with a nine-fold risk of progression.[21]

Location of IMH is also prognostic – ascending aorta has a high risk of progression to frank dissection and usually mandates repair. Exceptions to this seem to be Japanese and Korean series where there is a more benign course with Type A IMH treated with BP control, bed rest and serial imaging.[18]

Given recent advances in understanding of the contribution of genetic influences to MMP concentrations, elastin and collagen turnover and risk of syndromal AAS, it may be that the genotypic differences between Asian and European groups explains the differences in prognosis, progression and prevalence in IMH.

Penetrating Aortic Ulcer (Class 4 Aortic Dissection)

Pentrating aortic ulcer (PAU) was first described in 1934 by Shennan and then subsequently further characterized by Stanson et al in 1986. This condition can be defined as ulceration of an aortic atherosclerotic plaque penetrating through the internal elastic lamina into the aortic media. It is also classified as Class 4 Aortic Dissection.[9]

PAU comprises around 2.3–7.6% of acute aortic syndromes but in a series of 15 patients, 40% (n = 6) suffered aortic rupture, compared to a rate of 7.3% for Type A dissection and <4% for Type B, hence it is a morbid pathology when present.[22] It also appears that for a given aortic diameter the presence of PAU confers a worse prognosis than for classical or Class 1 TAD.[22]

PAU tends to occur in patients with extensive aortic atherosclerotic disease and in an older population than those affected by Class 1 dissection (a mean age of 77 years versus 54 for Type A dissection and 67 for Type B).[22] It appears that the pathological lesion (haemorrhage into/through an atherosclerotic plaque) is limited in its extent around the aorta by the transmural inflammation of extensive surrounding atherosclerosis. Penetration through, and dissection towards the adventitia can occur in the setting of medial penetration of the localised plaque haemorrhage.

Both IMH and PAU are recognised to be endpoints of a degenerative aortic pathology,

and largely occur in the descending thoracic aorta. In one series,[23] 90% of IMH and PAU were confined to the descending aorta.

Although such focal pathology as IMH and PAU seems ideally suited to treatment by endovascular means, it is probable that most patients in these groups with pathology in the descending aorta do not require intervention, unless they fulfil criteria that would categorise them in a treatment group for classical TAD (rupture/aneurysm etc). The presence of aneurysmal dilatation is a strong predictor of the requirement for intervention in the future.

COMPLICATIONS OF ACUTE AORTIC SYNDROME

Visceral ischaemia/malperfusion syndromes

Visceral ischaemia is one of the most significant and catastrophic consequences of TAD. In broad terms two main pathophysiological mechanisms of ischaemia have been described.

The first is a classical proximal entry tear in the absence of a distal fenestration. This leads to rapid and significant increases in mean false luminal pressure, leading to compression of the true lumen leading to distal ischaemia. In the context of considering treatment options, this pressure differential explains why the use of a bare stent alone in treating the entry tear is unlikely to succeed. It is unlikely that a bare stent would be able to compress the highly pressurised false lumen enough to divert flow without surpassing aortic rupture pressure.

A second pattern is where there is a distal fenestration present with relatively equivalent luminal pressures. This situation has a reduced risk of distal ischaemia as flow is not compromised, however an extension into a visceral vessel can cause branch ischaemia in one of two ways. These are described as either fixed or dynamic branch ischaemia. Fixed occlusion is caused by false luminal thrombus or pressurisation leading to a fixed impingement of the ostia. Dynamic occlusion occurs in the presence of a flap which acts as a floating valve across the ostia, particularly where there is differential pressurisation.

It is worthwhile remembering that aortic branch occlusion and malperfusion syndrome are not totally synonymous, given that partial or dynamic branch occlusion may not lead to significant end-organ ischaemia. There is some evidence that in recent times survival outcomes of branch occlusion and malperfusion syndromes have significantly improved in comparison to previous data from 1965–1985.[10] There is also good evidence to support mesenteric revascularisation prior to ascending aortic repair in particular, with improvements in mortality from 87% to 37% over time.

Ischaemic complications are present in up to 30% of cases of Type B dissection.[10] Rupture is less common but occurs in >20% of cases over the patient's lifespan. Recent literature still suggests 16–25% mortality in the patient group suffering ischaemic complications.[17] IRAD has demonstrated an overall mortality of 27.4% for all types of dissection, with mortality in distal dissections in the uncomplicated medical therapy cohort being 10.7%, rising to 31% in the complicated cohort. Malperfusion syndromes occur in 30–42% of dissection patients.[10] Spinal cord ischaemia is relatively rare as a complication of TAD but is still present in 2–3% of Type B dissections at presentation.[10]

Fate of the False Lumen

False lumen thrombosis occurs in 2–3% of medically managed patients, however even with surgical treatment distal false

lumen thrombosis only occurs in 15–30% of treated patients. This has implications for recurrent dissection and aneurysmal degeneration, with progression to aneurysm formation occurring in 30–50% of patients within 4 years of diagnosis of the initial dissection.[10] Long-term data show that aortic death is the ultimate outcome in 30–40% of patients with medically treated dissection, despite reasonable rates of early mortality with aggressive medical therapy.[17]

Aneurysmal Degeneration and Rupture

As outlined above, aneurysmal degeneration and rupture is one of the potential consequences of AAS. In medically managed patients who survive their intitial dissection event, 25–40% will continue onwards to develop aneurysmal dilatation. Out of this group 10–20% will rupture.[10] Degenerative disease rates in IMH are as high as 45%, with either contained rupture or aneurysm formation.[18] Similarly PAU rupture or aneurysm rates are in the region of 40% – with variable reports of severity/mortality risk. As discussed earlier, predictors of worse outcomes in IMH patients are initial aortic diameter above 4cm, and aortic wall thickness >1cm. These features conferred a 30-fold and 9-fold increase in progression to aneurysm and rupture respectively.[10] The prognostic features for development of aneurysmal degeneration include aortic size >4cm, persistent hypertension despite medical therapy, and persistent patency of the false lumen. Interestingly the recent data from IRAD also suggests a significant increase in 3 year mortality in the group with partial thrombosis of the false lumen – a relative risk of 2.69, with a 3 year mortality rate of 31.6%.[14]

CONNECTIVE TISSUE DISORDERS AND ACUTE AORTIC SYNDROMES

Heritable disorders such as Marfan's Syndrome (MFS), Ehlers-Danlos Syndrome (EDS) and Loeys-Dietz Syndrome (LDS) are well-recognised predisposing causes for TAD, however in large series only contribute 14–22% of dissections.[1] Their presence predisposes patients to early dissection and aneurysm formation compared to the atherosclerotic cohort, and identification of these patients early in life allows targeted medical therapy, surveillance and early intervention as appropriate to prevent rupture and its associated mortality.

Marfan Syndrome

Initially described by Antoine-Bernard Marfan in 1896 as a constellation of skeletal manifestations, Marfan Syndrome (MFS) has subsequently been more clearly codified into a recognised set of cardiovascular, skeletal and ocular findings. Marfan syndrome is responsible for approximately 5% of all aortic dissections and is the most common cause of dissection in patients under 40 years of age.

The classification of the syndrome was initially described in the Berlin Nosology (1986) but due to advances in molecular biology and further understanding of the disease and its sub-types, there was a new nosology developed in 1996 – the Ghent Nosology. This has recently been revised and was published in 2010.[24] This allows some differentiation from previously overlapping syndromes such as the Mitral Valve Prolapse Syndrome (MVPS) and the MASS (Myopia, Mitral Valve Prolapse, Non-progressive Aortic Root Dilatation, skeletal findings and striae), and also further differentiation from some of the more recently described overlap

syndromes such as Loeys-Deitz Syndrome. These other syndromes do not all carry the same vascular implications as MFS. Unfortunately in the past the laxity of diagnostic criteria meant that many people had the diagnosis of MFS applied, when in actuality their vascular mortality was lower than that of MFS.

The previously identified genetic basis of MFS is a mutation in the gene coding for fibrillin 1– a cysteine-rich glycoprotein important in the manufacture of connective tissues. In the aorta, fibrillin 1 is prevalent in the aortic adventitia, and to a lesser degree in the media where fibrillin 2, a similar but functionally different protein, is more prevalent.[25] The anatomic abnormalities in the MFS aorta are those of 'cystic medial degeneration' – loss of VSMC numbers, increased collagen content and elastic fiber disarray.[7]

The current diagnostic criteria include a series of major and minor criteria. Major criteria include aortic root dilatation/dissection, Ectopia Lentis, >= 4 particular skeletal manifestations and dural ectasia as defining characteristics, and the presence of the fibrillin 1 mutation as a component of diagnosis when these are not clear. In index patients the diagnosis must involve major involvement in at least two organ systems and minor involvement in one more. In the presence of a fibrillin 1 mutation that is known to cause the MFS, or when there has previously been a first degree relative with MFS based on the Ghent Nosology – there need be only one major and one minor manifestation in different organ systems. The presence of the fibrillin1 mutation is now given more weight in light of the increased ease of detection.

The 2010 Ghent nosology has been developed based on a more evidence-based approach, and in a more patient centric fashion with an alteration in the weighting of different criteria and development of more meaningful diagnostic thresholds.

Cardinal features such as aortic root dilatation and ectopia lentis are now given more weight, and in combination can be diagnostic. Other systemic manifestations (in other organ systems) contribute to an overall systemic risk score that assists in diagnosis.

MFS has variable time of onset, tissue distribution and severity of clinical manifestation, even in patients with identical fibrillin gene mutation.

Of interest in MFS, levels of proteases such as MMP-9 are actually quite low, particularly in comparison to AAA phenotypes, and there is some suggestion that MFS-related dissection and aneurysm are not as strongly influenced by proteases as had previously been thought.[6]

Fibrillin and Marfan Syndrome

Fibrillin1 is a fundamental component of the vascular ECM. Fibrillin-rich microfibrils play a significant role in linking vascular SMC to adjacent elastin fibrils. They are thought to regulate tissue development and turnover of elastin, and are a template for the construction of elastin microfibrils.[6]

The FBN-1 gene is a large gene on the q arm of Chromosome 15 at position 21.1. The description of this location is 15q21.1. It is closely associated with the gene for TGFβ1. There are more than 600 described mutations in the FBN-1 gene that can cause MFS with varying degrees of penetrance. The majority (around 2/3) of fibrillin 1 mutations are missense mutations that alter one amino acid out of the 2871 that make up the protein. Approximately 20% are frameshift mutations, and 12% are side splice mutations. Around 25% of the presentations of MFS have a new and previously unidentified mutation in the fibrillin 1 gene.[25]

It was previously felt that the abnormality of the fibrillin1 gene led to structural abnormalities in ECM and elastin through fibrillin 1 weakness which were the cause of the

aneurysmal degeneration seen in MFS. This rather simplistic concept has been partly refuted, particularly in light of syndromes such as Williams-Beuren syndrome where a microdeletion of the elastin gene is actually characterised by aortic stenosis rather than aneurysm and dissection.[6] It is now postulated that there is both a structural and a signalling component to the pathophysiology. This has led to a change in the definition of Marfan's from a purely connective tissue disorder to a developmental abnormality with effects on the development and morphogenesis of multiple organ systems.

There is some evidence from mouse knockout models that underexpression of the fibrillin 1 gene results in increased MMP expression and elastin fragmentation, leading to reduced structural integrity of the aorta.[26] There is also upregulation of MMPs when mutated fibrillin 1 (which is more prone to proteolysis) undergoes degradation. There is similarly an increase in macrophage numbers in the setting of elevated concentrations of fibrillin 1 fragments.[6] There is some evidence of a degree of a threshold phenomenon for the deterioration in vessel structure in relation to proportional amounts of normal and abnormal fibrillin 1 microfibrils.

One of the proposed mechanisms by which fibrillin 1 mutation causes loss of tissue integrity is the documented impact of abnormal fibrillin 1 on the action of normal fibrillin-1 in the formation of microfibrils. This is a case of a heterozygote disorder where there is a dominant-negative pattern of activity caused by the mutated gene product.[6,27]

Fibrillin 1 gene mutations on their own (ie in absence of Marfan's) that result in decreased fibrillin1 gene expression have also been linked to thoracic aortic aneurysm (TAA) and TAD. A reduction in fibrillin 1 abundance, but with normal protein structure may lead to similar but less severe phenotype to Marfan's that is restricted to the vascular system.[26] Similarly there appears to be a relationship between fibrillin 1 mutation and upregulation of TGFβ1 signalling pathways. This may be because of structural and functional similarities between latent TGFβ1 binding protein (LTBP) and fibrillin 1 and the possibility that abnormalities in the fibrillin 1 gene cause decreased affinity for TGFβ1 in LTBP – hence releasing greater amounts of TGFβ1 to increase signalling of TGFβ1 and therefore increase VSMC apoptosis.[6]

Given the large number of unique mutations, genotypic/phenotypic correlations are difficult. There are also additions to the fibrillin 1 gene which can cause the related phenotypic abnormality that is MASS. The recently described interplay between fibrillin 1 and TGFβ has similarly provided new insight into potential mechanisms for and explanations of the variable penetrance and phenotypic appearance of MFS and related disorders.

SMADs are intracellular proteins that transduce extracellular signals from TGFβ ligands to the nucleus where they activate TGFβ gene transcription. Some recent evidence suggests that the majority of the vascular end points in MFS are related to the influence of fibrillin 1 mutation on TGFβ1 levels and the subsequent TGFβ1/SMAD2 signalling interaction. The downstream effect of this on elastin manufacture and disorganisation via upregulation of TGFβ responsive gene expression leads to elastolysis and ECM degradation.

The Role of Transforming Growth Factor Beta (TGFβ) in the Development of Vascular System in Health and Disease

The TGFβ signalling pathway is important in both embryologic development – particularly in relation to body morphology and patterning, tissue differentiation and body

axis; as well as having a potent role in the differentiation and proliferation of a number of cell lines, and the stimulation of apoptosis – particularly in VSMCs. TGFβ mutations therefore have the potential to cause a number of congenitally acquired disorders of development and function of the skeletal, muscular and cardiovascular systems. This applies to both TGFβ and TGFβ receptors (TGFβR) or components of the signaling pathway. This includes a number of ligands that are currently of particular interest to researchers in TAA/AAA/TAD.

The TGFβ superfamily is large, and signals via two main pathways – TGFβ/activin/nodal receptor to downstream intracellular transduction proteins SMAD2 and 3, or alternatively bone morphogenetic proteins (BMPs) to SMAD1 and 5.[7,28]

TGFβ has three ligand subtypes -1, -2 and -3. These are secreted as what is termed a large latent complex (LLC) comprising 3 polypeptide chains. These chains are the small dimeric TGFβ ligand, a latency associated peptide (LAP) to which TGFβ is non-covalently bonded and a latent TGFβ binding protein (LTGFBP) of which there are three subtypes which are covalently bound to the LAP.[28]

After secretion, the LLC is sequestered in the ECM in association with fibrillin 1. This provides a pool of stable, largely inactive TGFβ which can be released under stimulus from a reservoir. The TGFβ receptors and ECM with a reservoir are widespread and the effects of TGFβ occur both locally and systemically. This explains why the regulation of TGFβ secretion at local level by variation in levels of ECM components such as fibrillin 1 can have significant effects.

The mechanism of TGFβ ligand signalling is reasonably well understood and appears to occur via two families of TGFβ receptor, type 1 and type 2 (of which there are further subtypes within each family). TGFβ binding to the type 2 receptor causes recruitment and subsequent phosphorylation of the type 1 receptor, leading to the formation of an activated receptor complex. TGFβ affinity for the receptor is variable, and there are a number of transmembrane co-receptors such as betaglycan (TGFBR3), which is high affinity but non-signalling, that enhance TGFβ binding to the type 2 receptor complex.[28]

The formation of the activated receptor complex leads to further cascading phosphorylation of receptor-associated SMADs (r-smads or smad 2/3). The type of SMAD activated depends on which TGFβ1 receptor subtype is activated. Once the r-smad is activated it binds with a co-smad (SMAD4) to shuttle it to the nucleus to allow transcription of the target TGFβ gene.

Clearly this is a complex pathway, and furthermore there are a number of other intracellular kinases which can influence the phosphorylation and hence activity of the SMAD family, all of which can have outside influences.[7,28,29]

Ehlers-Danlos Syndrome

Ehlers-Danlos Syndrome (EDS) is a hereditary disorder of connective tissue related to abnormalities in the gene for collagen, with six different types described.[30,31] It has an overall prevalence of approximately 1 in 25,000 live births.[31] Original descriptions were by Edward Ehlers in 1899 and Henri Alexandre Danlos in 1908. The most important subtype from the vascular perspective, and the most catastrophic in terms of presentation remains Type IV or what is now known as the vascular type, described by Andras Barbaras in 1967, in which he noted increased vascular fragility. The prevalence of the vascular type is <4% of all EDS cases. Presentations are heterogenous, and are not always entirely clear, and the distribution of clinical presentation reflects that of Type III collagen.

Children with EDS are often misdiagnosed as having coagulation disorders due to easy bruising.[30] Skeletal manifestations are consistent with abnormality in manufacture of Type III collagen with a defect in the pro-α-1 Type III collagen chain, coded for by the COL3A1 gene. Abnormalities in these collagens lead to ligamentous laxity.

The biochemical basis for EDS was described by Pope in 1975. It is a heterozygous mutation of the gene for Type III collagen, which is located on the long arm of chromosome 2, and is inherited in an autosomal dominant fashion.[31] Vascular EDS is best described as a monogenic orphan disease transmitted as an autosomal dominant trait. There is therefore a 50% chance of affected individuals passing on EDS to each child. Sporadic cases do however, account for up to half of all reported cases.

Diagnosis of Ehlers-Danlos Syndrome

Clinical diagnostic features of vascular EDS include typical facial features (although these may be absent in some cases), skin abnormalities with a thin translucent appearance and more visible veins, rupture of hollow viscera and vessels and easy bruising or ecchymoses. The classical facies of EDS are described as acrogeria, an appearance of slim face, thin long nose, sunken cheeks and often bulging or protruberant eyes. There may be periorbital pigmentation and/or telangiectasis. The upper lip is often fine and lacks puckering.[30] This is present in around 30% of patients.[31]

Skin manifestations vary from the other types of EDS, in particular the hypermobility and classical types, in that skin hyperextensibility is a less prominent feature. The skin does feel softer and is more translucent, and the subcutaneous vessels are often very prominent and visible – particularly on the trunk. Skin on the hands and feet is often more aged appearing than expected. With previous scarring, the appearance is quite stretched and there may be deposits of haemosiderin and enlargement of scars over mobile areas.

Easy bruising is a common finding, particularly in the younger group – in fact it is the most consistent clinical feature, being present is 66% of reported cases in one series.[32] These bruises and subcutaneous haematomas are often huge – much larger than the inciting injury would account for. The presence of these bruises often prompts investigation of platelet function and coagulation, however the underlying pathology is vascular fragility rather than any haematological abnormality.

Vascular complications in EDS are relatively rare in infancy but prevalent in teens and in the third and fourth decades of life. More than 80% of people have had a vascular complication by the age of 40.[31] Median survival of vascular type EDS patients is only 48 years. Vessel complications are the leading cause of mortality in patients with vascular EDS; with aortic tears, dissection, and arteriovenous fistulas being seen along with classical arterial rupture. These events are often spontaneous with no apparent cause and can occur in otherwise normal appearing blood vessels. The thoracic aorta and abdominal aorta are the main sites of involvement with more than 50% of these events occurring in the distal thoracic aorta.[31] Middle-sized arteries appear to be the main vessels in which these events otherwise occur. Extremity vessel events contribute approximately 25% of the complications that are seen in vascular EDS.[31] Spontaneous arterial rupture into closed spaces can lead to compartment syndrome and limb loss. Sudden onset of flank or abdominal pain should mandate non-invasive imaging as it is a common presentation of intestinal, uterine or arterial rupture.

The most commonly seen intra-cerebral

catastrophe related to vascular Ehlers-Danlos Syndrome is carotid-cavernous fistula.[30,32] This is an exceedingly uncommon presentation in the normal population, and is seen almost exclusively in patients with traumatic injury. The occurrence of this in an atraumatic setting should lead to a high clinical suspicion of the presence of a collagen-vascular disorder. The mean age of occurrence of carotid-cavernous fistula in this population is 31 years compared with 58 years in the general population. Less common presentations include intracranial haemorrhage in approximately 4% of cases.[7] Most of these cases have a previously identified intracranial aneurysm. Extracranial dissection of both the vertebral and carotid arteries is also seen. As with carotid-cavernous fistula the presence of this pathology in the absence of trauma should suggest the presence of a collagen vascular disorder. Other vascular anomalies include prominent varicose veins and these are widely seen in EDS patients. Surgical treatment of varicose veins is contraindicated.

Gastrointestinal and obstetric complications are also common-both of these tissue types being rich in type III collagen. From the gastrointestinal point of view, the sigmoid colon is the most regularly involved site of perforation. In contrast to the hypermobility of classical types of EDS, joint hypermobility is not a prominent feature in vascular EDS. In some studies there is an increased risk of congenital hip dysplasia and congenital clubfoot in comparison to the general population.[30] Differential diagnosis of EDS includes a number of paediatric coagulation disturbances and Silverman Syndrome, which can also have easy bruising and haematoma. In adults, Marfan's disease should also be considered.[30,33]

The diagnostic criteria for EDS were outlined most recently in the Villefranche nosology[34] that breaks the more classical description of EDS into six main types based on clinical presentation and the pathognomonic manifestations of each type. The presence of two or more major criteria is highly indicative of the disease and laboratory testing is recommended.[34]

Laboratory diagnosis involves the demonstration of structurally abnormal type III collagen produced by fibroblasts, and demonstration of an abnormal COL3A1 gene. The most reliable study is assessement of procollagen III deficiency using gel electrophoresis. This requires skin biopsy and has a risk of wound complications, thus site selection should be careful. Qualitative abnormalities in type III collagen can be seen by measuring secretion of type III collagen in cultured skin fibroblasts.[30]

Unfortunately although COL3A1 gene analysis is intuitively more accurate and would seem easier, it suffers the problem of lack of concordance between the mutation type and severity. There are numerous mutations possible in the gene given its large size, and there may be a greater prevalence of mutations than is currently recognised. Some mutations may have a degree of phenotypic correlation. Unfortunately the site of the mutation within the collagen gene has not been useful in predicting tissue integrity-one series of 135 EDS vascular type patients had no correlation between location of mutation and degree or presence of tissue fragility.[35]

From a biochemical perspective the most common point mutation that has been demonstrated is the replacement of a glycine in the collagen triple helix chain with another amino acid. Unfortunately the amino acid substitution does not correlate with phenotypic expression or clinical outcome. A proposed hypothesis in the aetiology of the clinical syndrome is that the abnormal procollagen components are either degraded in a form of 'protein suicide' [35] or are retained but their presence causes disruption of the

TABLE 14.5: The diagnostic criteria for the vascular subtype of EDS.

Inheritance Pattern	Autosomal dominant
Major Diagnostic Criteria	Thin, translucent skin
	Arterial/Intestinal/uterine fragility or rupture
	Extensive bruising
	Characteristic facial appearance
Minor Diagnostic Criteria	Acrogeria
	Hypermobility of small joints
	Tendon and muscle rupture
	Talipes equinovarus
	Early onset varicose veins
	Arteriovenous / carotid-cavernous sinus fistula
	Pneumothorax/haemopneumothorax
	Gingival recession
	Positive family history, sudden death in (a) close relative(s)

normal organisation and deposition of other types of collagens. This may explain the spectrum of differences in tissue fragility that are present with the same mutation. This is an evolving area of knowledge.[31]

Although controversial, some experts recommend ongoing surveillance to determine whether there is an incidental vascular lesion.[31] The controversy in this area arises from the fact that the majority of lesions will be treated conservatively and diagnosing them may increase patient anxiety unnecessarily. Arteriography is relatively contraindicated as an investigative modality in these patients as it is associated with a 67% complication rate and mortality rate of 17%,[32] however there is evolving discussion about its use as a treatment modality with the utilisation of endovascular coils or stents to minimise the use of arterial sutures. Similarly much of the previous evidence in relation to intervention in these patients was with larger sheaths and the advent of lower profile devices may minimise these risks.

The prognosis of Vascular EDS is dismal, with 92% of late deaths in a cohort of 220 index patients being due to vascular catastrophes, with median survival being 48–54 years, and patient survival to 60 years being as low as 55–68%.[31] The average age of first vascular or visceral complication is 24yrs with a 12% mortality. Pregnancy has a 25% risk of death with each pregnancy and an associated 50% chance of passing on the mutated COL3A1 gene.[32]

Loeys-Deitz Syndrome

Although MFS and EDS are by far the most commonly recognised connective tissue disorders (CTD) associated with AAS/TAD, recent understanding in the molecular biology of dissection has allowed recognition of a new disorder described by Bart Loeys and

Harry Deitz in 2005. This autosomal dominant CTD is recognised to be caused by mutation in the transforming growth factor beta (TGFβ) receptor 1 or 2 gene. LDS prevalence is currently not well-described. LDS does appear to be intimately related to MFS, and this becomes clear when it is understood that the genetic anomaly in LDS is a loss-of-function mutation in the TGFβR2 receptor – hence this mimics some of the effects of alteration in the levels of available TGFβ seen in fibrillin 1 deficiency syndromes such as MFS.

Phenotypic features of LDS include hypertelorism (90%), bifid uvula, cleft palate, generalised arterial tortuosity, craniosynostosis (50%), PDA and ASD. Mental retardation is often present. These features contrast with some of the features of MFS such as narrow high arched palate in MFS versus cleft, but there are a significant number of shared features such as ascending aortic aneurysm and dissection.

LDS falls into two types, LDS type 1 and LDS type 2. LDS1 has a number of similarities and overlap with MFS, including aortic root dilation, arachnodactyly (long thin fingers), dolichostenomelia (thin body and long extremities), pectus deformity and joint laxity. These patients are now discriminated from MFS patients in the new revised Ghent nosology by the absence of ectopia lentis (present in 40–56% of MFS patients) and other more stringent diagnostic criteria. LDS type 2 shares a number of similar features to vascular EDS, including soft, velvety skin and the presence of aortic aneurysm or dissection, however EDS lacks a significant number of features present in LDS including excessive vascular tortuosity, and bifid uvula. The table below outlines the major differences between the syndromes.

The vascular degenerative changes associated with LDS are significantly more malignant than those in MFS and occur at a younger age, hence early recognition is vital. Aortic root aneurysm is reported in patients as young as 6 years of age. The underlying genetic abnormality of LDS leads to altered signaling in the TGF-β cytokine family. TGF-β plays an important role in ECM formation and turnover, as well as cell proliferation and differentiation, along with apoptosis. This influence on proliferation and differentiation is particularly apparent in cardiovascular embryogenesis, including ventricular myocardial genesis.

TGF-β signal alterations lead to disarrayed elastin fibres, with loss of elastin content, and dilation and dissection as a consequence. In comparison to MFS, aortic and vascular aneurysms and dissections happen throughout the vascular tree, not just in the aortic root. Rupture has also been demonstrated in vessels <4.5 cm in diameter, in comparison to MFS where this would usually be considered a 'safe' size to manage conservatively.

Histological evaluation of vessels in LDS has shown excessive aortic wall collagen, along with increased levels of phosphorylated nuclear smad2. This indicates increased TGFβ signalling despite some degree of deficiency in the receptor.[29] In contrast to other CTD's, in particular EDS Vascular type, the surgical prognosis for LDS is quite good with reasonable surgical outcomes achievable as these patients lack the inherent tissue fragility of vascular EDS patients.[35]

Familial Thoracic Aortic Aneurysm Disease

Recent research shows that there are a significant number of patients who do not have typical named syndromes such as MFS but who have familial clustering of thoracic aortic aneurysm disease (TAAD). These patients display features of aortic pathology including ascending aortic or root dilation

TABLE 14.6: Major clinical features of Loeys-Dietz Syndrome (LDS) type 1 and 2, Marfan syndrome and the vascular type of Ehlers Danlos Syndrome (EDS type IV). Modified from Aalberts et al., 2008.

	Marfan	**LDS 1**	**LDS 2**	**EDS 4**
Vascular				
Aortic aneurysm/dissection	++	+++	+++	+++
Aortic Tortuosity	-	+++	+++	-
ASD	-	+	+	-
Skeletal				
Arachnodactyly[a]	+++	++	++	-
Dolichostenomelia[b]	++	+		-
Pectus abnormalities	++	++	++	-
Joint laxity	++	++	+++	+ (small joints)
Pes equinovarus[c]	-	+		+
Facial				
Craniosynostosis[d]	-	+/++	-	-
Hypertelorism[e]	-	+++	_	-
Cleft palate/bifid uvula	-	+++	+	-
Skin				
Excessive striae	+	-	-	-
Easy bruising	-		+++	++
Soft, velvety, translucent	-	+	++/+++	+++
Eyes				
Ectopia lentis[f]	++	-	-	-
Other				
Rupture large organs	-	-	+/++	++

The presence or absence of the features in italics might help to differentiate from Marfan syndrome, – infrequently, + around 25–50%, ++ around 50–75%, +++ >75%, [a]long slender fingers, [b]thin body habitus and long extremeties, [c]clubfeet, [d]premature closure of cranial sutures, [e]increased distance between pupils, [f]lens subluxation

and aneurysms or dissection of the ascending or descending aorta. They appear to have an autosomal dominant pattern of inheritance but with variable penetrance and expression. That is, some carriers do not demonstrate the pathology, and those that develop the pathology within a family do so at different severities. There is no gender bias. This is largely a diagnosis of exclusion, with a focus being on ruling out other named congenital or genetic anomalies that predispose to AAS/TAAD. The age of presentation provides some

hint as to the likely underlying pathology, with familial thoracic aortic aneurysm disease (FTAAD) patients presenting on average at older age than MFS patients, but younger age than sporadic TAAD patients.

Familial TAAD has been linked to a number of chromosomal abnormalities, some of which appear to be allelic with the mutations seen in well-characterised diseases such as MFS.

FTAAD type 1 is related to a gene defect on chromosome 11q23.3-4. This is an autosomal dominant inheritance pattern gene, but in comparison to a number of other syndromes, is a vascular-only syndrome with multiple segmental involvement of the thoracic and abdominal aorta commonly seen. This disease is highly penetrant in comparison to such diseases as MFS.

FTAAD type 2 has been linked to a 5q13-q14 mutation which is inherited in autosomal dominant fashion. In comparison to type 1 FTAAD, there is variable penetrance of this disorder, particularly in women. In comparison to type 1 FTAAD this process also carries with it a number of cardiovascular abnormalities which are similar to those seen in MFS. The postulation is that the 5q locus codes for a connective tissue protein of some kind.

FTAAD type 3 has a locus of mutation that maps to the 3p25-p24 site. This seems to be the site of the TGFBR2 gene, and the mutations that cause vascular anomalies appear to occur in the functionally important kinase domain leading to loss of function. This also overlaps a locus for a Marfan-like syndrome (the MFS2 locus). This mutation is found in approximately 5% of TAD patients. The phenotype is predominantly ascending aortic disease, however other site disease is also seen. Rupture and dissection at smaller sizes than usual is also recognised in the FTAAD type 3 patients.

Bicuspid Aortic Valve

Bicuspid aortic valve (BAV) is the most common congenital cardiac anomaly (1–2% of the population) and predisposes patients to increased risk of dissection, dilatation and aneurysm, independant of valvular function.[26] It displays an autosomal dominant inheritance pattern and has been shown to have a strong link with TAA in a cohort of 13 families.[25]

Similar medial degeneration is seen in BAV as that seen in fibrillin1 knockout mice. There is a measurable deficiency of fibrillin 1 and elevated levels of MMP-2 in bicuspid aortic valves compared to the control population.[26] There is similarly evidence of decreased amounts of fibrillin 1 in aortic media in BAV patients, but normal elastin and collagen levels. This is in comparison to atherosclerotic dilation where fibrillin 1 levels are increased. This suggests that the underlying pathology in BAV is similar to that in fibrillin 1 knockout mice – increases in TGFβ1 concentration and activity.

Turners Syndrome

Monosomy X, or Turners syndrome (TS) is a moderately common chromosomal disorder, (1 in 2500 live births), that carries with it an increased risk of aortic coarctation and dissection. The most commonly seen cardiovascular anomaly in TS is bicuspid aortic valve, and as discussed earlier, this independently increases the risk of TAD. BAV is seen in 13–34% of TS patients compared with 1–2% of the general population. The risk of aortic dissection in young Turners patients is significantly higher than the general population and dissection occurs at sizes below that at which dissection would normally be considered a risk (<5cm). Dissection is usually Type A, although there is a correlation between aortic coarctation

(present in 4–14% of TS patients) and Type B dissection. Given the short stature of TS patients, the aortic size index rather than the raw aortic size is preferred for calculating aortic dissection risk in these patients.

SUMMARY

Although previously considered a simple haemodynamic consequence of chronic hypertension, thoracic aortic dissection in the classical sense is now recognised as being part of a continuum of aortic pathologies that include intramural haematoma, penetrating aortic ulcer and a number of congenital connective tissue disorders. The recent advances in the molecular understanding of aspects of ECM turnover – particularly relating to fibrillin 1 and transforming growth factor – beta have allowed us to expand our diagnostic and treatment options into new areas.

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15 • Biomarkers in Vascular Disease

IAN M. NORDON, ROBERT J. HINCHLIFFE

St George's Vascular Institute, St James' Wing, St George's Hospital, Blackshaw Road, London, UK

INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of morbidity and mortality in the developed world. These diseases encompass the consequences of localized atherosclerosis and aneurysmal arterial degeneration. Evolution of risk factors contributes to the onset of subclinical disease; subclinical disease progresses to overt and often catastrophic clinical sequelae. Primary and secondary prevention strategies for CVD are public health priorities.

Whilst clinical assessment and cross-sectional imaging remain the cornerstones of patient management, they have limitations. There is increasing interest in the use of novel markers of cardiovascular disease as screening and risk-assessment tools to enhance the ability to identify the 'vulnerable' patients. Biomarkers are one tool to aid clinical assessment and identify high risk individuals, to ensure prompt and accurate disease diagnosis and to aid prognostic scoring of individuals with disease.

WHAT IS A BIOMARKER?

Initially described as a 'measurable and quantifiable biological parameter that could serve as an index for health assessment', the definition of a biomarker has since been standardized.

> *'A characteristic that is objectively measured as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention'* [1]

Biomarkers are indicators of disease trait (risk factor or risk marker), disease state (preclinical or clinical), or disease rate (progression).[2] They may also serve as surrogate end points used as an outcome measure to assess efficacy of therapy. A biomarker may be a recording taken from an individual (e.g. blood pressure), it may be an imaging test (CT / PET scan), or it may be a biosample (blood, serum, urine). Although each of these measurements constitutes a biomarker, the term biomarker has become synonymous with a novel protein, enzyme or cytokine with discriminatory value in clinical care.

TYPES OF BIOMARKER

Biomarkers found in body fluids may represent the active disease process or the

patient's reaction to the disease. A disease condition is a combination of biological changes directly due to disease (e.g. Disease Progression Biomarkers) and biological changes caused by host as it responds to disease (e.g. Host Response Biomarkers). Disease progression biomarkers are very specific to disease and tend to be proteins of low abundance. Conversely, host response biomarkers are less specific to the disease itself and are generally high abundance proteins. (Figure 15.1) When used in the correct clinical context both have discriminatory value.

A classical clinical example

Troponin is an established clinical biomarker. The diagnosis of myocardial infarction now stands on a convincing history, electrocardiogram changes and the detection of a protein biomarker for myocardial necrosis. The biomarker is a result of the systemic spillover of structural, myocardial specific myofilament proteins (Troponins). The levels of protein, due to the time course and extent of systemic release, correlate well with myocardial injury. First discovered by Ebashi in 1963, troponin's utility as a biomarker was highlighted in 1989 when a standardized immunoassay for circulating troponin T was developed. It underwent clinical validation against the then best marker of myocardial ischaemia, CK-MB, and was found to improve the efficiency of diagnosis of myocardial cell necrosis.[3] In 2000 the American Heart association incorporated a positive troponin T rise into its definition of myocardial infarction, and it remains the gold standard for the diagnosis of cardiac ischaemia.[4]

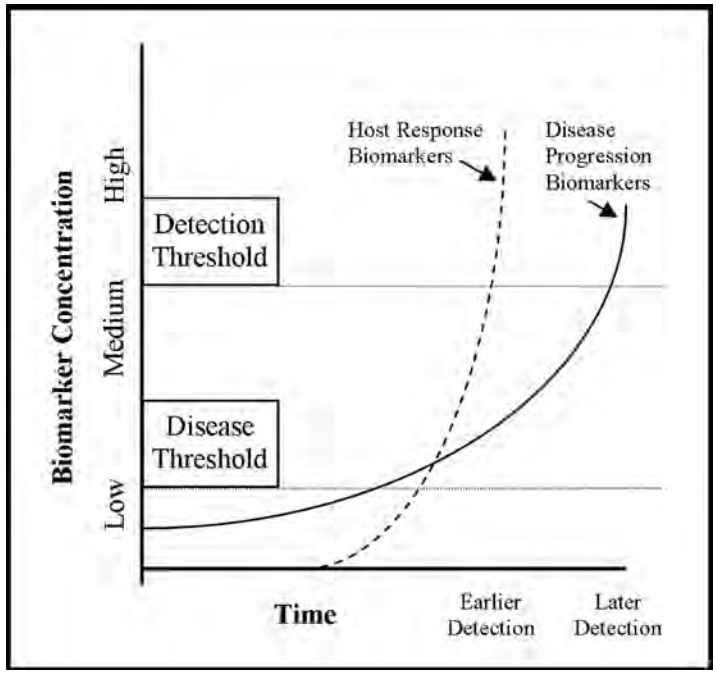

FIGURE 15.1: Comparison of Host Response and Disease Progression Biomarkers.

POTENTIAL VALUE OF BIOMARKERS IN VASCULAR DISEASE

Biomarkers have great potential to enhance all aspects of vascular care through AAA, carotid and peripheral vascular disease.

AAA development is likely to represent a product of genetic predisposition and environmental factors. AAA are characterized by local inflammation, matrix degradation and smooth muscle cell apoptosis.[5] Once established, AAAs grow at a rate of 2.6mm/year (95% range -1.0 to 6.1mm/year).[6] Generally this growth is insidious and asymptomatic until rupture. During this growth phase the active processes of AAA formation are on-going, both local and systemic cytokines and protein levels will be modified in response to or as a consequence of this pathology.

The principle challenge in the management of AAAs is that they generally remain asymptomatic until rupture. At rupture, survival is poor, with mortality rates up to 70%.[7] In order to make a significant impact on the outcome of AAA a number of significant advances are required. Improved detection of AAAs is the first step. Aneurysm screening is currently being rolled out in the UK and other countries. However there remains doubt over the cost-effectiveness of these ultrasound-based programs. Currently, maximum aortic diameter alone is generally the only means of assessing AAA rupture risk. However, the complications of AAA are not simply correlated to aortic diameter alone. Some small AAAs rupture and some large AAAs remain stable for prolonged periods.[8,9] Patients continue to undergo aneurysm repair on the probability of rupture, with the inevitability that some patients will undergo unnecessary repair. An improved risk model is required. Identification of blood-based biomarkers capable of identification and individual stratification of risk of progression and rupture would revolutionize the provision of care for AAA.

Endovascular AAA repair (EVAR) has significantly reduced the peri-operative mortality associated with elective AAA surgery.[10] The current standard of care requires regular post-deployment surveillance to ensure the aneurysm sac is excluded from the circulation and adequately depressurized. This surveillance is dependent on Duplex ultrasound and computed tomographic imaging. A blood test, for a biomarker of aneurysm expansion or aneurysm sac pressurization that could replace serial imaging would reduce the cost and morbidity attributed to graft surveillance.

Stroke is the third leading cause of death worldwide. Approximately 15% of strokes and transient ischaemic attacks (TIAs) are caused by unstable carotid artery plaque. Surgical treatment of a carotid artery stenosis by endarterectomy (CEA) can significantly reduce stroke risk, but is accompanied by morbidity and mortality. Equally, not all carotid plaques will become symptomatic and cause a stroke. Current evidence from a Cochrane systematic review states that CEA for asymptomatic carotid stenosis reduces the risk of ipsilateral, or any stroke, by 30% over 3 years. Fundamental to the selection of patients for intervention is the identification of plaques conferring an excess risk of neurological events. Currently, selection for carotid intervention is determined by the grade of stenosis and symptomatology. It is broadly accepted to treat high-grade symptomatic carotid stenosis, but in lower grade and asymptomatic patients interventions are still a matter of debate. There is growing evidence that stenosis alone is a poor guide. Molecular processes such as inflammation, lipid accumulation, apoptosis, thrombosis, proteolysis and angiogenesis have been shown to be highly related with plaque vulnerability.

Serum biomarkers reflecting these processes may distinguish unstable from stable carotid stenosis and be a powerful discriminator in the selection of patients for carotid surgery.

BIOMARKER DISCOVERY STEPS

Biomarkers must be measurable, add new information and aid the clinicians' management of patients**.** To apply the biomarker to a risk prediction model it must allow discrimination, calibration and risk stratification. (Table 15.1) Discrimination is the specificity and sensitivity of the marker, calibration denotes the ability of the marker to assign predicted risks that match actual observed risk, and risk stratification is the power to assign patients into clinically relevant categories.

There are two potential approaches to biomarker discovery. Firstly there is a knowledge-based approach exploring known candidates based on the understanding of disease pathophysiology. Alternatively an inductive approach can be undertaken, using non-hypothesis driven exploration to discover novel differences in genetic, proteomic or metabolomic expression. The two methodologies are complementary. Dependent on the understanding of molecular biology of disease and cell signaling pathways there is also cross-over between the 'omics' sciences used to trawl for novel candidates. (Table 15.2)

AAA BIOMARKERS

Candidate biomarkers have been studied based on present understanding of AAA pathogenesis. Examination of aneurysmal aortic wall biopsies has demonstrated pathological processes including medial arterial destruction, accumulation of inflammatory cells, elastin fragmentation, increased concentrations of proteolytic cytokines and in-situ thrombus. Consequently investigators have explored enzyme, protein and cytokine alterations on the basis of this understanding. The principle limitation of this approach being that all these features represent the end-stage of AAA development and may not be indicative of factors initiating AAA development or stimulating AAA growth.

The alternative 'hypothesis generating' approaches have been applied to AAA biomarker discovery. Samples of body fluids and vascular tissue have been compared between AAA patients and control subjects using genomic and proteomic array techniques.

TABLE 15.1: Translating biomarker discovery from the laboratory to patients

Phase	Title	Explanation	Estimated numbers required (n =)
P1	Discovery	Exploratory studies to identify potential biomarkers	50
P2	Validation	Capacity of biomarker to discriminate between health & disease	100
P3	Pre-clinical	Capacity of biomarker to detect pre-clinical disease	200
P4	Prospective	Prospective screening studies for sensitivity of biomarker	500
P5	Impact	Large scale study to asses impact of biomarker on survival	>1000

TABLE 15.2: Glossary of 'omics' methodologies used to discover novel biomarkers [SNP, single nucleotide polymorphism. NMR, nuclear magnetic resonance. BLAST, basic local alignment search tool. CT, computed tomography. MRI, magnetic resonance imaging. PET, positron emission tomography. SPECT, single-photon emission computed tomography]

Technology	Objective	Method	Tissue
Genetics	Gene identification	SNP genotyping Gene array analysis	Nucleated cells, diseased tissue
Proteomics	Protein or post-translational modified protein identification	2D-gel electrophoresis Mass spectrometry	Blood, saliva, tissue, urine
Metabolomics	Identification and characterization of small molecule	Mass spectrometry NMR spectroscopy	Blood, saliva, tissue, urine
Bioinformatics	Link array data to biological pathway	BLAST Hierarchical clustering	Data from combined methods
Molecular imaging	Non-invasive identification of molecular constituents of disease	CT MRI PET SPECT	Patients

These investigations have proposed novel potential circulating biomarkers of AAA. However, particularly in the proteomic studies, the studies have involved very small numbers of patients and similar numbers of control subjects. The challenge of finding appropriately matched controls can also reduce the value of some results with inbuilt confounding variables likely to diminish the power of any preliminary discovery. This is particularly the case when using aortic wall tissue for proteomic analysis as the availability of normal-aged aorta is limited and its method and timing of harvest and preservation will modify protein expression.

Circulating extracellular matrix markers

Collagen fragmentation is typically found in AAA biopsies. This is associated with synthesis of new type I and III collagen. During collagen synthesis both the carboxy-terminal and aminoterminal ends of the precursor molecule are released. These two fragments represent candidate biomarkers for increased extracellular matrix remodelling and consequent AAA formation. Small case control studies using radioimmunoassay for these peptide fragments have reported associations with AAA. However, contemporary series have failed to repeat these findings in a larger cohort.[10]

Tenascin-X was identified as a candidate biomarker due to its implication in Ehlers-Danlos syndrome, where patients are prone to aortic dissection and aneurysm formation. Elevated serum Tenascin-X has been demonstrated in AAA patients (n = 87) compared to controls. Notably, the highest quartile of serum Tenascin-X concentrations were associated with a 5-fold increase in AAA risk (OR 5.5; 95% CI, 2.0-13.8).[11]

TABLE 15.3: Substrates explored as possible biomarkers for AAA presence and growth

Related Process	Biomarker	Proposed significance	Reference
Circulating extracellular matrix markers	Tissue carboxyterminal propeptide of type I procollagen (PICP)	Plasma PICP levels are significantly decreased in AAA vs. controls ($p < 0.01$)	Nakamura M. et al. 2000[40]
	Aminoterminal propeptide of type III procollagen (PIIINP)	Acceleration of AAA growth is reflected in serum PIIINP ($r = 0.55$)	Satta J. et al. 1997[41]
	Tenascin-X	AAA is associated with high serum concentrations of tenascin-X	Zweers M.C. et al. 2006[11]
	Serum elastin peptides (SEP)	SEP levels higher in cases prone to rupture relative to controls (60% specificity) ($r = 0.40$)	Lindholt J.S. et al. 2001[13]
Matrix degrading enzymes	Cystatin-C	Negative correlation with expansion rate ($r = -0.24$)	Lindholt J.S. et al. 2001[42]
	MMP-9	Elevated in aneurysmal aortic walls – correlates with expansion of small AAAs ($r = 0.33$)	Linholt J.S. et al. 2000[14]
	Alpha-1 antitrypsin	Alpha-1 antitrypsin correlates with AAA growth ($r = 0.55$)	Vega de Ceniga et al. 2009[17]
	P-elastase	P-elastase is positively correlated with the mean annual AAA expansion rate ($r = 0.30$)	Lindholt J.S. et al. 2003[18]
Related to thrombus	Fibrinogen	Fibrinogen concentrations are significantly higher in AAA vs. controls (median: 2.89 vs. 2.53 g/L; $p<0.01$) and correlate with AAA size ($r = 0.32$)	Al-Barjas H.S. et al. 2006[19]
	D-Dimer	Annual AAA growth is positively and significantly associated with D-Dimer ($r = 0.39$)	Golledge J. et al. 2010[20]
	Homocysteine (HCY)	HCY levels correlate with AAA growth rate ($r = 0.28$). Hyper HCY is related to rapid AAA growth.	Halazun H.J. et al. 2007[43]
	Thrombin-antithrombin III complex (TAT)	Elevated serum TAT levels are associated with large AAA diameter ($r = 0.57$)	Yamazumi K. et al. 1998[44]

Related Process	Biomarker	Proposed significance	Reference
Inflammation	C-reactive protein (CRP)	CRP levels elevated in large AAAs	Norman P.E. et al. 2004[22]
	Osteopontin (OPT)	Osteopontin level correlates with AAA presence and growth (r = 0.24)	Golledge J. et al. 2007[26]
	IL-6	IL-6 level is independently associated with AAA and correlated with index diameter (r = 0.28)	Rohde L.E. et al. 1999[23]
	Osteoprotegerin (OPG)	Osteoprotegerin associated with AAA growth	Moran C.S. et al.[25]
	Resistin	Serum resistin concentration is independently associated aortic diameter (r = 0.19)	Golledge J. et al. 2007[26]
	Ig-G to *C. Pneumoniae*	Aneurysm progression correlated with IgG *C. Pneumoniae* infection (r = 0.45)	Lindholt et al. 2001[45]

Serum elastin peptide (SEP) is a degradation product of elastin. The role of SEP as a biomarker has been explored in two separate cohorts, the Viborg aneurysm screened cohort and the patients from the Chichester screened cohort who were unfit for surgery. Using a reproducible ELISA (enzyme linked immunosorbent assay) a clear correlation between SEP and aneurysm growth rate was reported (r = 0.4).[12] SEP was also found to be elevated in patients with symptomatic AAAs and those who went on to rupture.[13] This study was underpowered to identify a statistically significant biomarker and has not yet been repeated.

Matrix-degrading enzymes

Histological examination of aneurysm wall demonstrates fragmentation of the extracellular matrix. This has implicated elastases and matrix metalloproteinases (MMPs) in the pathophysiology of AAAs. Specifically, MMP-9 is abundantly expressed in AAAs and is considered to play a pivotal role in their formation. This candidate has been explored as a possible biomarker for AAA presence in case-control studies. The majority of studies confirm an elevated circulating MMP-9 concentration in patients with AAA compared to healthy controls or subjects with occlusive atherosclerotic disease.[14,15] Pooled analysis of these data has verified this finding,[16] however the variability in the findings, sample handling and analysis highlights the principle challenges in primary validation in biomarker discovery.

Alternative elastases have been explored as serum biomarkers. Small studies (n < 50) have raised the possibility of alpha-1 antitrypsin[17] and p-elastase[18] acting as serum biomarkers for aneurysm growth. They have not been repeated in larger cohorts, nor have these findings translated into a tool for prediction of rupture risk or the need for surgery.

Proteins associated with thrombosis

The role of the intraluminal thrombus commonly found in AAAs is yet to be fully

understood. Examination of this thrombus has identified a number of proteases that may be implicated in AAA progression. Proteins associated with thrombosis have been explored. These proteins may represent either end of the signaling pathway or be a by-product of degradation. The principle markers that have been evaluated are fibrinogen, D-Dimer, homocysteine and protein complexes implicit in the coagulation cascade.

A positive association between plasma fibrinogen concentration and AAA diameter has been demonstrated (r = 0.323).[19] The link between smoking and AAA is irrefutable, and raised plasma fibrinogen is induced by smoking. This association may only be a consequence of smoking and elevated fibrinogen has yet to be demonstrated independent of cigarette smoking.

D-Dimer level is a routinely used validated assay in general clinical practice to exclude a diagnosis of DVT. Plasma concentrations of D-Dimer reflect fibrin turnover in the circulation. Its role as a candidate biomarker for AAA has been explored. In a large cohort (n = 1260, 337 with AAA) average annual AAA growth was shown to be positively and significantly associated with D-dimer.[20] This study went on to propose possible diagnostic cut-off values for AAA presence were D-Dimer to be utilized as a screening tool. In their population, a level >400ng/ml for D-Dimer had an adjusted odds ratio (OR) of 12.1 (95% CI, 7.1-20.5) and >900ngml represented an OR of 24.7 ((%% CI, 13.7-44.6) for AAA presence. D-Dimer in combination with additional clinical risk stratification may have general value in AAA risk assessment.

Hyperhomocysteinaemia has been identified as a significant cardiovascular risk factor. These findings have evolved from studies into coronary heart disease and stroke. A review of the case-control studies found all series to report elevated homocysteine in subjects with AAA.[21] However this association was weak and failed to reflect a causal role for homocysteine in AAA development. It is likely that elevated homocysteine in AAA patients is reflective of dietary variability or renal clearance rather than the presence of an aneurysm.

Biomarkers to identify thrombosis are unlikely to translate into a universal clinical tool. The principle issue is that not all AAAs contain thrombus. Equally, in-situ thrombus is a dynamic substrate and findings from small studies may be a variable and not valid throughout the disease course.

Markers of inflammation

C-reactive protein (CRP) is the most commonly investigated biomarker in cardiovascular disease. It is an acute phase protein implicit in inflammation specifically to activate the complement cascade in cell death. Its elevation is inextricably linked to other inflammatory cytokines including interleukins (IL-6) and macrophage activation. CRP levels have been shown to be elevated in large aneurysms (40-54mm), but no association with AAA expansion has been shown.[22]

It has been suggested that the AAA itself is one source of IL-6. Circulating plasma levels of this inflammatory cytokine are elevated in AAA compared to controls (all series n<100). Also, plasma IL-6 has been correlated to aortic diameter in patients without AAA.[23] These findings are contributory to the understanding of AAA pathophysiology, supporting the role of inflammation and of macrophages in AAA progression. They lack the specificity to translate to a clinical biomarker.

Other candidates explored include osteopontin (OPN), osteoprotegerin (OPG) and resistin. These have been identified based on

the pathophysiology and epidemiology of AAA development. OPN and OPG are both cytokines associated with macrophage activity. Serum OPN levels show an independent but poor correlation with AAA growth ($r = 0.24$).[24] A similar finding has been reported for OPG; in a cohort of 146 men with small AAAs followed for 3 years, serum OPG showed a significant but weak correlation with AAA growth rate ($r = 0.2$).[25] The elevated risk of AAA associated with obesity has led to exploration of resistin as a putative biomarker. Serum resistin concentration is independently associated with AAA (OR 1.53; 95% CI, 1.32 - 1.76) and aortic diameter ($r = 0.19$, $P<0.0001$).[26]

BIOMARKERS OF AAA RUPTURE

Biomarkers capable of predicting AAA rupture would offer the greatest clinical value. Observing patients until rupture is rarely performed and unethical. As the rupture of a small aneurysm is a rare event, few ultrasound based studies have assessed the relationship between increasing biomarker levels and rupture. In the UK small aneurysm trial an association between cotinine and subsequent AAA rupture was reported.[27] This is a marker of smoking rather than any specific pathophysiological process.

Elevated MMP-9 levels have been reported in the plasma of patients with ruptured AAA compared to an elective non-ruptured population.[28] In this cohort, a 4-fold elevation in plasma MMP-9 was associated with non-survival at 30-days compared to those patients surviving surgery. Whether MMP-9 is important in the pathogenesis of rupture or simply a marker of an acute process is unclear.

BIOMARKERS FOLLOWING ENDOVASCULAR REPAIR

Endovascular repair (EVAR) has become the preferred strategy for the management of AAAs in many centers. Following stent graft deployment surveillance is required to ensure aneurysm exclusion and continued depressurization of the aneurysm sac. The role of biomarkers, to replace radiological imaging, has been explored. Decreases in MMP-3 and MMP-9 have been reported after successful EVAR with statistical differences compared to patients with active endoleak.[29] The principle problem with any biomarker will be its ability to discriminate between benign (type II) endoleaks and more significant (type 1 or type III) endoleaks.

BIOMARKERS OF CAROTID PLAQUE STABILITY

One current indication for carotid endarterectomy is Duplex derived grade of stenosis combined with clinical evaluation. There is growing awareness that in isolation this is a poor guide as to whether a patient should receive intervention. Biomarkers capable of discrimination between those carotid plaques which are either currently unstable or may become so in the future would revolutionize risk stratification in carotid surgery. Research into biomarkers for carotid plaque formation remains embryonic. The majority has come from subgroup analysis of large studies into coronary plaque risk analysis. Atherosclerosis is a multi-site disease process throughout the vasculature, therefore any biomarker for carotid plaque instability would require optimal specificity. This has led to early studies looking specifically at the carotid plaque tissue to identify possible candidates that would be particular to carotid atherosclerosis.

Atherosclerotic plaque development results from interaction between modified lipids, extracellular matrix, macrophages and activated vascular smooth muscle cells (VSMCs). Certain processes in the evolution of atherosclerotic lesions have been

TABLE 15.4: Substrates explored as possible biomarkers for carotid artery stenosis

Related Process	Biomarker	Proposed significance	Reference
Inflammation	C-reactive protein (hs-CRP)	Hs-CRP associated with progressive atherosclerosis, (upper quintile OR 3.65; 95% CI 1.65-8.08)	Schillinger M. et al. 2005[31]
	Seum amyloid A (SAA)	SAA associated with progressive atherosclerosis, (upper quintile OR 2.28; 95% CI 1.24-4.20)	Schillinger M. et al. 2005[31]
	IL-18	IL-18 expression found to be >3x greater in symptomatic plaques than assymptomatic	Mallat Z. et al. 2001[46]
	IL-6	Serum IL-6 elevated in symptomatic stenosis compared to asymptomatic	Koutouzis M. et al. 2009[33]
	Neopterin	Plasma levels (nmol/L) higher in complex plaques vs. non-complex plaques (24.2 vs. 19.4 ; P=0.01)	Sugioka K. et al. 2010[47]
	CD-36	Soluble CD36 elevated in patients with echolucent plaques vs. echogenic plaques	Handberg A. et al. 2008[48]
Lipid Accumulation	Lipoprotein-associated phospho-lipase A2(Lp-PLA2)	Symptomatic carotid plaques are characterised by elevated Lp-PLA2	Mannheim D. et al. 2008[34]
Apoptosis	Annexin V	Annexin V uptake associated with plaque instability	Keiselaer, B.L et al. 2004[35]
Thrombosis	Tissue plasminogen activator (t-PA)	Transient increase in t-PA gene expression associated with plaque instability	Sayed S. et al 2009[36]
	Fibrinogen	Elevated fibrinogen is associated with carotid disease progression	Sabeti S. et al. 2005[37]
	Plasminogen activator inhibitor-1 (PAI-1)	Transient increase in PAI-1 gene expression associated with plaque instability	Sayed S. et al 2009[36]
Proteolysis	MMP-9	MMP-9 level correlates with plaque instability. MMP-9 > 607ng/ml best predicted presence of unstable plaque (OR 19.2; 95% CI 3.9-94.2)	Alvarez B. et al. 2004[39]

associated with plaque vulnerability. These include inflammation, lipid accumulation, apoptosis, thrombosis, angiogenesis and proteolysis.[30] These changes are connected to the morphological characteristics of an unstable plaque. The search for a biomarker has focused on these processes.

Inflammation

Inflammation in the vessel wall is considered to play an essential role in the initiation, progression and the final steps of atherosclerosis, namely plaque destabilization and eventual plaque rupture. CRP may have direct pro-inflammatory effects and contribute to the initiation and progression of atherosclerotic lesions. In carotid artery stenosis hs-CRP correlates with morphological features of rapidly progressive carotid atherosclerosis.[31] CRP has also been shown to predict stroke risk in a healthy elderly population (Framingham Study).[32] Men in the highest quartile of CRP had double the risk of ischaemic stroke (RR 2.0; P = 0.03), and women had almost 3 times increased risk (RR 2.7; P = 0.0003) compared to the lowest quartile.

Serum amyloid A (SAA) is another acute phase protein. It is elevated in atherosclerotic lesions and has previously been shown to be a biomarker capable of predicting poor outcome in acute coronary syndromes. Serum SAA is associated with progressive carotid atherosclerosis, (upper quintile OR 2.28; 95% CI 1.24-4.20). The pro-inflammatory cytokine IL-6 has pro-atherogenic properties. Histology has demonstrated increased expression of IL-6 in unstable plaque regions. Elevated serum baseline IL-6 levels are associated with a greater stroke risk.[33]

Lipid accumulation

In atherosclerotic plaques, unstable lesions have a greater area occupied by lipid. Systemic lipid lowering in patients with cardiovascular risk using statins has shown a 25% proportional reduction in first event rate for stroke. OxLDL levels have been shown to be related to carotid plaque instability. One link between oxLDL and plaque instability is lipoprotein-associated phospholipase A2 (Lp-PLA2). In carotid artery disease, symptomatic carotid artery plaques express higher levels of Lp-PLA2 than asymptomatic plaques.[34] No serum studies have been performed on this possible biomarker.

Apoptosis

The necrotic core at the centre of advanced atherosclerotic plaques contains dead VSMCs and debris. Smooth muscle cells and inflammatory cells die as a consequence of programmed cell death (apoptosis). VSMC apoptosis may weaken the fibrous cap creating an unstable plaque prone to rupture. Apoptotic markers have been explored to identify vulnerable plaques. Annexin V, a marker of apoptosis, has been detected in symptomatic carotid artery plaques. This pilot study utilized exogenous radiolabelling and only examined 4 patients. The investigation did indicate that molecular imaging with the use of technetium-99m–labeled annexin A5 may be a new method for assessing plaque instability and identifying patients at risk for acute vascular events.[35]

Thrombosis

Thrombotic activity on carotid plaques is associated with stroke and transient ischaemic attacks (TIA). Examination of RNA from carotid plaques removed at endarterectomy has shown that expression of thrombomodulatory genes is increased in unstable plaques.[36] These include t-PA and

plasminogen activator inhibitor-1. To date no study has examined the possible role of these factors as biomarkers.

Plasma fibrinogen levels have been shown to be related to progressive atherosclerosis. In a cohort of 1268 assymptomatic patients progressive atherosclerosis was seen in 9.2%. The adjusted hazard ratio for atherosclerosis progression was 2.45 (P = 0.002) for the upper quartile compared to the lower quartile. Fibrinogen level at follow up was also shown to be associated with progressive disease (P = 0.004).[37]

Proteolysis

Plaque destabilization is associated with proteolysis. Proteolytic enzymes including matrix metalloproteinases appear important in the pathophysiology of atherosclerotic plaque cap rupture and consequent neurological events. It is likely that an imbalance in MMPs may lead to matrix degradation and plaque destabilization. In unstable carotid plaques there is a local increase in active MMP-9 concentration.[38] Elevation of MMP-9 has been shown in the serum of patients with symptomatic carotid artery disease in a small cohort of 40 patients undergoing carotid endarterectomy.[39]

CHALLENGES IN BIOMARKER DISCOVERY

A cautionary tail of biomarker exploration is described in the field of ovarian malignancy. Proteomic exploration was adopted early and with great enthusiasm in this field of cancer. Despite early reports citing proteins with 100% sensitivity, 95% specificity and a positive predictive value of 94% in a small cohort; these findings have failed to translate to a clinically applicable tool. The initial proteomic fervor was tempered and despite greater than 10 years exploration clinicians remain reliant on an older protein biomarker, CA-125.

Many candidate biomarkers, based on current understanding of vascular pathophysiology have been explored. None have translated to clinical practice. It is therefore the task of the discovery sciences i.e. proteomics and metabolomics to further this endeavor. Biomarkers continue to represent one of the most anticipated healthcare concepts. Yet before the potential can be fully realized, numerous challenges need to be resolved. It is unlikely that single biomarkers will be considered adequate for most applications. Multiple protein panels are the new paradigm. Because of variations in sample complexity, the approach to biomarker discovery will continue to be highly dependent on the intended application, each with its own discovery challenges. Body fluids are especially difficult to handle consistently. Serum is vulnerable to temperature and fasting state whilst variations in its protein content are difficult to identify as it is >90% albumin. Plasma is modified by the clotting cascade and haemoglobin breakdown, and urinary protein excretion is principally a product of renal filtration. High throughput consistent sample handling is essential if these biomarker panels are to be elucidated.

FUTURE WORK

The future of biomarker discovery lies in comparative proteomics combined with innovative bioinformatics and mathematical modeling. This review has demonstrated a large number of small independent scientific groups generating exciting and unique findings. The principle limitation consistent across the literature is a failure to develop these discoveries through validation in larger mixed populations. Different substrates (blood, plasma, serum) are being explored in

different conditions (Snap frozen, embedded, fresh), using varied assays, dependent upon the expertise of the scientific group. Large co-operatives tasked with biomarker discovery with defined consistent protocols across mixed populations offer the most appropriate environment for biomarker discovery.

CONCLUSION

Biomarkers will have increased utility in the future of vascular surgery. To date no biomarker for AAA or carotid stenosis has been translated into clinical practice. However with advancements in mass spectrometry and proteomic techniques combined with worldwide interest in this discovery science, a significant discovery cannot be far away. In 10 years time the decision to operate on a dilated aorta or carotid stenosis may be guided by the presence of a specific protein in the patient's serum, and no longer simply the morphology of the lesion.

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16 • Pathophysiology and Principles of Management of Vasculitides and Raynaud's Syndrome

MARTIN VELLER

VASCULITIDES

Occlusive arterial lesions in humans are usually caused by atherosclerosis. The primary and secondary vasculitides are rare inflammatory conditions that may also cause such ischaemia as well as occasional aneurysms. These pathologies usually present with unusual manifestations of ischaemia, but may also be the cause of common symptoms such as stroke, hypertension, intermittent claudication or Raynaud's phenomenon. Delayed recognition of these diseases is often associated with severe and irreversible complications. While the vasculitides are usually managed by physicians, vascular surgeons should be able to recognise them and assist in their management when appropriate.

INTRODUCTION

The vasculitides consist of primary and secondary pathologies in which non-specific transmural inflammation occurs within a blood vessel. The consequent vascular injury can cause vessel disruption, aneurysm formation or occlusion which can affect any blood vessel. The pathogenesis of each of these diseases is unclear, although they generally fall into one of the following groups:[1]

- Immune complex vasculitides: These are induced by circulating immune complexes or histamine. This results in the activation of the complement system, cytocines and monocytes in the vessel wall.
- Pauci-immune vasculitides: The anti-neutrophil cytoplasmic antibodies (ANCA) were first described in conjunction with rapidly progressing glomerulonephritis. Cytoplasmic ANCA (c-ANCA), perinuclear ANCA (p-ANCA) and x-ANCA (which is found in chronic inflammatory GIT pathologies) have been described. In the pauci-immune vasculitides, activation of neutrophils results in vascular endothelial damage.[2]
- T-cell vasculitides: In these, vessel wall damage is caused by CD4 lymphocyte mediated immune reactions.

The primary vasculitides occur rarely – between 20 and 100 cases per million. They usually present with non-specific clinical symptoms and signs – e.g. malaise, fever, weight loss – and generally evolve over a long duration. In addition, they may overlap with the manifestations of much more common infections, connective tissue diseases and malignancy. As a consequence making the

diagnosis is challenging and is confounded by the extensive overlap in the clinical and pathological manifestations between the vasculitides.

If unrecognised, these systemic diseases may be fatal. With appropriate treatment the majority of patients will improve but relapses are common. These relapses may be the result of recurrence, worsening of the inflammatory process or may be the consequence of complications of therapy.

CLASSIFICATION OF VASCULITIDES

The heterogeneous nature and extensive overlapping clinical and pathological features have made classification difficult. Currently, the most commonly used systems take into account the size of the vessel affected, the histological findings and the aetiology (Table 16.1).[1,3-5] There is also some value in differentiating between ANCA positive (e.g. Wegener's granulomatosis, microscopic polyangiitis, Churg-Strauss vasculitis, drug induced vasculitis) and ANCA negative vasculitides.[1]

Whilst, the classification systems attempt to highlight the differences among these diseases, in clinical practice these differences are not precise – polyarteritis nodosa for example normally considered to be a primary, medium vessel vasculitis, can also be caused by chronic hepatitis B or C infections and may involve small vessels.[1,5]

Table 16.1: The major vasculitides[3,22]

Primary vasculitis
Predominantly large vessel vasculitis
Giant cell arteritis (also temporal arteritis)
Takayasu's arteritis
Predominantly medium vessel vasculitis
Polyarteritis nodosa
Kawasaki's disease
Predominantly small vessel vasculitis
Churg-Strauss syndrome
Wegener's granulomatosis
Microscopic polyangiitis
Henoch-Schönlein purpura
Essential cryoglobulinaemic vasculitis
Hypersensitivity vasculitis
No predominant vessel size
Behçet's disease
Secondary vasculitis
Vasculitis secondary to connective tissue disorders
Rheumatoid vasculitis
Systemic lupus erythematosus (SLE)
Systemic sclerosis
Scleroderma (including the CREST syndrome)
Mixed connective tissue disease
Antiphospholipid antibody syndromes
Vasculitis secondary to viral disease
Hepatitis B and C virus
Human immunodeficiency virus (HIV)
Cytomegalovirus
Epstein-Barr virus
Drug induced vasculitis
Other vasculitides
Thromboangitis obliterans (Buerger's disease)
Purpura fulminans (Waterhouse-Friderichsen)
Thrombotic thrombocytopaenic purpura

CLINICAL PRESENTATION OF VASCULITIDES

In general, vasculitis should be considered to be present when chronic systemic symptoms of inflammation, including pyrexia, malaise, fatigue, and weight loss are associated with some form of organ dysfunction. Arthralgia, myalgia, pain in the digits, rashes, anaemia of chronic disorders, pericarditis and a raised ESR and CRP are also common. The common organ specific manifestations are listed in Table 16.2.

In general, the skin and peripheral nervous system signs are particularly useful because they tend to develop early in the course of

TABLE 16.2: The common clinical manifestations of the vasculitides

Skin	Livedo reticularis, palpable purpura, nodules, ulcers, gangrene
Peripheral nervous system	Mononeuritis multiplex, polyneuropathy
Central nervous system	Stroke, seizures, encephalopathy
Eyes	Blindness, scleritis
Heart	Myocardial infarction, cardiomyopathy, pericarditis, arrhythmia
Lung	Cough, chest pain, haemoptysis, shortness of breath
Kidney	Hypertension, proteinuria, haematuria, renal failure
Gastrointestinal tract	Haemorrhage, perforation
Genitals	Testicular atrophy, reduced ovarian mass

the disease and are easily detected. Small vessel vasculitis is often first noted when palpable purpura develops while the vasculitides affecting medium vessels commonly produce nodules, ulcers and gangrene.

The most common neurological manifestation of the vasculitides is mononeuritis multiplex. This is a distinctive peripheral neuropathy in which peripheral nerves infarct one at a time as a result of vasculitis in the vasa nervorum. Sudden, asynchronous and asymmetrical loss of function of individual nerves occurs, most frequently affecting sensory nerves. In the absence of diabetes and nerve compression syndromes, mononeuritis multiplex can usually be assumed to be due to a vasculitis such as polyarteritis nodosa or Wegener's granulomatosis.

INVESTIGATIONS OF VASCULITIDES

Haematological and serological changes will usually be present in individuals presenting with vasculitis. Chronic microcytic anaemia, a raised ESR and CRP are non-specific findings while red cell casts in urine, antinuclear antibody (ANA), complement, serum cryoglobulins and ANCA are more specific manifestations of the vasculitides.

X-rays are generally not useful other than in Wegener's granulomatosis where views of the nasal sinuses and chest, especially using computed tomography, may demonstrate diagnostic nodular lesions.

Biopsy of involved tissues is the most helpful method of making a definitive diagnosis, especially if the biopsy is taken from a symptomatic site. Occasionally a serological test, an angiogram or another investigation can be pathognomonic.

Some investigations are helpful in excluding secondary causes of a vasculitis or those conditions that may mimic a vasculitis. Examples include investigations to exclude drug reactions, syphilis, Human Immunodeficiency Virus (HIV), infective endocarditis, and antiphospholipid syndromes. By the very nature of these conditions this list is not exhaustive.

PRINCIPLES OF TREATMENT OF VASCULITIDES

While the vasculitides are usually treated by some form of immuno-suppression, the most important principle is to make sure that the treatment intensity is commensurate with

the severity of disease.[6] While most forms of vasculitis require aggressive treatment to prevent morbidity and mortality, some do not. For example, minor vasculitis limited to the skin or where the secondary cause of the vasculitis can be identified and subsequently can be withdrawn or treated, require no such therapy.

THE VASCULITIDES OF SPECIFIC INTEREST TO VASCULAR SURGEONS

Giant cell arteritis

Giant cell arteritis, which is also known as temporal arteritis, is the most common vasculitis found in adults. This panarteritis of the extracranial branches of the carotid artery occurs only in older individuals. The cause is unknown, but it is associated with the same HLAs found in rheumatoid arthritis and the disease process appears to be initiated by T cells in the adventitia responding to an unknown antigen.

The classic symptoms of giant cell arteritis are:[7]

- A temporal headache that is new or different (70%).
- Jaw claudication (50%).
- Polymyalgia rheumatica – which describes a condition that presents with acheing and stiffness of the shoulders, neck, and hip-girdle area (40%).
- Blindness (20%). This is the most significant complication of giant cell arteritis which can however be prevented by early treatment. The visual loss is caused by occlusion of the posterior ciliary branch of the ophthalmic artery. As a result, the blindness tends to be profound but fortunately is rarely the first manifestation of this complication and is usually preceded by episodes of diplopia and blurred vision.
- Malaise (50%).

The aorta and its major branches can also be involved (25%) leading to the development of thoracic aortic aneurysms some years after the first manifestation.

Giant cell arteritis is characterised by a raised ESR and CRP, and the presence of a normochromic, normocytic anaemia. Ultrasound of the affected temporal arteries can show a characteristic 'halo' of peri-vascular oedema or stenosis of the involved segments. Angiography (usually MRA or CTA) is required to demonstrate involvement of the thoracic aorta and its branches if this is suspected, while PET can demonstrate occult large-vessel inflammation. The definitive method of making the diagnosis of giant cell arteritis is by histology of the temporal artery. As a result of the risk of sudden and irreversible blindness, a temporal artery biopsy, which has a low morbidity, should be performed without hesitation to confirm the diagnosis.[8] As the artery may be affected by skip lesions a 3 to 5cm segment of artery should submitted for evaluation. The histology will demonstrate mononuclear cells infiltrating all layers of the artery with varying degrees of intimal proliferation and disruption of the internal elastic lamina.[7] In recent years the possibility of using duplex ultrasonograpy to identify characteristic features of giant cell arteritis has been raised and further research in this area is warranted.

Treatment with high doses of prednisone (40–60mg/day) and low dose aspirin should be started immediately when the diagnosis is suspected and before it has been confirmed by the temporal artery biopsy. If visual loss has been present for a few hours very high doses of intravenous methyl-prednisolone should be given as some vision may recover. Usually,

after more than 24 hours the visual loss is permanent. In the long-term, in the presence of a normal ESR and CRP, the prednisone can be tapered at a rate determined by the clinical picture and the ESR or CRP.[8] The effectiveness of methotrexate as a glucocorticoid-sparing drug for giant cell arteritis remains controversial while the role of biological therapies such as the tumour necrosis factor inhibitors, infliximab is unknown.

The majority of patients with giant cell arteritis will experience a relapse as the dose of prednisone is tapered and therefore prednisone will often need to be given for prolonged periods and with resultant frequent additional complications from the steroids therapy.

Surgical or endovascular interventions are occasionally indicated when the disease involves the aortic arch and its branches.

Takayasu's arteritis

This large vessel vasculitis affects mostly young adult women but has been found in infants and more rarely in the aged. The cause is unknown, yet the geographic distribution (predominantly south-east Asia, India and Africa) suggests environmental or genetic factors – e.g. HLA associations have been found in Japanese patients – while the predominance in women of childbearing age points to oestrogen and progesterone playing a role.[7]

Takayasu's arteritis is a T-cell driven, non-specific granulomatous inflammation of all layers of the vessel wall which pathologically cannot be distinguished from giant cell arteritis.[9] In response to the inflammation, cellular proliferation in the intima and media may lead to occlusion and stenosis of the artery, or weakening of the media and adventitia which can result in dilation and aneurysm formation. The most frequently affected vessels are the subclavian arteries, carotid arteries and the aorta, including the origin of its visceral branches. Dilatation or aneurysm formation is usually only found in the aorta. The pulmonary and coronary arteries can be involved, albeit rarely and myocarditis associated with Takayasu's arteritis has also been described.[9,10]

The disease is usually recognised by the manifestations of the vascular disease but constitutional symptoms of inflammation – fever, myalgia, arthralgia and weight loss – are commonly the earliest feature of this illness. The vascular manifestations include those associated with occlusion of arteries supplying the limbs, hypertension caused by aortic or renal artery stenosis, cerebrovascular manifestations due to occlusion of the carotid or vertebral arteries and aortic valve regurgitation. Involvement of the coronary arteries, myocardium and pulmonary arteries may cause angina and congestive cardiac failure.[9-11] Affected blood vessels are often tender and as a result, severe back pain, similar to that seen in patients with thoracic dissection, and pain in the carotid arteries is common.

No specific diagnostic test exists. An elevated ESR and CRP are usual during active phases of the disease.[9] Microcytic anaemia develops in many patients. Some patients have an elevated serum creatinine usually associated with longstanding hypertension, while glomerulonephritis occurs rarely.

Vascular imaging is essential to delineate the full extent of the vascular involvement. Many favour MRA, as this can demonstrate changes in the vessel wall prior to luminal changes being noted. PET may detect vascular inflammation but its accuracy in diagnosing the disease has not been determined but shows promise.[12]

The histological diagnosis is based on demonstrating the typical granulomatous vasculitis with giant cells in inflamed blood

vessels. This is however rarely possible, as biopsy of an affected artery while the disease is in an active phase is usually not advisable, and when specimens become available, while the disease is in a quiescent phase, only nonspecific transmural fibrosis can be found.

Initial treatment requires high doses of prednisone in the acute phase which is then tapered to 10mg/day and continued for 4 to 6 months. This regime is usually effective in addressing the inflammation in the vascular wall and abating the constitutional symptoms but two thirds of patients with Takayasu's arteritis experience relapse of symptoms or progression of vascular disease. As a consequence life-long monitoring of this condition is mandatory.

Methotrexate or mycophenolate mofetil in combination with prednisone are occasionally needed to reduce the inflammation and are also at times used without prednisone during phases of remission in order to minimise the corticosteroid side effects. Other drugs such as cyclophosphamide are used rarely because of their side effects but on occasion are all that some patients will respond to. Agents such as infliximab have shown promise but are usually unaffordable in the countries in which this disease predominates.[9] The use of statins and low-dose aspirin is encouraged.

The renal and cardiac dysfunction and hypertension that do not rapidly resolve after initial treatment for the acute inflammatory disease are managed using standard protocols. Diagnosing and managing these can, however, be challenging because of the diffuse vascular involvement. In patients who have cerebrovascular occlusive disease it is often also necessary to maintain a high blood pressure to avoid episodes of cerebral and brainstem hypoxia. Surgical and endovascular interventions play an important role in treating occlusive and aneurysmal manifestations.[11] The indications for intervention are the same as they are for other pathologies, i.e. usually only for life or limb threatening symptoms. Fortunately, few interventions are required in the acute phase, as failure rates are high for procedures performed when acute inflammation is present, are high. Revascularisation in our practice is deferred, if at all possible, until pharmacological therapy has completely suppressed the inflammation, which we believe to be indicated by a normal CRP and ESR. This experience is shared by others.[13]

In the long-term once the active disease has been treated, most patients return to near normal lifestyles and survival rates. A significant number of patients do however die as a consequence of renal or cardiac failure,[14] or from the complications of immunosuppressive treatment which is compounded by the high prevalence of HIV infections in some of the affected populations.[11]

Thromboangiitis obliterans (Buerger's disease)

This condition usually occurs in young individuals after extended and ongoing exposure to tobacco smoke, but can affect all age groups. The reason for the relationship between thromboangiitis obliterans and smoking has not been established. There are some indications that it has an immunological background, as increased levels of cell-mediated responses to vascular collagen and raised levels of anti-endothelial cell antibodies have been described.[15] Other forms of smoking, such as cannabis, may also cause similar disease patterns.

Thromboangiitis obliterans affects medium-sized arteries, veins and nerves. The lower limbs are most frequently affected but upper limb disease is common. Pathologically, the blood vessel wall architecture is usually

intact, with preservation of the internal elastic lamina and absence of necrosis. During the acute phase of the disease a highly cellular, inflammatory thrombus is found occluding the lumen. In time this becomes a band of fibrous tissue. Involvement of superficial veins is associated with a perivascular inflammatory cell infiltrate resulting in a thrombophlebitis.

Thromboangiitis obliterans is usually first recognised by the presence of extremely painful, severe, progressive digital ischemia (often first thought to be splinter haemorrhages).[15] Occasionally, the earliest lesion may be a superficial thrombophlebitis. In advanced cases the gangrene may extend well beyond the digits. Other organs are not involved. Prior to the development of gangrene, pain in the distal extremities may be due to a neuropathy. These symptoms are due to thickening of the tissues in the neurovascular bundles as a result of perivascular inflammation.

The diagnosis is generally confirmed by identification of the typical pattern of vascular involvement, exclusion of conditions that may mimic the disease, particularly atherosclerosis, and the presence of ongoing tobacco exposure. The demonstration of typical 'corkscrew' collaterals in the neurovascular bundles is characteristic but can also be found in other conditions such as diabetes mellitus and polyarteritis nodosa.[15] While most diagnostic systems for thromboangiitis obliterans are intended to exclude the presence of atherosclerosis, both these conditions often coexist, particularly in patients over the age of 40 years.

The only effective treatment for thromboangiitis obliterans is to completely stop smoking.[15] Immunosuppression and anticoagulation have no role. Occasional patients respond partially to prostaglandins. Revascularisation, using endovascular techniques or bypass grafts, is usually not feasible from a technical perspective, as a result of the inflammatory and thrombotic processes, and occlusion of distal runoff arteries, but may play a role if haemodynamically significant atherosclerotic lesions coexist in the patient. Effective pain control is essential as patients will not be able to stop smoking without it.

With any ongoing smoking, which tends to be the norm – such patients appear to be particularly nicotine dependent – arterial disease progresses. Limb loss therefore occurs frequently. On the other hand, complete abstinence is associated with remarkably good outcomes.

Behçet's disease

Behçet's disease was originally described as a syndrome consisting of recurrent oral aphthous ulcers, genital ulcers, and ocular inflammation. This idiopathic vasculitis can however, cause inflammation in almost any organ. The most significant morbidity associated with Behçet's disease is related to ocular inflammation, which can cause blindness. Most manifestations of the disease are however episodic and become less frequent over time, but mortality may occur – mostly as a result of thrombosis, aneurysms and de novo rupture of large vessels.

Behçet's disease is common in countries along the ancient Silk Road (eastern Mediterranean countries central Asia to China) – as many as 4 per 1000 population have been described – and rare elsewhere. It mostly affects young adults and is more frequent in men. The distinct geographic distribution suggests an environmental or genetic aetiology. The pathologic changes point to an abnormal reactivity of neutrophils and lymphocytes causing damage by a vasculitic process which affects all types of arteries. Venous thrombosis is also common.[16]

Clinically, oral ulceration tends to be the earliest manifestation and must be present to make the diagnosis of Behçet's disease. These painful ulcers, which affect the mucosa from the lips to the oropharynx, can be up to 2cm in diameter and usually have a white base and a red halo around the ulcer. Between two and five lesions are usually present at a time. The oral lesions tend to heal within two to three weeks without scarring while the genital ulcers tend to be larger and deeper, and often heal with scarring. In men the genital ulcers, which develop mostly on the scrotum and less commonly on the shaft of the penis, are associated with epididymitis. In women ulcers affect the vagina and vulva. Ocular inflammation is caused by an anterior and posterior uveitis. The anterior uveitis usually presents with a red eye, photophobia and blurred vision while the less common posterior uveitis, in combination with vasculitis affecting the carotid arteries and retina, can result in loss of vision. Other regularly encountered features include erythema nodosum, migratory thrombophlebitis, arthritis, spondylitis, gastrointestinal aphthous ulcers, meningoencephalitis, stroke (thrombotic or haemorrhagic), sagittal sinus thrombosis, seizures, hearing and vestibular impairment, dementia and psychiatric conditions. Vascular manifestations, include thrombophlebitis and venous thrombo-embolism, occur in a fifth of patients while the arterial vasculitic manifestations are much less common – this arterial process particularly affects mesenteric and pulmonary arteries and may cause occlusion, aneurysm formation or primary rupture.[17,18]

The diagnosis of Behçet's disease rests predominantly on the clinical features but raised nonspecific markers of inflammation are common while the disease is active. Serum IgD levels are also often elevated. Histology of the aphthous ulcers reveals an inflammatory vascular infiltrate but a true vasculitis is rare.[16]

The oral ulcers and other aphthous lesions are treated with topical steroids or Dapsone while vision- or life- threatening complications are treated with high doses of intravenous corticosteroids and other immunosuppressive agents. Infliximab is also effective.[16] Vascular interventions are required for symptomatic life threatening occlusive arterial disease, while aneurysms are treated according to their risk of rupture. All spontaneous arterial ruptures require emergency repair. Thrombophlebitis is treated symptomatically and venous thrombo-embolism is managed by anticoagulation.

Polyarteritis nodosa

This is a vasculitis confined to small and medium-sized arteries. Men and women are equally affected and the disease occurs in all age and ethnic groups. The association with chronic hepatitis B virus infection is well established.[19]

Polyarteritis nodosa can involve virtually all organs, but spares the lungs.[20] Commonly involved organs are:

- Skin – includes livedo reticularis, nodules, papules, ulceration, and digital ischemia often associated with splinter haemorrhages which can lead to gangrene.
- Peripheral nerves – usually mononeuritis multiplex of the sural, peroneal, radial, and ulnar nerves.
- Gastrointestinal tract – commonly postprandial abdominal pain, but also mesenteric infarction or aneurysmal rupture of visceral arteries due to multiple microaneurysms.
- Kidneys – excluding glomerulonephritis.

The constitutional symptoms of inflammation and pain, caused by myalgia, arthritis,

peripheral nerve infarction, testicular ischemia, or mesenteric vasculitis, are usually found. The other vascular presentations include renin-mediated hypertension, subclinical arteriolar involvement of the cardiac circulation with occasional congestive cardiac failure and myocardial infarction, and occasional stroke.

The diagnosis of polyarteritis nodosa is generally based on histology – most frequently of skin nodules, which specifically does not demonstrate granulomatous infarction – and the demonstration of microaneurysms in the visceral arteries. The ANA and rheumatoid factor (RF) are usually negative. The ANCA tends to be positive but specific enzyme immunoassays for antibodies to proteinase-3 or myeloperoxidase – the antigens known to be associated with systemic vasculitis – are negative. Polyarteritis nodosa is therefore not considered to be an ANCA associated vasculitis.

When polyarteritis nodosa is associated with hepatitis B virus, polyarteritis nodosa tends to occur soon after the initial infection and these patients also have low complement levels.

Treatment of idiopathic polyarteritis nodosa is similar to the other vasculitides already described.[20] Cyclophosphamide is required for approximately half the patients whose disease is refractory to corticosteroids or who have significant life threatening involvement of major organs. Hepatitis B virus if present is treated with antiviral therapy which is the reason that the frequency of polyarteritis nodosa is diminishing.

The prognosis is usually favourable. Bowel perforation and rupture of a mesenteric microaneurysm however requires emergency surgical intervention which is the reason that this disease still causes mortality. Recurrence rates after successful treatment of the initial clinical manifestation are unusual.

Vasculitis secondary to connective tissue diseases

Systemic lupus erythematosus (SLE)

In SLE, lupus vasculopathy is found in up to 40% of patients (mostly female). This usually is a typical vasculitis characterised by inflammation and necrosis in the vessel wall.[21] The aetiology of these changes are:

- Leucocytoclastic inflammation in 60%
- Cryoglobulinaemia in 30%
- Systemic vasculitis resembling polyarteritis nodosa in 6%.

Lupus vasculitis is also associated with thrombotic thrombocytopenic purpura, venous thrombosis, antiphospholipid syndrome and urticarial vasculitis.

Lupus vasculopathy is an immunological disease with various autoantibodies, directly or indirectly affecting endothelial cells and cell membrane phospholipids. These cause chronic vessel wall damage.[22] It is hypothesised that the endothelial deposition of circulating immune complexes causes activation of secondary inflammatory responses which then activate the complement cascade. This results in the destruction of vascular basal membranes. The expression and activation of adhesion molecules appear to also be the key factors in the pathogenesis of the vasculitis by enabling leukocyte adhesion to the vessel endothelium and allowing leukocyte infiltration into affected tissues. In addition, lupus vasculitis also initiates the development and progression of atherosclerosis.

The antibodies involved in lupus vasculitis include:[22,23]

- Anti-endothelial cell antibodies which occur in over 80% of SLE patients. It seems that their presence is typical of vasculitis, vascular thrombosis and lupus nephritis. These antibodies belong to

the IgG, IgM and IgA immunoglobins and bind to antigens through the F(ab)2 region. Multiple endothelial cell antigens react with these antibodies.
- Antiphospholipid antibodies which bind to exposed endothelial cell phospholipids. These antibodies cause vascular endothelial damage, an increased arterial and venous thrombosis risk and proliferative heart valve lesions.
- ANCA. Their role in lupus vasculitis remains unclear.
- Anti-double-stranded DNA antibodies may take part in vascular damage in SLE. They possess an anti-endothelial activity, which is directed against certain antigens on the endothelial surface.

The spectrum of the lupus vasculopathy ranges from the mild and most common form, affecting only vessels of the skin, to severe multiple organ dysfunction.[21] The cutaneous lupus vasculopathy manifests as purpura, urticaria or bullous lesions in the extremities, and livedo reticularis on the trunk. Occasionally necrosis of the nail bed and digital ulceration is also found. Lupus vasculitis in other organs can affect 20% of patients. Examples include:

- Focal segmental glomerulonephritis in the kidneys
- Necrotic inflammation in alveolar capillaries of the lungs
- Cognitive dysfunction, psychosis, convulsions and strokes in the brain
- Mononeuropathies in the peripheral nerves
- Gastrointestinal haemorrhage or perforation.

Antiphospholipid antibody syndrome (APS)

The APS is defined by the presence of at least one of the many plasma antiphospholipid antibodies and the occurrence of at least one clinical feature of which venous or arterial thromboses, recurrent foetal loss, or thrombocytopenia are the most common. The antiphospholipid antibodies, which are directed against plasma proteins bound to anionic phospholipids, are: [24,25]

- Lupus anticoagulants. These are antibodies directed against plasma proteins such as β2 glycoprotein-1 or prothrombin bound to anionic phospholipids. Despite their name, the presence of lupus anticoagulants is generally associated with thrombosis and they do not have an anticoagulant effect.
- Anticardiolipin antibodies.
- Other antibodies, e.g. those to β2 glycoprotein-I, prothrombin, annexin V, phosphatidylserine and phosphatidylinositol.

In addition to causing thrombosis, the above antibodies also increase vascular tone, thereby increasing the susceptibility to atherosclerosis, fetal loss and neurological damage.

The pathogenesis remains unclear. It appears that the antiphospholipid antibodies develop in susceptible individuals following incidental exposure to currently undefined, infectious agents. Once the antibodies are present a second hit, such as an infection, prolonged immobilisation, pregnancy, hormone replacement therapy, malignancy or nephrotic syndrome is required for the syndrome to develop.[24]

APS can either be a primary disease or can be associated with another secondary condition.[25] For example:

- Some healthy individuals have antiphospholipid antibodies but few ever get the APS.
- Autoimmune diseases – particularly SLE.

- Infections:
  - Bacterial infections: e.g. bacterial septicaemia, tuberculosis, leprosy and post-streptococcal rheumatic fever.
  - Viral infections: e.g. hepatitis A, B, and C, HIV, cytomegalovirus and Epstein-Barr virus.
  - Parasitic infections: malaria and visceral leishmaniasis
- Medications: e.g. phenothiazines, phenytoin, hydralazine, α-interferon, quinine, amoxicillin, chlorthiazide, oral contraceptives and propranolol.
- Malignancies including solid tumours of the lung, colon, cervix, prostate, kidney, ovary, breast, and bone; Hodgkin's and non-Hodgkin's lymphoma; myelofibrosis, polycythemia rubra vera, myeloid and lymphocytic leukemias.

The most common manifestations, in order of frequency, are deep vein thrombosis, thrombocytopenia, livedo reticularis, stroke, superficial thrombophlebitis, pulmonary embolism, fetal loss, transient ischemic attack and haemolytic anaemia. Rarely, APS results in ischaemic multiorgan failure. Other possible antiphospholipid antibodies related manifestations include migraine headache, Raynaud's phenomenon, pulmonary hypertension, avascular necrosis, cutaneous ulcers that resemble pyoderma gangrenosum, adrenal insufficiency and cognitive deficits.

The presence of APS should be considered in patients who have one or more otherwise unexplained thrombotic or thrombo-embolic events, one or more adverse outcomes related to pregnancy or otherwise unexplained thrombocytopenia or bleeding.

Rheumatoid arthritis

The vasculitis associated with rheumatoid arthritis occurs in patients with previously severe disease, typically in those with long-standing rheumatoid nodules, destructive joint disease, and high titres of rheumatoid factor. The development of a vasculitis is associated with new constitutional symptoms, skin ulceration, serositis, digital ischemia, and sensory and motor nerve dysfunction. It may also cause multiorgan dysfunction.

The vasculitis is caused by the deposition of immune complexes and antibody-mediated destruction of endothelial cells resulting in vascular necrosis and luminal thrombosis.[22] Cigarette smoking and other factors have a significant adjunctive role.

The diagnosis should ideally be established by tissue biopsy. Deep full-thickness skin biopsies from the edge of skin ulcers, that include some subcutaneous fat, can detect the presence of medium-vessel vasculitis.

As in all the vasculitides, therapy must reflect the severity of organ involvement. When small, relatively painless infarctions around the nail bed develop, these do not necessarily require treatment. While this vasculitis does respond to therapy it generally is associated with a poor prognosis. Fortunately, with the modern more effective treatment of rheumatoid arthritis, this condition is becoming rare.[26]

Scleroderma

Scleroderma refers to the presence of thickened, hardened skin which is a common feature of a heterogeneous group of connective tissue disorders. When the characteristic skin disorder is associated with visceral organ involvement, the disease is termed systemic sclerosis. This is further subcategorised into diffuse cutaneous systemic sclerosis and limited cutaneous systemic sclerosis on the basis of the extent and distribution of the skin involvement. Limited systemic sclerosis is commonly associated with the CREST syndrome (made up of cutaneous calcification, Raynaud's phenomenon, oesophageal dysmotility, sclerodactyly, and telangectasia).

The visceral manifestations of systemic sclerosis are varied, including fibrotic and/or vascular complications of the circulatory, musculoskeletal, renal, pulmonary, and gastrointestinal systems. The characteristic clinical manifestation of vascular dysfunction is Raynaud's phenomenon.[27] Episodes of Raynaud's phenomenon may be prolonged and can result in ischemic digital ulceration or infarction. In patients with limited cutaneous systemic sclerosis, Raynaud's phenomenon generally precedes other disease manifestations, often by many years. In contrast, in patients with diffuse cutaneous systemic sclerosis, the onset of Raynaud's phenomenon generally coincides with, and in some cases may even follow, the appearance of characteristic skin or musculoskeletal manifestations. Vascular injury and consequent chronic tissue damage underlies other serious complications of systemic sclerosis, including pulmonary artery hypertension, renal crisis and gastric antral vascular ectasia, and also contributes to the pathogenesis of cardiac and other gastrointestinal complications.[28]

The presence of systemic sclerosis is suggested by the presence of skin thickening and hardening that is not confined to one area. The diagnosis is supported by the presence of other non-cutaneous features such as:

- Heartburn and/or dysphagia
- Acute onset of hypertension and renal dysfunction
- Effort induced dyspnoea and interstitial pulmonary fibrosis
- Pulmonary hypertension
- Mucocutaneous telangiectasia on the face, lips, oral cavity, or hands
- Digital gangrene

Over 95% of patients have at least one autoantibody present. These include the presence of antinuclear antibodies and more specific antibodies such as anti-topoisomerase I, anti-centromere, anti-RNA polymerase III and anti-beta2-glycoprotein I antibodies which are frequently present in patients with systemic sclerosis. The anti-topoisomerase I, anti-centromere and anti-RNA polymerase III antibodies are specific but only moderately sensitive. A skin biopsy is generally not needed to confirm a diagnosis.

Infective vasculitides

Human immunodeficiency virus (HIV)

HIV infection can cause a range of vascular diseases.[29] In the arterial system, HIV positive patients have been found to have both aneurysms and occlusive disease, mostly thrombotic disease. These vessel wall pathologies have unique histological characteristics where the inflammation is found in the vasa vasora. The inflammatory process consists of neutrophils surrounded by a cuff of plasma cells and lymphocytes. In addition, there is marked endothelial swelling with fibrin deposits on the luminal surface which leads to occlusion of the vasa vasora. In the wall of large arteries there are patches with acute inflammatory infiltrate, while in other regions there are areas of extensive fibrosis without inflammation. This suggests that there is a sequence of events starting with inflammation concentrated in the adventitia. Later in the disease this may either cause trans-mural necrosis, possibly leading to aneurysm formation, or the transmural inflammation may induce luminal thrombus.

Both HIV associated aneurysms and HIV associated arterial occlusion are considered to be Acquired Immunodeficiency Syndrome (AIDS) defining conditions as they are usually associated with low CD4 counts in patients with other manifestations of the infection.

HIV associated aneurysms have been found in most major arteries and are usually saccular in nature as a result of a localized region of cytoclastic activity in all layers of the arterial wall. Patients tend to present with multiple aneurysms. Treatment is based on the usual principles of care for aneurysms but because of the poor general condition of affected patients, endovascular modalities are favoured.

Occlusive arterial disease most commonly involves the iliac arteries, but involvement of the coronary arteries is also found in young men who also smoke.[30] Treatment of these patients is hampered by the underlying vasculitis and is generally associated with a poor outcome unless the inflammatory process is reversed by treating the HIV infection.

The association between venous thrombo-embolism and the HIV infection has also been conclusively proven.[31] The reported incidence ranges from 0.2% to 18% which is well in excess of what one would expect in a non-infected population (0.05%). At the Johannesburg Hospital 84% of patients who presented with deep vein thrombosis were found to be HIV positive. The severity of the HIV infection appears to be of significance as there is a greater incidence of venous thrombosis in patients with low CD4 counts, while the risk is even higher when individuals have confirmed AIDS. The reason for HIV infection's relationship with venous thrombosis has not yet been conclusively elucidated, but appears to be multimodal, with all three limbs of Virchow's triad being involved.

PATHOPHYSIOLOGY AND PRINCIPLES OF TREATMENT OF RAYNAUD'S PHENOMENON

In order to preserve the core temperature, the normal physiological response to exposure to a cold environment is to reduce capillary blood flow to the skin, mostly in the limbs and digits. This response is regulated by a complex interaction of neural signals, circulating hormones, and the release of local mediators mostly derived from the vascular endothelium.

Raynaud's phenomenon describes an exaggerated vascular response to such a cold stimulus, or occasionally to emotional stress. The phenomenon is characterised by sharply demarcated colour changes of the skin of the digits. Typically these consist initially of a phase of vascoconstriction and therefore a pale appearance (white) followed in time by cyanosis (blue) as a result of deoxygenation of haemoglobin within the affected tissues. Finally, with reflex vasodilation as a result of the localised tissue ischaemia and the resultant inflow of oxygenated blood, a phase of hyperaemia (red) follows. Only the two initial phases, pallor and cyanosis, need to have been present for such an event to be recognised as a Raynaud's attack.[32]

Primary Raynaud's phenomenon describes this sequence of events in the absence of any associated disorders – occasionally called Raynaud's disease, which is inappropriate as the phenomenon only describes an exaggerated physiological response in these individuals – while secondary Raynaud's phenomenon is associated with a known disease –referred to occasionally as Raynaud's syndrome (Table 16.3).[33]

The defect in primary Raynaud's phenomenon is currently thought to be an increased vasoconstrictive response to $\alpha 2$ adrenergic stimuli, particularly at the level of $\alpha 2$ adrenergic receptors, in the digital arteries and cutaneous arterioles.[34] The exact mechanisms have however not yet been established. The vascular response to the $\alpha 2$ adrenergic agonists, serotonin and angiotensin II is increased during cooling and can be reversed by tyrosine kinase inhibitors.[35]

TABLE 16.3: Common secondary causes of Raynaud's phenomenon[32,33,36]

Connective tissue diseases and vasculitis:	
Systemic lupus erythematosus	Systemic sclerosis (scleroderma)
Rheumatoid arthritis	Giant cell arteritis
Thromboangiitis obliterans	Primary biliary cirrhosis
Diseases of arteries in the upper limbs:	
Atherosclerotic occlusive disease	Frost bite
Thoracic outlet syndrome	
Malignancies:	
Ovarian carcinoma	
Endocrine diseases:	
Phaeochromocytoma	Thyroid disease
Carcinoid syndrome	
Vasospastic conditions:	
Vibration syndromes	Migraine and Prinzmetal angina
Haematological disorders:	
Cryoglobulins and cold agglutinins	Polycythaemia
Paraproteinaemia	
Drugs and chemicals:	
Ergotamines	Polyvinyl chloride
Bleomycin and Vinblastine	

An underlying genetic mechanism is also suggested by the occasional appearance of Raynaud's phenomenon in family clusters, and because women are most frequently afflicted by this condition, suggesting that some of the relevant loci are located on the X chromosome. The effect of oestrogen on $\alpha 2$ adrenergic receptor expression may however also be a reason for the higher prevalence of this condition amongst women.[35]

In secondary Raynaud's phenomenon, the many diseases, drugs, and environmental factors that can cause the syndrome, disrupt the normal mechanisms responsible for control of vessel reactivity, each apparently in a unique manner. For example, in systemic sclerosis (scleroderma) the primary mechanism is considered to be associated with intimal fibrosis and endothelial dysfunction. In these patients, endothelin-1 levels are significantly increased. This potent vasoconstrictor is also involved in the development of fibrosis and other structural changes in blood vessels.[35] In addition, angiotensin II levels are increased, while nitric oxide levels are consistently found to be low.

Prevalence of Raynaud's Phenomenon

It is difficult to establish the prevalence of Raynaud's phenomenon because of the lack of standardisation in the diagnosis. However, when using at least pallor and cyanosis to define an episode of Raynaud's phenomenon, the prevalence ranges from 3 to 20% in women and 3 to 14% in men.[33] These wide ranges are explained by the great variation found across the world both in populations and in climate. For example Raynaud's phenomenon is common in central Europe when compared to populations in the Americas, Africa and Asia. It is also more common among women, in younger age groups (median onset 14 years), and in family members of individuals with

established Raynaud's phenomenon. About 25% of individuals first develop symptoms after the age of 40 years and rarely after the age of 60 years.[32]

Clinical Findings in Raynaud's Phenomenon

Raynaud's phenomenon most frequently affects the fingers and hands. While the toes and feet are often also affected this tends not to concern patients as much. A typical episode usually begins in a single finger and then spreads to all other digits but the thumbs are often spared. Vasospasm of the skin of the ears, nose, face, knees and nipples is also commonly described. The ischaemic phase (pallor and cyanosis) usually lasts for 15 to 20 minutes. Often when the initial phase of such an attack is prolonged, patients describe the feeling of pins and needles, numbness, and complain that the fingers are aching. These symptoms rapidly reverse in patients with primary Raynaud's phenomenon when the limb is rewarmed or the stress is alleviated. On the other hand in secondary Raynaud's phenomenon, asymmetry of symptoms, severe constant pain and ischaemic ulceration of the skin may occur, particularly when the mechanism of the underlying disease cannot be addressed.

Exposure to cold is the usual trigger and mostly occurs with rapid movement from a warmer to a cooler environment. As a consequence, air-conditioned rooms, or the mere washing of hands in cold water may be all it takes to bring on an episode. An attack is usually brought on by the distal limbs being exposed, but on occasion cooling of the trunk while the hands or feet areas are kept warm can also provoke an attack. As a consequence afflicted individuals should always ensure that all parts of their body are kept warm.

Diagnosis of Raynaud's Phenomenon

The diagnosis is mostly made when the classic symptoms are described. Confirming the diagnosis by using provocative manoeuvres such as a cold water challenge, is unhelpful due to inconsistency in producing the syndrome. As the majority of patients presenting with Raynaud's phenomenon have no other underlying disease little benefit is achieved by further evaluation.

In the small number of individuals who have a secondary cause or in whom symptoms persist, additional evaluation may be helpful, but this usually requires complex diagnostic tools that are generally not freely available. The tools used to assess the vascular responses to environmental stimuli in the skin, include nail fold capillaroscopy, angiography, laser Doppler flowmetry, and measurement of skin temperature. Generally, in patients with all forms of Raynaud's phenomenon these demonstrate that there is a delayed recovery phase of vascular flow after exposure to environmental stressors.[32,33]

Every patient with primary Raynaud's phenomenon should be carefully evaluated clinically, to exclude a secondary cause.[32] The criteria to diagnose primary Raynaud's phenomenon include the presence of symmetrical, episodic attacks, no evidence of occlusive arterial disease, no gangrene, and if there is doubt a negative nail fold capillary examination (if this is available), a negative ANA and normal ESR/CRP. The clinical clues that secondary Raynaud's phenomenon may be present include:

- Age of onset >40 years
- Male
- Pain and tissue ischemia (ulceration)
- Asymmetry

The presence of a raised ESR or CRP and autoantibodies suggests an underlying connective tissue disease.

PROGNOSIS

Approximately 50% of individuals with primary Raynaud's phenomenon will have a reduction in the frequency and severity of their symptoms over time, particularly if the onset of symptoms occurs in adolescence.

Occasionally, Raynaud's phenomenon is the first manifestation of an underlying disease. The frequency at which individuals thought to initially have primary Raynaud's phenomenon are found to have a underlying cause identified is in the region of 1% per year, but is usually only noted 10 years or more after the primary presentation. The best predictor that this may happen is an abnormal nail fold capillary pattern.[36]

TREATMENT

The initial management of patients with primary Raynaud's phenomenon is to promote lifestyle changes and to avoid the use of medication.[36] The advice usually given includes:

- Keeping the whole body warm by:
  - Avoiding sudden exposure to cold
  - Dressing warmly
  - Keeping all digits warm even in very cold environments
  - Avoiding rapidly changing temperatures
- Reducing emotional stress
- Avoid smoking and using drugs that cause vasoconstriction

If an attack occurs, the patient should be advised to go to a warm room and to place the hands under warm water, or to swing the upper limbs like a windmill.

In patients with secondary Raynaud's phenomenon the above measures are less helpful, as a result of more severe attacks. For this reason, and because of the progression of the ischaemia, they will usually require pharmacological intervention as do some patients with primary Raynaud's phenomenon in whom severe symptoms persist.

Multiple classes of drugs are used in the management of Raynaud's phenomenon. The medications used are:[37]

- Calcium channel blockers. Approximately 60% of patients have a clinically significant improvement of their symptoms (both in the reduction of the severity and frequency).[7] Not all calcium channel blockers are effective. Nifedipine in relatively small doses given three to four times per day is the one most frequently used. These drugs are introduced slowly in order to reduce the chance of patients experiencing headaches. Secondary Raynaud's phenomenon is also less likely to benefit from this class of drugs.
- Many direct and indirect vasodilators have some effect but few are freely available for the management of this condition. The serotonin reuptake inhibitors (such as fluoxetine), angiotensin receptor blockers (losartan) and other vasodilators such as buflomedil have demonstrated similar response rates when compared to the effective calcium channel antagonists.
- Prostaglandins. Prostaglandin E1 (PGE1), prostacyclin (PGI2), and iloprost (a PGI2 analogue) are the most frequently used. The response rate in severely affected patients is in the region of 60%. Infusions given over a few days often have benefit that often lasts several months.
- The phosphodiesterase inhibitors, sildenafil and tadalafil. The current data

supporting the use of these agents is sparse but promising.
- Endothelin receptor antagonists e.g. bosentan which acts as an antagonist of endothelin-B receptors, has been demonstrated to significantly reduce the number of new ulcers forming in patients with systemic sclerosis.
- Antioxidant agents, such as zinc gluconate and N-acetylcysteine have demonstrated some improvement in small studies
- Atorvastatin does reduce the severity and frequency of Raynaud's events and of ulcer formation in patients with scleroderma.

In addition, multiple antithrombotic agents have been utilised in patients with significant complications associated with Raynaud's phenomenon. These include aspirin, dipyridamole, systemic anticoagulation, and thrombolytic therapy. The benefit of antiplatelet therapy with aspirin (75 or 81mg/day) is uncertain but use of this agent should be considered in all patients with secondary Raynaud's phenomenon.

The role of sympathectomy has recently again been considered in the management of severe Raynaud's phenomenon mostly as a result of the development of less invasive and more focused procedures being available.[36] These include:

- A local chemical sympathectomy using a long acting local anaesthetic agent to achieve a wrist or digital nerve block which can reverse vasoconstriction and relieves pain.
- Intradigital botulinum toxin A has been used to achieve a chemical sympathectomy. While this is an interesting option, the current described experience is limited.[37]
- Cervical sympathectomy is likely to result in the immediate improvement in blood flow, but the degree and duration of improvement is very variable and long term outcomes particularly in patients with secondary Raynaud's phenomenon are poor.
- Localised microsurgical digital sympathectomy has been introduced as an alternative to proximal sympathectomy. This option appears to have more durable outcomes and is often now the choice when sympathectomy is the last treatment modality available, particularly only after vasodilator drugs and after specific treatments for any reversible cause, such as vasculitis, have failed.

RECOMMENDATIONS

All patients with Raynaud's phenomenon should avoid cold temperatures, stress, and vasoconstrictors, while always being dressed in warm clothing, and should warm their hands in order to terminate an attack.

Most importantly, patients with primary uncomplicated Raynaud's phenomenon should not be over-treated. Therapy should only be initiated in those patients with primary uncomplicated Raynaud's phenomenon in whom non-pharmacologic measures have failed. These medications should be introduced in a step wise manner and should only be continued if appropriate symptom relief is achieved. Parenteral medications and sympathectomy are usually not indicated.

Patients with secondary Raynaud's phenomenon may require more aggressive therapy. The major goal is to reduce the frequency of attacks, and to prevent digital ulceration. It is unlikely that any medical therapy on its own will completely terminate attacks. In general, a step wise escalation of therapy, initially using the oral vasodilators, followed by parenteral medications and lastly localised digital sympathectomy, should be used in accordance to the level of symptoms

and the extent of digital ulceration and gangrene. All such patients should also have the underlying disease addressed, which in patients with the vasculitides include the use of a statin and an antiplatelet agent. In addition appropriate and effective analgesia will be required. Such pain control and surgical amputation may be the only option in patients with late stage ischemia or severe structural arterial disease.

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17 • SIRS, Sepsis and Multiorgan Failure

VISHWANATH BIRADAR, JOHN L MORAN

The Queen Elizabeth Hospital, 28 Woodville Rd, Woodville South, Adelaide, South Australia

EPIDEMIOLOGY

Sepsis remains a common reason for intensive care unit (ICU) admission and is a leading cause of mortality. This disease is now recognized to be a time-sensitive emergency, because patients stand the best chance for survival when effective therapeutic interventions are delivered as early as possible. However, consistent data are lacking regarding the incidence and outcome of sepsis in ICUs globally. Data extrapolated from a cohort study conducted by Finfer *et al* in 2004 across twenty three closed multidisciplinary ICUs showed that the incidence of severe sepsis among adult patients was 0.77 patients per 1000 population (95% CI, 0.76–0.79). There were 752 episodes of severe sepsis identified in 691 patients, equating to 11.8 patients with severe sepsis per 100 ICU admissions (95% CI, 10.9–12.6).[1] The EPISEPSIS study group conducted a nationwide, prospective, multicentre survey of patients with severe sepsis in 206 French ICUs over two consecutive weeks. They estimated the incidence of severe sepsis to be 0.95 cases per 1000 in the French population.[2] In the United States between 1979 and 2000 an annualized increase of 8.7% in the incidence of sepsis was noted. (From about 164,000 cases (0.82 per 1000 population) to nearly 660,000 cases (2.4 per 1000 population)).[3]

Sepsis is estimated to affect 18 million people worldwide each year and kill 1400 people each day. According to an epidemiological study, sepsis affects about 700,000 people annually in the United States alone, with an overall mortality rate of 30%, or more than 50% in patients with septic shock and/or multiple system organ failure.[3] From a financial perspective, sepsis represents a major burden to the health care system in most developed countries since septic patients require admission and aggressive treatment in the ICUs. Table 17.1 gives the overall information on the incidence and mortality of severe sepsis in different parts of the world. Despite differences in study methodology, comparison between continents, countries and regions is possible, with some consistent themes emerging:

1) Severe sepsis represents a substantial health-care burden in all developed nations;

2) The overall incidence is increasing;
3) The overall mortality rate is declining; and
4) The nature of infections is changing, with infections caused by gram-positive organisms increasing in frequency.[4]

HISTORICAL PERSPECTIVE AND DEFINITION

The word 'sepsis' [σηψις], is the original Greek word for the 'decomposition of animal or vegetable organic matter in the presence of bacteria'.[9] The word was first noted in Homer's poems, where 'sepsis' is a derivative of the verb form sepo [σηπω], which means 'I rot'. The term is also found in the writings of the physician and philosopher Hippocrates (circa 400 BC) in his Corpus Hippocraticum. Hippocrates viewed sepsis as the dangerous, odiferous, biological decay that could occur in the body.[10]

In 1991, the American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM) convened a 'Consensus Conference' in an attempt 'to provide a conceptual and a practical framework to define the systemic inflammatory response to infection, which is a progressive injurious process that falls under the generalized term 'sepsis' and includes sepsis-associated organ dysfunction as well'.[11] They proposed the phrase 'Systemic Inflammatory Response Syndrome' (SIRS) which described the inflammatory process, independent of its cause. The systemic inflammatory response was seen in association with a large number of clinical conditions. Apart from infectious processes, other common non-infectious pathologic processes include pancreatitis, ischemia, multiple trauma, and tissue injury. When SIRS is the result of confirmed infectious process, it is termed 'SEPSIS', and is frequently associated with the development of multiple organ dysfunction and failure. This is the most common cause of death in patients who have severe sepsis.[12] The 1991 ACCP-SCCM Consensus Conference also introduced the term 'Multiple Organ Dysfunction Syndrome' (MODS). MODS was defined as 'the presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention.'[13]

In 2001, several North American and European intensive care societies agreed to revisit the definitions for sepsis and related conditions. This international sepsis definitions conference (Group of experts and opinion leaders represented by SCCM,

TABLE 17.1: Reported Incidence and Outcome of Severe Sepsis in Different Parts of the World.

Country of origin	Severe sepsis per 100 ICU admission	Incidence per 1000 population	ICU mortality	Hospital mortality
Australia & New Zealand[4]	11.8	0.77	26.5[1]	37.5[1]
United States[3]	11.8[5]	2.4	NR	17.9*
France[2]	15.3[6]	0.95	NR	41.9
United Kingdom[7]	28.7	0.66	30.8	44.7
Brazil[8]	17.4	NR	NR	47

* Sepsis overall (not severe sepsis), NR: Not Reported

ACCP, The European Society of Intensive Care Medicine (ESICM), The American Thoracic Society (ATS), and the Surgical Infection Society (SIS)) revisited the 1992 sepsis guidelines. They expanded the list of signs and symptoms of sepsis to reflect clinical bedside experience; no evidence exists to support a change to the definitions.[14] Infection was defined as a microbial phenomenon characterised by an inflammatory response to the presence of microorganisms or the invasion of normally sterile host tissue by microorganisms.

The SIRS concept is valid to the extent that a systemic inflammatory response is non specific and can be triggered by a variety of infectious and non-infectious conditions.

Current definitions do not allow for precise staging of the host response to infection and categorical definitions, such as SIRS, severe sepsis, and septic shock, have important limitations. Despite this, the SIRS concept has been globally adopted by clinicians and investigators, Table 17.2.

RISK FACTORS FOR SEPSIS

Factors which are potentially responsible for the growing incidence of sepsis and septic shock include:

1) Increased awareness and sensitivity of the diagnosis,
2) Increased use of cytotoxic and immunosuppressant agents,
3) Malnutrition,
4) Alcoholism,
5) Malignancy,
6) Diabetes mellitus,
7) Increasing number of transplant recipients and transplantation procedures,
8) Increasing number of patients who have compromised immune status,
9) Acquired immunodeficiency syndrome,
10) Increasing use of aggressive invasive procedures in patient management and diagnosis,
11) Increasing number of resistant microorganisms and
12) Increasing number of elderly patients.

Causative agents

Gram-positive organisms, endotoxin-containing Gram-negative organisms, fungi and other microbial pathogens can trigger sepsis. Gram-negative bacteria are responsible for most clinical sepsis, although in the past decade the spectrum of invading microorganisms appears to be shifting to Gram-positive bacteria and fungi. In a recent Australian study, infection was confirmed by positive culture in about 58% of episodes. Of the organisms cultured, 48.3% were gram-positive and 38.5% gram-negative; other organisms, including yeasts, fungi, legionellae and mycobacteria accounted for the remaining 13.2%.

PATHOPHYSIOLOGY OF SEPSIS

The pathophysiology of sepsis is a complex process. Pertinent factors include the bacterial density of contamination and the underlying immune status. SIRS is a result of uncontrolled activation of innate immune effector mechanisms which serve to localize and control bacterial invasion and to initiate repair of injured tissue. The innate immune system, comprising cellular (polymorphonuclear leukocytes, macrophages, natural killer cells, dendritic cells) and humoral components (complement and coagulation systems), is activated in early sepsis. Components of the innate immune response from the first line of defence are involved in the recognition and destruction of pathogens and allow time for acquired immune response to be effective. The host–microbe interaction leads to the

TABLE 17.2: Definitions of Systemic Inflammatory Response Syndrome (SIRS) and Different Degrees of Severity of Sepsis.[15]

Condition	Description
SIRS	Two or more of the following conditions: • Temperature >38.5°C or <35.0°C; • Heart rate of >90 beats/min; • Respiratory rate of >20 breaths/min or $PaCO_2$ of <32mmHg; and • White blood cell count of >12,000cells/mL, <4000cells/mL, or >10% immature (band) forms
Sepsis	SIRS in response to documented infection (culture or Gram stain of blood, sputum, urine, or normally sterile body fluid positive for pathogenic microorganism; or focus of infection identified by visual inspection)
Severe sepsis	Sepsis and at least one of the following signs of organ hypoperfusion or organ dysfunction: • Areas of mottled skin; • Capillary refilling of ≥3 seconds; • Urinary output of <0.5mL/kg for at least 1 hour or renal replacement therapy; lactate >2mmol/L; • Abrupt change in mental status or abnormal EEG findings; • Platelet count of <100,000cells/mL or disseminated intravascular coagulation; • Acute lung injury/Acute Respiratory Distress Syndrome; and • Cardiac dysfunction (echocardiography)
Septic shock	Severe sepsis and one of the following conditions: • Systemic mean blood pressure (BP) of <60mmHg (<80mmHg if previous hypertension) after 20 to 30mL/kg starch or 40 to 60mL/kg saline solution, or pulmonary capillary wedge pressure (PCWP) between 12 and 20mmHg. • Need for dopamine of >5mcg/kg/min, or nor adrenaline or epinephrine of <0.25mcg/kg/min to maintain mean BP at >60mmHg (80mmHg if previous hypertension)
Refractory septic shock	Need for dopamine at >15mcg/kg/min, or nor adrenaline or adrenaline at >0.25mcg/kg/min to maintain mean BP at >60mmHg (80mmHg if previous hypertension)

activation of several mediators within the innate immune system, including proinflammatory and anti-inflammatory cytokines and the coagulation cascade. The consequences of a systemic proinflammatory reaction include endothelial damage, microvascular dysfunction, impaired tissue oxygenation and organ injury. The consequences of an excessive anti-inflammatory response include anergy and immunosuppression. In addition, pro- and anti-inflammatory processes may interfere with each other, creating a state of what has been termed destructive immunologic dissonance.[16] The entire process is often described as uncontrolled, maladaptive, or dysregulated. Sepsis may therefore pathologically be described as an autodestructive process that permits the extension of a normal pathophysiologic response to infection that can result in MODS.[17]

Innate immunity and toll-like receptors (TLRs)

SIRS is a consequence of uncontrolled activation of innate immune responses triggered when 'pattern recognition' receptors sense the presence of molecular signatures that are present in the pathogens. Pre-eminent among such pattern recognition receptors are TLRs which serve as primary sensors of the innate immune system.[18] Various TLRs have been identified in humans; TLR4 is expressed on the cell surface and serves as the primary sensor for endotoxins (also called lipopolysaccharides (LPS)) which are a constituent of the outer membrane of Gram-negative bacteria. The exoskeleton of Gram-positive bacteria is comprised of peptidoglycan (PGN) and lipoteichoic acid (LTA) which is sensed by TLR2.[19]

Proinflammatory response

The primitive, but effective, local inflammatory processes (adherence, chemotaxis, phagocytosis, bacterial killing) are highly regulated at various levels, mainly through the production of macrophage cytokines. Once a macrophage has been triggered and activated during the invasion of tissue by bacteria, it secretes cytokines (tumor necrosis factor (TNF), interleukins (IL)) and other mediators into the cell's microenvironment. These cytokines and other multiple mediators act in concert, initiating and then amplifying the resultant generalised inflammatory processes. The overwhelming systemic inflammatory response that follows manifests itself in the shock syndrome characterised by endothelial damage, coagulopathy, loss of vascular tone, myocardial dysfunction, tissue hypoperfusion, and MODS. However, several randomised human clinical trials involving antagonism of pro-inflammatory cytokines and anti-endotoxin strategies have either failed to improve survival, or reported worsened survival. A potential reason for failure of these immunomodulatory strategies could have been that sepsis is a heterogeneous disorder, and the timing of the intervention(s) may have been inappropriate.

Coagulation cascade

Inflammatory mediators, such as TNF, initiate coagulation through the induction of tissue factor expression, primarily on monocyte/macrophages, polymorphonuclear and endothelial cells. The activation of the coagulation cascade leads to fibrin and clot formation. There is also loss of native anticoagulant function, indicated by decreased activity and circulating levels of protein C. A cross–talk between inflammatory and coagulation pathways leads to self-amplifying loops of activation of endothelium, leading to the formation of microthrombi and further endothelial damage, thus setting the stage for the development of consumptive coagulopathy. Despite improved understanding of the coagulation pathway, it remains unclear why Activated Protein C improved 'survival' in a landmark clinical trial,[21] while strategies targeted at other components of the coagulation cascade, such as tissue factor pathway inhibitor and antithrombin III, had no impact on mortality.[22] In addition to the complex coagulation cascade and hyperpermeable state of endothelium, vasomotor tone of the vessel is also affected. Vasoconstrictive (endothelin, thromboxane A2, and platelet activating factor (PAF)) and vasodilatory (nitric oxide and prostacyclin) metabolites are produced in certain circumstances with important consequences in terms of microcirculatory homeostasis and maintenance of tissue perfusion.[19].

MULTIORGAN DYSFUNCTION SYNDROME (MODS)

The precise mechanisms of cell injury and resulting organ dysfunction in sepsis are not clearly understood. Multiorgan dysfunction syndrome is associated with widespread endothelial and parenchymal cell injury, some of which can be explained by hypoxic hypoxia, direct cytotoxicity or apoptosis.

Epithelial and endothelial dysfunction

Epithelial cells line the organs involved in MODS, including liver, lung, and intestines. Increased permeability and loss of the epithelial cell barrier is hypothesized to play a role in MODS. Increased permeability of the lung epithelial cells leads to acute lung injury (ALI) or acute respiratory distress syndrome (ARDS). The reactive oxygen species, lytic enzymes, and vasoactive substances (nitric oxide, endothelial growth factors) lead to microcirculatory injury, which is compounded by the inability of the erythrocytes to navigate the septic microcirculation. These changes are accompanied by peripheral vasodilatation, hypotension, tissue hypoperfusion, increased permeability, and increased peripheral oedema leading to hypoxic hypoxia frequently observed in severe sepsis. Direct cytotoxicity due to endotoxin, TNF-alpha, and nitric oxide may cause damage to mitochondrial electron transport, leading to disordered energy metabolism. This is called cytopathic or histotoxic anoxia which is an inability to utilize oxygen even when it is available.

Immune suppression and apoptosis

Few patients die shortly after the onset of sepsis due to profound hypotension or hypoxemia whilst many will have prolonged ICU course and may die following nosocomial infection. The interaction between proinflammatory and anti-inflammatory mediators plays an important role in determining the outcome of sepsis. Activated CD4 T cells are programmed to secrete cytokines with either of two distinct and antagonistic profiles. They secrete cytokines with inflammatory (type 1 helper T-cell [Th1]) properties, including TNF, interferon-1 and interleukin-2, or cytokines with anti-inflammatory (type 2 helper T-cell [Th2]) properties for example, interleukin-4 and interleukin-10. The factors that determine whether CD4 T cells have Th1 or Th2 responses are currently unknown but may be influenced by the type of pathogen, the size of the bacterial inoculum, and the site of infection.[17] Programmed cell death (apoptosis) is a normal cellular process. Sepsis is accompanied by increased apoptosis of lymphoid cells, and, to a lesser extent, parenchymal cells. Ingestion of apoptotic cells by macrophages may lead to a Th2 response, while ingestion of necrotic cells favours a Th1 response, thus apoptosis contributes to immunosuppression. The proinflammatory cytokines may delay apoptosis in activated macrophages and neutrophils, but other tissues, such as the gut epithelium, may undergo accelerated apoptosis. Endogenous release of steroids during stress increases apoptosis. Therefore, derangement of apoptosis appears to play a critical role in the tissue injury involved in sepsis.

Sepsis, circulatory failure and organ dysfunction

The widespread disruptions in severe sepsis can result in profound cardio-circulatory dysfunction. This manifests itself as shock. The dysfunction involves the cardiac, peripheral vascular (macrovascular) and

microcirculatory elements of the circulation, depending on the degrees of cardiac or vascular dysfunction and the volume status of the patient. The clinical picture ranges from cold, clammy and under-perfused to one of hyperdynamic shock. However in clinical practice, hyperdynamic shock is seen much more frequently.[23]

Landry and Oliver[20] enumerated the primary mechanisms for vascular smooth muscle relaxation in sepsis to include activation of ATP-sensitive potassium channels in the plasma membrane, activation of inducible nitric oxide synthase, and vasopressin deficiency. There are numerous vasoregulatory mediators in septic shock, and distant organs, including the brain, adrenal glands, liver, and heart; all influence vascular tone.[22] Another potential factor that may contribute to persistence of vasodilation is impaired compensatory secretion of anti-diuretic hormone (vasopressin). Low doses of vasopressin may be effective in raising blood pressure in patients refractory to other vasopressors and may have other potential physiologic benefits. However, the recent VASST trial, a randomized, controlled trial comparing norepinephrine alone to norepinephrine plus vasopressin at 0.03units/min, showed no difference in outcome in the intent to treat population.[24]

The situation in septic shock is further complicated by widespread microcirculatory dysfunction, further impairing tissue oxygen delivery, and diminished mitochondrial activity resulting in impaired oxygen extraction. The microcirculation is a key target organ for injury in the sepsis syndrome. Sepsis is associated with a decrease in the number of functional capillaries (capillarity), which causes an inability to extract oxygen maximally. These changes may be due to extrinsic compression of the capillary by tissue oedema, endothelial swelling, and plugging of the capillary lumen by leukocytes or red blood cells (which lose their normal deformability properties in sepsis). Nitric oxide plays a pivotal and multifaceted role in the complex pathophysiology of sepsis in maintaining microcirculatory homeostasis and patency, especially when the microcirculation sustains an insult (as with sepsis).[25] In the healthy state and under pathologic conditions, NO maintains microcirculatory homeostasis by regulating microvascular tone, leukocyte adhesion, platelet aggregation, microthrombi formation, and microvascular permeability. Direct or indirect effects of one or more circulating myocardial depressing substances results in myocardial depression, ventricular dilatation and/or decreased left ventricular ejection fraction further affecting circulation.[26]

Endothelial injury and the inflammatory process due to neutrophil entrapment in the pulmonary vasculature leads to disturbed capillary blood flow and enhanced microvascular permeability, resulting in interstitial and alveolar oedema.[27] ARDS is a frequent and well described manifestation of severe sepsis. Mechanisms by which sepsis and endotoxinemia might lead to acute renal failure are incompletely understood. Sepsis often results in acute renal failure due to acute tubular necrosis and systemic hypotension, direct renal vasoconstriction and release of various cytokines are contributing factors.[27] Nervous system involvement in sepsis can be central, causing encephalopathy, or peripheral resulting in neuropathy. At least 25% of patients admitted to medical or surgical intensive care units for more than seven days have some degree of acquired paresis. Neurological manifestations of sepsis includes limb muscle weakness and atrophy, reduced or absent deep tendon reflexes, loss of peripheral sensation to light touch and pin prick with relative preservation of cranial nerve function.[28]

MANAGEMENT

Treatment of sepsis and septic shock rests upon the triad of hemodynamic resuscitation, antimicrobial therapy and source control. Establishing vascular access and initiating aggressive fluid resuscitation should be the initial priority when managing patients with severe sepsis or septic shock. Relative intravascular hypovolaemia is common and rapid large volume infusions of intravenous fluids, appropriate vasopressor and inotropic support are indicated as initial therapy unless there is coexisting clinical or radiographic evidence of heart failure. Rivers *et al.*[29] in a single centre randomised controlled trial (RCT) demonstrated decreased mortality by initiating protocolized resuscitation of patients with sepsis induced shock in the first 6 hours. The goals of initial resuscitation involved the use of crystalloids or colloids to maintain central venous pressure of 8–12mmHg and a mean arterial pressure (MAP) of at least 65mmHg with fluid and norepinephrine or dopamine as the initial vasopressor of choice. Dobutamine may be indicated in patients with myocardial dysfunction as indicated by elevated cardiac filling pressures and low cardiac output. Treatment goals, assuring vital organs are perfused are; to maintain a urine output 0.5mL/kg/hr and a superior vena caval oxygen saturation ($ScvO_2$) or mixed venous oxygen saturation (SvO_2) less than 70% or 65% respectively. Rivers et al reported a mortality reduction from 47% in the control group to 31% in the treatment group. There is no evidence for the use of dopamine to increase urine output as a treatment goal. The Saline versus Albumin Fluid Evaluation (SAFE Study) was the largest randomised controlled trial ever performed in the critical care population. It involved almost 6997 critically ill patients (that is, not specifically with sepsis), run by the Australian and New Zealand Intensive Care Society Clinical Trials Group (ANZICS CTG). Patients were eligible if clinicians judged that fluid was needed to treat intravascular volume depletion, and were treated with either 4% albumin (n = 3497) or 0.9% (n = 3500) saline. The two groups had similar baseline characteristics. Death from any cause during the 28 days after randomisation was the primary outcome measure. There were 726 deaths in the albumin group, as compared with 729 deaths in the saline group (relative risk of death, 0.99; 95% CI, 0.91 to 1.09; P = 0.87). There were no significant differences between the groups in the mean (±SD) numbers of days spent in the ICU (6.5 ± 6.6 in the albumin group and 6.2 ± 6.2 in the saline group, P = 0.44), days spent in the hospital (15.3 ± 9.6 and 15.6 ± 9.6, respectively; P = 0.30), days of mechanical ventilation (4.5 ± 6.1 and 4.3 ± 5.7, respectively; P = 0.74), or days of renal-replacement therapy (0.5 ± 2.3 and 0.4 ± 2.0, respectively; P = 0.41).[30]

The Surviving Sepsis Campaign (SSC), an initiative of the ESICM, the International Sepsis Forum and SCCM was developed to improve the management, diagnosis, and treatment of sepsis. The most recent version was published early in 2008.[31] As per these SSC guidelines, the Rivers study was considered as Grade B evidence. However, there were also concerns raised regarding the widespread implementation of this study into practise in other jurisdictions. One of these was the high mortality in the control group (47%). Mortality in other studies reporting severe sepsis has been quoted as 30–35%,[7,32] which could suggest that while Early Goal Directed Therapy may have a beneficial effect when baseline mortality is high, it may be less effective when baseline outcomes are better. The other concern was that introduction of this treatment paradigm would have huge implications for staffing and infrastructure

in the emergency department and ICU. The $ScvO_2$ may be used as warning signal in critically ill patients and act as a marker instead of SvO_2 in emergency departments and ICU in the early stages of hemodynamic optimisation. Following initial resuscitation, it is uncertain whether goal- directed therapy should be based on $ScvO_2$ instead of SvO_2. Studies have provided indirect support for the use of lactate in goal-directed therapy, but there is as yet insufficient evidence for its use as a resuscitation end point. Single centre studies frequently either lack the scientific rigor or external validity required to support widespread changes in practice and their premature incorporation into guidelines may make the conduct of definitive studies more difficult.[33] ARISE (Australasian Resuscitation In Sepsis Evaluation) is a phase III, multi-centre, ANZICS CTG (Australia, New Zealand Intensive Care Society- Clinical Trails Group) endorsed, randomised, controlled study evaluating early goal-directed therapy in 1600 patients presenting to the Emergency Department with severe sepsis across Australian, New Zealand and Hong Kong hospitals. The study is being conducted over 2.5 years through the Australian and New Zealand Intensive Care Research Centre, Department of Epidemiology and Preventive Medicine, Monash University. This study will hopefully provide more directions towards this topic.

Appropriate cultures should properly be obtained before initiating antibiotic therapy but this should not prevent administration of antimicrobial therapy. It is recommended that empiric antibiotic therapy be administered within 1 hour of the identification of severe sepsis. In the presence of septic shock, each hour delay in achieving administration of effective antibiotics appears to be associated with a measurable increase in mortality.[34] Rapid diagnostic methods (polymerase chain reaction, micro-arrays) might aid in the earlier identification of pathogens.[35] Specific anatomical diagnosis of infection and measures to control the source within the first 6 hours following presentation is recommended.[31] A procalcitonin-guided strategy to treat suspected bacterial infections in non-surgical patients in intensive care units could also reduce antibiotic exposure with no apparent adverse outcomes. A multicentre, prospective, parallel-group, open-label, randomised trial demonstrated procalcitonin guided strategy resulted in significantly more days without antibiotics when compared with control group (14.3 days [SD 9.1] versus 11.6 days [SD 8.2]; absolute difference 2.7 days, 95% CI .0.4 to 4.1, $p<0.0001$).[36]

Steroids

Use of corticosteroids in patients with septic shock has been controversial for several decades and continues to be controversial despite the publication of several trials including two recent large RCT's. Sepsis may be associated with relative adrenal insufficiency in a substantial subset of patients.

Annane *et al*[37] in a multi-centre French trial, randomised 300 patients with septic shock to receive either placebo or hydrocortisone 50mg intravenously every six hours (plus fludrocortisone 50mcg enterally once a day) within eight hours of onset of septic shock. Treatment continued for seven days. Based upon a high-dose (250mcg) ACTH (adrenocorticotropic hormone) stimulation test, the patients were classified as having adequate adrenal reserve (maximum increase in serum cortisol of >9mcg/dL) (248nmol/L) or inadequate adrenal reserve (maximum cortisol increase of ≤9mcg/dL (248nmol/L). This study showed significant shock reversal and reduction

of mortality rate in patients with relative adrenal insufficiency. Based on this study many clinicians still use steroids in certain subsets of septic patients.

However, a recent large, European multicenter trial CORTICUS,[38] which was a double blinded randomised trial assigning 499 patients with septic shock to receive hydrocortisone or placebo intravenously every six hours for five days, followed by a tapering regimen, failed to show a survival benefit with steroid therapy for septic shock irrespective of the presence or absence of relative adrenal insufficiency. The therapeutic guiding role of the ACTH stimulation test was cast into doubt by this trial.

Similarities between the two studies included a beneficial steroid effect on time to shock reversal, no evidence for increased risk of neuromuscular weakness, and no hyperglycaemia. Differences between the two studies Annane[37] and CORTICUS trial[38] respectively include: Entry window (8 vs. 72 hours; SBP <90mmHg (>1 hour vs. <1 hour); additional treatment with (fludrocortisone vs. no fludrocortisone); treatment duration (7 vs. 11 days); weaning (none vs. present); differences in steroid effects according to the response to ACTH test (yes vs. no) and increased risk of superinfection (no vs. yes).

Another major difficulty regarding the use of steroid is the lack of definitive data regarding the appropriate cutoff values for 'relative' adrenal insufficiency in the shock state. Significant variability exists in the results of cortisol assay among research centres and whether they estimate free or total cortisol assay. Free and total cortisol may vary significantly based upon the protein concentration. Which steroid is also a pertinent question; there is little evidence for steroids other than hydrocortisone.

Recombinant human activated protein C (rhAPC)

Coagulation plays a central role during inflammatory processes, particularly those due to infection. Drotrecogin alfa (activated) or recombinant human activated protein C is a 54 kilodalton recombinant glycoprotein with antithrombotic, profibrinolytic, and anti-inflammatory properties. Protein C is an inactive zymogen synthesized in the liver. When coupled to thrombomodulin on the endothelial surface, protein C is converted to Activated Protein C by thrombin. Significant decreases in protein C levels have been well documented in sepsis and specifically in septic shock.[39] The conversion of protein C to activated protein C may be impaired during sepsis as a result of the down-regulation of thrombomodulin by inflammatory cytokines. This led to interest in therapeutic administration of activated protein C (and similar agents) in early sepsis. It has now become the first biological therapy to report a mortality benefit in human RCT of sepsis. The Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis (PROWESS)[21] was a randomized, double-blind, placebo-controlled, multi-center trial. Patients with systemic inflammation and organ failure due to acute infection were randomised to placebo or to receive rhAPC (24μg/kg/hr) for 96 hours. The primary end point was death from any cause at 28 days. Nearly 1700 patients were randomized but the study was stopped early when an interim analysis showed a survival benefit in the treatment arm. Based upon post-hoc analyses of the study data, drotrecogin alpha was of greater benefit in the most severely ill patients, including those with an APACHE (Acute Physiology and Chronic Health Evaluation) II score ≥25 and patients with multiple organ dysfunction. This formed the basis for the

FDA decision to license rhAPC for use in sepsis.

A subsequent trial of rhAPC in patients with a low risk of death was halted after an interim analysis for lack of effectiveness.[40] Another trial, involving the paediatric population who had severe sepsis, was stopped after approximately 400 patients had been enrolled, again because of futility. The Surviving Sepsis Guidelines[31] suggest its use (if there are no contraindications) in adult patients with sepsis- induced organ dysfunction associated with a clinical assessment of high risk of death, most of whom will have Acute Physiology and Chronic Health Evaluation (APACHE) II >25. However, there has been considerable criticism of the PROWESS trial and Australian and New Zealand Intensive Care Society does not recommend the use of rhAPC within practice guidelines.[41] The decision regarding administration of rhAPC is likely best made based upon clinicians assessment of high risk of death due to multiorgan failure versus the risk of bleeding complications.

Currently another trial 'Efficacy and Safety of Drotrecogin Alfa (Activated) in Adult Patients with Septic Shock' is in progress. The purpose of this placebo-controlled study is to determine if drotrecogin alfa (activated) treatment provides significant mortality reduction and organ function improvement in patients with septic shock compared with placebo treatment in patients receiving the current standard of care for septic shock. This study will also assess the effectiveness of drotrecogin alfa (activated) in reducing 28-day mortality in patients with septic shock and concomitant severe protein C deficiency at baseline.

Glucose control

Hyperglycaemia is reported to be associated with poor clinical outcomes in critically ill patients. In 2001, Van Den Berghe and colleagues[42] demonstrated significant mortality benefit by intensive insulin infusion titrated to strict euglycaemia in critically ill surgical patients. However, a second study by the same author which targeted medical ICU patients using the same strict glycaemic control failed to show survival benefit. With the available evidence, most clinicians agree that glycaemic control is a desirable intervention in critically ill patients although the optimal blood glucose range is still controversial. A blood glucose level of 140 to 180 mg/dL (7.7 to 10mmol/L) appears to be an acceptable target. A more stringent target (80 to 108mg/dL [4.5 to 6mmol/L]) was associated with higher incidence of hypoglycaemia and significantly higher 90-day mortality in the recently published (Australasian based) NICE SUGAR trail. This study randomised 6104 patients; 3054 were assigned to undergo intensive control 81 to 108mg/dL (4.5 to 6.0mmol/L) and 3050 to undergo conventional control blood glucose $\leq$180mg/dL (<10.0nmol/L). Severe hypoglycaemia was reported in 6.8% of the intensive-control group and 0.5% of the conventional-control group ($P<0.001$).[43]

Renal replacement therapy

Continuous Renal Replacement Therapy (CRRT) involves either dialysis based solute removal) or filtration (convection-based solute and water removal) treatments that operate in a continuous mode. Haemofiltration (HF) refers to the use of a hydrostatic pressure gradient to induce the filtration (or convection) of plasma water across the membrane of the hemofilter. Hemofiltration has been described as a technique which can lower cytokine levels. In a single-centre, randomized, controlled study in which continuous renal-replacement therapy was the sole treatment approach, survival improved

when the intensity of therapy was increased from an assigned effluent rate of 20ml/kg/hr to either 35 or 45ml/kg/hr.[44] Bellomo and colleagues recently reported the results of the Randomized Evaluation of Normal versus Augmented Level (RENAL) Replacement Therapy Study, which was conducted at multiple centers in Australia and New Zealand. In the RENAL Study,[45] 1508 patients with severe acute kidney injury who required intensive care were randomly assigned to receive continuous venovenous hemodiafiltration at a total effluent flow rate of either 25ml or 40ml/kg/hr. In both treatment groups, 44.7% of patients died in the first 90 days after randomization (odds ratio, 1.00; 95% CI, 0.81 to 1.23). Overall, 94.4% of patients who were alive after 90 days no longer required dialysis, with similar rates of recovery of kidney function in both treatment groups.

3-hydroxy-3-methylglutaryl-coenzyme reductase inhibitors (HMG-CoA)

The therapeutic use of HMG-CoA reductase inhibitors, also known as statins, has become widespread as lipid lowering agents in the prevention and treatment of major cardiovascular diseases. There is evidence that statins have beneficial effects on the perioperative risk of cardiac complications and sepsis. Statins appear to have actions on vascular nitric oxide through the balance of inducible and endothelial nitric oxide synthase. Statins also have anti-inflammatory properties, exemplified by reduced plasma concentrations of the inflammatory cytokines tumour necrosis factor (TNF-α) and interleukin (IL-6). Various cohort studies have been published in favour of statins reducing mortality in sepsis. A meta-analysis of cohort studies (including one randomised trial) demonstrated a protective effect for statins in patients with sepsis and/or other infections compared to placebo for various infection-related outcomes; (0.61 (95% CI, 0.48-0.73) for 30-day mortality).[46] Current ongoing RCTs of statins in sepsis are to be watched with interest.

Other adjuvant therapies in sepsis

Cytokines and anticytokine therapies

Granulocyte Colony Stimulating Factor (G-CSF) is a cytokine involved in myelopoiesis with a predominant effect on the polymorphonuclear leukocyte (PMN). Studies in humans with pneumonia have had encouraging results but with no mortality benefit. TNF plays a central role in the inflammatory process; but phase III clinical trials of TNF antibodies and TNF fusion proteins led to negative results. Similarly, studies on antagonising interleukin-1, a cytokine with similar properties, also led to negative results.

Pooled immunoglobulin (IVIG)

Immunoglobulin has been used for sepsis states such as meningococcal and pneumococcal infections with some documented survival benefit. The mechanism of action is most likely immunomodulatory and binding and inactivation of the bacterial derived super-antigen. Its use has been suggested in toxic shock syndrome due to *Streptococcus pyogenes* and *Staphylococcus aureus*. A large randomised trial of 653 patients with severe sepsis failed to demonstrate any benefit of IVIG. The 28-day mortality rate was 37.3% in the placebo group and 39.3% in the IVIG group and thus not significantly different ($p = 0.6695$).[47] Many clinical studies and meta-analyses have examined the utility of IVIG, but there exists insufficient data to make a firm recommendations for its use in sepsis and septic shock.

Acute respiratory distress syndrome (ARDS)

ARDS is an acute (rapid onset) syndrome with bilateral infiltrates on chest x-ray; no evidence of elevated left atrial pressure (the pulmonary capillary wedge pressure is ≤18 mmHg if measured) and a ratio of arterial oxygen tension to fraction of inspired oxygen (PaO_2/FiO_2) is less than 201mmHg. Conventional therapy, aimed at tidal volumes (V_t) of 12–15ml/kg, generated lung volumes that overstretched alveoli resulting in volutrauma (secondary lung injury). The landmark Acute Respiratory Distress Syndrome Network multicenter trial randomly assigned 861 mechanically ventilated patients with ARDS and acute lung injury to receive low tidal volume ventilation (tidal volume of 6 mL/kg) or conventional mechanical ventilation (tidal volume of 12mL/kg). Mechanical ventilation using a lower tidal resulted in decreased mortality and an increase in the number of days without ventilator use.[48] The overall goal was to maintain acceptable gas exchange and avoid alveolar over-distension, tolerating hypercapnia if indicated; thus minimizing the adverse effects of mechanical ventilation.

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18 • Pathophysiology of Reperfusion Injury

PRUE COWLED, ROBERT FITRIDGE

Discipline of Surgery, The University of Adelaide, The Queen Elizabeth Hospital, Woodville South, South Australia, Australia

INTRODUCTION

Ischaemia-Reperfusion Injury (IRI) is defined as the paradoxical exacerbation of cellular dysfunction and death, following restoration of blood flow to previously ischaemic tissues. Reestablishment of blood flow is essential to salvage ischaemic tissues. However reperfusion itself paradoxically causes further damage, threatening function and viability of the organ. IRI occurs in a wide range of organs including the heart, lung, kidney, gut, skeletal muscle and brain and may involve not only the ischaemic organ itself but may also induce systemic damage to distant organs, potentially leading to multi-system organ failure. Reperfusion injury is a multi-factorial process resulting in extensive tissue destruction. The aim of this review is to summarise these molecular and cellular mechanisms and thus provide an insight into possible windows for effective therapeutic intervention.

ISCHAEMIA

ATP and mitochondrial function

Ischaemia occurs when the blood supply is less than the demand required for normal function, resulting in deficiencies in oxygen, glucose and other substances required for metabolism. Derangements in metabolic function begin during this ischaemic phase. Initially, glycogen breakdown by mitochondrial anaerobic glycolysis produces two molecules of adenosine triphosphate (ATP) along with lactic acid, resulting in a decrease in tissue pH, which then acts by negative feedback to inhibit further ATP production. (Figure 18.1) ATP is then sequentially broken down into adenosine diphosphate (ADP), adenosine monophosphate (AMP) and inosine monophosphate (IMP) and then further into adenosine, inosine, hypoxanthine and xanthine. (Figure 18.2 upper panel)

At the cellular level, a lack of ATP production causes ATP-dependent ionic pumps, including the Na^+/K^+ and Ca^{2+} pumps, to fail and the transmembrane ionic gradients are lost. Consequently, cytosolic sodium content rises, drawing with it, a volume of water to attempt to maintain the osmotic equilibrium and resulting in hydroponic swelling of the cells. To maintain the ionic balance, potassium ions escape from the cell into the interstitium (reviewed in[1]). Calcium is released from the mitochondria into the cytoplasm and into extracellular spaces, thereby activating mitochondrial calcium-dependent cytosolic proteases including calpain, which then converts the

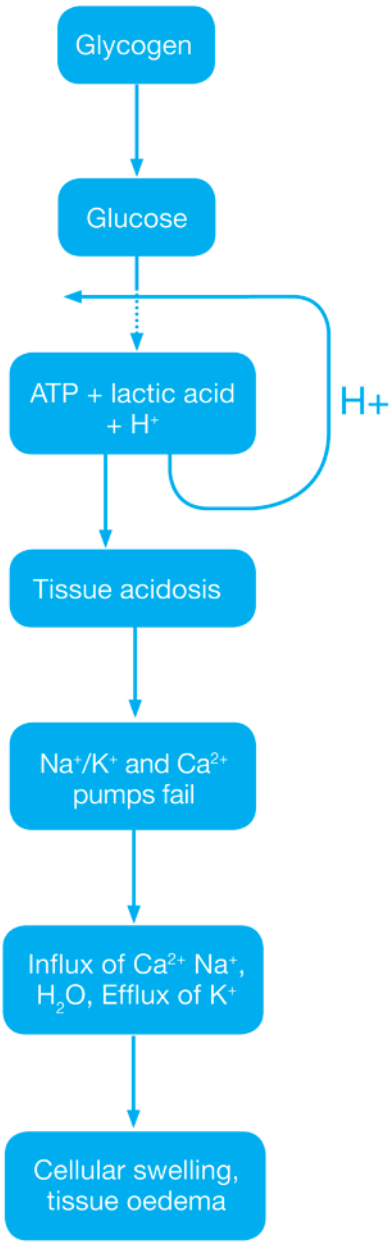

FIGURE 18.1: Dysregulation of metabolic pathways during ischaemia
Anaerobic glycolysis during ischaemia results in negative feedback which inhibits ATP production, thereby inducing tissue acidosis, calcium influx and tissue oedema.

cellular enzyme xanthine dehydrogenase to xanthine oxidase (Figure 18.2 upper panel). Phospholipases are also activated during ischaemia, degrading membrane lipids and increasing the levels of circulating fatty acids.

Gene expression during ischaemia

As well as metabolic derangements, ischaemia induces expression of a large number of genes, which play a major role in the tissue's response to ischaemic damage. An RNA expression microarray analysis, using mouse soleus muscle rendered ischaemic by femoral ligation, found that expression of 962 genes was induced and 327 genes were repressed.[2] The activated genes were largely clustered into cytokine genes and mediators of inflammation and immune cell infiltration. The repressed genes were largely involved in energy production, including mitochondrial respiration and fatty acid oxidation.

Hypoxia itself also activates a number of genes, particularly transcription factors, including activating protein-1 (AP-1), hypoxia-inducible factor-1 (HIF-1) and nuclear factor-kappaB (NF-kB). HIF-1 then activates transcription of other genes such as vascular endothelial growth factor (VEGF), erythropoietin and glucose transporter-1, which all play an important role in the cells' adaptive responses to hypoxia (reviewed in[3]). Expression of both HIF-1 and cyclo-oxygenase-2 (COX-2) are also induced in the lungs of rats subjected to haemorrhagic shock. COX-2 may promote the inflammatory response through the rapid and exaggerated production of nitric oxide and prostaglandins, contributing to organ damage.[4] Activation of NF-kB occurs during both the ischaemic and reperfusion phases and will therefore be discussed below.

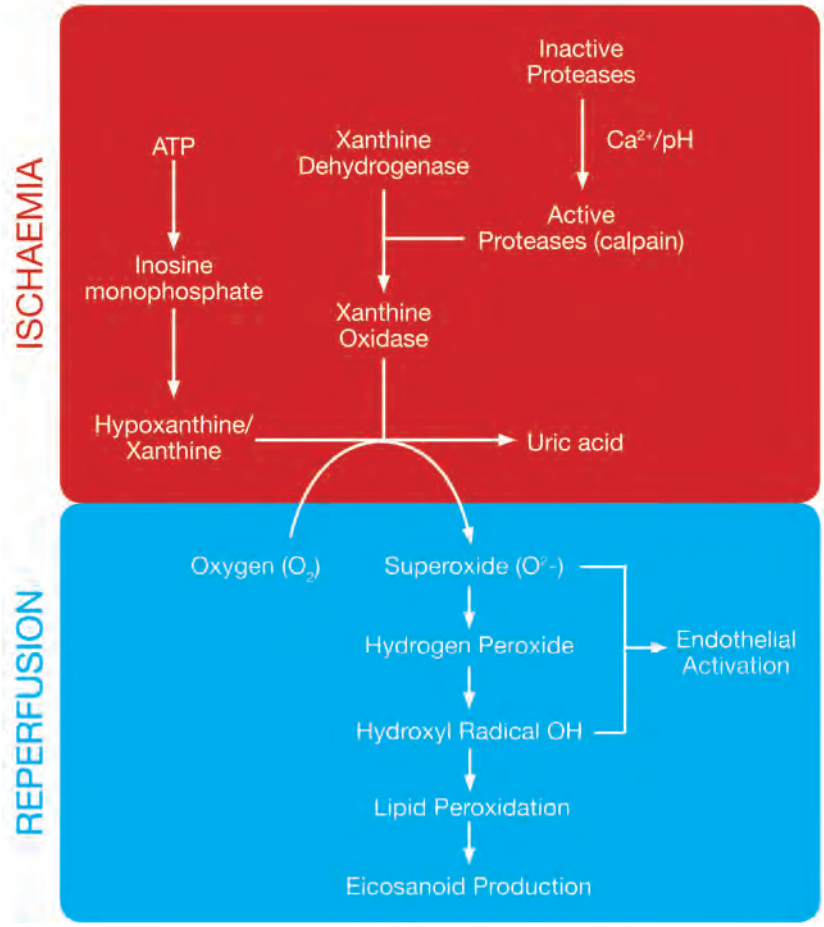

FIGURE 18.2: Generation of reactive oxygen species during reperfusion
During ischaemia, ATP is degraded and xanthine dehydrogenase converted to xanthine oxidase. In the presence of fresh oxygenated blood, xanthine oxidase catalyses the conversion of hypoxathine to highly reactive and toxic superoxide anions with urea as a by-product. Superoxide then reacts with H^+ to initiate the production of both hydrogen peroxide and the hydroxyl radical, which ultimately mediate lipid peroxidation and tissue damage.

REPERFUSION

Reactive oxygen species

Table 18.1 illustrates the major reactive oxygen species (ROS), which play a role in tissue damage during IRI and the sources of generation of these species. Reactive oxygen species have a destructive role in mediating tissue damage during IRI. During ischaemia, the degradation of ATP produces hypoxanthine (Figure 18.2, upper panel). Once the ischaemic tissue is reperfused, an influx of molecular oxygen catalyses xanthine oxidase to degrade hypoxanthine to uric acid and thereby liberating the highly reactive superoxide anion (O_2^-) (Figure 18.2, lower panel). Superoxide is subsequently converted to hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^\bullet$) (Figure 18.2, lower panel). The major consequence of hydroxyl radical production is peroxidation of the lipid structures of cell membranes

TABLE18.1: Reactive Oxygen species involved in IRI

Reactive oxygen species involved in IRI
Major ROS
Superoxide anion (O_2^-)
Hydrogen Peroxide (H_2O_2)
Hydroxyl radical ($OH^•$)
Nitric Oxide (NO)
Peroxynitrite ($ONOO^-$)
Minor ROS
Lipid hydroperoxide
Lipid peroxyl radical
Lipid alkoxyl radical
Thiol radical
Sources of ROS during IRI
Xanthine oxidase system
Activated neutrophils
Mitochondrial electron transport chain
Arachidonic acid metabolism
Auto-oxidation of catecholamines

resulting in the production and systemic release of proinflammatory eicosanoids, disruption of cell permeability and ultimately cell death. During IRI, ROS also activate endothelial cells, elevating the activity of the transcription factor, NF-κB. Once activated, the endothelial cell produces E-selectin, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), endothelial-leukocyte adhesion molecule (ELAM-1) plasminogen activator inhibitor-1 (PAI-1), tissue factor and interleukin-8 (IL-8). These adhesion molecules contribute to important interactions between the neutrophil and the endothelium and will be discussed in more detail later.

Superoxide anions can be detected within ischaemic muscle and also in the venous effluent of reperfused limbs,[5] suggesting an additional role for superoxide in inducing damage to distant organs during skeletal muscle reperfusion injury. Xanthine oxidase is located within a spectrum of cell types and tissues to varying degrees, indicating widespread distribution and differing susceptibility to oxidant-mediated IRI. Inhibition of xanthine oxidase activity, by administration of allopurinol prior to ischaemia, reduces the production of superoxide and hence reduces the severity of reperfusion injury in animal models using a range of tissues including skeletal muscle, brain and gut. Results in humans are also promising. A systematic review[6] provided evidence that allopurinol was effective in some studies in reducing the severity of post-operative cardiac dysfunction and arrhythmias after coronary artery bypass grafting, although larger trials are needed. Studies in other clinical settings of IRI remain limited.

Eicosanoids

As discussed above, ROS initiate lipid peroxidation of cellular membranes, releasing arachidonic acid, the main substrate for the production of prostaglandins, thromboxanes and leukotrienes (Figure 18.2, lower panel). These derivatives of arachidonic acid are collectively known as the eicosanoids and play a major role in the pathophysiology of IRI.

Prostaglandins, synthesised from arachidonic acid via the cyclo-oxygenase pathway, have a protective vasodilatory effect in IRI. However, since prostaglandins are short-lived molecules, their rapid depletion subsequently leads to uninhibited vasoconstriction, reduced local blood flow and exacerbation of ischaemia. The potential of prostaglandins to ameliorate the degree of metabolic and tissue derangement following IRI has been demonstrated in various tissues. In a placebo-controlled trial of human liver transplantation, administration

of prostacyclin was shown to improve postoperative graft function.[7] Patients who received prostacyclin demonstrated better post-operative myocardial oxygen consumption after coronary artery bypass surgery[8] and improved muscle blood flow following skeletal muscle IRI.[9]

Plasma thromboxane A_2, also synthesised from arachidonic acid, increases within minutes following skeletal muscle IRI, thus promoting vasoconstriction and platelet aggregation. These events coincide with a rapid rise in pulmonary artery pressure and a subsequent increase in pulmonary microvascular permeability,[10] which correlates with sequestration of polymorphonuclear cells in the lungs. In animal models of lower limb IRI, thromboxane synthase inhibitors and synthetic thromboxane A_2 receptor antagonists prevented pulmonary leuko-sequestration, thereby increasing blood flow to reperfused tissues and preserving tissue viability and function.[11] Together these studies suggest that administration of thromboxane A2 antagonists may offer therapeutic potential to improve limb salvage rates after surgery for acute ischaemia.

Leukotrienes are also synthesised from arachidonic acid through the activation of 5-lipoxygenase and participate in the inflammatory cascade of IRI. Leukotrienes lead to local and systemic injury by their direct proinflammatory action on endothelial and smooth muscle cells and indirectly by their effects on neutrophils. The leukotrienes C_4, D_4, and E_4 modify the endothelial cytoskeleton, leading to increased vascular permeability and also enhance smooth muscle contraction, resulting in vasoconstriction. The lung produces leuko-trienes following remote IRI. The direct effects of leukotrienes on pulmonary microvessels lead to increased permeability, transient pulmonary hypertension and the activation of the endothelium to produce thromboxane, resulting in additional vaso-constriction. The leukotriene B_4, released by activated neutrophils, leads to further pulmonary neutrophil accumulation.

The administration of 5-lipoxygenase synthesis inhibitors has been successfully used in animal studies to attenuate IRI. Such agents abolish the elevations in leukotrienes B_4 and C_4 and inhibit neutrophil infiltration normally induced by IRI, reducing mucosal permeability.[12] However, there is currently very little up to date information on their use in a clinical situation.

Nitric oxide

Nitric oxide (NO) is a signalling molecule synthesised from L-arginine by the nitric oxide synthase enzyme (NOS) of which there are three types, constitutive (cNOS), inducible (iNOS) and endothelial (eNOS). An initial surge in NO level in the first 15 minutes of the ischaemic phase is due to transient eNOS activation. This is followed during early reperfusion by a general decline in endothelial function and loss of functional eNOS, so that NO production falls, along with an increased production of reactive oxygen species. eNOS-derived NO is also necessary for the maintenance of vascular tone. The reduction in eNOS levels that occurs in IRI may therefore predispose to vasoconstriction, a common response seen in IRI. The second surge in NO production is largely due to cytokine-mediated up-regulation of iNOS after about three hours of reperfusion.

The pathophysiological role of nitric oxide in reperfusion injury is variable, being dependent on the nature of its generation and appears to be tissue specific. In some instances, NO acts as an anti-oxidant and, in others, combines with the superoxide anion to form the peroxynitrite radical, a potent promoter of lipid peroxidation and hence

cellular membrane disruption (Reviewed in[13]). Manipulation of nitric oxide production during IRI, using a range of techniques, has recently provided considerable evidence for a principal role for nitric oxide in the aetiology of IRI. Myocardial IRI has been well studied, with paradoxical results, where low doses of NO were found to be protective and high doses harmful. The influence of NO in skeletal muscle IRI has been less well characterized, with some studies suggesting that NO may potentiate cytotoxicity and others suggesting a beneficial role for NO in extremity IRI. In skeletal muscle IRI, NO production may be deleterious and inhibition of NOS activity using a non-specific NOS inhibitor greatly reduced the severity of muscle damage.[14]

The assessment of experimental data derived from pharmacological NOS inhibition is difficult due to the non-specificity of NOS inhibitors; administration of these inhibitors at differing times during the injury merely adds to the complexity. In essence, augmentation of NO delivery may be beneficial with respect to protection, particularly in the ischaemic and early reperfusion phase. Inhibition of the iNOS-induced surge in NO production at later times during reperfusion also mediates defense against IRI-induced tissue damage. However, in the clinical setting, systemic distortion of NO kinetics by administering NOS inhibitors would be likely to induce wide-ranging physiological disturbances. Further investigations will be needed to define a role for NOS inhibition in ameliorating the severity of IRI and local administration of these inhibitors may be required.

Endothelin

Endothelins are potent peptide vaso-constrictors produced by the vascular endothelium. Hypoxia, growth factors, angiotensin II and noradrenaline all stimulate their production resulting in Ca^{2+}-mediated vasoconstriction. Endothelin-1 is elevated following skeletal muscle IRI during both the ischaemic and reperfusion phases and mediates capillary vasoconstriction, neutrophil aggregation and neutrophil-endothelial interactions. Endothelin-1 inhibitors, including bosentan and tezosentan, inhibit neutrophil infiltration, increase functional capillary density, microvascular perfusion and hence tissue viability and function following IRI.[15] However these inhibitors are not in widespread clinical use.

Cytokines

Hypoxia and IRI both induce the expression of numerous cytokines, including tumour necrosis factor-alpha (TNF-α,) interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8) and platelet activating factor (PAF), in association with elevations in activity of the transcription factor, NF-kB (reviewed in[16]) These cytokines are released systemically and are thus important in the development of systemic inflammatory response syndrome and ultimately multi-system organ failure.

TNF-α is a 17-kilodalton pro-inflammatory cytokine produced by activated macrophages, monocytes, T-lymphocytes, natural killer cells and fibroblasts. It is a potent chemoattractant and early response cytokine, which subsequently induces expression of IL-1, IL-6, IL-8 and PAF. Elevated serum levels of TNF-α have been detected during cerebral and skeletal muscle IRI and are known to increase neutrophil sequestration and permeability following pulmonary IRI. Serum TNF-α levels increased rapidly in an animal model of aortic clamping, thus inducing up-regulation of iNOS, which increased NO production in the lungs, leading to more severe lung damage.[17] In the same study, inhibition of TNF-α activity prior to limb ischaemia decreased pulmonary

NO production and reduced the severity of IRI. TNF-α can also induce the generation of ROS and enhance the susceptibility of the vascular endothelium to neutrophil mediated injury, by inducing the expression of ICAM-1, which mediates binding of neutrophils to the activated endothelium.

Numerous studies in animal models attest to the potential of TNF-α blockade as a therapeutic modality to reduce the severity of IRI. Anti-TNF-α antibody protected against IRI-induced pulmonary injury in a rat model by preventing microvascular damage. The introduction of humanised antibodies including etanercept and infliximab, has provided encouraging results in the treatment of other TNF-α-mediated inflammatory diseases, including a number of forms of arthritis and inflammatory bowel disease (reviewed in[18]). However, clinical trials to test the efficacy of TNF-α blockade in human IRI have not yet been reported.

The cytokines IL-1α and IL1β are produced during IRI by tissue macrophages, neutrophils and the vascular endothelium. IL-1α is a potent chemotactic agent and stimulates neutrophil infiltration during hepatic IRI. Both IL-1α and TNF-α also increase levels of expression of ICAM-1 on the vascular endothelium. Exposure of endothelial cells in culture to IL-1α and TNF-α induces synthesis of E-selectin, which then interacts with L-selectin on the neutrophil surface leading to rolling on the endothelial surface. Permanent adhesion of the neutrophil to the endothelium is then mediated by expression of ICAM-1, IL-8 and PAF in the endothelial membranes (Figure 18.3).

Numerous activating stimuli synthesised during IRI include H_2O_2, thrombin, leukotrienes C_4, and D_4, IL-1b, histamine, bradykinin and ATP; all of which induce the synthesis of PAF by monocytes, macrophages, neutrophils, eosinophils, basophils, platelets and endothelial cells. PAF functions as both an inter- and intra-cellular messenger, having three major effects, vasoconstriction, chemoattraction and increased microvascular permeability. PAF is rapidly produced

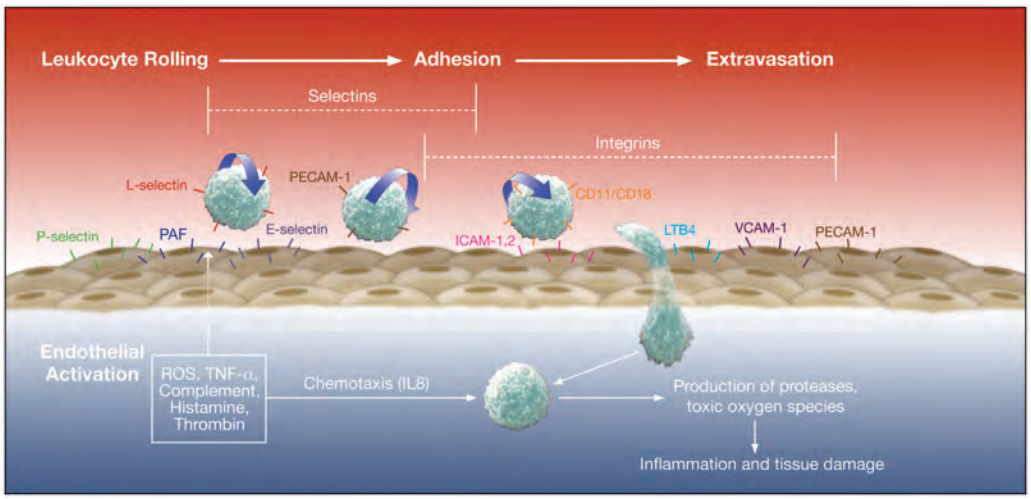

FIGURE 18.3: Neutrophil rolling, adhesion to endothelium and extravasation
During reperfusion, activated neutrophils adhere to the activated endothelium and subsequently extravasate into surrounding tissue, resulting in proteolytic degradation of basement membranes. Activated neutrophils also generate toxic reactive oxygen species from molecular oxygen, contributing to tissue degradation during reperfusion.

following skeletal muscle and renal IRI with peak levels after 15 minutes of reperfusion. PAF enhances the binding of neutrophils to endothelial cells since a PAF-receptor antagonist blocked adhesion to endothelial cells during IRI.[19] Similarly pre-treatment with the PAF inhibitor lexipafant reduced the severity of intestinal barrier dysfunction and pulmonary and liver permeability in a rat model of intestinal IRI.[20] However lexipafant is unlikely to be clinically useful as a pharmacotherapy for IRI since, alone, it failed to completely inhibit pulmonary endothelial damage after small bowel IRI.[21]

IL-6 is a proinflammatory 19-26kDa protein produced by monocytes, fibroblasts, keratinocytes and endothelial cells in response to IL-1 and TNF-α. IL-6 primes and stimulates the respiratory burst in neutrophils, stimulates endothelial cell expression of ICAM-1 and increases endothelial permeability. IL-6 is produced in hypoperfused skeletal muscle in patients with peripheral arterial disease and is released from the gut into the systemic circulation during reperfusion in aortic aneurysm surgery.[22] In the setting of renal transplantation, IL-6 was released in large amounts from the reperfused transplanted kidney during the first 30 minutes of reperfusion.[23]

IL-8 is a potent neutrophil chemotactic and activating factor. It is produced by monocytes, T cells, NK cells, fibroblasts, endothelial cells, eosinophils and neutrophils in response to IL-1, TNF-α, endotoxin, histamine and hypoxia. The chemotactic activity of IL-8 induces diapedesis of activated neutrophils through the endothelium. (Figure 18.3) Elevated levels of serum IL-8 have been detected during early reperfusion following human lung transplantation and predict poor graft function.[24] An anti IL-8 antibody prevented pulmonary neutrophil infiltration and tissue injury in a rabbit model of lung IRI.[25]

Neutrophil and endothelial interactions

Neutrophils play a major role in tissue damage incurred during IRI. Activated neutrophils are a major source of ROS, which are generated through the activity of the membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex. Whilst oxidizing NADPH to NADP+, NADPH oxidase also reduces molecular oxygen to form the superoxide anion. Myeloperoxidase, stored in the azurophilic granules of neutrophils, converts hydrogen peroxide to toxic hypochlorous acid, which, in addition to its direct effects, is also capable of activating proteases. The activated neutrophils also secrete a number of proteases, including matrix metalloproteinases, which will degrade basement membrane and other tissue structures, contributing to the severity of tissue destruction.

Neutrophil infiltration is observed at sites of tissue damage[26,27] and the depletion of neutrophils reduces the severity of organ damage in a mouse model of liver IRI.[28] Depletion of neutrophils during cardiac surgery has been extensively investigated as a modality to reduce the severity of post-operative cardiac dysfunction with inconsistent results. Some studies have shown a reduction in markers of cardiac damage while others have been less successful in demonstrating a clinically relevant effect.

Selectins are a family of transmembrane molecules, expressed on the surface of leukocytes, activated endothelial cells and in platelets. Selectins mediate the initial phase of neutrophil–endothelial cell interactions, often termed rolling (Figure 18.3), which is essential for their subsequent adhesion and extravasation. L-selectin is expressed constitutively on the surface of neutrophils and initiates the reversible attachment of neutrophils to endothelial cells and platelets.

Antibody-mediated blocking of L-selectin impairs the ability of neutrophils to roll on endothelial cells and reduces neutrophil infiltration following skeletal muscle and pulmonary IRI.[29]

P-selectin is stored in the α-granules of platelets and the Wiebel-Palade bodies of endothelial cells and is rapidly translocated to the cell surface along with PAF in response to thrombin, histamine, reactive oxygen species, complement and TNF-α. Typically, peak levels of endothelial P-selectin are detected 6 hours after reperfusion. Endothelial P-selectin plays a vital role in the rolling of neutrophils along the activated endothelium. Activation of the endothelium by pro-inflammatory mediators also results in de novo transcription and synthesis of E-selectin. Expression of endothelial E-selectin is induced during both renal and cerebral IRI. The focal expression of E-selectin at sites of endothelial activation promotes neutrophil adhesion and infiltration into adjacent tissues. In support of a vital role for E-selectin in mediating tissue damage during IRI, a study showed that antibodies against E-selectin reduced infarct size following cerebral IRI in mice.[30] Blocking the activity of selectins shows promise in ameliorating the severity of tissue damage in a number of animal models of IRI. Although some promising selectin inhibitors have been tested in animal models of IRI, this therapy has yet to be tested in a clinical situation (reviewed in[31]).

The integrin and immunoglobulin supergene families of adhesion molecules mediate the strong adhesion of activated neutrophils to the endothelium and hence allow their subsequent extravasation during IRI. The integrins form a large family of cell surface adhesion molecules that mediate intercellular recognition and cellular binding to the extracellular matrix. The neutrophil β_2-integrin adhesion glycoprotein complex consists of a common polypeptide chain, CD18, which is non-covalently linked to three different α-polypeptide chains (CD11a, CD11b, CD11c). CD11a/CD18 is expressed on all leukocytes and mediates the attachment of stimulated neutrophils to the vascular endothelium through a specific interaction with ICAM-1 and ICAM-2. Chemotactic cytokines (IL-1, TNF-α) and ROS all induce neutrophil adherence to the endothelium by CD11/CD18-dependent mechanisms. The CD11b/18 complex on activated neutrophils interacts with ICAM-1 on the surface of the endothelial cell to mediate firm adhesion of neutrophils prior to their extravasation (reviewed in[32]). All of these molecules are required for the development of lung injury following skeletal muscle IRI. Using an anti-CD18 monoclonal antibody, inhibition of CD18-mediated leukocyte adhesion prevented vasoconstriction, inhibited vessel leakage and reduced vascular resistance in animal models of skeletal muscle IRI. However, despite encouraging animal studies, the clinical efficacy of blocking CD11/CD18-mediated interactions in IRI remains doubtful (reviewed in[33]). Clinical trials in humans failed to demonstrate any effect of CD11/CD18 in reducing infarct size following primary coronary angioplasty in the setting of acute myocardial infarction. A more recent review[34] summarised the results from a number of clinical trials using antibodies to CD11/CD18, including for myocardial infarct and stroke, all of which failed to show any significant benefit to the patient.

The immunoglobulin supergene family (ligands for integrins) contains a large number of molecules with multiple immunoglobulin-G-like domains. Several members of this family are involved in leukocyte-endothelial cell interactions including ICAM-1, VCAM-1 and platelet-endothelial cell adhesion molecule-1 (PECAM-1). Levels of expression

of ICAM-1 on endothelial cells are enhanced by exposure to circulating TNF-α that is generated in response to IRI. VCAM-1 was elevated during renal IRI in a mouse model but, unlike ICAM-1, was independent of TNF-α since renal IRI in TNF-α knockout mice also upregulated VCAM-1. PECAM-1 is expressed constitutively on platelets, leukocytes and endothelial cells. IRI induces elevated PECAM-1 levels thereby enhancing activation of neutrophil-endothelial interactions mediated by β-integrins and exacerbating neutrophil extravasation and tissue damage.

The therapeutic potential of blocking the activity of adhesion molecules has been tested in a number of animal models with encouraging results. Using monoclonal antibodies, inhibition of ICAM-1 activity attenuated neutrophil adhesion in the liver, reduced pulmonary sequestration and oedema following skeletal muscle IRI and also reduced intestinal dysfunction following IRI.[35] Antisense oligonucleotides to ICAM-1 ameliorated renal IRI and prevented delayed graft dysfunction in a rat model of renal transplantation.[36] However, results obtained in clinical trials have not been as positive. A recent clinical trial of anti-ICAM-1 antibody therapy in ischaemic stroke (Enlimomab Acute Stroke Trial) concluded that this was not an effective treatment and may significantly worsen stroke outcome, raising significant doubts regarding the efficacy of this therapeutic modality.[37]

Complement activation

Complement activation and deposition also contribute significantly to the pathogenesis of IRI. Rubin and colleagues have demonstrated that reperfusion of skeletal muscle is associated with systemic depletion of the complement protein, factor B, indicative of activation of the alternative complement pathway.[38] The complex C5b-9 is also deposited into the endothelial cell membrane after IRI, leading to osmotic lysis.[39] Pulmonary damage following bilateral hind limb ischemia was significantly reduced when the soluble complement receptor (sCR1) was administered to rats, thus inhibiting complement activity.[40] In the clinical setting, a relationship has been demonstrated between the severity of multi-system organ dysfunction and degree of complement activation after aortic cross clamping.[41]

Inhibition of the complement cascade has been demonstrated to improve outcomes following IRI in a number of different animal models. Complement depletion of circulating plasma improved the initial blood flow and decreased muscle necrosis and injury after ischaemia and prolonged reperfusion in dogs. Complement blockade also prevented leukocyte adhesion, leading to better capillary perfusion and muscle cell viability and attenuated the increase in permeability index in tissues.[42] Unequivocal evidence for the importance of complement activation during skeletal muscle IRI has been provided from experiments where limb ischaemia was induced in C5-deficient mice. These mice had approximately 50% less tissue damage than the wild-type animals.[39] An additive role of both complement and neutrophils in mediating skeletal muscle IRI has also been observed, with a greater reduction in histological damage in neutropenic C5-deficient animals than in neutropenic or C5-deficient mice alone.[39] These data continue to demonstrate the multifactorial nature of tissue damage induced during IRI since complement blockade failed to completely ameliorate tissue damage.

TISSUE DESTRUCTION

Proteases and metalloproteinases

The matrix metalloproteinases (MMPs) are a family of zinc dependent enzymes that have the ability to degrade components of the extracellular matrix. Together with their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), they are the major physiological regulators of the extracellular matrix. MMPs are intimately involved in all processes that necessitate degradation or synthesis of the extracellular matrix and important roles for these enzymes have been identified in wound healing, periodontal disease, cancer metastasis and, of particular relevance, vascular disease including the development of aneurysms, atherosclerotic plaques and reperfusion injury.

Elevations of MMP-2 and MMP-9 have been detected following pulmonary, hepatic and cardiac IRI. MMPs are also elevated following cerebral IRI, corresponding with opening of the blood-brain barrier, degradation of the basal lamina, increased capillary permeability and cerebral oedema.[43] Definitive roles for MMP-9 in the pathophysiology of cerebral IRI have been demonstrated by using both selective MMP-9 inhibitors and MMP-9 knockout mice, which both significantly reduce cerebral infarct size.[44] The role for MMPs in renal IRI is less clear. MMP-2 may have a late role in renal IRI with an elevation detected as late as 8 weeks after IRI.[45] However the MMP inhibitor (Batimastat) did not alter the severity of IRI induced renal dysfunction.[46]

Barr and co-workers[47] carried out a study examining acute ischaemic stroke patients by MRI and correlated systemic plasma MMP-9 levels with a hyperintense acute reperfusion injury marker (HARM), measured by MRI 24 hours later. Plasma MMP-9 was a significant predictor of elevated HARM measures, supporting the hypothesis that elevated MMP-9 is associated with disruption of the blood brain barrier after ischaemic stroke. These results raise the possibility that inhibition of MMP-9 may be a useful modality to reduce the severity of cerebral damage.

Studies in our laboratory have demonstrated both a local and systemic role for MMP-2 and MMP-9 in the degradation of type IV collagen in pulmonary tissues and in skeletal muscle following lower limb IRI.[27] Permanent ischaemia alone, without reperfusion, also results in elevation of MMP-2 and MMP-9, correlating with destruction of the basement membrane components, type IV collagen and laminin.

Apoptotic cell death during ischaemia-reperfusion injury

Tissue destruction resulting from IRI can be due to either necrotic or apoptotic cell death. Apoptosis or programmed cell death is an active process characterized by a series of gene-directed events leading to a characteristic cell morphology, controlled DNA fragmentation and eventually death of the cell. The role of apoptosis in IRI-induced tissue damage has been widely investigated in recent years. Oxidative stress and the production of ROS will induce apoptosis, the characteristics of which can readily be recognised following cerebral IRI. Similarly, renal and cardiac IRI all result in detectable levels of apoptosis in the damaged tissue. Apoptosis therefore appears to play a fundamental role in cellular damage occurring during IRI in a number of tissues. However the role of apoptosis in skeletal muscle IRI remains controversial. Studies conducted in our laboratory,[26] in agreement with Knight and co-workers,[48] have failed to detect any evidence of apoptosis in rat skeletal myocytes following IRI. This implicates a tissue-specific mechanism of cell death following IRI. Blocking the apoptotic

cascade, using specific inhibitors directed against pro-apoptotic caspase enzymes, have been partially effective in animal models, reducing the severity and infarct size following hepatic and cardiac IRI.

No reflow phenomenon

No reflow is the failure of microvascular perfusion, following restoration of flow to previously ischaemic tissue. The cause of this phenomenon has not been fully elucidated (reviewed in[49]) but is certainly multifactorial. Cytokines and activated neutrophils act synergistically to produce microvascular barrier dysfunction. The resultant increase in permeability leads to the exudation of fluids and proteins, increasing the interstitial pressure and decreasing the net intravascular pressure. In addition, CD18-dependent leukocyte plugging produces partial occlusion of post-capillary venules, further contributing to no-reflow. Neutrophil depletion virtually abolishes the phenomenon in the myocardium, brain and skeletal muscle, confirming a vital role for neutrophils in no-reflow.

THERAPEUTIC APPROACHES TO IRI

Ischaemic preconditioning

Ischaemic preconditioning consists of brief and repetitive episodes of IRI before the induction of sustained organ ischaemia and is effective in reducing the severity of tissue damage. The preconditioning effect can be delivered remotely instead of to the target organ. This treatment could be useful in a number of operative settings including transplantation, coronary bypass grafting and elective major vascular surgical procedures where the onset of ischaemia can be tightly controlled. In these settings, brief extremity IRI (10 minutes) administered by tourniquet before surgery has been widely investigated and shows promise as a therapy to reduce the severity of IRI.

Animal models of a number of settings of IRI have been used to investigate mechanisms of ischaemic preconditioning but the basic molecular mechanisms remain unclear, probably due to the multiple signal transduction pathways involved in this phenomenon. However it is generally recognised that brief ischaemic preconditioning induces a cascade of intracellular kinases, which subsequently modify mitochondrial function. A recent study in a rat model of lower limb IRI illustrated clearly that two brief 10 minute episodes of IRI before a full 60 minutes of ischaemia was effective in reducing pro-inflammatory neutrophil-endothelium interactions. This effect was noted in both the lower limb itself and in remote tissues, illustrating the systemic nature of this phenomenon.[50] In a mouse model of hind limb IRI, preconditioning significantly reduced tissue damage in the limb itself and also in lung and small bowel. Preconditioned animals were also significantly protected against post-operative mortality.[51]

A large number of clinical trials have also been reported investigating the efficacy of ischaemic preconditioning but with varying degrees of success (reviewed in[52]). A small randomised clinical trial aimed to determine if remote lower limb ischaemic preconditioning before EVAR could reduce the severity of renal and cardiac damage.[53] A significant reduction in urinary biomarkers of renal injury was detected in the preconditioning cohort but this small pilot trial was unable to detect any effect on clinical endpoints. However, in the setting of open AAA repair where operative ischemia is profound, promising results were obtained. Remote preconditioning

significantly protected against post-operative myocardial injury, myocardial infarction, and renal impairment.[54] An excellent 'proof-of concept' study of ischaemic preconditioning was recently reported in the setting of evolving ST-elevation acute myocardial infarction. Subjects were randomised while in the ambulance and received intermittent arm ischaemia during transport to hospital (four cycles of 5 minute inflation and 5 minute deflation of a blood-pressure cuff). The primary endpoint was the myocardial salvage index 30 days after primary percutaneous coronary intervention, measured by myocardial perfusion imaging. The data showed convincingly that remote ischaemic conditioning before hospital admission increased myocardial salvage.[55] Further studies are needed to verify the effect of remote conditioning on clinical outcomes but this therapeutic modality currently appears very promising.

Ischaemic post-conditioning

Ischaemic post-conditioning is defined as rapid sequential intermittent interruption of blood flow applied during the early moments of reperfusion. This technique is particularly relevant where the initial ischaemic insult could not have been predicted, thus a preconditioning approach to limiting tissue damage could not have been applied. Experimental animal models have been used to successfully show attenuation of organ injury, including the heart, spinal cord, brain, kidney, liver, muscle, lung and intestines (reviewed in[56]). The mechanisms of post-conditioning are not yet entirely clear but appear to involve multiple signalling pathways and molecules, including protein kinases, ROS, pro-inflammatory cytokines and NO, as well as alterations in mitochondrial function (reviewed in[57]).

Animal models of particular relevance to vascular surgical procedures have been tested widely and results show promise for post-conditioning as an effective therapy to reduce the severity of IRI. In a rat model of lower limb ischaemia induced by aortic clamping, rats underwent 180 minutes of ischaemia followed by post-conditioning consisting of six cycles of 10 seconds aortic occlusion followed by 10 seconds declamping at the beginning of reperfusion. Post-conditioning caused a significant reduction in both the severity of systemic inflammatory responses and degree of remote pulmonary and renal damage.[58] In a similar study in the rat,[59] 60 minutes infrarenal aortic cross-clamping followed by intermittent 4 times 15 seconds reperfusion-15 seconds ischaemic episodes before reperfusion, was effective in reducing production of ROS, leukocyte-endothelial activation and cytokine production.

Based on the experimental models, ischaemic postconditioning thus appears to show promise as an effective therapy in vascular surgery to reduce reperfusion injuries after aortic surgery and revascularization procedures (reviewed in[60]). Some clinical studies have verified these findings, although this has been largely limited to cardiac IRI. However, the duration of the occlusion and reperfusion periods will be critical to the degree of protection and further studies are needed to calculate useful algorithms to plan therapeutic strategies after a significant ischaemic insult.

Conditioning effects of volatile anaesthetics

Anaesthetics have been widely demonstrated to reduce the severity of IRI-induced damage in the setting of myocardial ischaemia and reperfusion during cardiac surgery (reviewed in[61]). However, there is conflicting evidence regarding the relative contributions of preconditioning, conditioning during

ischaemia and postconditioning to the significant cardioprotection provided by anaesthetics. The molecular mechanisms and signal transduction pathways involved in protection are an area of active investigation. A proteomic study demonstrated that volatile anaesthetics (isoflurane, sevoflurane or desflurane) induced long lasting changes in the expression of 106 proteins in the rat myocardium.[62] Evidence also suggests that inhibition by anaesthetics of the opening of the mitochondrial permeability pore may be a key mechanism of anaesthetic-induced preconditioning. Anaesthetic-induced postconditioning mechanisms are also multifactorial. Volatile anaesthetics are known to inhibit neutrophil adhesion in the coronary arteries during the reperfusion phase, thereby inhibiting the inflammatory action of activated neutrophils in post-ischaemic tissues (Figure 18.3).

There is good clinical evidence for the cardioprotective effects of volatile anaesthetics during cardiac surgery. A meta-analysis examined randomized trials comparing volatile with non-volatile anaesthesia in coronary bypass surgery. There was no significant difference in myocardial ischaemia, myocardial infarct, intensive care unit length of stay or in-hospital mortality. However, patients receiving volatile anaesthetics had significantly higher cardiac indices, lower troponin I serum concentrations and a lower requirement for inotropic support.[63] A more recent large multicentre study provided excellent evidence that volatile anaesthesia significantly reduced mortality after coronary bypass grafting.[64] Evidence for anaesthetic protection in vascular surgical settings other than in cardiac IRI is not currently available but is likely to be equally significant and should be actively investigated in the future.

Pharmacological treatments

As discussed in many of the sections above, a wide range of pharmacological therapies have been tested in both animal models and in the clinic. Although many of the animal models show considerable promise in reducing the severity of IRI, results from clinical trials have uniformly been disappointing. A recent Cochrane Review reported on treatments to reduce IRI during liver resection under vascular control.[65] They identified 15 randomised trials, which examined 11 pharmacological interventions (methylprednisolone, multivitamin antioxidant infusion, vitamin E infusion, amrinone, prostaglandin E1, pentoxifylline, mannitol, trimetazidine, dextrose, allopurinol and a thromboxane A2 synthetase inhibitor). Although some therapies improved liver enzyme levels, there were no significant differences between the groups for mortality, liver failure, or perioperative morbidity. A second Cochrane review from the same authors[66] examined the effects of prostaglandin E1, pentoxifylline, dopexamine, dopamine, ulinastatin, gantaile, sevoflurane, and propofol during liver IRI and reached the same conclusion that there were no significant differences.

Statin therapies have been widely accepted into clinical practice and there is also considerable evidence, both experimental and clinical, that statins will reduce the severity of IRI in a range of settings. Statins inhibit a range of cellular responses to IRI-induced inflammation, including inhibition of NFkB activity, which leads to decreased transcription of MMPs, adhesion molecules and cytokine genes. Binding of adhesion molecules on activated neutrophils to endothelial cell surface receptors is also blocked. Secretion of MMPs from activated neutrophils is also inhibited by statins. In the endothelium, levels of expression of eNOS mRNA are increased and the eNOS

protein is activated, while expression of endothelin-1 is inhibited. All of these effects will ameliorate the severity of tissue damage during IRI (reviewed in[67]).

Trials of lower limb IRI in the rat were carried out in our laboratory and illustrated convincingly that pre-treatment for a week with simvastatin before IRI markedly protected both skeletal muscle and remote organs including the lungs and kidneys.[14,68] In the clinical setting, a recent review[69] discussed the efficacy of statins in patients undergoing a range of vascular surgical procedures. Symptomatic patients with carotid artery stenosis and taking statins appear to have better outcomes after carotid endartarectomy than those not on statins, although the difference between the cohorts is not marked. In the setting of infrainguinal bypass for peripheral arterial disease, the indications that statins may protect against IRI during surgery are less definitive with some conflicting results although 1-year mortality was improved. Evidence for any effect of statin treatment on the severity of postoperative complications after AAA repair is lacking, although a retrospective observational study showed that all-cause mortality was reduced in those on long term statin therapy.[70] However, since all vascular patients should be receiving statin treatment for secondary prevention of cardiovascular disease, prospective randomized trials to obtain definitive results can no longer ethically be performed.

SUMMARY

In summary, IRI is a highly complex series of interwoven pro-inflammatory and pathological events. The production, release and activation of cytokines, ROS, proteases and complement if left unchecked, leads to both local and systemic injury with potentially fatal consequences. The failure of therapeutic interventions to translate into clinical practice is a reflection of this complexity and redundancy within the system. New therapeutic agents directed towards multiple areas within this cascade may be required to overcome this difficult clinical challenge.

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19 • Compartment Syndromes

EDWARD CHOKE, ROBERT SAYERS, MATTHEW BOWN

Department of Vascular Surgery, University of Leicester, UK

DEFINITION

Compartment syndrome is a clinical and pathological syndrome where the pressure within an anatomical tissue compartment rises above the normal physiological value for that compartment and detrimentally alters the function of the tissues either temporarily or permanently. Acute compartment syndromes affecting the abdominal cavity and the fascial compartments of the limbs are those encountered in vascular surgery.

ACUTE LIMB COMPARTMENT SYNDROME

The importance of acute limb compartment syndrome (LCS) is that, if left untreated, it results in rhabdomyolysis with resultant release of potassium, myoglobin and other toxins into the systemic circulation, which can lead to renal and/or multi-organ failure. The mortality of acute renal failure and multi-organ failure is high. These patients require critical care which may involve dialysis and other organ support. Untreated LCS often necessitates amputation to prevent further acute systemic deterioration. If the immediate insult is survived without the need for amputation a permanently disabling ischaemic contracture may result.[1]

LCS arises due to the anatomical arrangement of muscles surrounded by restrictive, inelastic osteofascial envelopes. Increased pressure within these fascial compartments can occur as a result of extrinsic compression such as that from plaster casts or bandages, or increased volume of the contents of the compartment. The volume within the compartment can be increased as a result of enlargement of those tissues contained within the compartment (e.g.muscle oedema) or due to the presence of a pathological space-occupying mass (such as a haematoma or abscess). In the field of vascular surgery LCS is most commonly encountered following delayed revascularisation of an ischaemic limb, fractures with/without vascular injury or following radiological complications such as perforation during angioplasty. It is occasionally seen in conditions such as phlegmasia caerulea dolens where venous hypertension exists.

Incidence

The incidence of LCS after revascularisation depends primarily on the type of insult. After elective vascularisation of chronically ischaemic limbs the incidence is very

low, from 0% to 0.5%.[2,3] The incidence increases in revascularised acutely ischaemic limbs to approximately 10% to 20%.[4-6] Revascularisation following vascular trauma is the most significant risk factor for LCS, with a reported incidence of up to 62%,[7] particularly if this is associated with either a vascular injury at or below the popliteal artery, or a fracture (Figure 19.1).

Anatomy/Physiology

LCS develops in the limbs because of their particular anatomical and physiological characteristics. Anatomically this is principally the arrangement of muscles within envelopes of dense, inelastic fibrous fascia. In addition to muscles, each compartment also contains peripheral nerves and blood vessels that traverse these compartments to supply distal parts of the limb and/or supply the structures within that compartment.

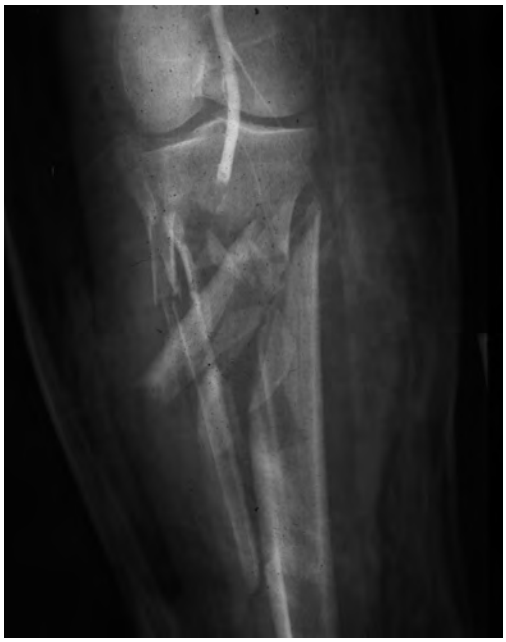

FIGURE 19.1: Severe comminuted fracture of the tibia and fibula with distal popliteal artery injury demonstrated angiographically. An injury with a high risk of acute limb compartment syndrome.

The anatomical arrangement of the capillary beds and physiological processes occurring within these also contributes to the development of LCS. Figure 19.2a shows the normal anatomical arrangement and physiological passage of fluid across the capillary wall. Fluid exchange across a capillary wall is affected by hydrostatic pressure and oncotic pressure. The extra-cellular compartment has low hydrostatic and oncotic pressure due to the draining action of lymphatics. In capillaries hydrostatic and oncotic pressure varies along their length. At the arterial end of the capillary, hydrostatic pressure (35mmHg) is greater than tissue hydrostatic pressure (0mmHg). Oncotic pressure within the capillary (28mmHg) is higher than tissue oncotic pressure (3mmHg) (therefore acting to draw fluid back into the capillary) but since the net hydrostatic pressure acting to filter fluid out of the capillary is greater than the net oncotic pressure, fluid is filtered into the extra-cellular space. As blood passes along the capillary to the venous end fluid is lost. At the venous end of the capillary, hydrostatic pressure is much lower (15mmHg) and the resultant net force now acts to draw fluid back into the capillary (net oncotic pressure is greater than the net hydrostatic pressure).

Aetiology/Pathophysiology

LCS causes tissue damage due to ischaemia within the affected compartment. This ischaemia is not due to interruption of the regional blood supply but due to the failure of the micro-circulation caused by the elevated compartment pressure (the initial insult may, of course, be due to regional ischaemia). The initial injury causes swelling of the tissues within the compartment that, in turn, results in increased intra-compartmental pressure (ICP).

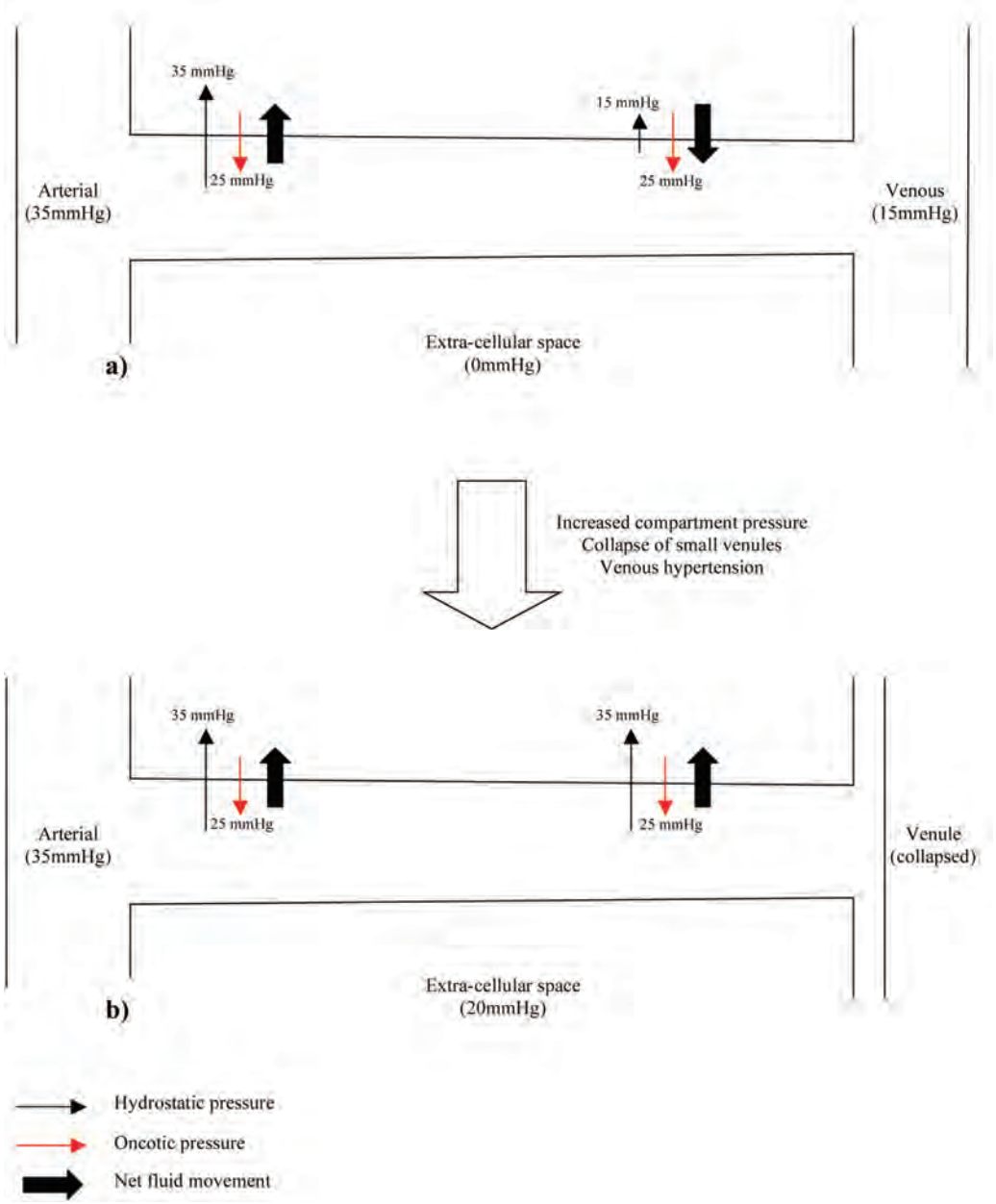

FIGURE 19.2: Capillary fluid exchange, **a)** in normal physiological circumstances, **b)** in the case of raised extracellular tissue pressure, as in acute limb compartment syndrome.

As ICP increases above physiological levels the first part of the circulation to be affected are the small venules, since these vessels have the lowest pressure. These venules collapse and since the arterial side of the capillary beds remains open, the hydrostatic pressure within the capillaries continues to filter plasma out of the blood vessels into the tissues. However, since the venous side of the capillary bed is closed, the normal return of extracellular fluid back into the circulation by combined oncotic and hydrostatic forces

cannot occur and actually reverses due to the venous hypertension in the capillary (Figure 19.2b). Lymphatic drainage is also impaired by the high tissue pressures. The net result is further tissue swelling, with a subsequent further increase in ICP and thus the initiation of a vicious cycle. As ICP increases further the arterial side of the capillary bed and eventually the arterioles become affected causing frank ischaemia and permanent tissue damage shortly follows.

In the initial stages of LCS the principal pathological changes in muscles affected is oedema within and around muscle fascicles. As LCS progresses frank ischaemic changes occur in the muscle fibres.[8]

The most common cause of compartment syndrome in vascular surgery is tissue oedema due to the ischaemia-reperfusion injury caused by limb revascularisation. The re-establishment of a blood supply to ischaemic tissues has been observed to cause more damage than ischaemia alone. During ischaemia, anti-oxidant mechanisms are capable of dealing with any oxygen free radicals produced. However, the return of oxygenated blood to ischaemic tissues results in a burst in production of oxygen free radicals due to the action of xanthine oxidase (XO) on tissue hypoxanthine, both of which are produced in ischaemic tissues. The return of oxygen upon reperfusion supplies the final substrate for this reaction to proceed and this produces a burst of superoxide production (Figure 19.3). Free radicals are produced from this superoxide and mediate cell damage largely via lipid peroxidation of cell membranes. Oxygen free radicals also cause activation of microvascular neutrophil polymorphs and endothelial cells. Activated endothelium produces arachidonic acid metabolites, nitric oxide (NO), endothelins, complement and cytokines. These various mediators contribute to the continuation and extension of a local inflammatory response and the production of a cellular and acellular inflammatory infiltrate with corresponding tissue swelling (Figure 19.4).[9]

Clinical presentation

Different tissues within the osteo-fascial compartments of the limbs are able to tolerate ischaemia to different degrees. The most sensitive to ischaemia are unmyelinated nerve fibres followed by myelinated nerve fibres, skeletal muscle, skin and then bone. Large arteries are relatively resistant to acute hypoxia and blood flow within them is maintained until late after the onset of LCS since ICP only exceeds systemic blood pressure at the latter stages of the disease process. It is these different hypoxic tolerances of each tissue that lead to the symptoms and clinical signs of LCS.

The most common symptom of LCS is severe pain that is unresolved by analgesics and the degree of which is out of proportion to the injury sustained. Symptoms due to neurological dysfunction include weakness and paraesthesia in the myotomes and dermatomes associated with the peripheral nerves that pass through the affected compartment. These symptoms will progress steadily over a short period of time.

Clinical signs associated with LCS are pain on passive movement of the muscles in the affected compartment, tenderness of the muscle bellies lying within the affected compartment and tenseness of the compartment. There may be muscle weakness and sensory loss (particularly two-point discrimination due to the early loss of the unmyelinated nerve fibres). Commonly foot drop occurs in LCS affecting the lower leg. There may be signs of the injury that has caused LCS to develop such as a fracture, surgical dressings or bruising. Peripheral pulses will be maintained until long after LCS has become established. Signs suggestive

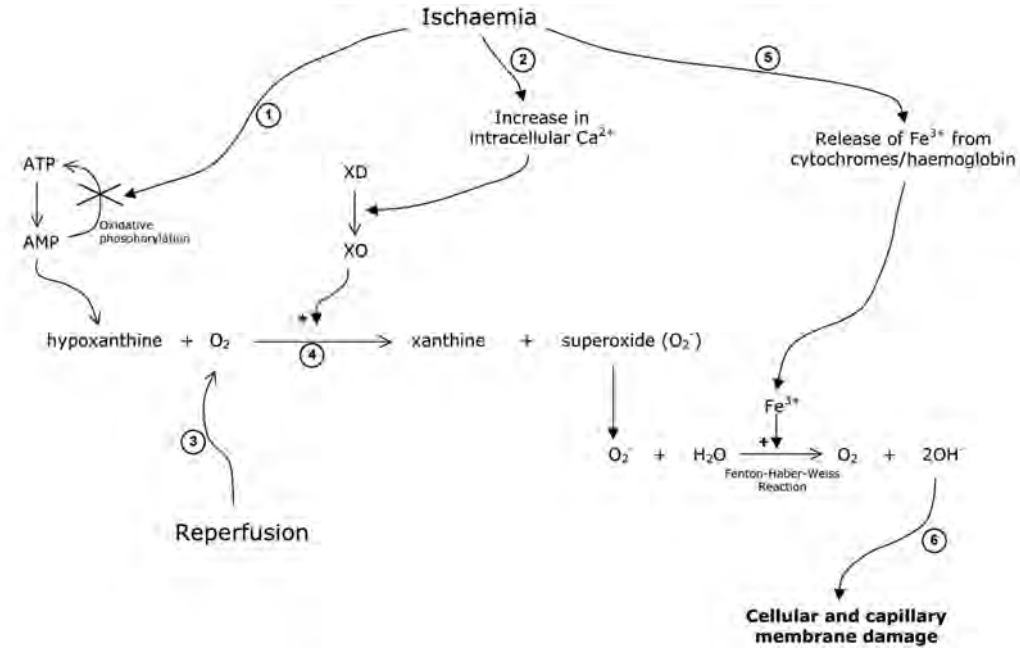

FIGURE 19.3: Xanthine Oxidase (XO) pathway activation by ischaemia-reperfusion and the production of reactive oxygen species. Ischaemia prevents oxidative phosphorylation **(1)** and cellular ATP cannot be regenerated. This leads to the accumulation of AMP and, in turn, hypoxanthine. Ischaemia also causes the accumulation of intracellular calcium **(2)**. This catalyses the conversion of xanthine dehydrogenase (XD) to xanthine oxidase (XO) Upon reperfusion oxygen is supplied **(3)** and this provides the final substrate to allow XO to convert hypoxanthine to xanthine, producing superoxide as a by-product **(4)**. During ischaemia iron is released from cytochrome, haemoglobin and other haem containing molecules **(5)**. This iron catalyses the Fenton-Haber-Weiss reaction producing hydroxyl radicals from superoxide. These hydroxyl radicals damage to cellular and capillary membranes leading to loss of function and cell death **(6)**.

of irreversible ischaemic change include fixed, non-blanching skin staining or frank gangrene. In these cases therapy should be directed towards preventing systemic complications and death.

Clinical assessment of suspected LCS is difficult. The majority of symptoms and signs are only reliably assessed in a fully conscious patient; those at highest risk of LCS often have an altered level of consciousness due to the injury or operation that has placed them at risk. Also, many patients may have some of these clinical signs present due to the injury that has caused the LCS such as the pulseless, painful, paraesthetic limb of acute ischaemia. The most useful guide to LCS is to maintain a high index of clinical suspicion.

Investigation

Since clinical assessment of a limb at risk of LCS is difficult, a test to give an objective measurement of ICP is desirable. Many methods for measuring ICP exist. Described techniques are wick catheters,[10] slit catheters,[11] needle manometry[12] and infra-red spectroscopy (Figure 19.5).[13] These various methods all have relative advantages and disadvantages. Catheter techniques allow continuous monitoring of limbs at risk and are more accurate than simple needle manometry[10] but they are more complex and require prior training in their use. Needle manometry can be simply performed using an 18g needle connected

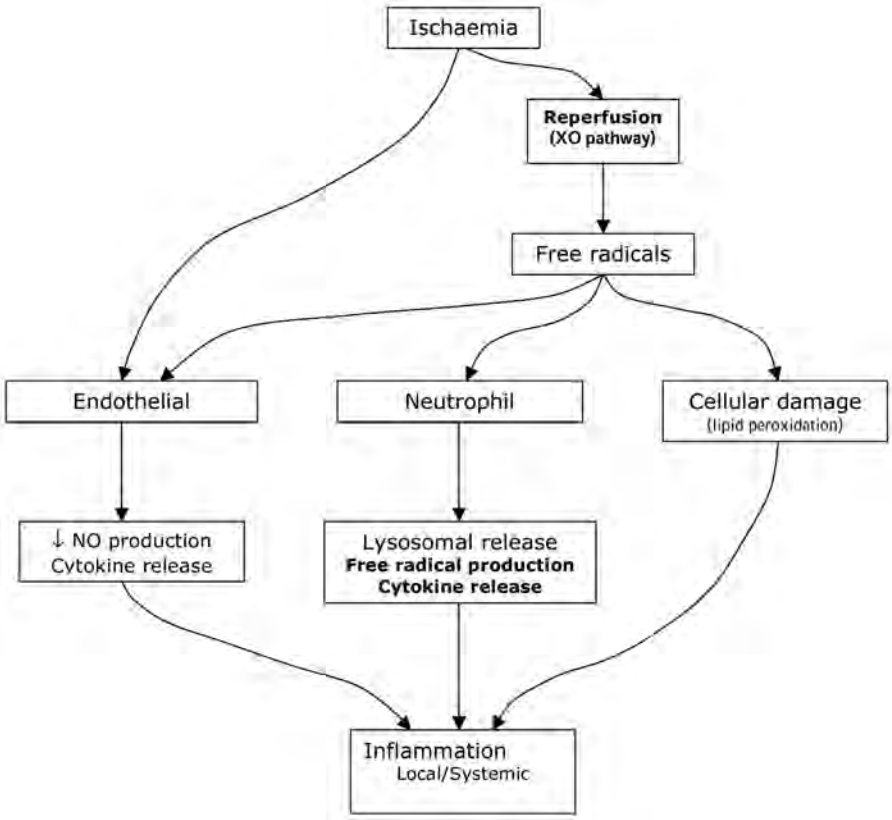

FIGURE 19.4: Pathways and effects of ischaemia-reperfusion injury. XO: xanthine oxidase, NO: nitric oxide.

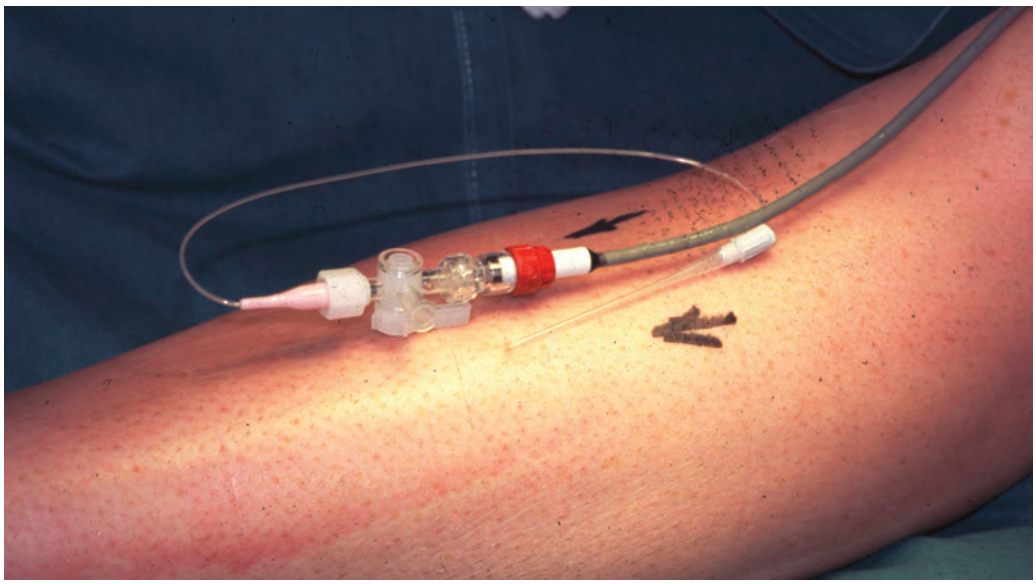

FIGURE 19.5: Slit catheter inserted into anterior tibial compartment and connected to a pressure transducer. (Photograph courtesy of Mr MJ Allen.)

to a mercury manometer, or small handheld electronic devices are available. Near-infrared spectroscopy is still under evaluation. Whilst this technique is non-invasive it requires relatively expensive equipment and the interpretation of the readings from this method are less intuitive than a figure for absolute compartment pressure expressed in mmHg.

Normal resting ICP is 0-10mmHg.[2,14] Capillary blood pressure varies from 30–40mmHg at the arterial side of the capillary bed to 10–15mmHg at the venous side.[15] Given the suggested pathophysiological processes underlying the development of LCS , it would be expected that symptoms would first occur at compartment pressures somewhere between these two values. Many authors have suggested absolute cut-off levels of ICP to diagnose LCS. Mubarak suggested a value of 30mmHg[16] whilst Allen suggested a value of 50mmHg or a value above 40mmHg for longer than 6 hours.[17] However, in several clinical studies clinical symptoms and signs of LCS have not correlated well with measured compartment pressures. Tissue perfusion is dependent, not only on the interstitial pressure but also the arterial perfusion pressure. Because of this, Whitesides et al suggested that the difference between diastolic blood pressure and ICP should be used to diagnose LCS, with a difference of less than 30mmHg being diagnostic.[18] It has been shown that this definition for diagnosing LCS results in less unnecessary fasciotomies that using absolute ICP levels of either 30mmHg or 40mmHg.[19] An alternative method is to calculate the difference between mean blood pressure and ICP, with a value of less than 40mmHg as a diagnostic cutoff.[20]

The measurement of ICP may allow more accurate diagnosis in those patients in whom clinical assessment is difficult due to co-existent injury or physical attributes but time should not be wasted on ICP measurement in patients who have clinically obvious LCS. In addition, many hospitals will not have the equipment or expertise available to accurately measure ICP. It is also thought that measured ICP may only reflect the ICP at the tip of the needle/catheter and not reflect the pressure change in the whole compartment. Since any delay in initiating treatment for LCS may result in a worse outcome once diagnosed, LCS should be treated expediently.

Apart from the measurement of ICP there are no specific tests to diagnose LCS. In clinically advanced cases where there is tissue damage, the resulting inflammation may be manifest as a leucocytosis or, if there is significant tissue necrosis, serum creatine phosphokinase will be elevated and a metabolic acidosis occurs.

Treatment

The treatment of acute LCS is urgent fasciotomy of all affected compartments (Figure 19.6). In patients who present acutely (within 12 hours of onset of LCS) this should be performed immediately. Delayed fasciotomy (longer than 12 hours after the onset of LCS) results in a significantly poorer outcome in terms of functional loss.[21] In those patients whose presentation is delayed, consideration has to be given as to whether the limb is unsalvageable and the possibility that a fasciotomy in this group of patients may lead to significant morbidity without ultimately improving functional outcome. If fasciotomy is delayed longer than 12 hours but less than 36 hours infection rates increase but limb salvage rates are similar.[22] Beyond 36 hours rates of amputation, infection, neurological injury and death increase such that early amputation rather than futile attempts at limb salvage should be considered in this group.[22,23]

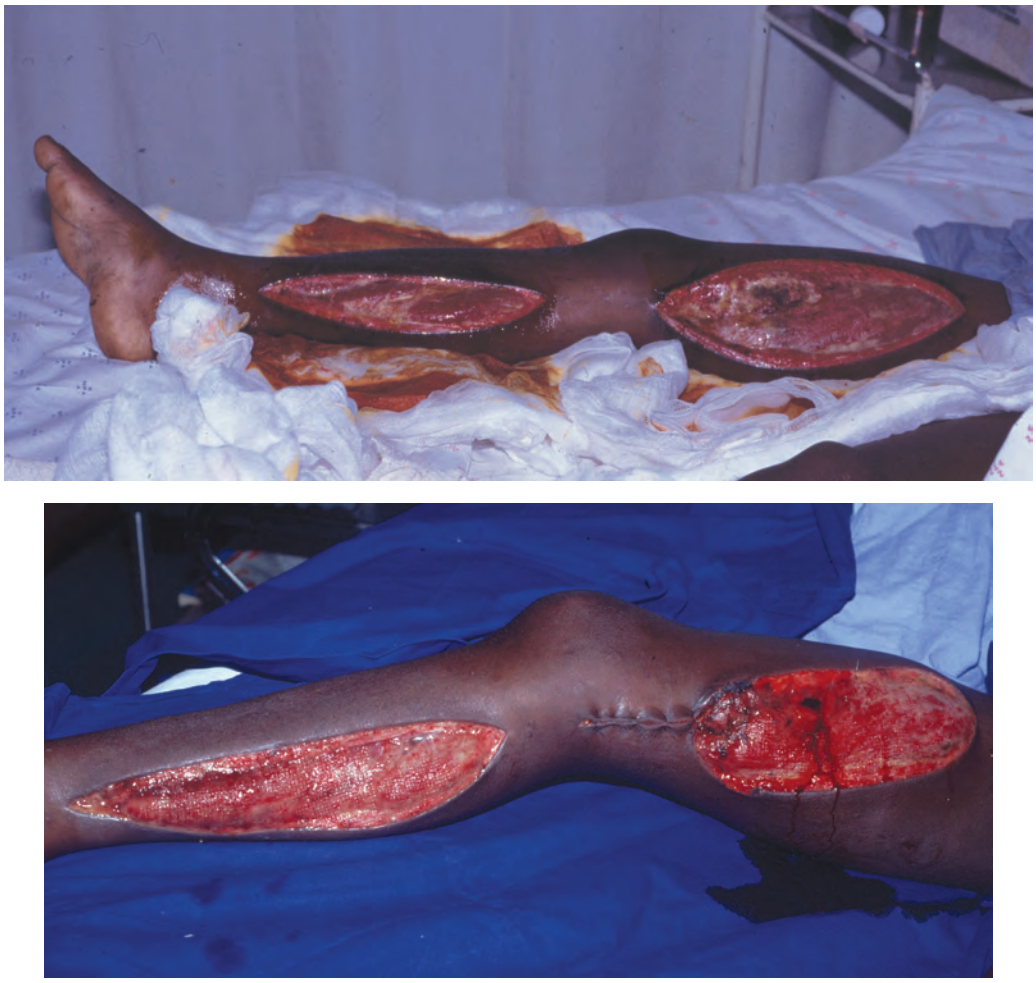

FIGURE 19.6: a) Medial thigh and calf fasciotomies following lower limb ischaemia after traumatic vascular injury. **b)** The same wounds 5 days showing healthy granulation tissue.

Fasciotomy should be performed in such a manner so that all constrictive elements surrounding a compartment are released. In the limbs this is the skin and the deep fascia, which encloses four separate compartments, the anterior, peroneal (lateral), superficial posterior and deep posterior. These can be decompressed either via a single lateral incision[24] or via two incisions, one lateral and one medial (Figure 19.7).[25] The lateral incision is made over the peroneal compartment one finger-breadth anterior to the fibula from the fibular head to the ankle. The anterior and peroneal compartments are then opened along the length of the incision. The posterior compartments can then be opened either through this incision by dissecting behind the fibula or by removing a piece of the fibula. Alternatively the deep posterior compartment can be opened by dissecting anteriorly to the fibula through the interosseous membrane. If two incisions are used the second is made on the medial aspect of the lower leg and the two posterior compartments decompressed. The thigh is usually decompressed medially and/or laterally.

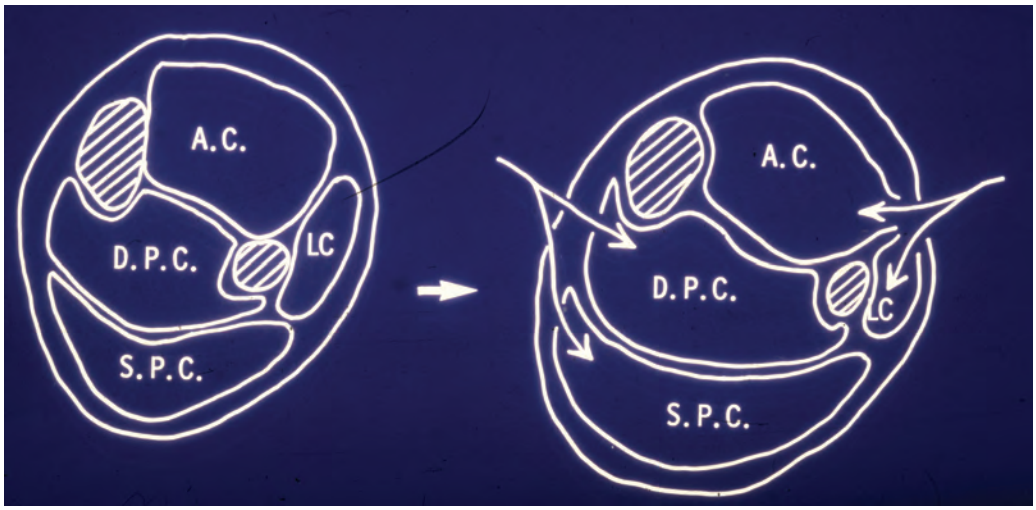

FIGURE 19.7: Diagrammatic cross-section through mid-calf showing four osteo-fascial compartments (left), arrows indicating medial and lateral incisions required for four-compartment fasciotomy. Shaded areas represent the tibia and fibula. **AC:** anterior compartment, **DPC:** deep posterior compartment, **SPC:** superficial posterior compartment, **LC:** lateral (peroneal) compartment.

In the upper limb the forearm is the most commonly affected by LCS. Both the volar and dorsal compartments can usually be decompressed via a single volar incision over the whole length of the forearm made in a curved fashion to avoid contractures. In other areas of the limbs the incisions should be made based on the anatomy of that region and positioned so as to open the whole length of the affected compartment. After the fasciotomy has been performed any devitalised or necrotic muscle should be debrided.

Several alternatives exist for the management of fasciotomy wounds after the compartment syndrome has resolved: skin grafting, delayed primary closure, secondary closure, healing by secondary intention and the use of skin flaps. Skin grafting is usually performed between 7 and 21 days and has been suggested to reduce wound complications when compared to other methods (Figure 19.8).[26]

In addition to fasciotomy, methods directed towards reducing the degree of initial tissue injury causing LCS have been suggested. Free radical scavengers such as mannitol and superoxide dismutase have been shown to be of benefit in LCS caused by ischaemia-reperfusion injury in animals.[27,28] Some benefit has been shown using these agents in humans[29] although no comparative studies between these treatments and fasciotomy have been performed. Lysine-acetyl-salicylate, a thromboxane A2 inhibitor has shown some benefit in animals but has not been studied in humans.[30] At present there is not enough evidence to justify the routine use of these compounds in clinical practice. Hyperbaric oxygen therapy has been suggested to be of use in improving the outcome of fasciotomy.[31]

Complications of LCS

Locally, LCS, if left untreated, will cause ischaemia and subsequent limb loss. If infection occurs in the devitalised tissues systemic sepsis can occur and may result in multi-organ failure. In addition to infective

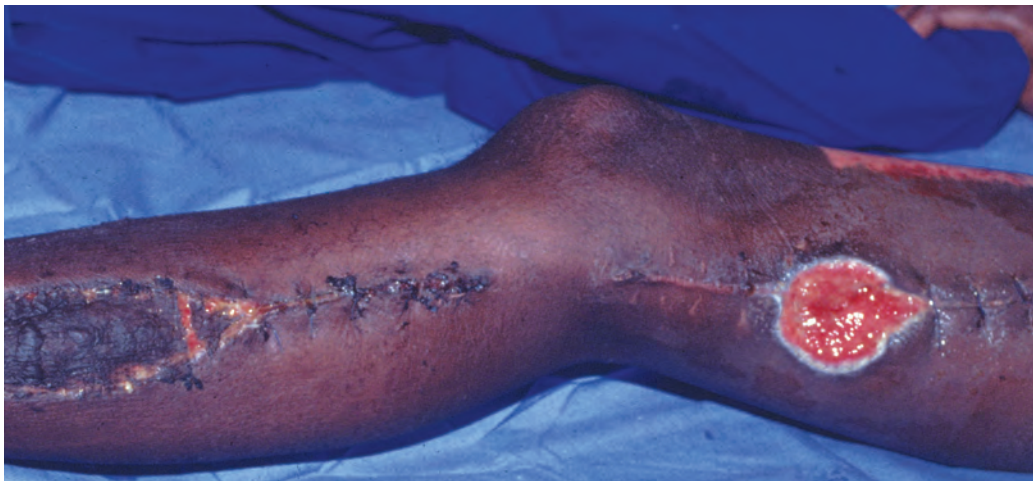

FIGURE 19.8: Split skin grafting to calf fasciotomy 2 weeks post-injury (same patient as figure 19.6). The thigh fasciotomy has been treated by delayed closure leaving a small defect to heal by secondary intention.

complications the metabolic consequences of a devitalised limb have to be considered. As skeletal muscle becomes ischaemic and necroses, myoglobin and potassium are released into the circulation. These have nephrotoxic and cardiotoxic effects if released in large enough quantities, and may cause remote organ dysfunction or failure.

Following decompression of LCS, devitalised muscle released myoglobin can cause renal failure due to tubular blockage. Treatment involves optimal fluid therapy and consideration of alkalisation of the urine using intravenous sodium bicarbonate, aiming for a urinary pH greater than 6.5 and a plasma pH of greater than 7.4.

In some situations, amputation above the level of ischaemia may be required, a procedure associated with a high risk of mortality and morbidity in an already severely compromised patient.

Outcome

Whilst fasciotomy wounds are associated with a moderate degree of morbidity,[26] fasciotomy does not appear to have any effect on long-term calf-pump function.[32] If fasciotomy is performed without delay in LCS the outcome in terms of preventing limb loss, systemic complications and long term functional disability is good.[33,34] Failure to promptly treat LCS risks the development of systemic complications such as multi-organ failure with a corresponding high risk of death.

ACUTE ABDOMINAL COMPARTMENT SYNDROME

The abdominal compartment syndrome (ACS) was first described by Kron in 1984.[35] ACS is a clinical syndrome characterised by progressive intra-abdominal organ dysfunction resulting from increased intra-abdominal pressure (IAP). In 2004, the World Society of Abdominal Compartment Syndrome gathered at the International ACS Consensus Definitions Conference in 2004 to produce internationally accepted definitions.[36,37] The consensus statement defined intra-abdominal hypertension (IAH) as IAP more than or equal to 12mmHg and ACS as a sustained IAP more than or equal

to 20mmHg that is associated with new organ dysfunction or failure. The severity of IAH was characterised into 4 grades (summarised in Table 19.1). The concept of intra-abdominal perfusion pressure (IAPP) was also defined by the society as mean arterial pressure minus the IAP, as a measure of net pressure available for perfusion of intra-abdominal organs. The normal value of IAPP is greater than 60mmHg.

Acute elevation of intra-abdominal pressure causes not only dysfunction of those organs within the abdominal cavity (hepatic, gastro-intestinal and renal dysfunction) but has effects on more distant organ systems such as the cardiovascular, respiratory and central nervous systems. Whilst it has most commonly been described following abdominal trauma,[38] vascular surgical procedures are the next most common cause.[39] ACS is important since it results in dysfunction of multiple organ systems in patients who are already significantly compromised and is a contributory factor in the development of multi-organ failure.

In addition to acute ACS, intra-abdominal pressure may become chronically elevated in patients with obesity or ascites. In this situation, the rise in intra-abdominal pressure occurs over a prolonged period of time and abdominal wall compliance increases concurrently, thus preventing the detrimental physiological effects of acute ACS.[40] This condition is largely irrelevant, except that it may result in falsely high readings when assessing acute changes in intra-abdominal pressure in these patients.

Incidence

Papavasiliou *et al*[41] reported that IAP was significantly higher after ruptured AAA (ruptured AAA) repair than either open or endovascular elective repair of non-ruptured AAA. In the ruptured AAA group, 55% developed IAP values of greater than 15mmHg. Djavani *et al*[42] reported that in their series of 17 ruptured AAA patients who underwent IAP monitoring, 9 (53%) patients developed IAP pressures greater than 20mmHg (IAH grade III and above) and 7 (41%) developed clinical ACS. However a limitation of this study was that IAP monitoring was restricted to complicated cases. As not all patients had IAP monitoring, the true incidence of ACS may be overestimated in their study. Mehta *et al*[43] reported a 20% incidence of ACS in their 30 patients who underwent endovascular repair for ruptured AAA, and management of this syndrome is assuming increasing importance after endovascular surgery for ruptured AAA. ACS is rare following elective aortic surgery.[44]

Aetiology

In a similar fashion to LCS, raised intra-abdominal pressure can occur as a result of increased intra-abdominal volume (either retroperitoneal or intra-peritoneal) or extrinsic compression, which is usually due to changes in the abdominal wall, either iatrogenic or pathological.

Expansion of retroperitoneal volume can be caused by traumatic bleeding, pancreatitis

TABLE 19.1: Intra-abdominal hypertension grades as defined by the World Society of Abdominal Compartment Syndrome

Grade I	IAP between 12 and 15mmHg
Grade II	IAP between 16 and 20mmHg
Grade III	IAP between 21 and 25mmHg
Grade IV	IAP greater than 25mmHg

or sepsis.[45,46] More commonly intra-abdominal volume expansion is caused by intra-peritoneal expansion either by traumatic or iatrogenic bleeding, peritonitis, visceral oedema, or intra-abdominal packing for uncontrollable haemorrhage.[47-51] Rarely visceral oedema can occur following non-abdominal trauma that is thought to be due to large volume fluid resuscitation.[52]

Extrinsic compression of the abdominal cavity can be caused by tight abdominal closure following laparotomy incisions, burns eschars, pneumatic anti-shock trousers and the repair of large hernias which results in an effective reduction of abdominal cavity volume.[51,53-55] In addition high intra-thoracic pressure may lead to high intra-abdominal pressure.[56]

The pathophysiology of ACS after ruptured AAA is multifactorial.[41,57-59] The space occupying effect of large retroperitoneal haematoma (primary ACS) is a significant factor contributing to IAH, as dictated by the inverse relation between pressure and volume. In endovascular treatment of ruptured AAA, any ongoing type II endoleak bleeding from the lumbar and inferior mesenteric arteries into the disrupted aneurysm sac may contribute to the size of the haematoma and exacerbate the existing IAH. This is aggravated in the setting of severe coagulopathy.[43] Furthermore, modifications in microvascular permeability associated with the shock state in ruptured AAA can lead to visceral and soft tissue oedema, worsening the IAH.[41,57-59] Secondary ACS (where pathology lies outside of the abdomen) is caused by massive fluid resuscitation-induced bowel oedema, ascites or through reperfusion injury associated with ruptured AAA.[60]

Pathological effects of raised intra-abdominal pressure

Elevated intra-abdominal pressure causes a reduction in mesenteric and hepatic arterial, intestinal and hepatic micro-circulatory, and portal venous blood flow.[61,62] This reduction in the visceral blood flow occurs at pressures as low as 10mmHg and further impairment occurs with further increases of intra-abdominal pressure. As visceral blood flow reduces, ischaemia occurs, resulting in impaired cellular respiratory function and subsequent cellular damage. The acidosis which follows can be assessed by gastric tonometry. Reduction in gastric pH has been shown to occur early in ACS and this can be reversed by abdominal decompression.[63] Whilst severe tissue damage has been shown to only occur at high intra-abdominal pressures (>40mmHg),[64] gastrointestinal bacterial translocation occurs at much lower pressures (25mmHg).[65] Since bacterial translocation has been implicated as a significant contributory factor to the development of multi-organ failure, this is an important effect of relatively low pressure ACS.

Renal impairment was one of the earliest noted effects of ACS.[35] Progressive deterioration in renal function occurs as intra-abdominal pressure increases. Oliguria occurs at pressures above 15mmHg whereas pressures of greater than 30mmHg cause absolute anuria.[44,66,67] Compression of the renal veins and direct renal parenchymal compression causes increased vascular resistance with a secondary reduction in renal perfusion.[46,66] These changes cause a reduction in glomerular filtration rate and a subsequent increase in renin, aldosterone and anti-diuretic hormone occurs. Further increases in renal vascular resistance occur as a result and lead to retention of sodium and water. Ureteral compression does not appear to cause renal dysfunction in ACS since the placement of ureteral stents has been shown not to improve renal function in ACS.[68]

Elevated intra-abdominal pressure affects not only those organs within the abdominal cavity but also has detrimental effects on

distant organ systems. Cardiac output decreases as intra-abdominal pressure increases as a result of decreasing preload and increasing afterload.[57] Preload is reduced due to direct compression of the abdominal inferior vena cava and compression of the superior vena cava due to increased intra-thoracic pressure as a result of direct transmission of elevated intra-abdominal pressure across the diaphragm. Elevated intra-thoracic pressure also directly compresses the heart, reducing end diastolic volume. All of the above results in reduced stroke volume. A compensatory increase in heart rate occurs which only partially restores cardiac output.[69] The reduced cardiac output caused by ACS also causes further impairment of renal function beyond that caused by ACS alone.

Respiratory dysfunction also occurs as intra-abdominal pressure increases.[69] Direct transmission of elevated intra-abdominal pressure across the diaphragm causes elevations in intra-thoracic pressure, which in turn increases pulmonary vascular resistance. The volume of the thoracic cavity is also decreased due to elevation of the diaphragm, compressing the lungs. This compression results in reduced lung volume and compliance.[70] These changes in the vasculature and physical properties of the lungs reduce respiratory function.

Raised intra-abdominal pressure causes increased intra-cerebral pressure and reduced cerebral perfusion that is thought to be due to reduced cerebral venous drainage.[71,72] Also, abdominal wall blood flow is reduced in ACS due to direct compression and leads to ischaemia and muscle swelling.[73] This, in turn, reduces abdominal wall compliance, exacerbating ACS.[74]

Clinical presentation

Clinical evaluation of patients with ACS is not reliable[75] and the only physical sign due to ACS per se may be a tense, distended abdomen. The majority of clinical signs of ACS are due to the compromise of those organs systems affected by ACS – respiratory, renal, gastrointestinal and cardiovascular dysfunction. The classic collection of clinical presentations associated with ACS includes a tense abdomen on physical exam with oliguria and increased airway pressure.[35] However these signs and symptoms are extremely nonspecific in critically ill patients, in whom ACS occurs most frequently. Critically ill patients frequently undergo large-volume fluid resuscitation and therefore commonly have impaired tissue perfusion, hypotension, and oliguria. Acute lung injury or pulmonary oedema are often seen in these same patients, either of which may result in increased airway pressure.[76] Clinical examination therefore has a limited role in diagnosing ACS. In these patients the most important factor to consider is a history of an abdominal injury or intervention that places them at risk of developing ACS leading to active monitoring of IAP to detect ACS.

Investigation

The investigation of choice in a patient with suspected ACS is the measurement of intra-abdominal pressure. The most commonly applied technique is that described by Kron.[35] This utilises an indwelling urinary catheter to obtain a direct measurement of intra-vesical pressure and has been shown to correlate well with intra-abdominal pressure.[77] 50ml of saline is introduced into the bladder via the aspiration port of the catheter which is clamped distal to this point. After allowing the pressure within the bladder to equilibrate with that in the abdominal cavity a pressure transducer (such as that used for measuring central venous pressure) is attached to an 18g needle inserted into the aspiration port and the pressure measured using the symphysis

pubis as a reference point ('zero'). The intra-abdominal pressure can then be measured. Modifications to this technique have been proposed to avoid the repeated disturbance of a closed system with the potential to introduce infection.[78] This method has been shown to be inaccurate at low pressures (<15mmHg).[79] Alternative techniques include intra-gastric pressure measurement[80] and inferior vena caval pressure measurement via femoral vein catheterisation.[81] Whilst gastric pressure has shown good correlation with bladder pressure measurements at low intra-abdominal pressure neither of these methods have been validated against the high bladder pressure measurement in humans with established ACS.[82]

Treatment

The principles of management for ACS are prevention, early recognition and definitive treatment of fully manifested ACS by surgical decompression. Prevention of ACS involves identification of at-risk patients by appreciating the risk factors, setting, and pathogenesis of ACS. Risk factors for ACS in vascular cases are massive fluid resuscitation (>5L/24hr), sepsis or bacteraemia, mechanical ventilation including use of positive end expiratory pressure, polytransfusion (>10U packed red blood cells/24hr) and acidosis.[37] Intra operative risk factors for grade III or IV IAH for open ruptured AAA repair were noted to be longer cross clamping time, increased operative bleeding and increased operative time,[42] and the following risk factors identified for endovascular repair of ruptured AAA: use of aortic occlusion balloon, presence of severe coagulopathy, massive transfusion requirements, and the emergent conversion of modular bifurcated stent grafts to aorto-uniiliac devices.[43] Presence of these risk factors should alert the clinician to more aggressive monitoring of IAP and the introduction of preventive measures before full-scale ACS develops. In general the at-risk patients in whom IAP should be monitored are detailed in Table 19.2 as set out by the World Society of Abdominal Compartment Syndrome.[37]

There are various nonsurgical treatment options available for dealing with elevated IAP[83] to prevent manifestation of ACS. Some of these methods (nasogastric decompression, effective pain management and sedation) are used routinely in critically ill patients. Body positioning (avoidance of acute flexion at the hips and reverse Trendelenburg) can relieve pressure on the abdomen. Neuromuscular blockade can alleviate abdominal wall tensions leading to dramatic decrease in IAP.[84,85] This can be administered quickly and safely to intubated ICU patients[84,85] and is a useful adjunct to other nonoperative measures, allowing measures such as removal of excess

TABLE 19.2: Indications for intra-abdominal pressure monitoring as defined by the world consensus definitions

Post operative abdominal surgery with a distended abdomen
Abdominal trauma
Mechanically ventilated patients with other organ dysfunction
Patient with a distended abdomen and signs or symptoms consistent with ACS and including oliguria, hypoxia, hypotension, unexplained acidosis, mesenteric ischaemia, elevated intracranial pressure
Patients with an open abdomen or abdominal packing after temporary closure
Patients who have undergone massive fluid resuscitation, secondary to fluid loss due to leaky capillaries

fluid to become effective. Rectal decompression with enemas or rectal tubes, and prokinetic agents may potentially be used in selective cases such as suspected toxic megacolon or inflammatory bowel syndrome.

Other preventive measures include avoidance of excess fluid resuscitation therapy. The end points of resuscitation need to be accurately determined and unnecessary over-vigorous fluid administration should be averted by meticulous and precise intensive care monitoring.[86] Fluid resuscitation should be goal-directed and titrated aggressively against achieving end points such as decrease in lactate, adequate mixed venous oxygen saturation and reductions in base deficit. Another important preventive measure against ACS is the use of prophylactic delayed abdominal closure and maintenance of an open abdomen. Delayed wound closure in ruptured AAA repair patients in whom primary closure was not possible demonstrated a trend towards decreased mortality from 73% in primary closure to 50% with delayed closure.[87] An in-vitro abdominal simulation model demonstrated that the 'Bogota bag' technique which involves utilising a sterile fluid administration bag, cut flat and sewn to close the defect was most effective at preventing increases in abdominal pressures and proposed that vacuum dressing is contraindicated in the initial phase following decompression.[88] However vacuum-pack temporary closure has advantages of ease of mastery, effectiveness in patient care and comfort, consistently low associated complication rate, and low cost in both general and vascular surgery and trauma patients. A series involving 258 surgical patients undergoing open abdomen management with the sutureless negative pressure dressing reported an overall primary fascial closure rate of 68.4%.[89] It is still imperative that the ICU team avoid overresuscitation in patients who are admitted to the ICU with a temporary abdominal closure. Monitoring the IAPP during this period may provide guidance and if it is not possible to maintain an IAPP >60, then additional surgical intervention may be necessary.

The definitive treatment of ACS is expedient decompressive laparostomy. This has been shown to rapidly and effectively reverse the detrimental effects of ACS in the gastrointestinal, renal, cardiovascular, respiratory and central nervous systems.[45,46,57,69,90-92] It has been shown that in clinically unstable patients with ACS, surgical decompression can be safely performed at the bedside in the ICU.[93] Temporary containment of the abdominal contents is achieved using a mesh (silastic, polypropylene or polygalactin), plastic bag (intravenous fluid container or bowel bag) or vacuum systems (Figure 19.9a). Alternatively the skin can be closed leaving the fascia un-apposed. Following formation of the laparostomy consideration has to be given as to the method used to close the resulting defect. After the patient has recovered from the organ dysfunction associated with ACS delayed primary closure can be considered. The rate of unsuccessful closure of the abdomen has been reported between 20% and 78%.[94,95] Coverage of the bowel, prevention and management of enterocutaneous fistulas, and management of large ventral hernias are some of the potential problems of an open abdomen. The incidence of failed closure and additional complications increases with delay and therefore efforts should be made to close the abdomen as soon as the underlying cause of the ACS has been dealt with. This window of opportunity is in general about 7 days but varies greatly from patient to patient, and actual timing should be tailored to the individual assessment of the abdomen. After this time the body's attempt to heal secondarily will lead to development of adhesions and early granulation tissue culminating in a 'frozen abdomen' that prevents

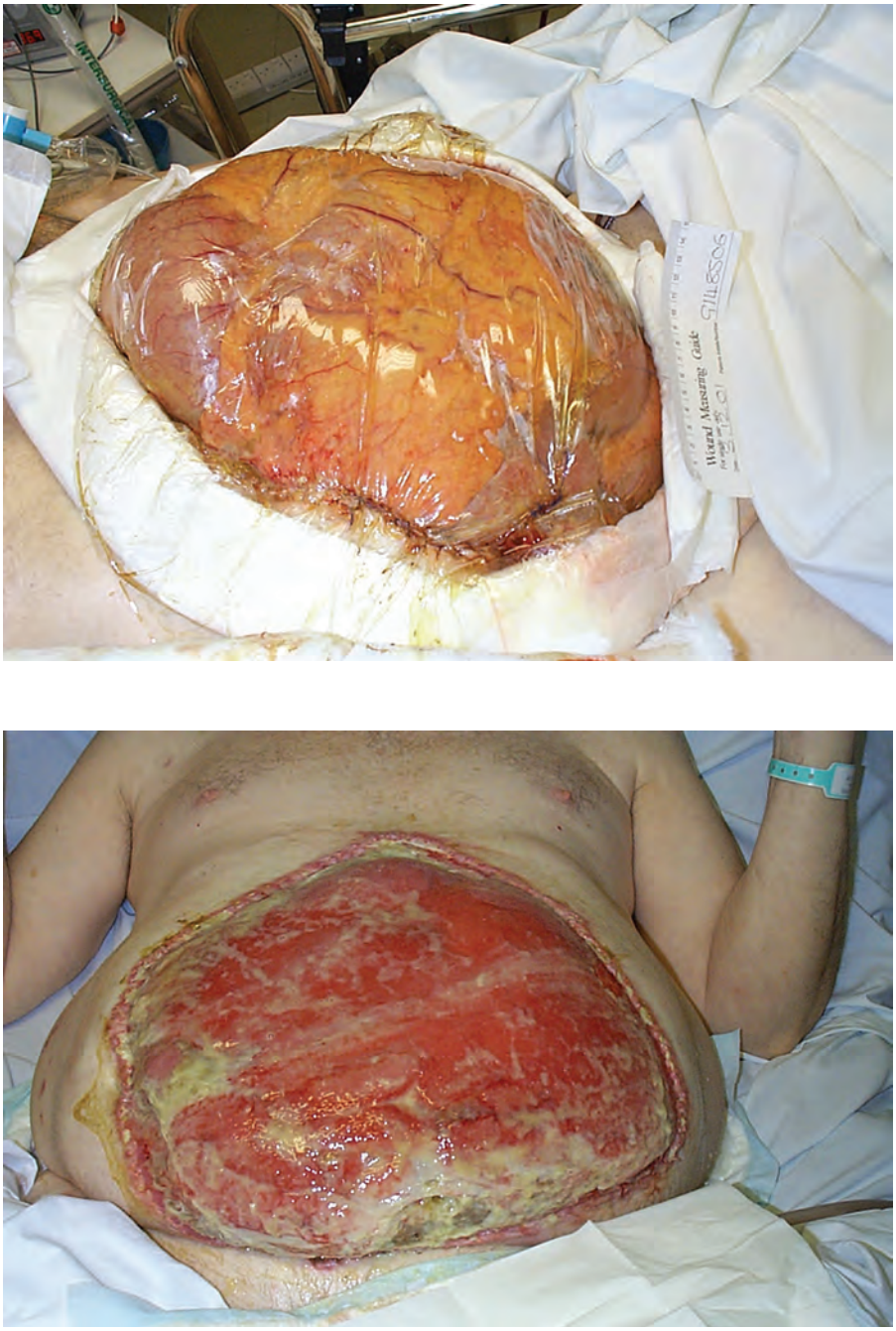

FIGURE 9: a) Immediate containment of abdominal contents using plastic bowel bag after decompressive laparostomy following ruptured abdominal aortic aneurysm repair. **b)** The same wound after 4 weeks, the viscera having granulated over.

fascial closure. In cases where primary closure of the fascia is not possible, the abdominal contents should be left to granulate over and then either heal by secondary intention or skin grafts applied (Figure 19.9b). The resultant ventral hernia will then need to be repaired at a later date.

Normal intra-abdominal pressure is less than 10.5mmHg in men and 8.8mmHg in women.[96] Historical studies[35,50] that proposed threshold pressures above 25mmHg to start definitive decompressive treatment have largely been disregarded. In patients with ruptured AAA, Papavassiliou *et al*[41] demonstrated that an IAP threshold of 15mmHg was associated with significant physiological dysfunction, lower than ACS of other aetiology. The algorithm for the assessment and management of patients after abdominal aortic aneurysm surgery as proposed by Loftus *et al*,[58] suggested immediate bladder pressure measurement of IAP in patients at risk after surgery and if the pressure is greater than 20mmHg, abdominal closure should be delayed. Following surgery patients should be monitored, if the IAP rises above 30mmHg urgent decompression should be mandatorily performed. In those with IAP between 21 to 30mmHg, urgent decompression should be considered. In those with IAP between 16 and 20mmHg urgent decompression should be considered in the presence of organ dysfunction. In those patients with IAP less than 15mmHg physiological support should be continued. Ganeshanatham *et al*[60] proposed a lower threshold of IAP more than or equal to 25mmHg for which to perform mandatory decompression in post-operative vascular patients. At IAP of between 16 and 25mmHg, these authors suggested decompressive laparotomy if there has been no recovery of deranged physiological parameters despite optimisation.

Complications of surgical decompression

Much of the morbidity of ACS is due to the decompression surgery itself. Once healed the patient is frequently left with a large ventral hernia. With mesh closures, entero-laparostomy fistulas are not uncommon (7%) and the rate of mesh dehiscence from the fascia or skin was 22%.[97] Enterocutaneous fistula arose in 5% of patients with negative pressure vacuum dressings.[89] Infection is a potential complication of the temporary abdominal wound closures and this risk increases if more than one dressing application is needed.[89] The temporary open wounds are not only an obvious point of entry for pathogens but are also a potential source of excessive fluid loss. In hypovolaemic patients, decompression may result in haemodynamic instability. Rebleeding can occur if coagulopathies are not reversed prior to the decompression. The increased pulmonary compliance from rapid reduction in IAP can lead to increased minute ventilation resulting in a respiratory alkalosis. The toxic metabolites entering the systemic circulation can precipitate a cardiac event as with any reperfusion injury.[60]

Outcome

ACS is associated with an overall high mortality of between 60% and 70%, not solely due to the development of ACS itself but in addition to the insult that caused it.[98,99] A combined analysis of 18 papers with total of 250 patients who underwent decompressive surgery demonstrated that surgical decompression significantly reduced the mean IAP from 34.6mmHg to 15.5mmHg with associated improvements in respiratory function and cardiac output.[100] With an average mortality of 49.2%, it has not been

definitively demonstrated whether surgical decompression for ACS confers any beneficial overall effect on survival although in many studies, ACS without intervention results in mortality approaching 100%. Given this consideration and the potential risks of decompressive surgery, the best treatment for ACS is therefore prevention where possible.

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20 • Pathophysiology of Pain

STEPHAN A. SCHUG, HELEN C. S. DALY,
KATHRYN J.D. STANNARD

School of Medicine and Pharmacology, University of Western Australia, Royal Perth Hospital, Perth, WA.

INTRODUCTION

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage.[1]

The mechanism by which a damaging stimulus in the body is perceived as painful by the brain is a complex one which is not yet fully understood. The complexity of the process results from the nervous system not being a 'hard wired' system, but exhibiting plasticity that enables it to modify its function under different conditions.

As shown by the definition, pain serves the purpose to prevent tissue damage and protect the body while it is healing. Under certain conditions, pain can become maladaptive and persist as chronic pain. This pain serves no protective function and is described as pathological pain as opposed to physiological pain;[2] it is then no longer a symptom of another disease, but a disease in its own right.[3] Another term for pathological pain has been suggested recently: dysfunctional pain.[4]

In order to adequately treat physiological, but even more pathological pain, an understanding of pain mechanisms is required.

PERIPHERAL MECHANISMS

Nociception/Transduction

Painful stimuli are detected by nociceptors, which are free nerve endings located in tissues and organs. They have high thresholds and, under normal circumstances, only respond to noxious stimuli.

There are two distinct types of nociceptors

- High threshold mechanoreceptors which stimulate small myelinated Aδ-fibres and transmit a well-localised sharp or pricking sensation that lasts as long as the stimulus.
- Polymodal nociceptors that stimulate small unmyelinated slowly conducting C fibres. As well as responding to mechanical stimuli they are activated by thermal and chemical stimuli e.g. hydrogen ions, potassium ions, bradykinin, serotonin, adenosine triphosphate and prostaglandins.

The ion channels for noxious stimuli have been partially identified; the transient receptor potential (TRP) family of these ion channels and in particular the vanilloid-type

TRP 1 (TRPV1) have been studied in most detail.[5] This receptor is sensitive to higher temperatures, acidity and capsaicin, an exogenous ligand (extract of chilli pepper) and receptors like this one are currently investigated as therapeutic targets for pain therapy.

Nerve growth factor (NGF) is also involved in the transduction process, as it binds to its receptor TrKa and thereby triggers increased transduction in pain states, in particular inflammatory pain. A monoclonal antibody against NGF, tanezumab, has shown very promising effects in early stage trials in osteoarthritis and chronic low back pain.[6]

Conduction

Voltage-gated sodium channels mediate conduction along primary sensory afferents. As for all other impulses throughout the body, action potential propagation is dependent on these channels. There are two types of sodium channels, differentiated by their sensitivity to tetrodotoxin. Both types are present in nociceptive neurons, with the tetrodotoxin-resistant type only present in nociceptors, which makes it a potential target for novel analgesics. Further research has identified two such sodium channels, labelled NaV1.7 and NaV1.8, which seem to have a specific role in pain modulation.[7] Mutations of these channels are linked to congenital insensitivity to pain and erythromyalgia and attempts are made to identify blockers of these channels, which might be therapeutic in chronic or neuropathic pain.

Nociceptors also have voltage-gated calcium channels, which are found on the presynaptic membrane and are involved in neurotransmitter release at the dorsal horn. These are modulated by alpha-2-delta compounds such as gabapentin and pregabalin, new first-line treatments of neuropathic pain and central sensitisation.[8]

Pain is transmitted by primary afferents, which have their cell bodies in the dorsal root ganglion (DRG). They terminate in the dorsal horn of the spinal cord. The dorsal horn cells are divided into specific regions or laminae called Rexed's laminae with lamina I being the most superficial.[9]

- Aδ-fibres are fast conducting and transmit the first sharp pain on initial stimulation. They terminate mainly in lamina I, but also send some fibres to lamina V of the dorsal horn where they synapse with second order neurones. They contain the neurotransmitter L-glutamate.
- C fibres are unmyelinated slow conducting fibres which transmit a less well localised persistent aching pain that lasts after the initial stimulus has gone. They terminate in lamina II of the dorsal horn. As well as glutamate they contain several other neurotransmitters including neuropeptides, such as substance P, and calcitonin gene-related peptide (CGRP), cholecystokinin, brain-derived neurotrophic factor and glial-derived neurotrophic factor. C fibres express several presynaptic receptors that modulate transmitter release. These include cholecystokinin (CCK), opioid and gamma-aminobutyric acid subtype B (GABA B) receptors. Apart from the CCK receptor, they inhibit the release of transmitter.
- Aβ-fibres conduct low intensity mechanical stimuli which convey touch and not pain, however in chronic pain states they are involved in the transmission of pain. They terminate deeper in the dorsal horn in laminae III-VI.

SPINAL CORD MECHANISMS

Primary sensory afferents terminate in the spinal cord where they synapse with cells

of the dorsal horn. Nococeptive specific neurons are located mainly in laminae I and II but also lamina V and respond only to noxious inputs under normal conditions

There are a number of different cells involved in the relay of painful stimuli including nociceptive specific cells and wide dynamic range neurons. Wide dynamic range neurons are located mainly in lamina V, but also in III and IV to a lesser extent, where they respond to stimuli from Aβ-, Aδ- and C-fibres.[9]

The cells of the dorsal horn involved in nociception express a number of receptors.

- AMPA (a-amino-3 hydroxy-5-methylisoxazole) receptors which bind glutamate.
- NMDA (N-methyl-D aspartate) receptors which also bind glutamate, neurokinin receptors NK-1 which bind substance P.
- GABA-A receptors which are ligand-gated calcium channels that hyperpolarize the cell and reduce responsiveness to stimulation.
- Voltage-gated calcium channels.
- Glycine receptors that provide an inhibitory function

The ability to detect a potentially damaging noxious stimulus is mediated by glutamate acting on the AMPA receptor following stimulation of Aδ-fibres. The other receptors and neurotransmitters are involved in the modulation of the response.

When a high intensity noxious stimulus arrives at the dorsal horn via C-fibres, initially glutamate is released which acts via the AMPA receptor. As stimulus intensity increases, then other neurotransmitters are released such as Substance P. Slow post-synaptic currents are set up which are mediated by a number of receptors including the NMDA receptor. These are also involved in the modulation of the pain response.[10]

Ascending systems

Noxious information is conveyed from the dorsal horn to the brain via several ascending tracts in the spinal cord. The majority of the wide dynamic range neurons and nociceptive specific neurons are conveyed anterolaterally in three pathways:[11]

- The spinothalamic tract: Its fibres cross over to the contralateral side and pass through the brainstem to nuclei in the thalamus, finally terminating in the somatosensory cortex where pain is perceived and localised.
- The spinoreticular tract: It terminates in the reticular formation and has projections, which terminate in the pons, medulla and periaqueductal grey matter. It is involved in descending inhibition of pain.
- The spinomesencephalic tract: It is also involved in the modulation of descending control.

Descending control

The dorsal horn receives inputs from higher centres that modulate the response to nociceptor input.[12]

The descending control of output from the dorsal horn comes mainly from areas in the brainstem, namely the periaqueductal grey matter, the raphe nuclei and the locus coeruleus.[13] Inhibitory tracts descend in the dorsolateral fasciculus and synapse in the dorsal horn. The key neurotransmitters involved are noradrenaline and serotonin. Noradrenaline acts via post synaptic α-2 receptors, the action of serotonin is less specific. Endogenous opioids are also involved in descending inhibition at a spinal and supraspinal level.[13] These endorphins and enkephalins acting via the descending system are thought to be responsible for the analgesia induced by stress.

As well as descending control from the brainstem, nociceptive impulses are also attenuated by input via Aβ-fibres (transmitting information on touch), which is the basis for the use of Transcutaneous Electrical Nerve Stimulation (TENS) for analgesia, but also for simply rubbing a hurting body part. This observation formed the basis for the initial gate-control theory of pain.[14]

PAIN MODULATION

The above description of pain explains the initial sensation of pain immediately following injury, however it does not explain the more complex phenomena associated with pathological pain due to neuroplastic changes.

These phenomena have a number of different causal mechanisms, which occur initially in the periphery, but later mainly in the dorsal horn as the main site modulation of painful stimuli.

Peripheral sensitisation

Tissue injury results in the release of inflammatory mediators, such as bradykinin, histamine, K+, H+, 5-Hydroxytryptamine (5-HT), ATP and nitric oxide from damaged cells.[15] Breakdown of arachidonic acid by cyclo-oxygenase produces leukotrienes and prostaglandins. Immune cell activation results in the release of further mediators including cytokine and growth factors. These mediators provide an 'inflammatory soup' which produces a painful area of primary hyperalgesia. These inflammatory mediators spread into the tissues surrounding the initial area of injury to produce an area of secondary hyperalgesia.

They act either by stimulating nociceptors themselves or by acting via inflammatory cells to stimulate release of additional pain inducing agents. They also modify the response of primary afferents to subsequent stimuli either by changing the sensitivity of the receptors or by modulating the voltage-gated ion channels. For example, after tissue and nerve injury, N-type calcium channels become more active resulting in greater release of glutamate in the spinal cord.[16] The magnitude of the current generated by sensory-neuron specific sodium channels is also increased.

Chronic inflammation and also nerve injury has an effect on the presence and distribution of voltage-gated sodium channels, which can become concentrated in areas of injury and produce ectopic discharges. Sensory-neurone-specific sodium channels have a significant role in chronic pain states. Studies have shown them to become concentrated in neurones proximal to a site of nerve injury and play a role in the hyperalgesia and allodynia of chronic pain states.[9] In addition, NGF binding to TrKa receptors increases peripheral sensitivity as discussed before.[6]

Not all sensory neurons are active all the time and this peripheral sensitisation will recruit 'dormant' nociceptors, thus increasing the receptive fields of dorsal horn neurons and increasing the intensity and the area of pain.[17]

Central sensitisation in the dorsal horn

Central sensitisation is an increase in the excitability of the dorsal horn so that the dorsal horn cells have a lower threshold and respond to low intensity stimuli that are not usually painful. It also results in a greater response to supra threshold stimuli thus producing the symptoms of allodynia and hyperalgesia.

There are several mechanisms, which occur at the dorsal horn and contribute to

chronic pathological pain states by central sensitisation. These will be discussed in the context of neuropathic pain, as they are most relevant there.

NEUROPATHIC PAIN

Neuropathic pain arises following disease or injury to nerves from a number of aetiologies eg, ischaemic, traumatic, infection. Characteristics of neuropathic pain include spontaneous stimulus-independent pain and pain that is stimulus dependent and exhibits the features of allodynia and hyperalgesia. There are a variety of different mechanisms responsible for the generation of symptoms, which may be quite different from patient to patient.[18]

Mechanisms of neuropathic pain

The pathophysiology of neuropathic pain involves central and peripheral mechanisms and is in principle a 'maldaptive response of the nervous system to damage'.[4] Usually more than one mechanism may be involved and producing a unifying hypothesis for all neuropathic pain states is inappropriate.[19]

Peripheral mechanisms

Electrophysiological evidence over the last 25 years shows that activity in sensory neurones after injury is necessary for the development of neuropathic symptoms. Some proposed mechanisms are:

Spontaneous ectopic discharge

Normal primary afferent neurones require the input of a stimulus in order to reach firing potential. It has been shown that after a nerve injury spontaneous firing in the afferent neurone occurs. A and C fibres have been shown to demonstrate oscillatory activity resulting in ectopic firing. Cross excitation of other neurones increases this effect. These phenomena may be particularly relevant to the development of hyperalgesia, allodynia and chronic pain after nerve injuries.

Reorganisation of expression of ion channels in the peripheral nerves is responsible for the ectopic discharge. Both sodium and calcium channels have been shown to be involved with their altered expression increasing the excitability of neurones. The afferent barrage provided by spontaneous discharge from neurones provides a constant input to the central nervous system that may induce central sensitisation.[20]

Altered gene expression

Damaged peripheral sensory neurones undergo Wallerian degeneration and lose contact with peripheral targets and the supply of neurotrophic factors. The sensory neurones undergo altered gene expression, the result of which is a change in the type and level of neurotransmitters released in the spinal cord.[21] For example, some A-β fibres appear to release transmitters normally associated with nociceptors such as substance P. This seems to contribute to central sensitisation.[22] A change in gene expression also results in either up or down regulation of ion channels, in particular different types of sodium channel involved in ectopic spontaneous activity.

Spared sensory neurons

Changes have also been found in uninjured sensory fibres that are along side those with a lesion. They frequently show the opposite gene expression changes from their damaged neighbours; possibly due to increased bioavailability of neurotrophic factors. This can result in increased activity in the spared afferents, although the exact mechanism is not understood.[21]

Involvement of the sympathetic nervous system

Some patients exhibit neuropathic pain that is dependent on activity in the sympathetic nervous system.[23] After a peripheral nerve injury, a coupling develops between the sympathetic nervous system and the sensory nervous system. Axons involved develop increased α-adrenoceptors and therefore have an exaggerated response to circulating catecholamines. Morphological changes to the nerve follow with sympathetic axons sprouting into the dorsal root ganglion forming baskets around the cell bodies of sensory neurones. These changes lead to sympathetically maintained pain. Evidence for a sympathetic component to a patient's pain include sympathetically maintained, often unilateral limb pain, oedema, vasomotor and sudomotor asymmetries.

Collateral sprouting

Sprouting of fibres from sensory axons in the skin has been shown to occur in denervated areas, for example after crush injuries. However this does not occur in proportion to the degree of neuropathic pain experienced and is likely to be a small if at all significant factor in its pathophysiology.

Effects of bradykinin

This main plasma kinin, a vasodilator peptide, is involved in hyperalgesia associated with inflammatory pain, with a change in expression of its binding sites within the dorsal root ganglion after nerve injury.

Central mechanisms

The central mechanisms potentially involved in the generation of neuropathic pain are thought to result in neuroplastic changes in the CNS. A phenomenon termed central sensitisation occurs after peripheral nerve injury. Central sensitisation changes the way the neurones respond to subsequent inputs.[4] This may result in spontaneous ongoing pain and abnormally evoked pain (allodynia and hyperalgesia).[17] The mechanisms that are thought to be responsible occur primarily in the dorsal horn.

Wind-up

The term wind up describes the altered response of the dorsal horn neurones to repeated input from C-fibres.[10,17] Following brief, repetitive C-fibre stimulation, the dorsal horn cells respond in a linear fashion. However if the stimulus continues, further C-fibre activation produces an amplified response in the dorsal horn to the same intensity of stimulus.

This phenomenon is mediated by the NMDA receptor. Activation by sustained C fibre input leads to opening of the channel, an increased intracellular calcium concentration and an increased response to glutamate. Glutamate is the main excitatory neurotransmitter released from primary afferent neurones that acts at postsynaptic receptors. The NMDA receptor in its resting state is blocked by magnesium which is released when the cell is depolarised thus opening the channel in the receptor and allowing an influx of sodium and calcium and further depolarisation. When a painful stimulus arrives at the dorsal horn, the cells are initially depolarised by glutamate acting at the AMPA receptor thus allowing removal of the magnesium block. Once the stimulus is removed, the dorsal horn cells continue to fire for several seconds.

There is potential for this to be modified pharmacologically and a number of studies suggest that NMDA antagonists may prevent these phenomena and prevent hyperalgesia.[10]

Wind up is relatively short lived (seconds to minutes), whereas central sensitisation persists so the exact relationship remains unclear.

Central sensitization

Central sensitisation is also mediated by the NMDA receptor. Under conditions of prolonged C-fibre activation, depolarisation of the dorsal horn cells causes the NMDA receptor to lose its magnesium block.[10] Substance P acting via its receptor, the neurokinin-1 receptor, prolongs this depolarisation and allows further influx of calcium. The increase of calcium in the dorsal horn activates calcium dependent kinases such as protein kinases A and C, which are then able to phosphorylate amino acids within the NMDA receptor to produce a conformational change in the structure. This permanently removes the magnesium block in the receptor and allows it to be activated by glutamate. The process of central sensitisation differs from windup in that the changes remain long after the C-fibre input has ceased. Furthermore, the magnesium is removed by posttranslational changes in the NMDA receptor and is not just depolarisation induced.[17,22,24]

Central disinhibition

Central disinhibition results from loss of modulatory control mechanisms, which may lead to abnormal excitation of central neurones.[25] The main inhibitory neurotransmitter is γ-aminobutyric acid (GABA). It has been shown that suppression of this pathway results in allodynia. Within two weeks after a peripheral nerve injury, GABA receptor levels are reduced. So it seems that down regulation of GABA mediated pathways may be in part responsible for central sensitisation.

Expansion in receptive field size (recruitment)

Receptive fields of dorsal horn neurones contain subliminal areas; these represent a reservoir of activity.[26] With ongoing activation after injury there is an expansion of receptive field size leading to increased perception of pain, resulting in secondary hyperalgesia. This expansion of receptive fields does not reflect peripheral nerve or nerve root distribution, but spinal cord architecture. It might therefore be confusing from a diagnostic point of view, as it transgresses the boundaries imposed by a hard-wired model of the CNS.[27]

Immediate early gene expression

Immediate changes in gene expression in dorsal horn cells occur in response to Aδ- and C-fibre stimulation. These changes persist for a variable length of time and may contribute to central neuroplasticity. Noxious stimulation mediated by Aδ- and C-fibres produces an immediate change in the expression of certain genes within the dorsal horn cells.[28] These changes are detected within minutes of stimulation and may last for months or even years. The gene c-*fos* encodes for a protein, *fos*, which forms part of a transcription factor which may control the expression of other genes which produce long term changes in the dorsal horn. *C-fos* activation occurs as a result of increases in intracellular calcium following release of neurotransmitters like substance P and glutamate involved in relay of nociceptive information.[29] This is followed rapidly by the appearance of *fos* protein which can be detected in laminae I, II and V of the dorsal horn. The presence of *fos* protein can be used as a marker of noxious stimulation and thus also to determine the effect of agents to reduce noxious stimulation.

Anantomical re-organisation of the spinal cord

Primary afferent neurones synapse in the laminae of the dorsal horn with second order ascending neurones. Under normal conditions, Aδ- and C-fibres terminate in laminae I and II, whereas Aβ-fibres terminate in laminae III and IV.

Following C-fibre injury, the large unmyelinated Aβ-fibres sprout terminals into lamina II. Aβ-fibres, which are activated by low intensity non-painful stimuli can thus stimulate the dorsal horn neurons present in lamina II, usually associated with noxious sensation.[30] This observation could explain allodynia, as Aβ-fibres form synapses with second order neurones and their low-threshold non-noxious inputs will be signalled as nociceptive in origin.

However, doubt surrounds this theory as a main mechanism of allodynia as sprouting is not fully established until 2 weeks after the injury. Furthermore it has been suggested that this sprouting only occurs in a small subgroup of Aβ neurones.[21]

As well as sprouting fibres into lamina II, Aβ-fibres also undergo phenotypic switching and produce the neurotransmitter substance P and calcitonin gene related peptide. These neurotransmitters are usually produced only by C-fibres, but after nerve injury their expression by C-fibres is down-regulated. Aβ-fibres begin to release these at the dorsal horn following low intensity stimulation. This release of substance P can maintain the central sensitisation changes in the dorsal horn at the NMDA receptor that is usually only maintained by continued C-fibre input.

Contribution of glial cells to pain conditions

There is now increasing recognition that neuropathic pain is not only the result of changes in neuronal cells and pathways, but also that glial cells, thought to be not relevant to neuronal function, play a major role here.[31] Schwann cells, spinal microglia and astrocytes, more regarded as components of the immune system, seem to have relevant contributions to the development of chronic pain states and make it plausible that these have features of a neuroimmune disorder and may offer new approaches to treatment.

Symptoms of neuropathic pain

Patients with neuropathic pain usually experience persistent and/or paroxysmal pain.[18] The pain often has an abnormal quality, for example burning, electric-shock like, shooting, lancinating or numbing. Neuropathic pain can occur in an area of neurological deficit, but might also arise from areas still innervated normally.[32] Neuropathic pain exhibits often one or more of the following characteristic features:

- *Dysaesthesia*, an unpleasant abnormal sensation, whether spontaneous or evoked.
- *Hyperalgesia*, an increased response to a painful stimulus.
- *Allodynia*, pain elicited by a normally non noxious stimulus
- *Hyperpathia,* a painful syndrome characterised by increased reaction to a stimulus, especially a repetitive stimulus, as well as an increased threshold.
- *Hypoalgesia,* diminished pain in response to a normally painful stimulus.

Clinical features of neuropathic pain are often summarised as stimulus-dependent, stimulus-independent and sympathetically-maintained pain:[32]

Stimulus-dependent pain

Following nerve injury, increased C-fibre activity causes central sensitisation within the dorsal horn via activation of the NMDA receptor as described earlier.

Central sensitisation produces three main effects:

1) enlargement of the sensory field of a dorsal horn neuron (secondary hyperalgesia)
2) increase of the response to a suprathreshold stimulus (hyperalgesia)
3) generation of a response to a subthreshold stimulus (allodynia)

These phenomena represent stimulus-dependent pain, although the relationship between stimulus and response might be widely varying.

Stimulus independent pain

As mentioned earlier, there are two types of sodium channels present on sensory neurons. The tetrodotoxin resistant channels are implicated in the generation of the spontaneous pain of pathological pain states.

Following injury there is reorganisation of the expression and location of the various types of sodium channel within the neuron. The tetrodotoxin resistant channels relocate to the neuroma, where it produces areas of hyperexcitability and ectopic discharges. After nerve injury, both injured nerves and uninjured nerves close to the site of injury display spontaneous discharges. The alterations in expression of sodium channels are thought to be due to alterations in the supply of neurotrophins such as nerve growth factor and glial-derived neurotrophic factor.[33]

Sympathetically maintained pain (SMP)

In a small but significant proportion of chronic pain sufferers the pain has a definite sympathetic system element to it and is said to be sympathetically maintained.

Following partial nerve injury in these patients, both injured and uninjured primary afferents express alpha-2 adrenoceptors on their membranes so they become sensitive to circulating catecholamines and noradrenaline release from sympathetic nerve terminals.[17]

Direct coupling also occurs between the sympathetic and peripheral nervous systems with sympathetic nerves sprouting axons into the dorsal root ganglion to form baskets around the cell bodies of nociceptor neurons, where they form functional synapses. This sprouting is thought to occur under the influence of nerve growth factor. Other more central mechanisms of somatosensory-sympathetic coupling are also investigated.[23]

Neuropathic pain syndromes

There are many causes of neuropathic pain including a number of disease states.

Peripheral neuropathies

Metabolic/Endocrine: Diabetics can develop different types of neuropathies, these include polyneuropathies, autonomic neuropathy, compression neuropathy and focal neuropathies. There are more than 15 million diabetics in the USA and more than half of them over 60 years have neuropathic pain.

Many diabetics, especially those with poor blood glucose control develop a distal, symmetrical, proximally spreading and painful neuropathy.[34] Severe pain is often a feature and may be described as burning, aching or have lightning components to it. It seems that the main cause is demyelination and to a lesser extent axonal degeneration. The first stage in prevention and treatment of early neuropathies is good glycaemic control. Additionally, hyperglycaemia may have a direct effect on neuropathic pain by altering pain thresholds, tolerance and affecting opioid receptors.

Mononeuropathies, usually involve the motor supply to extraocular muscles and also nerve supply to the limbs. The third cranial nerve is most frequently affected. Pain is often a symptom. Additionally, an asymmetrical proximal predominantly motor neuropathy can occur, especially in older patients with poor glycaemic control.

Untreated hypothyroidism may result in neuropathic pain.

Toxic: Well-known neuropathies here include those caused by alcohol, chemotherapy (where the neuropathy maybe the dose

limiting factor) and, more recently anti-AIDS drugs e.g. isoniazid.

Postinfectious: The most common problem here is Post Herpetic Neuropathy (PHN), which increases in incidence, intensity and persistence with age.[35] The pain persists in the distribution of a peripheral nerve after herpes zoster infection (shingles). It is thought that chronic inflammatory changes result in damage to sensory nerves resulting in deafferentation of nociceptive fibres. The pain is persistent and can become intolerable with associated allodynia. Treatment is very difficult, particularly in later stages.

Hereditary: Fabry's disease, a rare lipid storage disorder, often presents with a painful neuropathy.

Malignant: Neuropathies can occur as a non-metastatic complication of malignant disease, usually a sensory neuropathy that can sometimes be painful. Neuropathic pain can also be caused as the result of direct tumour invasion involving nearby nerves.

Idiopathic: Trigeminal neuralgia (tic douloureux) is defined by the IASP as a 'sudden, usually unilateral, severe, brief, stabbing, recurrent pains in the distribution of one or more branches of the fifth cranial nerve'.[1] It can occasionally be secondary to an underlying condition e.g. tumour, multiple sclerosis. The diagnosis is made on clinical grounds as the patients describe a characteristic pain. It occurs most frequently in the maxillary division and least in the ophthalmic division of the trigeminal nerve. The pain is triggered by light touch, eating, talking and the cold. Examination in the absence of other neurological symptoms reveals very little. There is no definitive diagnostic test. Magnetic resonance imaging (MRI) can reveal an underlying cause or nerve compression, but is otherwise not highly sensitive or specific. Magnetic resonance tomographic angiography (MRTA) has been used in delineating the pathophysiological process and may provide a helpful diagnostic image but there is insufficient evidence at present.

The pathophysiology is not completely understood. The most evidence supports vascular compression of the nerve, resulting in hyperexcitability and abnormal neuronal activity, causing pain. This theory is supported by the fact that patients often experience immediate pain relief from microvascular decompression and has been confirmed radiologically in a study utilising MRTA.[36]

Spontaneous remission may occur so surgery is reserved for those refractory to medical treatment. Complications of surgical microvascular decompression include facial nerve damage, haematoma, cerebrospinal fluid leak and infection. However, despite its risks microvascular decompression is currently the best option from a variety of surgical procedures. The other surgical techniques include gasserian ganglion surgery, radiofrequency thermocoagulation, percutaneous retro Gasserian glycerol injection or microcompression, posterior fossa surgery and gamma knife irradiation.

Patients should be made aware of the risks associated with the chosen surgical technique and warned that the pain relief may not be permanent and balance these against the benefit from potential long term analgesia in cases refractory to medical management.[37]

Vascular: Vascular pain is a complex issue. Pain can be arterial, microvascular or venous in origin. Neuropathy can in particular follow venous insufficiency.[38] In every vascular disease, sympathetic changes may develop which contribute a neuropathic element to the ischaemic pain. The patient may develop skin hyperalgesia, dystrophic skin with a shiny appearance, muscle atrophy and vasomotor

phenomena. Sympathetic blocks may be beneficial.[39]

Posttraumatic: Posttraumatic neuropathies are common and can develop after any nerve injury. Even minor demyelination injuries without neurological sequelae can result in neuropathies. Examples are sciatica, neuroma or nerve entrapment after surgery or trauma, phantom limb pain, complex regional pain syndromes (CRPS) type I (without neurological deficit, previously called Reflex Sympathetic Dystrophy RSD) and Type II (with neurological deficit, previously called causalgia) and post-thoracotomy pain.

Central neuropathies

Central pain is due to a lesion or dysfunction of the CNS.[40] These lesions may have associated symptoms that affect the patient and their pain eg ataxia, motor weakness and hearing/visual loss. Epilepsy and depression are also common with cerebral lesions. These aspects need to be addressed along with treatment of the pain.

Central pain is associated with spinothalamocortical dysfunction and may develop over a length of time and varies widely between individuals regardless of aetiology.

Vascular lesions in the brain and spinal cord: The aetiology here includes infarction, haemorrhage, and vascular malformation. Stroke is the most common cause of central pain due to its high incidence.[41] Around 8% of patients with acute stroke have been shown to suffer from central pain in the following 12 months.

Multiple sclerosis: This demyelination process can result in neuropathic pain by a variety of mechanisms. Cranial nerve neuropathies, but also widespread central pain syndromes are common consequences and often difficult to treat.[42]

Trauma, tumours and infections: Brain injury, but by far more commonly spinal cord injury, can result in a variety of central pain syndromes.[43] Syringomyelia and syringobulbia as a consequence of such injuries can cause further central pain. Tumours of the brain and spine as well as infections and abscesses can cause similar symptoms.[40]

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21 • Post-amputation Pain

STEPHAN A. SCHUG, GAIL GILLESPIE

School of Medicine and Pharmacology, University of Western Australia, Royal Perth Hospital, Perth, WA

INTRODUCTION

The phenomenon of pain in a missing limb has puzzled patients, doctors and the lay public for centuries. In the 16th Century the French military surgeon Ambroise Paré published a medical description of the enigmatic affliction, while in the 17th century the great philosopher Rene Descartes looked at its potential pathophysiology. The most famous 'first' description of the condition is attributed to the great neurologist Charles Bell,[1] but it was only in the later part of 19th Century, that the US military surgeon Silas Weir Mitchell introduced the term 'phantom limb': '*There is something almost tragical, something ghastly, in the notion of these thousands of spirit limbs, haunting as many good soldiers, and every now and then tormenting them . . .*'

We now know that post-amputation syndromes can occur with any amputated body part apart from limbs e.g. breast, tongue, teeth, genitalia and even inner organs such as the rectum.[2-4]

CLASSIFICATION AND INCIDENCE OF POST-AMPUTATION PAIN SYNDROMES

Following amputation (or deafferentiation injury such as brachial plexus avulsion) a number of phenomena can develop, which require differentiation.

Stump Pain

Stump pain is pain localized to the site of amputation. Stump pain can be acute (usually nociceptive) or chronic (usually neuropathic). Stump pain is most common in the immediate post-operative period.[5] The overall incidence of chronic stump pain is in the range of 45%.[6] The incidence of early stump pain is increased by the presence of severe pre-amputation pain[7] and severe acute stump pain.[8] The cause is unclear but probably multifactorial. Stump pain is problematic and can interfere with prosthesis use.

Phantom Sensation

Phantom sensation is defined as any sensory perception of the missing body part with the exclusion of pain. Almost all patients who have undergone amputation experience such phantom sensations.[9] These sensations range from a vague awareness of the presence of the organ via associated paraesthesia to complete sensation including size, shape, position, temperature and movement.[10] Phantom sensations usually diminish in intensity or size over time, but may persist for a long time. 'Telescoping' of the phantom part can occur with time such that the phantom limb gradually shrinks proximally to approach the stump.[11] Eventually the phantom limb is felt to be within the stump itself.

Phantom Limb Pain

Phantom limb pain (PLP) is defined as any noxious sensory phenomenon of the missing limb or organ. The incidence of phantom limb pain is estimated to be 60–80% after limb amputation.[5,6] The pain is independent of gender, level or side of amputation.[5] There is, however, a lower incidence among children and congenital amputees.[12]

Pain can be immediate or delayed in onset. It is typically intermittent and changes with time. Typical characteristics of phantom limb pain are burning, shooting, crushing, throbbing, cramping, aching, tingling, boring; often the limb is described as being in a hyperextended or otherwise unnatural position. The pain usually occurs in the distal portion of the missing limb.[5] The incidence of pain may be increased if pre-amputation pain was present and may then resemble the pre-amputation pain in character and localisation.[8,13] However, the exact relationship between pre-amputation pain and PLP is not a simple one, especially as patients' pain perceptions alter and may be exaggerated with time.[5] The incidence of phantom pain diminishes with time after amputation, as does the frequency and intensity, being highest immediately following surgery.[5]

It is important to realise, that the terms for noxious syndromes, 'stump pain' and 'phantom limb pain', are subjective descriptive terms that do not make assumptions on differences in pathophysiology. There is, in fact, a strong correlation between phantom pain and stump pain and they may be inter-related phenomena.[14] All three phenomena can co-exist. [7]

PATHOPHYSIOLOGY OF POST-AMPUTATION PAIN SYNDROMES

The pathophysiology of post-amputation pain syndromes is most likely based on a combination of peripheral and central factors, which interplay subsequent to the significant trauma of an amputation.

Peripheral Factors

The following changes occur after peripheral nerve injury such as cutting of a nerve:[15]

1) Sensitization of peripheral nociceptors with a decreased threshold to noxious stimulation;
2) Increased response to supra-threshold stimulation;
3) Spontaneous activity of peripheral receptors due to sensitisation including ectopic pacemaker sites, possibly as a result of the increase in sodium channels, α-adrenergic channels, calcium channels and stretch-activated channels that follows nerve injury;
4) Sensitization of non-nociceptive receptors to nociceptive impulses.

These changes contribute to hyperalgesia and allodynia in the stump; therefore stump

manipulation and revision can worsen pain due to repeated deafferentation injuries. The dorsal root ganglion may also be the site of ectopic neuronal activity subsequent to deafferentiation and thereby contributing to pain syndromes.

Furthermore, regrowth of severed nerves often produces nodules called 'neuromas'. Neuronal activity originating from peripheral neuromas either spontaneously or in response to mechanical, chemical or electrical stimulation, may cause increased sensitivity of the stump to different stimuli.[15]

Other peripheral factors include increased muscle tension in the stump correlated with cramping and spasmodic pain and decreased blood flow to stump correlates with descriptions of phantom pain such as burning or tingling. Low stump temperature correlates with burning pain.

Overall, while physical stimulation of the stump may accentuate phantom limb pain, current evidence suggests that peripheral mechanisms do not cause, but at most modulate or perpetuate phantom limb pain.

Spinal Factors

The combination of increased afferent input from sensitised nerve endings and the dorsal root ganglion may contribute to central sensitisation. The following changes occur in the dorsal horn of the spinal cord after nerve injury:[15]

1) Increased spontaneous activity of dorsal horn neurones
2) Increased response to afferent input
3) After-discharges following repetitive stimulation
4) Expansion of peripheral receptive fields
5) Wind-up (increased neuronal activity in dorsal horn neurons following repetitive C-fibre stimulation), mainly mediated by N-methyl D-aspartate (NMDA) receptors.

These factors play an important role in many chronic pain syndromes, but to which extent these factors are involved in perpetuation of phantom syndromes is currently unclear, although involvement is likely.

Supraspinal Factors

The presence of pain prior to amputation is thought to increase the likelihood of phantom pain.[13] In 1971, Melzack proposed that the painful extremity had created a painful central 'engram'. An engram is the schematic representation of body parts in the CNS caused by consistent sensory input. This engram was thought to persist after amputation causing phantom pain.

On the basis of these observations, the *neuromatrix theory* was proposed by Melzack in 1990.[16] In this theory, the body's physical self is represented by a matrix, a complex network of neurones connecting somatosensory cortex, thalamus and limbic system. This *neuromatrix* is genetically determined and subsequently modulated by sensory input, thereby creating a *neurosignature* for each body part. This neurosignature determines how a body part is consciously perceived; phantom sensations are the result of persistence of the neurosignature after the loss of the limb. The genetic determination of the neurosignature is supported by the observation that children who are born with a missing limb may feel phantom sensations of the missing part.

In this theory, phantom limb pain is the result of abnormal reorganisation in the matrix, either due to a preexisting pain state or the amputation process itself.[16]

By analysing neuromagnetic fields, the group around Flor has been able to show a close correlation between the degree of neuromatrix reorganisation and the development of phantom limb pain;[17] reorganisation

of somatosensory cortex occurs with neighbouring representation zones moving into the deafferented zone.[18] Here it is also of note that many of the sites of amputation that commonly lead to phantom sensation and pain are sites with a relatively large cortical somatosensory representation.

An alternative theory discussed in the literature is the *dynamic reverberation theory.* This originated from the observation that selective stereotactic cortectomies of the corona radiata or focal brain lesions in the parietal cortex, thalamus or cortico-thalamic fibres on the contralateral side have resulted in permanent relief of phantom pain. This led Canavero, in 1994, to the theory that phantom pain and sensation were a result of a localized dynamic reverberation loop between cortex and thalamus. He postulated that this loop could operate with or without sensory activation.[19]

CURRENT PATHOPHYSIOLOGICAL MODEL OF POST-AMPUTATION PAIN SYNDROMES

A comprehensive model incorporating the current state of knowledge has been proposed by Flor et al. It includes peripheral and central factors as relevant contributors to the development and perpetuation of phantom limb pain.[18] In principle, it suggests that somatosensory pain memories and a subsequently altered homuncular representation in the somatosensory cortex are the underlying factors of phantom limb pain, which can be sustained by peripheral factors. In more detail, it assumes that memories of pain established before an amputation are powerful causative contributors to phantom limb pain generation. In analogy to findings in other chronic pain patients, such pain memories increase the representation zone in the primary somatosensory cortex. The changes are then perpetuated after the amputation by selective C-fibre deafferentiation, random input from stump neuromas, abnormal changes in the dorsal root ganglia and the dorsal horn of the spinal cord and sympathetic activation.[20]

PREVENTION OF POST-AMPUTATION PAIN

In view of the immense difficulties in treating phantom limb pain once it is established, considerable efforts have been made to identify techniques to prevent the syndrome. Regrettably, the evidence on none of the methods tried has been conclusive, although overall results of epidural anaesthesia and possibly ketamine administration are promising.

Perioperative Lumbar Epidural Blockade

In 1988, Bach *et al*. demonstrated that lumbar epidural blockade (LEB) with bupivacaine plus morphine, started 72 hours *prior to surgery*, reduced the incidence of phantom limb pain in the first year after surgery.[21] This promising result initiated a number of similar studies; Schug *et al*. investigated the use of pre-emptive lumbar epidural anaesthesia/analgesia preoperatively for 24 hours and postoperatively for 72 hours in a small sample of patients. At an interview one year after amputation, those patients with epidural analgesia had significantly less severe phantom limb pain than those receiving general anaesthesia.[22]

Another study comparing pre-operative and intra-operative analgesia using LEB showed no difference between groups. The duration of pre-operative LEB, however was variable between patients with a median pre-operative infusion of 18 hours.[23] However, this study has been criticised for its quality

of analgesia and the inclusion of pain of any intensity in the results.

More recently, Lambert and colleagues compared pre-operative epidural with perineural analgesia. The LEB was started 24 hours before surgery and continued for three days post-operatively. No difference was found in stump or phantom pain or in phantom sensation at six and 12 months.[24] Although this was a randomised trial, the numbers were small and it is questionable whether the study had sufficient power for phantom pain outcome measurements.

In conclusion, there have been several studies looking at preventive analgesia using LEB. The results are conflicting,[25] however a meta-analysis showed that perioperative epidural analgesia reduced the incidence of severe phantom limb pain with an NNT of 5.8.[26] Overall the results are promising and a protective effect has again been confirmed in a recent audit at our institution.

Peripheral Nerve Blockade

Infusions of local anaesthetics via peripheral nerve sheath catheters, usually inserted by the surgeon during the amputation, are a safe method providing excellent analgesia for the immediate post-operative wound pain. They are, however, of no proven benefit in the prevention of phantom pain or stump pain.[25]

NMDA Antagonists

The use of pre-incision ketamine as pre-emptive analgesia has been described previously in other settings. A small observational study suggests that the incidence of severe phantom limb pain may be reduced with the use of ketamine as a bolus followed by an infusion started prior to skin incision and continued for 72 hours post-operatively.[27] This promising study was small and used historical controls. A randomised controlled trial could not confirm these results, but was underpowered.[28] Epidural ketamine had no preventive effect in an RCT.[29] However, a trial of memantine in combination with regional analgesia showed a preventive effect.[30] Overall, further investigations are justified.

EVALUATION OF THE PATIENT WITH POST-AMPUTATION PAIN SYNDROMES

Phantom sensation requires pre- and post-operative counselling and education but it should not generally pose a clinical problem.

Examination

Examine stump to exclude common causes:

1) Prosthogenic Pain
   a) Due to an improperly fitting prosthesis:
      i) Poor socket fit, cushioning or alignment
   b) Inappropriate suspension resulting in pistoning
   c) Painful adductor roll in the above-knee amputee
   d) Distal residual limb weight-bearing
   e) Poor trim line
2) Neuropathic Pain
   a) Caused by neuroma formation
   b) Test for presence of wind-up by examining for Tinel's sign – shooting pains elicited by repeated tapping over the area
3) Arthrogenic Pain
   a) Pain originating in neighbouring joint or surrounding soft tissue, ligaments or tendons.
4) Referred Pain

Excessive biomechanical stress in amputees may cause painful musculoskeletal conditions such as sacroiliac dysfunction, piriformis syndrome, facet syndrome or radiculopathy.[31] Therefore it is important to examine posture and gait.

Furthermore, it is of value to examine skin for areas of ulceration and infection, palpate for areas of tenderness, bony exostosis, heterotropic ossification and adherent scar tissue and evaluate muscle strength and range of movement of neighbouring joints to exclude contracture formation.

THERAPY OF POST-AMPUTATION PAIN SYNDROMES

A survey by Sherman *et al*. in 1980 identified over 50 different therapies currently in use for the treatment of phantom limb pain.[32] This suggests clearly that an effective treatment of phantom limb pain has not been established and that 'the results are poor and usually below the expected rate of cure of pain with placebo treatment alone.'

Randomised controlled trials have only established the effectiveness of a small number of therapies.

However, as early treatment is more effective and often multidisciplinary approaches are needed, patients with severe postamputation pain should be promptly referred to a multidisciplinary pain clinic to ensure optimal and timely pain management.

Calcitonin

The parenteral administration of calcitonin is a proven treatment for phantom limb pain and in our experience the most effective in early stages,[33] while there is no benefit in chronic phantom limb pain.[34] After initial anecdotal reports,[35] a randomised double-blind cross-over study by Jaeger and Maier showed excellent effectiveness.[36] 200 IU of salmon calcitonin was given as an intravenous infusion over 20 minutes and provided complete pain relief for 76% of patients; 71% did not experience recurrence of their phantom pain. Calcitonin may also be given subcutaneously or intra-nasally.[33]

The mechanism of action of calcitonin in inhibition or modulation of pain perception is unknown; however anecdotal descriptions of its effectiveness in a number of states of central sensitisation are published. Side effects including dysaesthesia, nausea and vomiting have been described, but most are transient and can be prevented by prophylactic use of anti-emetics.[36] The risk of an anaphylactic reaction is most likely minimal, but needs to be considered.

Ketamine

Ketamine, an antagonist of the NMDA receptor, is another proven treatment of stump and phantom limb pain. In a randomised trial of patients with existing pain, ketamine has been shown to significantly reduce phantom pain and stump pain. It was also shown to decrease wind-up like pain (pain evoked by repetitive mechanical stimuli), and to increase the pain-pressure threshold in patients with phantom pain and stump pain. It was given as a bolus of 0.1mg/kg/5minutes followed by an infusion of 7mcg/kg/minute. Pain recurred 30minutes after discontinuation of the infusion in most patients.[15] This was confirmed in a more recent trial.[34]

Over-activity of NMDA receptors may be a factor in the maintenance of stump pain and phantom pain.

Analgesic and Co-analgesics Compounds

As outlined in the chapter 'Treatment of Neuropathic Pain', these agents can play an

important role in pain due to nerve injury, but have only variable effects in phantom limb pain.

Opioids

Generally, neuropathic pain is less responsive to opioids than nociceptive pain.[37] However, a randomised double-blind study of oral retarded morphine sulphate (MST) showed a significant reduction in phantom pain in opioid-sensitive patients, with pain reduction of over 50% occurring in 42% of patients. Neuromagnetic source imaging of three patients suggested reduced cortical reorganization may be occurring with the use of MST.[38]

A study comparing the effects of intravenous lidocaine with intravenous morphine showed that morphine given as 0.05mg/kg bolus followed by 0.2mg/kg infusion over 40 minutes, given on three consecutive days, significantly reduced both stump and phantom pain.[39] An NNT of 5.6 and superiority over mexilitine was found for morphine in a more recent trial.[40] Tramadol was as effective as amitriptyline in treating post-amputation pain.[41]

Gabapentin

In a randomized, double-blind, placebo-controlled, cross-over study, six weeks of gabapentin was better than placebo in relieving phantom limb pain. Pain intensity difference was significantly greater for gabapentin than for placebo.[42] These findings were not confirmed by a later study.[43]

Clonazepam

Anecdotal evidence suggests that clonazepam may be useful in the treatment of phantom pain.[44] There are however no studies to confirm this.

Lidocaine

A randomised double blind study comparing the effects of intravenous lidocaine with intravenous morphine showed that lidocaine given as 1mg/kg bolus followed by 4mg/kg infusion, given on three consecutive days, significantly reduced stump pain but had no effect on phantom pain.[39]

Carbamazepine

There is only anecdotal evidence for the use of carbamazepine in the treatment of post-amputation syndromes.[45] It has been extensively used in the treatment of other neuropathic pain states. Side effects can be problematic. Randomised trials are required.

Tricyclic Antidepressants (TCA)

In a randomised trial, chlorimipramine, a serotonin reuptake inhibitor, was found to be significantly better than nortriptyline, a noradrenaline reuptake inhibitor, in the treatment of central pain syndromes.[46] Amitriptyline and tramadol were equally effective in the treatment of phantom limb pain.[41]

Selective Serotonin Reuptake Inhibitors

Venlafaxine is a serotonin and noradrenaline reuptake inhibitor with a side-effect profile significantly better than TCAs.[47] There are no RCTs on its effect on post-amputation pain syndromes.

Baclofen

This gamma-aminobutyric acid (GABA) agonist, when given intrathecally, has been shown to reduce chronic musculoskeletal pain.[48] It may therefore be of some benefit if muscle spasm is the source of the pain. It has not been proven to be of use in phantom limb pain.

Capsaicin

Capsaicin depletes the neurotransmitter, substance P from sensory nerves and may give relief to some patients with stump pain when used topically.[49]

Symptomatic Treatment of Pain Components

The burning component of phantom limb pain alone can be decreased by pharmacological and behavioural therapies that increase the temperature of the stump such as sympathectomy, α- or β-blockade or biofeedback.

Cramping can be relieved by treatments that reduce muscle tension, for example with the use of baclofen or again biofeedback.

Nonpharmacological Therapies

The following therapies are thought to relieve phantom pain by causing increased sensory inflow into the stump area:

- TENS
- Acupuncture
- Physical therapy

With the development of theories on cortical reorganisation as a cause for phantom limb pain, there are now therapies tried, which are based on the concept of reversing such reorganisation.[18] Sensory discrimination training programs show promise here; this is a process during which patients have to discriminate the frequency or location of high intensity, non-painful electrical stimuli applied through electrodes on their stump in an attempt to separate merged regions on their cortical somatosensory map.

A recent study using this technique showed that phantom limb pain was significantly decreased in the group who underwent the training process compared to controls. Cortical reorganisation, assessed by neuroelectric source imaging and structural magnetic resonance imaging, was also reduced in this group.[50] Mental imagery of limb movement[51,52] and a combination of laterality recognition, mirror movements and imagined movements are other successful approaches based on this concept.[53]

Similarly, there are now first data, that use of a myoelectric prosthesis may prevent cortical reorganisation and phantom limb pain.[54]

Invasive Therapies

Electroconvulsive Therapy (ECT)

This psychiatric treatment is thought to interrupt the dynamic reverberations that maintain central and phantom pain in the thalamocortical pathway[19] and has been used in the treatment of refractory phantom pain.[55] There have been no trials in this area.

Nerve Blockade

There is only anecdotal evidence for the use of peripheral nerve blockade in the treatment of phantom pain syndromes.[56] There have been no trials in this area.

Spinal Cord Stimulation

This treatment, thought to facilitate inhibitory descending pathways, has been described in the treatment of phantom pain. The overall success rate of this expensive and invasive approach in this indication is in the range of less than 50%.[57,58]

Implantable Intrathecal Delivery Systems

Infusing clonidine, local anesthetic, baclofen or opioids, usually in a combination, may be beneficial in selected patients with phantom limb pain, although there are no definitive studies.

Dorsal Root Entry Zone (DREZ) Lesions

DREZ lesioning has a limited effect for a limited time only in phantom limb pain;[59] this is in line with clinical experience with this neurodestructive approach. Other types of surgery and neuroablation often makes pain worse because of repeated stimulation and/or deafferentation of the affected nerves.

Psychological Therapy

Pre-amputation counselling is mandatory as amputees go through normal grieving processes. It is important to identify anxiety and depression early, as these can magnify pain perception. Behavioural, cognitive, group therapy and pain management programs are all useful methods of helping patients cope with their pain. Hypnosis, biofeedback and muscular relaxation training to disrupt the pain-anxiety-tension cycle are important components of chronic pain therapy. [60]

FUTURE AIMS

Future aims in the management of post amputation syndromes focus on:

1) Further prospective randomised trials to evaluate the benefits of current pharmacological therapies.
2) Clarification of the role of pre-emptive analgesia in the prevention of phantom pain.
3) Evaluation of the promising methods that attempt to revert the cortical reorganization that occurs following amputation towards normal.
4) A multi-modal and multi-disciplinary approach to pain management.

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22 • Treatment of Neuropathic Pain

STEPHAN A SCHUG[1], KATHRYN JD STANNARD[2]

School of Medicine and Pharmacology, University of Western Australia, Royal Perth Hospital, Perth, Western Australia.

INTRODUCTION

Neuropathic pain is defined by The International Association for the Study of Pain (IASP) as pain following a primary lesion or dysfunction of the nervous system.[1] It is caused either by peripheral damage with lesions involving peripheral nerves, dorsal root ganglia and the dorsal roots (peripheral neuropathic pain) or by central damage, which may involve injury caused by infarction or trauma of spinal cord or brain (central neuropathic pain).

Neuropathic pain results in persistent pain syndromes that have no biological function, but are difficult to treat and cause great distress to the individual. Neuropathic pain is also referred to as neurogenic pain, deafferentation pain, neuralgia, neuralgic pain and nerve pain.

Neuropathic pain may develop immediately after a nerve injury or after a variable interval. It may be maintained by factors different from the initial cause. It can persist for a long time and is frequently not explained by underlying pathology. Patients are frequently seen by many different specialists and their treatment often fails to resolve the pain. As the pain persists other factors such as environmental, psychological and social stressors become relevant contributors to the overall presentation.

PRINCIPLES OF TREATMENT

Treatment of neuropathic pain is not straightforward. The pain is often refractory to conventional analgesic regimens such as Non-Steroidal Anti-Inflammatory Drugs (NSAIDs). Opioids have only limited efficacy in neuropathic pain as outlined later in this chapter; therefore so called co-analgesics, medications which are not typically used as analgesics, are often the first-line treatment of neuropathic pain.

Increasingly, data from randomised controlled trials (RCTs) and meta-analyses are leading to improvements in management and a more evidence-based approach. A number of recent evidence-based guidelines have been published by various societies and organisations. Of particular value are the guidelines published by the European Federation of Neurological Societies (EFNS)[2] and by the Special Interest Group Neuropathic Pain of the IASP (NeuPSIG).[3]

It is important that patients have realistic expectations regarding treatment efficacy and potential side effects in order to improve

compliance with medication.[4] A balance between these should be achieved on an individual basis. A single drug therapy should be tried before combinations of drugs are started. Non-pharmacological treatments are available that may be appropriate for certain cases. For optimal results a multidisciplinary approach to treatment should be adopted that addresses affective and behavioural changes and disability.

PHARMACOLOGICAL TREATMENT

Opioids

The use of opioids to treat neuropathic pain is controversial. In 1988 a study implied that patients with neuropathic pain did not experience pain relief from opioids.[5] However, this study has been criticised for possible selection bias as most non-responders had previously used morphine without effective results and there was no individual titration of morphine. A number of reasons have been suggested for the relative opioid resistance; these include among others, down-regulation of peripheral and spinal opioid receptors[6,7] and physiological antagonism with up-regulation of cholecystokinin.[8] There is also evidence in animal experiments that long term use of opioids induces a state of CNS hyperexcitability implying that tolerance to opioids may have a pharmacology similar to hyperalgesia.[9] Studies in humans have so far not confirmed these experimental data.

Since then multiple RCTs in neuropathic pain have been conducted with morphine, fentanyl, oxycodone and other opioids. The general consensus is that pain intensity may be relieved by opioids titrated for that individual. This concept was confirmed in a study in which dose responses of opioids in nociceptive and neuropathic pain were compared and higher doses were indeed required in neuropathic pain.[10] A Cochrane Review of the multiple RCTs confirmed significant efficacy of opioids in neuropathic pain.[11]

Recommendations for clinical use of opioids in neuropathic pain

There is now a general consensus that a subset of patients with neuropathic pain benefit from treatment with opioids.[12] However, current guidelines for neuropathic pain treatment do not recommend opioids as a first-line option due to the potential adverse effects and risks of these drugs.[2,3] If a decision to use opioids is made, then the approach to identify these patients and manage their treatment should follow guidelines for the use of opioids in chronic noncancer pain (CNCP).[13,14]

Summarising such guidelines is beyond the scope of this chapter; in brief they usually include a past/present history of drug addiction as a relative contraindication, the need for regular follow-up visits and opioids to be prescribed and supervised by the same doctor. Legal issues with opioid prescriptions are associated with their controlled status, the risk of addiction and abuse and the potential for diversion into illegal channels by selling or passing on to others.[15] Methadone, due to its additional monaminergic and NMDA receptor effects, might be a particularly useful opioid in the setting of neuropathic pain.[16,17]

Tramadol

Mechanism of action

Tramadol is a centrally acting synthetic analogue of codeine; however it is not a conventional opioid as it has relatively low affinity for μ-opioid receptors and is classified by the FDA as an atypical centrally acting analgesic. Tramadol together with its primary active metabolite has

three synergistic mechanisms of action to provide analgesia. It combines weak effects on opioid receptors with monoaminergic mechanisms. Reuptake inhibition of 5-HT and noradrenaline contribute to the anti-nociceptive action of tramadol.[18,19] Tramadol is a racemic mixture with opioid receptor activity and 5-HT reuptake inhibition mainly associated with the (+)-tramadol enantiomer, whereas (-)-tramadol is a reuptake inhibitor of noradrenaline.[20,21] The monaminergic effects suggest a higher analgesic potency of tramadol in neuropathic than in nociceptive pain states; this has been recently confirmed by our group.[22]

Efficacy

Tramadol has been investigated for the treatment of chronic pain in the past. A number of RCTs of tramadol in patients with neuropathic pain have been completed. The first involved 131 patients with diabetic neuropathic pain, 37% patients withdrew, more from adverse effects in the tramadol group and more from lack of efficacy in the placebo group. The tramadol group had significantly more pain relief than placebo.[23] In a subsequent randomised placebo-controlled crossover trial 45 patients with neuropathic pain were studied. Significant reductions in spontaneous and touch-evoked pain were achieved with tramadol.[24]

A meta-analysis of these and other RCTs identified tramadol as an effective treatment for neuropathic pain; or with number needed to treat (NNT) for 50% pain relief of 3.8 with therapeutic effects on paraesthesia and allodynia and a number needed to harm (NNH) of 8.3.[25]

Adverse effects

Tramadol causes less respiratory depression[26] and constipation[27] than conventional opioids. Physical dependence to tramadol use is extremely rare[28] and occurs in the range of 1:100,000 users.[29] Similarly, tramadol has a low abuse potential[30] and its risk of addiction has been rated in the range of 1:100,000.[29,31] For these reasons, tramadol is not under special regulatory control in most countries. Phase IV clinical trials have reported the overall incidence of side-effects from tramadol to be 15.3%.[32] The majority of side-effects were found to be dose dependent.

Recommendations for clinical use of tramadol in neuropathic pain

Experimental evidence and a meta-analysis have shown tramadol to be a particularly useful analgesic in neuropathic pain with a low incidence of adverse effects, mainly of a benign nature. In our experience (and that of many other pain clinics) it is the preferred opioid-like drug in this indication, in particular as a background analgesic in a slow-release preparation. However, it is not a first-line drug in neuropathic pain.[2,3]

Antidepressants

Tricyclic antidepressants (TCAs)

In 1960 Paoli *et al* made the incidental discovery that the tricyclic antidepressant (TCA) imipramine had an analgesic effect.[33] Since then other TCAs and other antidepressants have been evaluated and used for the treatment of neuropathic pain. TCAs are the first class of medication to be proven to be effective for neuropathic pain in a double blind placebo controlled trial.[34] The role of TCAs in the treatment of neuropathic pain is now well established and has the best documented evidence.[35] The overall NNT for neuropathic pain is 3.6 with better efficacy in diabetic neuropathy than in postherpetic neuralgia (PHN).

Amitriptyline is established as the 'gold standard' as it has the most evidence available, especially for the treatment of painful diabetic neuropathy and PHN.[36] However,

amitriptyline and other TCAs have also been evaluated for the relief of pain in peripheral neuropathies and central post stroke pain. In comparative trials no single TCA has been found to be superior for neuropathic pain except in PHN where amitriptyline was found to be superior to maprotiline.[34]

Mechanism of action

Initially it was thought that the analgesic action of TCAs was related to their antidepressant activity. However, it is now clear that there is an independent specific analgesic effect, as the doses used to relieve neuropathic pain are smaller and the onset of analgesic efficacy is faster than an antidepressant effect and analgesia does not appear to depend upon mood improvement in depressed patients.[37,38] In addition, pain relief was found to be independent of any sedative effect.

TCAs are inhibitors of the reuptake of monoaminergic transmitters and this mechanism mediates their analgesic effect by the following presumed mechanisms: [36]

1) Central blockade of monoamine re-uptake, particularly serotonin and noradrenaline, leads to enhancement of the descending inhibitory monoaminergic pathways in the dorsal horn of the spinal cord.
2) Anticholinergic activity reduces firing of central neurones involved in pain, especially after deafferation.

Additionally there may be a number of other contributing mechanisms: moderation of NMDA receptor activity, opioid receptor activity, increase in dopamine or endorphin levels, blockade of central or peripheral histamine receptors, sodium channel blockade and blockade of adrenergic receptors on regenerating sprouts.[36,39]

Adverse effects

The optimum analgesic dose of TCAs can often not be reached due to unpleasant side effects. A systematic review of randomised controlled trials of TCAs used to treat neuropathic pain found that out of 100 patients, 30 had significant pain relief, 30 had minor side effects and 40 had to discontinue their therapy due to side effects.[40] These include:

1) Anticholinergic: dry mouth, constipation, urinary retention and blurred vision.
2) Antihistaminergic: confusion and sedation (the latter may be of benefit)
3) Anti α-adrenergic: postural hypotension and sexual dysfunction.

Cardiac conduction abnormalities may also arise due to the muscarinic anticholinergic actions. Patients at risk should have a pre-treatment ECG as cardiac conduction defects are a contraindication to treatment with TCAs. Another potential problem is overdose where TCAs are more dangerous than other groups of antidepressants and maybe fatal due to severe cardiac arrhythmias and convulsions.

Desipramine, imipramine and nortriptyline are more specific to noradrenergic blockade and are associated with less anticholinergic and antihistamine side effects. They may be useful in patients who are not able to tolerate amitriptyline, before progressing to another class of drug. In PHN and painful diabetic neuropathy, they were both found to be as effective as amitriptyline,[34,37,41] but associated with less side effects. Physical withdrawal reactions have been described for most antidepressants, but psychological addiction is not an issue.[36]

Selective serotonin re-uptake inhibitors (SSRIs)

Selective serotonin re-uptake inhibitors such as fluoxetine and paroxetine have only

limited efficacy in neuropathic pain.[35] They alter serotonergic (5-HT) far more than noradrenergic (NE) neurotransmission. However, due to their selectivity they do not interfere as much with adrenergic, histaminergic or muscarinic receptors and therefore have fewer side effects. There is currently insufficient evidence to make generalisations regarding their use in this indication.[42]

Serotonin/Noradrenaline reuptake inhibitors (SNRIs)

Venlafaxine and duloxetine are novel antidepressants, which belong to the class of SNRIs. They have similar mechanisms of action as TCAs, but no anticholinergic effects. While data on venlafaxine are currently inconclusive, duloxetine has indications in the treatment of diabetic polyneuropathy (NNT = 6) and fibromyalgia (NNT = 8) and is approved for this indication in some countries.[43] There are no direct comparisons to other antidepressants published, but SNRIs are thought to be better tolerated than TCAs.

Recommendations for clinical use of antidepressants as analgesics

Evidence-based decisions to use antidepressants in neuropathic pain states are usually based on a NNT approach:[35,43,44]

- TCAs for PHN: NNT 2.7
- TCAs for atypical face pain: NNT 2.8
- TCAs for diabetic neuropathy: NNT 1.3
- Duloxetine for diabetic neuropathy: NNT 5.8 - 6

It is difficult to generalise a dosage regimen for antidepressants in neuropathic pain, due to significant inter-individual variability.[45] McQuay *et al* have demonstrated a dose response relationship for amitriptyline with a greater analgesic response at 75mg/d than 25 or 50mg/d.[46]

Current recommendations for prescribing TCAs are:[4]

1) Start with a low dose (5–10mg/d) especially in the elderly and increase this weekly to analgesic efficacy or unacceptable side effects.
2) Once the optimal dose is achieved analgesic efficacy usually takes up to a week to achieve.
3) There have not been any trials conducted for longer than 6 weeks, so there is no evidence base for optimum duration of treatment. The current practice is to continue the same effective dose for several months and then to try and reduce it.
4) If a therapeutic dose of a TCA fails to provide pain relief, other antidepressants are also likely to fail.
5) If a TCA provides pain relief at the expense of unacceptable side effects then other antidepressants (in particular SNRIs) with a lower side effect profile should be tried.
6) If due to contraindications or unacceptable side effects a patient is unable to be treated with TCAs, other antidepressants (in particular SNRIs) should be tried before excluding this drug category.

Anticonvulsants

In the 1960s phenytoin was found to have an analgesic effect in the treatment of painful diabetic neuropathy.[47] Since then anticonvulsants have been evaluated and used in neuropathic pain states, including old agents: carbamazepine, sodium valproate, phenytoin and newer agents: gabapentin, lamotrigine, felbamate and pregabalin. Anticonvulsants have a specific indication in the treatment of trigeminal neuralgia with carbamazepine the first line therapy. They

may prove effective in conditions that have proved intractable to other treatments.

Mechanism of action

The neuronal hyperexcitability and corresponding molecular changes in neuropathic pain have many features in common with the cellular changes in certain forms of epilepsy.[48] The pain relieving effect of anticonvulsants is thought to be due to dampening of abnormal central nervous activity that follows nerve damage.[49] This may occur by: [50,51]

1) Sodium channel blockade resulting in a reduction of ectopic firing in both peripheral nerves and the dorsal root ganglion
2) Indirect or direct enhancement of inhibitory GABAergic neurotransmission
3) Inhibition of excitatory glutaminergic neurotransmission

Overall effects may be due to a combination of these mechanisms and longer term neuroplastic effects.[51] The process of ectopic impulse generation is so sensitive to sodium channel blockade that these agents have an action at much lower concentrations than that required to block normal neuronal transmission.[52]

Individual medications

Clonazepam

Clonazepam is a benzodiazepine anticonvulsant acting as a GABA agonist. Lorazepam, nitrazepam and diazepam have also been used in chronic pain. They have anxiolytic and anticonvulsant properties. However, with the exception of clonazepam, benzodiazepines are not generally felt to have specific analgesic activity and their use is not encouraged for this purpose due to their addictive nature, tolerance and cognitive impairment.[53]

However, for clonazepam several studies suggest a role in lancinating neuropathic pain. The old cross-over trial by Swerdlow shows clonazepam to be superior to carbamazepine, phenytoin and sodium valproate with regard to efficacy in neuropathic pain and adverse effects.[54]

This reflects our past clinical experience, where clonazepam was an easy to use agent with excellent efficacy and minimal side effects, in particular sedation. However, clonazepam is a benzodiazepine and thereby closely linked to risks of tolerance, dependence and addiction/abuse and should no longer be used as a first-line agent in neuropathic pain.

Gabapentin

Gabapentin is a relatively new anticonvulsant, available in the USA since 1995. It is a lipophilic GABA analogue but does not interact with $GABA_A$ or $GABA_B$ receptors or directly affect GABA uptake.[55] It is now clear that this drug has a modulating effect on the $\alpha 2\text{-}\delta$ subunit of voltage-gated calcium channels, an unexpected pharmacological target.[56] By modulating the calcium influx into hyperexcitable primary afferent neurons, gabapentin reduces the release of excitatory amino acids, in particular glutamate, and thereby reduces the excitation of secondary neurons. This explains its effects in neuropathic pain, but also in other conditions presenting with hyperalgesia and allodynia including fibromyalgia, even postoperative[57] and burns pain[58] and its anxiolytic effect with efficacy in generalised anxiety disorder. Large scale RCTs have demonstrated efficacy in PHN and diabetic neuropathy at target doses of 3600mg/day.[59-61]

The Cochrane review reports NNT of 3.9 in PHN and 2.9 for painful diabetic neuropathy.[62] Results indicate a similar efficacy of gabapentin and TCAs.[63]

A case report cited a significant improvement with gabapentin treatment in a patient with central post stroke pain that had failed

to respond to a variety of analgesics.[64] Gabapentin was also effective in the treatment of central neuropathic pain after spinal cord injury.[65]

The most commonly reported side effects are somnolence, fatigue, ataxia and dizziness. A dose adjustment is required in renal failure, but not in hepatic disorders as gabapentin is excreted unchanged by the kidneys.

The effective analgesic dose of gabapentin is variable, with some patients responding at low doses and others requiring high doses (more than 3600mg/day) for the same benefit. This is partially due to uptake by an active carrier process, showing saturation kinetics. It has been suggested that treatment failure maybe due to inadequate dosage, although rapid dose escalation can be responsible for the high incidence of CNS side effects.[66] The development of pregabalin with better kinetics and higher efficacy has reduced the usage of gabapentin.

Pregabalin

Pregabalin, an analogue to gabapentin, has been developed with an indication for neuropathic pain. It has a similar pharmacodynamic effect to gabapentin, i.e. modulates the $\alpha 2$-δ subunit of voltage-gated calcium channels and thereby reduces excitatory amino acid release.[56] It differs from gabapentin insofar as it has a higher potency, a better bioavailability, linear absorption kinetics and a longer half-life permitting twice instead of three times daily dosing.

Pregabalin is used successfully in a number of neuropathic pain states of peripheral and central origin including PHN, diabetic neuropathy (NNT 3.24)[67] and spinal cord injury pain.[68] It has also been used successfully in fibromyalgia[69] and generalised anxiety disorder[70] and has these three conditions as an indication in many countries. It is not only superior to gabapentin from a pharmacokinetic point of view, but also in clinical practice achieving better pain relief and quality of life.[71]

Adverse effects of pregabalin include sedation, drowsiness, disturbance of balance and unexplained peripheral oedema. However, these adverse effects are often mild and can be partially avoided by slow and careful titration of the dose. Starting doses of 75mg in ambulatory patients (with 25mg in the frail), starting with an evening dose and higher evening than morning doses are useful recommendations for the titration process.

The efficacy of pregabalin and its mild adverse effects have made it a viable first-line alternative to antidepressants in the setting of neuropathic pain. An interesting aspect from a surgical perspective is its perioperative use, which leads to improved postoperative pain, reduced opioid consumption and opioid side effects.[57] Two more recent studies suggest further benefit from its perioperative use by improving recovery after laminectomy[72] and reducing chronic neuropathic pain after knee joint replacement.[73]

Carbamazepine

Carbamazepine has been the first line treatment for trigeminal neuralgia for many years.[74] A recent Cochrane review found that three placebo-controlled trials of carbamazepine in trigeminal neuralgia demonstrated a combined NNT of 2.5.[75] It has not however been shown to be efficacious in PHN or central pain and its use in other neuropathic states has been reported only in small uncontrolled studies. Evidence shows carbamazepine inhibits spontaneous and evoked responses of spinal neurones and increases brain serotonin. Doses of up to 1200mg/day can be used.

Side effects are the main limitation to its use and include sedation, ataxia, drug interactions and liver dysfunction.[76] Serious but rare side effects are irreversible aplastic anaemia and Stevens-Johnson-Syndrome.

With carbamazepine therapy regular haematological and liver function monitoring is required. Occasional monitoring of serum sodium is also recommended because hyponatraemia can occur. The sustained release preparations of carbamazepine may limit the side effects.

Sodium valproate

This is structurally unrelated to other anticonvulsants and does not block sodium channels. The exact mechanism of action is unknown but may be related to increased GABA synthesis and release and hence potentiated GABAergic inhibition. In addition valproate attenuates the neuronal excitation caused by glutamate activation of NMDA receptors.[77] There is evidence for its use in migraine prophylaxis[78] and some for second line therapy in trigeminal neuralgia.[79] It can be used in doses up to 800mg/day.

Again side effects and the risk of serious toxicity limit its use. These include sedation, gastrointestinal disturbance, altered liver function with potentially fatal hepatotoxicity, decreased platelet aggregation and other haematological effects and drug interactions. Close follow up is mandatory.

Phenytoin

Phenytoin can be of help in patients with neuropathic pain but less so than carbamazepine. It has fallen from favour mainly due to its extensive side effect profile, complex kinetics and drug interactions and a lack of supportive studies.

Side effects include sedation, gingival hypertrophy, hirsutism and coarsening of facial features. At high blood levels neurotoxicity occurs and cardiac conduction is affected and thus close blood drug level monitoring is required. Results from RCTs have shown an analgesic effect in diabetic neuropathy and Fabrys disease.[80,81] The Cochrane review found that NNT for diabetic neuropathy with phenytoin was 2.1.[75]

Lamotrigine

This new anticonvulsant appears to act on voltage-gated cation channels (calcium and potassium) as well as inhibiting glutamate release.[82] Studies (open and double blind) have indicated that lamotrigine can be effective in diabetic neuropathy, central post stroke pain, HIV associated polyneuropathy and trigeminal neuralgia.[48,53,83] It may be useful in cases of trigeminal neuralgia that have proven refractory to carbamazepine and phenytoin, in doses of 50–400mg/day.[53] However, other evidence suggests that it may not be more effective than placebo in many other cases of neuropathic pain.[82]

Side effects have restricted the use of lamotrigine: dizziness, constipation, nausea, somnolence and diplopia.[53] Lamotrigine is associated with Steven-Johnson-Syndrome with 1:1000 patients requiring hospitalisation and can be rarely fatal. These side effects can be lessened and the incidence of rash significantly decreased by slow titration of lamotrigine, starting at a dose of 12.5 to 25 mg per day and slowly increasing to 100 to 200 mg per day over 1 to 2 months.

Recommendations for clinical use of anticonvulsants as analgesics

Anticonvulsants are typically used for neuropathic pain that has a shooting, burning or lancinating character. Empirically they are often used in combination with a TCA, although the evidence for using both classes of drug in combination is not strong.

For peripheral neuropathic pain and spinal cord injury pain pregabalin is the anticonvulsant of choice.[2] For trigeminal neuralgia only, carabamazepine is the first choice.[2] Although few trials exist for the treatment of central post-stroke pain, current opinion is that lamotrigine and gabapentin may be helpful.[84]

As with antidepressants, titration should start with low doses, gradually increasing to

a dose that either produces analgesic efficacy or unacceptable side effects.

Local anaesthetics and antiarrhythmics

In 1948 systemic procaine was identified as beneficial in the treatment of neuropathic pain. This led to the evaluation of other local anaesthetics for the treatment of neuropathic pain.

Mechanism of action

The mechanism of analgesic action is thought to be due to membrane stabilising effects by blockade of voltage-dependent sodium channels and hence reduced ectopic activity in damaged afferent nerves.[50] In addition there maybe a central action on sodium channels and at the spinal level, blocking the actions of glutamate.[85,86]

Lignocaine

Over the last 35 years there have been reports of analgesic efficacy of intravenous lignocaine in a wide range of neuropathic pain states, including diabetic neuropathy, peripheral nerve lesions, PHN and central pain.[87-92] Sakuri and Kanazawa have reported its effectiveness in multiple sclerosis associated pain.[93] There is large variation in reported duration of analgesic effect, varying from no residual effect to 20 weeks benefit in patients with central pain. A beneficial response to a lignocaine infusion may suggest a similar benefit from oral mexiletine, but does not predict this reliably.[94]

Mexiletine

This antiarrhythmic is an oral analogue of lignocaine that has been used in neuropathic pain with mixed results. Some effectiveness has been demonstrated in treating pain after peripheral nerve injuries and painful diabetic neuropathy, although these findings are not consistent and the effects are less than that provided by TCAs and anticonvulsants with an NNT of 10.[95] Optimal dosing may be a problem with a poor therapeutic ratio and potential cardiotoxicity.[4] Mexiletine should only be regarded as a last resort in the treatment of neuropathic pain.

Recommendations for clinical use of lignocaine and mexilitine in neuropathic pain

Side effects of both substances are CNS (dizziness, nausea, perioral numbness, convulsions & coma) and CVS effects (arrhythmias). Contraindications therefore include, cardiac conduction abnormalities, left ventricular failure and ischaemic heart disease. An ECG should be obtained before and during treatment to monitor any cardiac effects. If there is a question regarding safety in a patient, a cardiologist's opinion should be sought, prior to starting treatment.

For lignocaine the recommended starting dose is 1 –1.5mg/kg as a slow IV bolus; this is an ideal agent for the neuropathic pain emergency. Maintenance is by IV infusion of 1-3mg/min with measurement of blood concentrations.The recommended starting dose for mexiletine is 150mg three times a day with a slow increase to 600mg to 1200mg per day to optimal results.

N-methyl-D-aspartate-receptor (NMDA) antagonists

NMDA receptors are activated by the excitatory neurotransmitter glutamate. NMDA antagonists are thought to play an important role in the development of central sensitisation following a peripheral nerve lesion. They may block this hyperactivity responsible for the maintenance of the pain. Drugs with NMDA receptor antagonist activity include ketamine, dextromethorphan, memantine and amantadine.

Ketamine

Ketamine is the most commonly used NMDA antagonist. Its original use was as an anaesthetic agent, particularly 'in the field' and other difficult locations and situations. It has also been used for the treatment of severe asthma and for sedation. However, ketamine is known to have analgesic properties at subanaesthetic doses.[96]

Analgesic efficacy of ketamine has been demonstrated in RCTs for PHN, peripheral nerve injuries, phantom limb pain and post stroke central pain.[96] In peripheral[97] and central neuropathic pain states,[98] low-dose IV ketamine was superior to IV lignocaine. Ketamine may in part provide analgesia by reversing opioid tolerance.[99] In opioid-tolerant patients low-dose ketamine improves postoperative analgesia and reduces opioid requirements.

Unpleasant side effects limit its use, although they occur rarely with the low doses commonly used to treat neuropathic pain. These are mostly psychomimetic: sedation, hallucinations, dysphoria, unpleasant sensations (dissociation) and paranoid feelings. It is important to warn patients in advance of these potential effects; they can be reduced by co-prescribing benzodiazepines such as midazolam if needed. The pharmacokinetics of a sublingual and oral dosing form have been documented[100] and a nasal spray of ketamine is under development.[101]

Other NMDA antagonists

Dextromethorphan, amantidine and memantine have been shown to have weaker actions than ketamine. In a blinded trial, Nelson *et al* demonstrated an analgesic effect with high dose dextromethorphan in painful diabetic neuropathy,[102] but this has not been reproduced in other neuropathic pain states.[103] Memantine was also shown to be ineffective in phantom limb pain treatment;[104] its routine use in neuropathic pain can currently not be recommended.[105]

Miscellaneous compounds for systemic use

Clonidine

Clonidine is an α_2-agonist with analgesic activity. Its analgesic action is thought to occur centrally and at a spinal level, mediated by activation of α_2-adrenoceptors in the dorsal horn of the spinal cord. This results in direct inhibition of postsynaptic spinal dorsal horn neurones or by decreasing the release of noradrenaline from sympathetic nerve terminals.

Efficacy

Only a small number of studies have been conducted to look at a potential role in the treatment of neuropathic pain. Significant improvement was reported in patients with PHN treated with clonidine.[106] Transdermal clonidine (0.1 to 0.3mg per day) has been used with success in patients with diabetic neuropathies.[107,108] A double blind crossover study in 20 chronic pain patients, comparing epidural clonidine and an epidural combination of morphine and lignocaine found epidural clonidine to be as effective as epidural morphine in 20 chronic pain patients.[109] It is registered in the USA as an adjuvant in combination with epidural local anaesthetics and opioids for resistant neuropathic pain. Side effects include drowsiness, dizziness and dry mouth.

Baclofen

Baclofen is a gamma-aminobutyric acid (GABA) receptor agonist, capable of crossing the blood-brain barrier. It is an agonist at GABA-B receptors and has presynaptic action in the spinal cord preventing the release of excitatory neurotransmitters.[110]

Baclofen causes muscle relaxation and is used to treat muscle spasticity. It has been shown to have antinociceptive action and has been used to treat neuropathic pain.[111] It was

first used for this purpose to treat trigeminal neuralgia.[112] Its efficacy has not however been confirmed in other neuropathic pain conditions.[113] Baclofen has been administered intrathecally and may be useful for pain related to spinal cord injuries.[114] Side effects include sedation, nausea, confusion, convulsions, hypotension, GI upset, visual disturbances and occasionally hepatic impairment (A to Z). After prolonged use, baclofen requires a gradual dose reduction in order to minimise the risk of a withdrawal syndrome.[110]

Levodopa

Ertas *et al* found levodopa to be better than placebo in treating painful diabetic neuropathy.[115] A review of placebo-controlled trials by Sindrup and Jensen in patients with diabetic neuropathy showed that NNT was 3.4 for levodopa, compared with 6.7 for SSRIs.[95] A placebo-controlled trial has demonstrated efficacy in acute herpes zoster pain.[116]

Cannabinoids

There has been increasing interest in the use of cannabis and cannabinoids as analgesics in chronic pain. Cannabinoid receptors are located in the central and peripheral nervous system. Animal models have shown that cannabinoid receptors do not undergo down-regulation after nerve lesions (unlike opioid receptors) and that cannabinoids may attenuate the associated sensory changes.[8]

Cannabis has been used for thousands of years for medicinal and recreational purposes. There is much interest surrounding its legalisation and its potential role as an analgesic. The data situation here remains unclear; however, overall there is a trend to show some efficacy by some cannabinoids in some neuropathic pain states. A meta-analysis found efficacy in neuropathic pain states including multiple sclerosis.[117] Similarly, a randomised trial of smoked cannabis in neuropathic pain reduced pain intensity and improved sleep quality.[118] However, a trial in spinal cord injury pain failed to show efficacy.[119] Adverse effects associated with cannabinoids are common, the main being sedation, disorientation, ataxia, memory impairment, dry mouth and blurred vision.

Larger blinded, randomised controlled trials are required before it can be ascertained whether cannabinoids are efficacious in neuropathic pain. The development of new safe and effective agonists that separates the psychotropic effects from the therapeutic ones would improve trial designs.

Topical treatments

Allodynia is frequently a feature of neuropathic pain especially in PHN, traumatic neuropathies and causalgia. It may therefore be helpful to consider the use of topical medications for the treatment of cutaneous hyperalgesia in these cases. There are a few options in the form of capsaicin, local anaesthetics (and NSAIDs with some reports of good pain relief from post herpetic neuralgia with topical aspirin preparations).[120-122]

Lignocaine 5% medicated plaster

In patients with PHN, success has been reported using lignocaine patches or topically applied gel to painful areas.[52,123,124] The mechanism of action is thought to involve suppression of ectopic discharges from sensory afferents and from providing mechanical protection to underlying allodynic skin.[125] In 1999 the FDA approved the use of 5% topical lignocaine patches for treatment of PHN. It is recommended as a first-line approach for this indication and other localised neuropathic pain states.[126] A meta-analysis showed superiority of the plaster over capsaicin and pregabalin and

similar efficacy to gabapentin, however with significantly fewer systemic side effects.[127]

The advantages of this route of administration are its effectiveness, duration of analgesia, ease of application without dose titration and lack of systemic side effects. The safety profile is particularly advantageous in the elderly population whom are most affected by PHN. The area of pain has however to be of limited size for practical application.

Capsaicin

Capsaicin is the pungent component to chilli peppers. The chilli pepper has been recognised by various cultures for many years for its medicinal qualities.[128] It is neurotoxic and has analgesic properties. When capsaicin is applied topically it initially causes a burning sensation and heat hyperalgesia that decreases with subsequent applications.

Mechanism of Action

Capsaicin acts on receptors at the terminals of primary nociceptive afferents. In 1997 a specific receptor on C fibres was cloned, a vanilloid receptor, the VR-1 receptor. When capsaicin binds to this receptor, it induces initial activation of the nociceptors, hence the burning sensation. It depletes substance P from the sensory nerve terminals of peripheral nociceptors. With repeat or prolonged application, this is followed by desensitisation and inactivation of the receptive terminals of the nociceptors.[129] There is also evidence that it causes depletion of substance P in epidermal nerve fibres.[130]

Efficacy

In a meta-analysis of RCTs, low-dose capsaicin cream (0.075%) repeatedly administered had an NNT of 6.6 for any pain relief.[131] A commercially available patch with high-dose capsaicin (8%) had an NNT of 12 for 30% pain relief. These not very impressive results have to be seen in the context of a potentially high placebo effect, as the burning feeling after administration results in the patient effectively being unblinded. This criticism is confirmed by one double blind trial using a placebo with a similar burning sensation and finding no difference in analgesic efficacy between placebo and capsaicin.[132]

The use of capsaicin has been limited by this unpleasant burning sensation occurring in 60–70% patients, need for frequent applications and uncertain efficacy.[4] Co-administration of lignocaine gel has been used in order to improve compliance.[4]

Capsaicin can only be seen as an adjuvant treatment and second line therapy.

NON PHARMACOLOGICAL THERAPY

Transcutaneous electrical nerve stimulation (TENS)

This technique applies cutaneous electrodes to stimulate peripheral nerves to relieve pain. [133] This is based on the gate control theory of pain transmission, so that by stimulating Aβ and Aδ fibres pain transmission by C-fibres is inhibited. It utilises a pulse generator that provides a range of currents, frequencies and pulse widths. The surface electrodes are placed on either side of the painful area or alternatively over the nerves supplying that area. The current is then increased until a tingling sensation is felt in the painful area, the timing and duration of pulses is a matter of titration to maximal response. TENS may reduce analgesic requirements.[134] A meta-analysis shows improvement in neuropathic symptoms in patients with diabetic polyneuropathy.[135]

It has few side effects and complications, allergic dermatitis may occur at the contact sites and its use is contraindicated in patients with pacemakers. Its efficacy can be assessed

quickly (there is a significant placebo effect) and can therefore be easily trialled for any potential benefit to an individual.[136] Unfortunately tolerance may develop resulting in loss of previously effective analgesia, changing the stimulation variables can sometimes attenuate this.

Spinal cord stimulation (SCS)

This technique requires an implantable device with electrodes positioned under direct vision at open laminectomy or via a needle in the epidural space percutaneously. The electrodes are placed above the level of the pain and connected to an inductance coil placed on the abdominal wall; an implantable power source can also be used. The mechanism of action is not yet clear, but it seems to be not effective in nociceptive pain, but only in neuropathic pain.[137] It seems that this device can be as useful for a variety of neuropathic and chronic pain states.[138] A systematic review describes efficacy in refractory neuropathic back and leg pain, failed back surgery syndrome and chronic regional pain syndrome (CRPS) Type 1.[139]

Complications include infection, bleeding, dural puncture and hardware failure; decisions on use of this invasive and expensive approach should be made ideally by a multidisciplinary team experienced in the use of such techniques. Deep brain stimulation and motor cortex stimulation have also been used to treat neuropathic pain.[140]

Sympathetic nerve blocks

The diagnosis of sympathetically maintained pain can be confirmed by the response to a sympatholytic procedure. This may be helpful, if there is a significant sympathetic component to the patient's pain. A patient should receive sustained pain relief after administration of a sympathetic chain or sympathetic plexus local anaesthetic block or accumulative relief from a number of procedures. If the patient fails to respond, a systematic pharmacological approach is tried. However, a block may be incorrectly thought to be successful. This can happen for one of two reasons: either, the local anaesthetic is absorbed and provides a systemic analgesic effect or it diffuses locally and acts on nearby somatic nerves.[141]

In case of effectiveness, progression to a sympatholytic procedure can be chosen. Techniques then involve the use of neurolytic substances or radiofrequency ablation; regrettably the current scientific basis for this approach is poor.[142]

Neurosurgical destructive techniques

There has been increasing awareness that destructive techniques may in fact increase pain in the long term due to the plasticity of the nervous system, sometimes resulting in incapacitating side effects. Therefore these techniques, which include neurectomy and dorsal root entry zone lesions, are now rarely used.[143] The exception being in the treatment of trigeminal neuralgia, which has proved refractory to pharmacological treatments. In this situation a variety of surgical procedures can provide rapid pain relief, the most effective option being microvascular decompression. Recurrence is still a risk but appears to be more frequent after percutaneous radiofrequency rhizotomy or compression with a percutaneously positioned balloon than the more invasive microvascular decompression technique.[144]

Cognitive-behavioural therapy

Chronic neuropathic pain is best managed in a multidisciplinary pain clinic.[145] This is because the patient often has cognitive,

affective and behavioural factors influencing their pain; however new understandings of the physiology of cortical reorganisation in chronic pain might also lead to new psychological approaches.[146] A multidisciplinary approach will address both the somatic and psychological aspects of the patient's condition. The main methods are cognitive-behavioural therapy and operant conditioning.

The cognitive-behavioural approach aims to identify and modify the patient's thoughts, feelings, beliefs and behaviour. Common problems are anxiety, depression and the development of fear-avoidance. Behavioural therapy procedures are utilised to bring about change. The aims are to enable patients to take a positive and active role in coping with their pain and to change maladaptive behaviour that may be aggravating the problem.

Operant conditioning uses firstly continuous re-enforcement to encourage positive behaviour from the patient that is then stepped down later on. This is based on the belief that the consequence of certain behaviour determines whether it is likely to recur.

Psychotherapy may increase levels of activity and decrease medication requirements but an actual reporting in pain reduction may be more modest. In addition the therapy may need to be extended to the carers of the patient in order to change their response to the patient's beliefs and behaviour. The current data situation on this approach to neuropathic pain is insufficient to draw any conclusions on efficacy.[147]

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23 • Principles of Wound Healing

GREGORY S. SCHULTZ[1], GLORIA A. CHIN[2], LYLE MOLDAWER[2], ROBERT F. DIEGELMANN.[3]

[1]Department of Obstetrics and Gynecology, University of Florida, Gainesville, Florida, USA

[2]Department of Surgery, University of Florida, Gainesville, Florida, USA

[3]Department of Biochemistry, Medical College of Virginia, Richmond, Virginia, USA

INTRODUCTION

Acute wounds normally heal in an orderly and efficient manner, and progress smoothly through the four distinct, but overlapping phases of wound healing: *haemostasis*, *inflammation*, *proliferation* and *remodelling* (Figure 23.1).[1,2,3] In contrast, chronic wounds will similarly begin the healing process, but will have prolonged inflammatory, proliferative, or remodelling phases, resulting in tissue fibrosis and in non-healing ulcers.[4] The process of wound healing is complex and involves a variety of specialized cells, such as platelets, macrophages, fibroblasts, epithelial and endothelial cells. These cells interact with each other and with the extracellular matrix. In addition to the various cellular interactions, healing is also influenced by the action of proteins and glycoproteins, such as cytokines, chemokines, growth factors, inhibitors, and their receptors. Each stage of wound healing has certain milestones that must occur in order for normal healing to progress. In order to identify the differences inherent in chronic wounds that prevent healing, it is important to review the process of healing in normal wounds

PHASES OF ACUTE WOUND HEALING

Haemostasis

Haemostasis occurs immediately following an injury.[5] To prevent exsanguination, vasoconstriction occurs and platelets undergo activation, adhesion and aggregation at the site of injury. Platelets become activated when exposed to extravascular collagen (such as type I collagen), which they detect via specific integrin receptors, cell surface receptors that mediate a cell's interactions with the extracellular matrix. Once in contact with collagen, platelets release the soluble mediators (growth factors and cyclic AMP) and adhesive glycoproteins, which signal them to become sticky and aggregate. The key glycoproteins released from the platelet alpha granules include fibrinogen, fibronectin, thrombospondin, and von Willebrand factor. As platelet aggregation proceeds, clotting factors are released resulting in the

deposition of a fibrin clot at the site of injury. The fibrin clot serves as a provisional matrix.[6] The aggregated platelets become trapped in the fibrin web and provide the bulk of the clot (Figure 23.2). Their membranes provide a surface on which inactive clotting enzyme proteases are bound, become activated and accelerate the clotting cascade.

Growth factors are also released from the platelet alpha granules, and include platelet derived growth factor (PDGF), transforming growth factor beta (TGF–β), transforming growth factor alpha (TGF-α), basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor (VEGF). Major growth factor

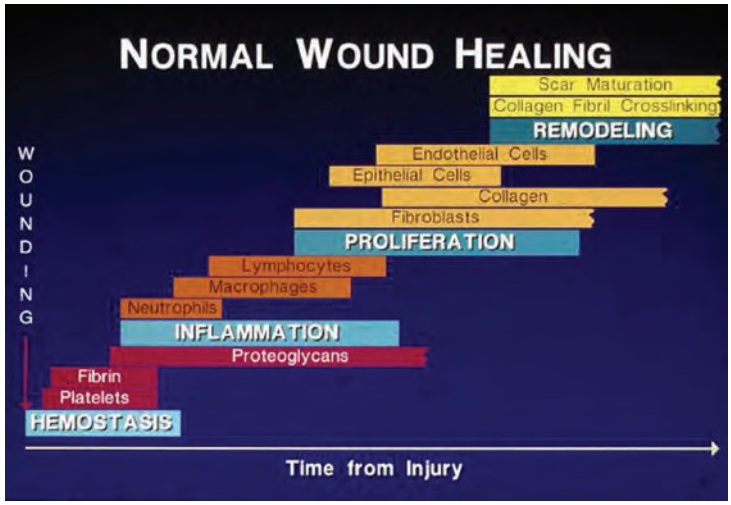

FIGURE 23.1: Phases of Normal Wound Healing. Cellular and molecular events during normal wound healing progress through four major, integrated, phases of haemostasis, inflammation, proliferation and remodelling.

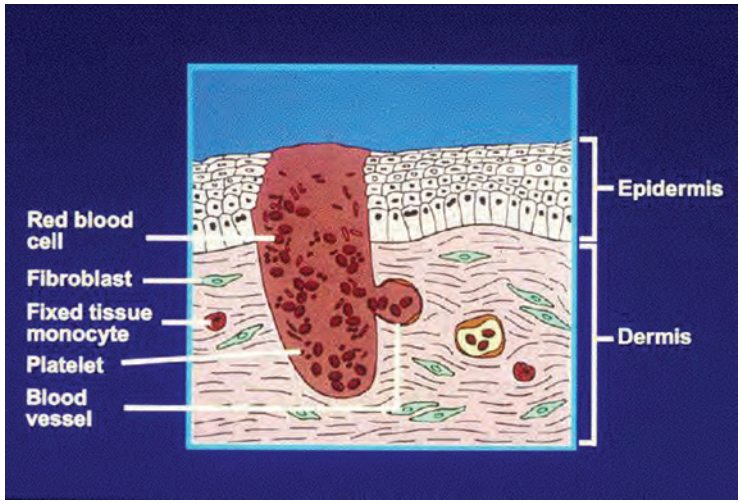

FIGURE 23.2: Haemostasis Phase. At the time of injury, the fibrin clot forms the provisional wound matrix and platelets release multiple growth factors initiating the repair process.

families are presented in Table 23.1. Neutrophils and monocytes are then recruited by PDGF and TGF-β from the vasculature to initiate the inflammatory response. A breakdown fragment generated from complement, C5a, and a bacterial waste product, f-Met-Leu-Phe, also provide additional chemotactic signals for the recruitment of neutrophils to the site of injury. Meanwhile, endothelial cells are activated by VEGF, TGF-α and bFGF to initiate angiogenesis. Fibroblasts are then activated and recruited by PDGF to migrate to the wound site and begin production of collagen and glycosaminoglycans, proteins in the extracellular matrix which facilitate cellular migration and interactions with the matrix supporting framework. Thus, the healing process begins with hemostasis, platelet deposition at the site of injury, and interactions of soluble mediators and growth

TABLE 23.1: Major growth factor families

Growth factor family	Cell source	Actions
Transforming Growth Factor β TGF-β1, TGF-β2 TGF-β3	Platelets Fibroblasts Macrophages	Fibroblast Chemotaxis and Activation ECM Deposition ⇑ Collagen Synthesis ⇑ TIMP Synthesis ⇓ MMP Synthesis Reduces Scarring ⇓ Collagen ⇓ Fibronectin
Platelet Derived Growth Factor PDGF-AA, PDGF-BB, VEGF	Platelets Macrophages Keratinocytes Fibroblasts	Activation of Immune Cells and Fibroblasts ECM Deposition ⇑ Collagen Synthesis ⇑ TIMP Synthesis ⇓ MMP Synthesis Angiogenesis
Fibroblast Growth Factor Acidic FGF, Basic FGF, KGF*	Macrophages Endothelial Cells Fibroblasts	Angiogenesis Endothelial Cell Activation Keratinocyte Proliferation and Migration ECM Deposition
Insulin-like Growth Factor IGF-I, IGF-II, Insulin	Liver Skeletal Muscle Fibroblasts Macrophages Neutrophils	Keratinocyte Proliferation Fibroblast Proliferation Endothelial Cell Activation Angiogenesis ⇑ Collagen Synthesis ECM Deposition Cell Metabolism
Epidermal Growth Factor EGF, HB-EGF**, TGF-α, Amphiregulin, Betacellulin	Keratinocytes Macrophages	Keratinocyte Proliferation and Migration ECM Deposition
Connective Tissue Growth Factor CTGF	Fibroblasts Endothelial Cells Epithelial Cells	Mediates Action of TGF-βs on Collagen Synthesis

*KGF - keratinocyte growth factor
**HB-EGF - Heparin-binding EGF-like growth factor

factors with the extracellular matrix to set the stage for subsequent healing events.[1,2,7]

Inflammation

Inflammation, the next stage of wound healing occurs within the first 24 hours after injury and can last for up to 2 weeks in normal wounds and significantly longer in chronic non-healing wounds (Figure 23.3). Mast cells release granules filled with enzymes, histamine and other active amines, which are responsible for the characteristic signs of inflammation, the *rubor* (redness), *calor* (heat), *tumor* (swelling) and *dolor* (pain) around the wound site. Neutrophils, monocytes, and macrophages are the key cells during the inflammatory phase. They cleanse the wound of infection and debris and release soluble mediators such as proinflammatory cytokines (including IL-1, IL-6, IL-8, and TNF-α), and growth factors (such as PDGF, TGF-β, TGF-α, IGF-1, and FGF) that are involved in the recruitment and activation of fibroblasts and epithelial cells in preparation for the next phase in healing. Cytokines that play important roles in regulating inflammation in wound healing are described in Table 23.2.

In addition to the growth factors and cytokines, a third important group of small regulatory proteins, listed in Table 23.3, has been identified, and are collectively named chemokines, from a contraction of chemoattractive cytokine(s).[8,9,10] The structural and functional similarities among chemokines were not initially appreciated, and this has led to an idiosyncratic nomenclature consisting of many acronyms that were based on their biological functions, (e.g., monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1, MIP-1), their source for isolation (platelet factor-4, PF-4) or their biochemical properties (interferon-inducible protein of 10 kDa (IP-10), or regulated upon activation normal T-cell expressed and secreted, RANTES). As their biochemical properties were established, it was recognized that the approximately 40 chemokines could be grouped into four major classes based on the pattern of cysteine residues located near the N-terminus. In fact, there has been a

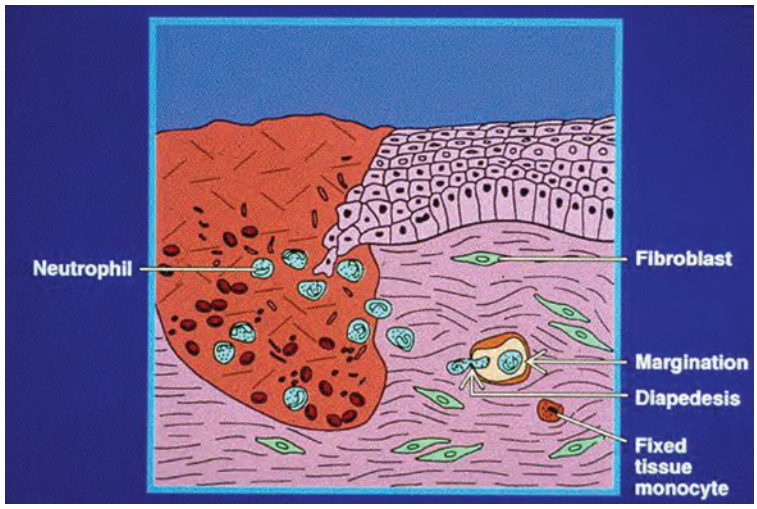

FIGURE 23.3: Inflammation Phase. Within a day following injury, the inflammatory phase is initiated by neutrophils that attach to endothelial cells in the vessel walls surrounding the wound (margination), change shape and move through the cell junctions (diapedesis), and migrate to the wound site (chemotaxis).

TABLE 23.2: Cytokines involved in wound healing

Cytokine	Cell source	Biological activity
Pro-inflammatory Cytokines		
TNF-α	Macrophages	PMN margination and cytotoxicity, ± collagen synthesis; provides metabolic substrate
IL-1	Macrophages Keratinocytes	Fibroblast and keratinocyte chemotaxis, collagen synthesis
IL-2	T lymphocytes	Increases fibroblast infiltration and metabolism
IL-6	Macrophages PMNs Fibroblasts	Fibroblast proliferation, hepatic acute-phase protein synthesis
IL-8	Macrophages Fibroblasts	Macrophage and PMN chemotaxis, keratinocyte maturation
IFN-γ	T lymphocytes Macrophages	Macrophage and PMN activation; retards collagen synthesis and cross-linking; stimulates collagenase activity
Anti-inflammatory Cytokines		
IL-4	T lymphocytes Basophils Mast cells	Inhibition of TNF, IL-1, IL-6 production; fibroblast proliferation, collagen synthesis
IL-10	T lymphocytes Macrophages Keratinocytes	Inhibition of TNF, IL-1, IL-6 production; inhibits macrophage and PMN activation

recent trend to re-establish a more organized nomenclature system based on these four major classes. In general, chemokines have two primary functions: 1) they regulate the trafficking of leukocyte populations during normal health and development, and 2) they direct the recruitment and activation of neutrophils, lymphocytes, macrophages, eosinophils and basophils during inflammation.

Neutrophils

Neutrophils are the first inflammatory cells to respond to the soluble mediators released by platelets and the coagulation cascade. They serve as the first line of defense against infection by phagocytosing and killing bacteria, and by removing foreign materials and devitalized tissue. During the process of extravasation of inflammatory cells into a wound, important interactions occur between adhesion molecules (selectins, cell adhesion molecules (CAMs) and cadherins) and receptors (integrins) that are associated with the plasma membranes of circulating leukocytes and vascular endothelial cells.[11,12] Initially, leukocytes weakly adhere to the endothelial cell walls via their selectin molecules which causes them to decelerate and begin to roll on the surface of endothelial

TABLE 23.3: Chemokine familes involved in wound healing

Chemokines	Cells affected
α-CHEMOKINES (CXC) with glutamic acid-leucine-arginine near the N-terminal Interleukin-8 (IL-8)	Neutrophils
α-CHEMOKINES (CXC) *without* glutamic acid-leucine-arginine near the N-terminal Interferon -inducible protein of 10 kd (IP-10) Monokine induced by interferon-γ (MIG) Stromal-cell-derived factor 1 (SDF-1)	Activated T lymphocytes
β-CHEMOKINES (CC) Monocyte chemoattractant proteins (MCPs): MCP-1,-2,-3,-4,-5 Regulated upon activation normal T-cell expressed and secreted (RANTES) Macrophage inflammatory protein (MIP-1α) Eotaxin	Eosinophils Basophils Monocytes Activated T lymphocytes
γ-CHEMOKINES (C) Lymphotactin	Resting T lymphocytes
δ-CHEMOKINES (CXXXC) Fractalkine	Natural killer cells

cells. While rolling, leukocytes can become activated by chemoattractants (cytokines, growth factors or bacterial products). After activation, leukocytes firmly adhere to endothelial cells as a result of the binding between their integrin receptors and ligands such as VCAM and ICAM that are expressed on activated endothelial cells. Chemotactic signals present outside the venule then induce leukocytes to squeeze between endothelial cells of the venule and migrate into the wounded tissue using their integrin receptors to recognize and bind to extracellular matrix components. The inflammatory cells release elastase and collagenase to help them migrate through the endothelial cell basement membrane and to migrate into the extracellular matrix (ECM) at the site of the wound. Neutrophils also produce and release inflammatory mediators such as TNF-α and IL-1 that further recruit and activate fibroblasts and epithelial cells. After the neutrophils migrate into the wound site, they generate oxygen free radicals, which kill phagocytized bacteria, and they release high levels of proteases (neutrophil elastase and neutrophil collagenase) which remove components of the extracellular matrix that were damaged by the injury. The persistent presence of bacteria in a wound may contribute to chronicity through continued recruitment of neutrophils and their release of proteases, cytokines and reactive oxygen species. Usually neutrophils are depleted in the wound after 2 to 3 days by the process of apoptosis, and they are replaced by tissue monocytes.

Macrophages

Activated macrophages play pivotal roles in the regulation of healing, and the healing process does not proceed normally without

macrophages. Macrophages begin as circulating monocytes that are attracted to the wound site beginning about 24 hours after injury (Figure 23.4). They extravasate by the mechanisms described for neutrophils, and are stimulated to differentiate into activated tissue macrophages in response to chemokines, cytokines, growth factors and soluble fragments of extracellular matrix components produced by proteolytic degradation of collagen and fibronectin.[13] Similar to neutrophils, tissue macrophages have a dual role in the healing process. They patrol the wound area ingesting and killing bacteria, and removing devitalized tissue through the actions of secreted MMPs and elastase. Macrophages differ from neutrophils in their ability to more closely regulate the proteolytic destruction of wound tissue by secreting inhibitors for the proteases. As important as their phagocytic role, macrophages also mediate the transition from the inflammatory phase to the proliferative phase of healing. They release a wide variety of growth factors and cytokines including PDGF, TGF-β, TGF-α, FGF, IGF-1, TNFα, IL-1, and IL-6. Some of these soluble mediators recruit and activate fibroblasts, which will then synthesize, deposit, and organize the new tissue matrix, while others promote angiogenesis. The absence of neutrophils and a decrease in the number of macrophages in the wound is an indication that the *inflammatory* phase is nearing an end, and that the *proliferative* phase is beginning.

Proliferative phase

The milestones during the *proliferative phase* include replacement of the provisional fibrin matrix with a new matrix of collagen fibers, proteoglycans, and fibronectin to restore the structure and function to the tissue. Another important event in healing is angiogenesis, the in-growth of new capillaries to replace the previously damaged vessels and restore circulation. Other significant events in this phase of healing are the formation of granulation tissue and epithelialization.

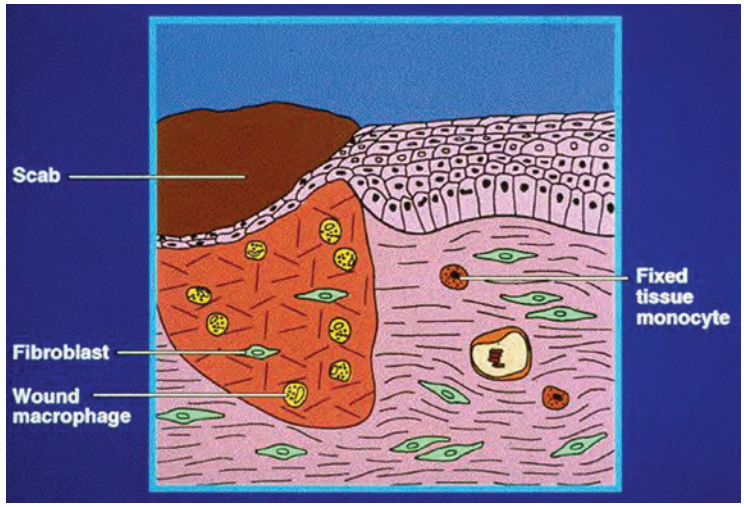

FIGURE 23.4: Proliferation Phase. Fixed tissue monocytes activate, move into the site of injury, transform into activated wound macrophages that kill bacteria, release proteases that remove denatured ECM, and secrete growth factors that stimulate fibroblasts, epidermal cells and endothelial cells to proliferate and produce scar tissue.

Fibroblasts are the key cells in the *proliferative phase* of healing.

Fibroblast migration

Fibroblasts migrate into the wound in response to multiple soluble mediators released initially by platelets and later by macrophages (Figure 23.4). Fibroblast migration in the extracellular matrix depends on precise recognition and interaction with specific components of the matrix. Fibroblasts in normal dermis are typically quiescent and sparsely distributed, whereas in the provisional matrix of the wound site and in the granulation tissue, they are quite active and numerous. Their migration and accumulation in the wound site requires them to change their morphology and to produce and secrete proteases to clear a path for their movement from the ECM into the wound site.

Fibroblasts begin moving by first binding to matrix components such as fibronectin, vitronectin and fibrin via their integrin receptors. Integrin receptors attach to specific amino acid sequences (such as R-G-D or arginine-glycine-aspartic acid) or binding sites in these matrix components. While one end of the fibroblast remains bound to the matrix component the cell extends a cytoplasmic projection to find another binding site. When the next site is found, the original site is released (apparently by local protease activity), and the cell uses its cytoskeleton network of actin fibers to pull itself forward.

The direction of fibroblast movement is determined by the concentration gradient of chemotactic growth factors, cytokines and chemokines, and by the alignment of the fibrils in the ECM and provisional matrix. Fibroblasts tend to migrate along these fibrils as opposed to across them. Fibroblasts secrete proteolytic enzymes locally to facilitate their forward motion through the matrix. The enzymes secreted by the fibroblasts include three types of MMPs, collagenase (MMP-1), gelatinases (MMP-2 and MMP-9) which degrade gelatin substrates, and stromelysin (MMP-3) which has multiple protein substrates in the ECM.

Collagen and extracellular matrix production

The collagen, proteoglycans and other components that comprise granulation tissue are synthesized and deposited primarily by fibroblasts. PDGF and TGF-β are two of the most important growth factors that regulate fibroblast activity. PDGF, which predominantly originates from platelets and macrophages, stimulates a number of fibroblast functions including proliferation, chemotaxis, and collagenase expression. TGF-β, also secreted by platelets and macrophages is considered to be the master control signal that regulates extracellular matrix deposition. Through the stimulation of gene transcription for collagen, proteoglycans and fibronectin, TGF-β increases the overall production of matrix proteins. At the same time, TGF-β down-regulates the secretion of proteases responsible for matrix degradation and also stimulates synthesis of tissue inhibitor of metalloproteinases (TIMP), to further inhibit breakdown of the matrix. Recent data indicate that a new growth factor, named connective tissue growth factor (CTGF), mediates many of the effects of TGF-β on the synthesis of extracellular matrix.[14]

Once the fibroblasts have migrated into the matrix they again change their morphology, settle down and begin to proliferate and to synthesize granulation tissue components including collagen, elastin and proteoglycans. Fibroblasts attach to the cables of the provisional fibrin matrix and begin to produce collagen. At least 20 individual types of collagen have been identified to date. Type III collagen is initially synthesized

at high levels, along with other extracellular matrix proteins and proteoglycans. After transcription and processing of the collagen mRNA, it is attached to polyribosomes on the endoplasmic reticulum where the new collagen chains are produced. During this process, there is an important step involving hydroxylation of proline and lysine residues. Three protein chains associate and begin to form the characteristic triple helical structure of the fibrillar collagen molecule, and the nascent chains undergo further modification by the process of glycosylation. Hydroxyproline in collagen is important because it plays a major role in stabilizing the triple helical conformation of collagen molecules. Fully hydroxylated collagen has a higher melting temperature. When levels of hydroxyproline are low, for example in vitamin C-deficient conditions (scurvy), the collagen triple helix has an altered structure and denatures (unwinds) much more rapidly and at lower temperatures. To ensure optimal wound healing, wound care specialists should be sure patients are receiving good nutritional support with a diet with ample protein and vitamin C.

Finally, procollagen molecules are secreted into the extracellular space where they undergo further processing by proteolytic cleavage of the short, non-helical segments at the N- and C-termini. The collagen molecules then spontaneously associate in a head-to-tail and side-by-side arrangement forming collagen fibrils, which associate into larger bundles that form collagen fibers. In the extra-cellular spaces an important enzyme, lysyl oxidase, acts on the collagen molecules to form stable, covalent, cross-links. As the collagen matures and becomes older, more and more of these intramolecular and intermolecular cross-links are placed in the molecules. This important cross-linking step gives collagen its strength and stability, and the older the collagen the more cross-link formation has occurred.

Dermal collagen on a per weight basis approaches the tensile strength of steel. In normal tissue, it is a strong molecule and highly organized. In contrast, collagen fibers formed in scar tissue are much smaller and have a random appearance. Scar tissue is always weaker and will break apart before the surrounding normal tissue.

Angiogensis

Damaged vasculature must be replaced to maintain tissue viability. The process of angiogenesis is stimulated by local factors of the microenvironment including low oxygen tension, low pH, and high lactate levels.[15] Also, certain soluble mediators are potent angiogenic signals for endothelial cells. Many of these are produced by epidermal cells, fibroblasts, vascular endothelial cells and macrophages, and include bFGF, TGF-β, and VEGF. It is now recognized that oxygen levels in tissues directly regulate angiogenesis by interacting with oxygen sensing proteins that regulate transcription of angiogenic and anti-angiogenic genes. For example, synthesis of VEGF by capillary endothelial cells is directly increased by hypoxia through the activation of the recently identified transcription factor, hypoxia-inducible factor (HIF), which binds oxygen.[16] When oxygen levels surrounding capillary endothelial cells drop, levels of HIF increase inside the cells. HIF-1 binds to specific DNA sequences and stimulates transcription of specific genes such as VEGF that promote angiogenesis. When oxygen levels in wound tissue increase, oxygen binds to HIF, leading to the destruction of HIF molecules in cells and decreased synthesis of angiogenic factors. Regulation of angiogenesis involves both stimulatory factors like VEGF and anti-angiogenic factors like angiostatin, endostatin, thrombospondin, and pigment epithelium-derived factor (PEDF).

Binding of angiogenic factors causes endothelial cells of the capillaries adjacent to the devascularized site to begin to migrate into the matrix and then proliferate to form buds or sprouts. Once again the migration of these cells into the matrix requires the local secretion of proteolytic enzymes, especially MMPs. As the tip of the sprouts extend from endothelial cells and encounter another sprout, they develop a cleft that subsequently becomes the lumen of the evolving vessel and complete a new vascular loop. This process continues until the capillary system is sufficiently repaired and the tissue oxygenation and metabolic needs are met. It is these new capillary tuffs that give granulation tissue its characteristic bumpy or granular appearance.

Granulation

Granulation tissue is a transitional replacement for normal dermis, which eventually matures into a scar during the remodelling phase of healing. It is characterized from unwounded dermis by an extremely dense network of blood vessels and capillaries, elevated cellular density of fibroblasts and macrophages and randomly organized collagen fibers. It also has an elevated metabolic rate compared to normal dermis, which reflects the activity required for cellular migration and division and protein synthesis.

Epithelialization

All dermal wounds heal by three basic mechanisms: contraction, connective tissue matrix deposition and epithelialization. Wounds that remain open heal by contraction; the interaction between cells and matrix results in movement of tissue toward the center of the wound. As previously described, matrix deposition is the process by which collagen, proteoglycans and attachment proteins are deposited to form a new extracellular matrix. Epithelialization is the process where epithelial cells around the margin of the wound or in residual skin appendages such as hair follicles and sebaceous glands lose contact inhibition and by the process of *epiboly* begin to migrate into the wound area. As migration proceeds, cells in the basal layers begin to proliferate to provide additional epithelial cells.

Epithelialization is a multi-step process that involves epithelial cell detachment and change in their internal structure, migration, proliferation and differentiation.[17] The intact mature epidermis consists of 5 layers of differentiated epithelial cells ranging from the cuboidal basal keratinocytes nearest the dermis up to the flattened, hexagonal, tough keratinocytes in the uppermost layer. Only the basal epithelial cells are capable of proliferation. These basal cells are normally attached to their neighboring cells by intercellular connectors called desmosomes and to the basement membrane by hemi-desmosomes. When growth factors such as epidermal growth factor (EGF), keratinocyte growth factor (KGF) and TGF-α are released during the healing process, they bind to receptors on these epithelial cells and stimulate migration and proliferation. The binding of the growth factors triggers the desmosomes and hemi-desmosomes to dissolve so the cells can detach in preparation for migration. Integrin receptors are then expressed and the normally cuboidal basal epithelial cells flatten in shape and begin to migrate as a monolayer over the newly deposited granulation tissue, following along collagen fibers. Proliferation of the basal epithelial cells near the wound margin supply new cells to the advancing monolayer apron of cells (cells that are actively migrating are incapable of proliferation). Epithelial cells in the leading edge of the monolayer produce and secrete proteolytic enzymes (MMPs) which enable the cells to penetrate scab, surface necrosis, or eschar. Migration continues until the

epithelial cells contact other advancing cells to form a confluent sheet. Once this contact has been made, the entire epithelial monolayer enters a proliferative mode and the stratified layers of the epidermis are re-established and begin to mature to restore barrier function. TGF-β is one growth factor that can speed up the maturation (differentiation and keratinization) of the epidermal layers. The intercellular desmosomes and the hemi-desmosome attachments to the newly formed basement membrane are also re-established. Epithelialization is the clinical hallmark of healing but it is not the final event – remodelling of the granulation tissue is yet to occur.

Recent studies by Sen, *et al.* have demonstrated that under conditions of hypoxia, HIF-1alpha is stabilized which in turn induces the expression of specific micro RNAs that then down-regulate epithelial cell proliferation (1). Therefore it appears that there are very complex mechanisms involved in the role of oxygen and hypoxia during the process of wound healing.

Remodelling

Remodelling is the final phase of the healing process in which the granulation tissue matures into scar and tissue tensile strength is increased (Figure 23.5). The maturation of granulation tissue also involves a reduction in the number of capillaries via aggregation into larger vessels and a decrease in the amount of glycosaminoglycans and the water associated with the glycosaminoglycans (GAGs) and proteoglycans. Cell density and metabolic activity in the granulation tissue decrease during maturation. Changes also occur in the type, amount, and organization of collagen, which enhance tensile strength. Initially, type III collagen was synthesized at high levels, but it becomes replaced by type I collagen, the dominant fibrillar collagen in skin. The tensile strength of a newly epithelialized wound is only about 25% of normal tissue. Healed or repaired tissue is never as strong as normal tissues that have never been wounded. Tissue tensile strength is enhanced primarily by the reorganization of collagen fibers that were deposited randomly

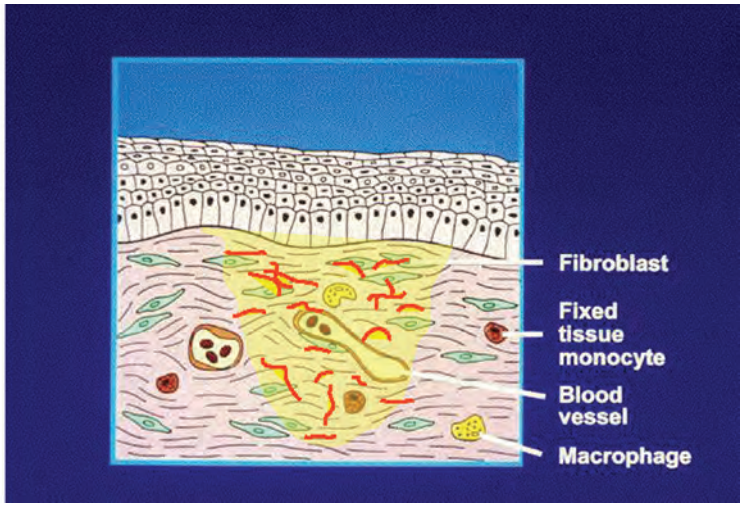

FIGURE 23.5: Remodelling Phase. The initial, disorganized scar tissue is slowly replaced by a matrix that more closely resembles the organized ECM of normal skin.

during granulation and increased covalent cross-linking of collagen molecules by the enzyme, lysyl oxidase, which is secreted into the ECM by fibroblasts. Over several months or more, changes in collagen organization in the repaired tissue will slowly increase the tensile strength to a maximum of about 80% of normal tissue.

Remodelling of the extracellular matrix proteins occurs through the actions of several different classes of proteolytic enzymes produced by cells in the wound bed at different times during the healing process. Two of the most important families are the matrix metalloproteinases (MMPs) (Table 23.4), and serine proteases. Specific MMP proteases that are necessary for wound healing are the collagenases (which degrade intact fibrillar collagen molecules), the gelatinases (which degrade damaged fibrillar collagen molecules)

TABLE 23.4: Matrix metalloproteinases and tissue inhibitors of metalloproteinases

Protein	Pseudonym	Substrates
MMP-1	Interstitial Collagenase Fibroblast Collagenase	Type I, II, III, VII, and X Collagens
MMP-2	72 kDa Gelatinase Gelatinase A Type IV Collagenase	Type IV, V, VII, and X Collagens
MMP-3	Stromelysin-1	Type III, IV, IX, and X Collagens Type I, III, IV, and V Gelatins Fibronectin, Laminin and Pro-collagenase
MMP-7	Matrilysin Uterine Metalloproteinase	Type I, III, IV and V Gelatins Casein, Fibronectin and Pro-collagenase
MMP-8	Neutrophil Collagenase	Type I, II, and III Collagens
MMP-9	92 kDa Gelatinase Gelatinase B Type IV Collagenase	Type IV and V Collagens Type I and V Gelatins
MMP-10	Stromelysin-2	Type III, IV, V, IX, and X Collagens Type I, III, and IV Gelatins Fibronectin, Laminin and Pro-collagenase
MMP-11	Stromelysin -3	Not determined
MMP-12	Macrophage Metalloelastase	Soluble and insoluble elastin
MT-MMP-1	Membrane type MMP-1	Pro-MMP-2
MT-MMP-2	Membrane type MMP-2	Not determined
TIMP-1	Tissue inhibitor of Metalloproteinases-1	Collagenases
TIMP-2	Tissue inhibitor of Metalloproteinases-2	Collagenases
TIMP-3	Tissue inhibitor of Metalloproteinases-3	Collagenases

and the stromelysins (which very effectively degrade proteoglycans). An important serine protease is neutrophil elastase which can degrade almost all types of protein molecules. Under normal conditions, the destructive actions of the proteolytic enzymes are tightly regulated by specific enzyme inhibitors, which are also produced by cells in the wound bed. The specific inhibitors of the MMPs are the tissue inhibitors of metalloproteinases (TIMPs) and specific inhibitors of serine protease are α1-protease inhibitor (α1-PI) and α2 macroglobulin.

Summary of acute wound healing

There are four phases of wound healing:

- Haemostasis – establishes the fibrin provisional wound matrix and platelets provide initial release of cytokines and growth factors in the wound.
- Inflammation – mediated by neutrophils and macrophages which remove bacteria and denatured matrix components that retard healing, and are the second source of growth factors and cytokines. Prolonged, elevated inflammation retards healing due to excessive levels of proteases and reactive oxygen that destroy essential factors.
- Proliferation – fibroblasts, supported by new capillaries, proliferate and synthesize disorganized ECM. Basal epithelial cells proliferate and migrate over the granulation tissue to close the wound surface.
- Remodelling – fibroblast and capillary density decreases, and initial scar tissue is removed and replaced by ECM that is more similar to normal skin. ECM remodelling is the result of the balanced, regulated activity of proteases.

Cellular functions during the different phases of wound healing are regulated by key cytokines, chemokines and growth factors. Cell actions are also influenced by interaction with components of the ECM through their integrin receptors and adhesion molecules. MMPs produced by epidermal cells, fibroblasts and vascular endothelial cells assist in migration of the cells, while proteolytic enzymes produced by neutrophils and macrophages remove denatured ECM components and assist in remodelling of initial scar tissue.

COMPARISON OF ACUTE AND CHRONIC WOUNDS

Normal and pathological responses to injury

Pathological responses to injury can result in non-healing wounds (ulcers), inadequately healing wounds (dehiscence), or in excessively healing wounds (hypertrophic scars and keloids). Normal repair is the response that re-establishes a functional equilibrium between scar formation and scar remodelling, and is the typical response that most humans experience following injury. The pathological responses to tissue injury stand in sharp contrast to the normal repair response. In excessive healing there is too much deposition of connective tissue that results in altered structure, and thus, loss of function. Fibrosis, strictures, adhesions, keloids, hypertrophic scars and contractures are examples of excessive healing. Contraction is part of the normal process of healing but if excessive, it becomes pathologic and is known as a contracture. Deficient healing is the opposite of fibrosis. It occurs when there is insufficient deposition of connective tissue matrix and the tissue is weakened to the point where scars fall apart under minimal tension. Chronic non-healing ulcers are examples of severely deficient healing.

Biochemical differences in the molecular environments of healing and chronic wounds

The healing process in chronic wounds is generally prolonged, incomplete and uncoordinated, resulting in a poor anatomic and functional outcome. Chronic, non-healing ulcers are a prime clinical example of the importance of the wound cytokine profile and the critical balance necessary for normal healing to proceed. Since cytokines, growth factors, proteases, and endocrine hormones play key roles in regulating acute wound healing, it is reasonable to hypothesize that alterations in the actions of these molecules could contribute to the failure of wounds to heal normally. Several methods are used to assess differences in molecular environments of healing and chronic wounds. Messenger ribonucleic acid (mRNA) and protein levels can be measured in homogenates of wound biopsies. The proteins in wounds can be immunolocalized in histological sections of biopsies. Wound fluids collected from acute surgical wounds and chronic skin ulcers are used to analyze the molecular environment of healing and chronic wounds. From these studies, several important concepts have emerged from the molecular analyses of acute and chronic wound environments.

The first major concept to emerge from analysis of wound fluids is that the molecular environments of chronic wounds have reduced mitogenic activity compared to the environments of acute wounds.[4] Fluids collected from acute mastectomy wounds when added to cultures of normal human skin fibroblasts, keratinocytes or vascular endothelial cells, consistently stimulated DNA synthesis of the cultured cells. In contrast, addition of fluids collected from chronic leg ulcers typically did not stimulate DNA synthesis of the cells in culture. Also, when acute and chronic wound fluids were combined the mitotic activity of acute wound fluids was inhibited. Similar results were reported by several groups of investigators who also found that acute wound fluids promoted DNA synthesis while chronic wound fluids did not stimulate cell proliferation.[18,19,20]

The second major concept to emerge from wound fluid analysis is the elevated levels of pro-inflammatory cytokines observed in chronic wounds as compared to the molecular environment of acute wounds. The ratios of two key inflammatory cytokines, TNFα and IL-1β, and their natural inhibitors, P55 and IL-1 receptor antagonist, in mastectomy fluids were significantly higher in mastectomy wound fluids than in chronic wound fluids. Trengove and colleagues also reported high levels of the inflammatory cytokines IL-1, IL-6 and TNFα in fluids collected from venous ulcers of patients admitted to the hospital.[21] More importantly, levels of the cytokines significantly decreased in fluids collected two weeks after the chronic ulcers had begun to heal. Harris and colleagues also found cytokine levels were generally higher in wound fluids from non-healing ulcers than healing ulcers.[20] These data suggest that chronic wounds typically have elevated levels of pro-inflammatory cytokines, and that the molecular environment changes to a less pro-inflammatory cytokine environment as chronic wounds begin to heal.

The third concept that emerged from wound fluid analysis was the elevated levels of protease activity in chronic wounds compared to acute wounds.[4,22,23] For example, the average level of protease activity in mastectomy fluids determined using the general MMP substrate, Azocoll, was low (0.75μg collagenase equivalents/ml, n = 20) with a range of 0.1 to 1.3μg collagenase equivalents/ml.[24] This suggests that protease activity is tightly controlled during the early phase of wound healing. In contrast, the average level

of protease activity in chronic wound fluids (87µg collagenase equivalents/ml, n = 32) was approximately 116-fold higher ($p<0.05$) than in mastectomy fluids. Also, the range of protease activity in chronic wound fluids is rather large (from 1 to 584µg collagenase equivalents/ml). More importantly, the levels of protease activity decrease in chronic venous ulcers two weeks after the ulcers begin to heal.[24] Yager and colleagues also found 10-fold higher levels of MMP-2 protein, 25-fold higher levels of MMP-9 protein, and 10-fold higher collagenase activity in fluids from pressure ulcers compared to surgical wound fluids using gelatin zymography and cleavage of a radioactive collagen substrate.[25] Other studies using immunohistochemical localization observed elevated levels of MMPs in granulation tissue of pressure ulcers along with elevated levels of neutrophil elastase and cathepsin-G.[26] TIMP-1 levels were found to be decreased while MMP-2 and MMP-9 levels were increased in fluids from chronic venous ulcers compared to mastectomy wound fluids.[27] Recently, Ladwig and colleagues reported that the ratio of active MMP-9/TIMP-1 was closely correlated with healing outcome of pressure ulcers treated by a variety of protocols (Figure 23.6).[28]

It is interesting to note that the major collagenase found in non-healing chronic pressure ulcers was MMP-8, the neutrophil-derived collagenase. Thus, the persistent influx of neutrophils releasing MMP-8 and elastase appears to be a major underlying mechanism resulting in tissue and growth factor destruction and thus impaired healing. This suggests that chronic inflammation must be decreased if pressure ulcers are to heal.

Other classes of proteases also appear to be elevated in chronic wound fluids.

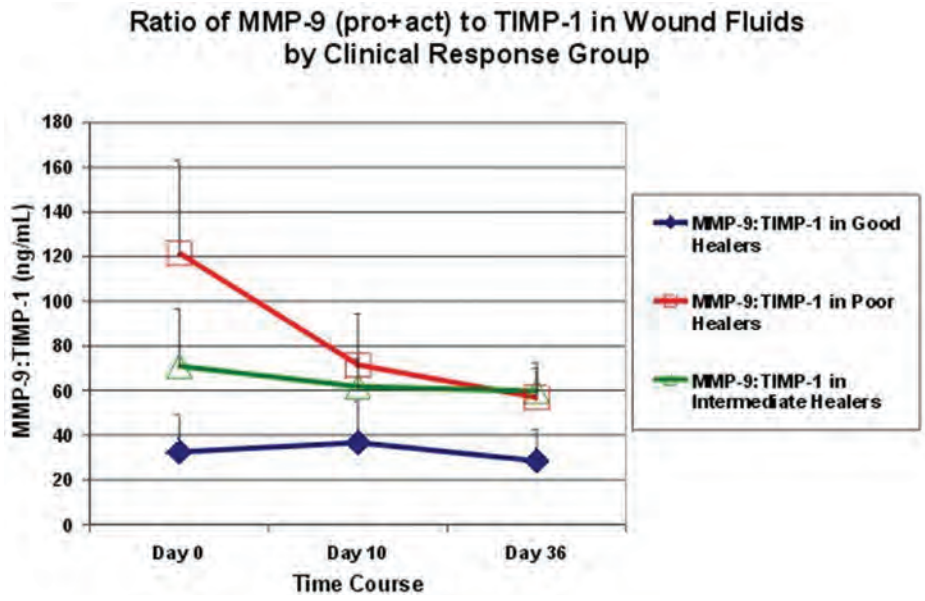

FIGURE 23.6: Low Protease/Inhibitor Ratios Correlate with Healing. Low values of the ratio of MMP-9/TIMP-1 in wound fluids from patients with chronic pressure ulcers correlate with healing of chronic pressure ulcers over 36 days of treatment, supporting the concept that high protease/inhibitor ratios prevent healing of chronic wounds.

It has been reported that fluids from skin graft donor sites or breast surgery patients contained intact α1-antitrypsin, a potent inhibitor of serine proteases, very low levels of neutrophil elastase activity, and intact fibronectin.[29] In contrast, fluids from the chronic venous ulcers contained degraded 1-antitrypsin, and 10-fold to 40-fold higher levels of neutrophil elastase activity, and degraded fibronectin. Chronic leg ulcers were also found to contain elevated MMP-2 and MMP-9, and that fibronectin degradation in chronic wounds was dependent on the relative levels of elastase, α1-proteinase inhibitor, and α2-macroglobulin. [30,31]

Besides being implicated in degrading essential extracellular matrix components like fibronectin, proteases in chronic wound fluids also have been reported to degrade exogenous growth factors *in vitro* such as EGF, TGF-α, or PDGF.[1,24,32,33] In contrast, exogenous growth factors were stable in acute surgical wound fluids *in vitro*. Supporting this general concept of increased degradation of endogenous growth factors by proteases in chronic wounds, the average immunoreactive levels of some growth factors such as EGF, TGF-β and PDGF were found to be lower in chronic wound fluids than in acute wound fluids while PDGF-AB, TGF-α and IGF-1 were not lower.[32,34]

In general, these results suggest that many chronic wounds contain elevated MMP and neutrophil elastase activities. The physiological implications of these data are that elevated protease activities in some chronic wounds may directly contribute to the failure of wounds to heal by degrading proteins which are necessary for wound healing such as extracellular matrix proteins, growth factors, their receptors and protease inhibitors. Interestingly, Steed and colleagues[35] reported that extensive debridement of diabetic foot ulcers improved healing in patients treated with placebo or with recombinant human PDGF (Figure 23.7). It is likely that frequent sharp debridement of diabetic ulcers helps to convert the detrimental molecular environment of a chronic wound into a pseudo-acute wound molecular environment.

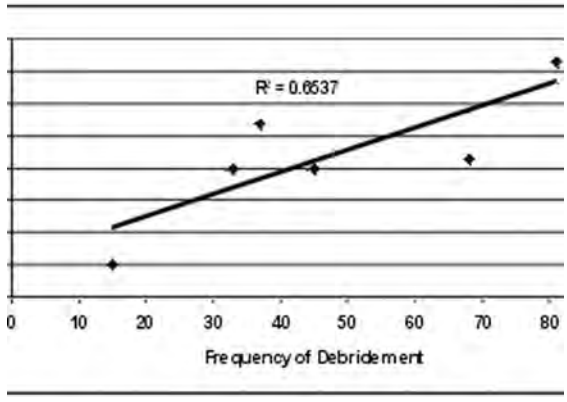

FIGURE 23.7: Frequency of Wound Debridement Correlates with Improved Healing. There was a strong correlation between the frequency of debridement and healing of chronic diabetic foot ulcers, supporting the concept that the abnormal cellular and molecular environment of chronic wounds impairs healing.

Biological differences in the response of chronic wound cells to growth factors

The biochemical analyses of healing and chronic wound fluids and biopsies have suggested that there are important molecular differences in the wound environments. However, these data only indicate part of the picture. The other essential component is the capacity of the wound cells to respond to cytokines and growth factors. Interesting new data are emerging which suggest that fibroblasts in skin ulcers which have failed to heal for many years may not be capable of responding to growth factors and divide as fibroblasts in healing wounds. Ågren and colleagues[36] reported that fibroblasts from chronic venous leg ulcers grew to lower density than fibroblasts from acute wounds from uninjured dermis. Also, fibroblasts from venous leg ulcers that had been present greater than three years grew more slowly and responded more poorly to PDGF than fibroblasts from venous ulcers that had been present for less than three years. These results suggest that fibroblasts in ulcers of long duration may approach senescence and have a decreased response to exogenous growth factors.

FROM BENCH TO BEDSIDE

Role of endocrine hormones in the regulation of wound healing

Classical endocrine hormones are molecules that are synthesized by specialized tissue and secreted into the blood stream which are then carried to distant target tissue where they interact with specific cellular receptor proteins and influence the expression of genes that ultimately regulate the physiological actions of the target cell. It has been known for decades that alterations in endocrine hormones can alter wound healing. Diabetic patients frequently develop chronic wounds due to multiple direct and indirect effects of the inadequate insulin action on wound healing. Patients receiving anti-inflammatory glucocorticoids for extended periods are also at risk of developing impaired wound healing due to the direct suppression of collagen synthesis in fibroblasts and the extended suppression of inflammatory cell function. The association of oestrogen with healing was recently reported by Ashcroft and colleagues[37] when they observed that healing of skin biopsy sites in healthy, postmenopausal women was significantly slower than in healthy premenopausal women. Molecular analyses of the wound sites indicated that TGF-β protein and mRNA levels were dramatically reduced in postmenopausal women in comparison to sites from premenopausal women. However, the rate of healing of wounds in postmenopausal women taking oestrogen replacement therapy occurred as rapidly as in premenopausal women. Furthermore, molecular analyses of wounds in postmenopausal women treated with oestrogen replacement therapy demonstrated elevated levels of TGF-β protein and mRNA that were similar to levels in wounds from premenopausal women. Aging was also associated with elevated levels of MMPs and decreased levels of TIMPs in skin wounds, which were reversed by oestrogen treatment.[38,39] The beneficial effects of oestrogen on wound healing could be achieved with topical oestrogen and were also observed in healthy aged men.[40] These data indicate the significant interactions that can occur between endocrine hormones and growth factors in the regulation of wound healing.

Molecular basis of chronic non-healing wounds

Conditions that promote chronic wounds are repeated trauma, foreign bodies, pressure

necrosis, infection, ischemia, and tissue hypoxia. These wounds share a chronic inflammatory state characterized by an increased number of neutrophils, macrophages, and lymphocytes which produce inflammatory cytokines, such as TNF-α, IL-1 and IL-6. *In vitro* studies have shown that TNF-α and IL-1 increase expression of MMPs and down-regulate expression of TIMP in a variety of cells including macrophages, fibroblasts, keratinocytes, and endothelial cells. All MMPs are synthesized as inactive proenzymes, and they are activated by proteolytic cleavage of the pro-MMP. Serine proteases, such as plasmin, as well as the membrane type MMPs can activate MMPs. Another serine protease, neutrophil elastase, is also present in increased concentrations in chronic wounds, and is very important in directly destroying extracellular matrix components and in destroying the TIMPs, which indirectly increases the destructive activity of MMPs.[4,22,25,33] Thus, the general molecular profile that appears in various types of chronic ulcers is (1) increased levels of inflammatory cytokines, which leads to (2) increased levels of proteases and decreased levels of protease inhibitors, which (3) degrade molecules that are essential for healing, including growth factors, their receptors and ECM proteins, which (4) prevent wounds from healing normally. Nwomeh and colleagues[23] further describe this common pathway in chronic wounds as a self-perpetuating environment in which chronic inflammation produces elevated levels of reactive oxygen species and degradative enzymes that eventually exceed their beneficial actions of destroying bacterial and debriding the wound bed and produce destructive effects that help to establish a chronic wound.

Based on these biochemical analyses of the molecular environments of acute and chronic human wounds, it is possible to propose a general model of differences between healing and chronic wounds. As shown in Figure 23.8, the molecular environment of healing wounds promotes mitosis of cells, has low levels of inflammatory cytokines, low levels of proteases and high levels of growth factors and cells capable of rapid division. In contrast, the molecular environments of chronic wounds generally have the opposite characteristics, i.e., the molecular environment does not promote mitosis of cells, has elevated levels of inflammatory cytokines, has high levels of proteases

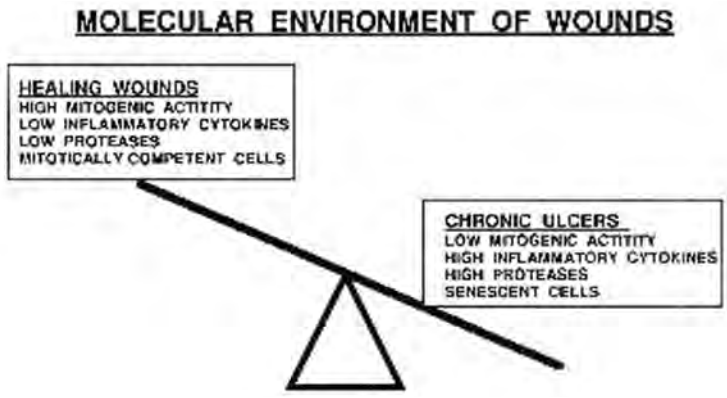

FIGURE 23.8: Comparison of the Molecular and Cellular Environments of Healing and Chronic Wounds. Elevated levels of cytokines and proteases in chronic wounds reduce mitogenic activities and response of wound cells, impairing healing.

and low levels of growth factors and cells that are approaching senescence.[41,24,21] If these general concepts are correct, then it may be possible to develop new treatment strategies which would re-establish in chronic wounds the balance of cytokines, growth factors, proteases, their natural inhibitors and competent cells found in healing wounds.

Chronic venous stasis ulcers

Mechanisms involved in the creation and perpetuation of chronic wounds are varied and depend on the individual wounds. In general, the inability of chronic venous stasis ulcers to heal appears to be related to impairment in wound epithelialization. The wound edges show hyperproliferative epidermis under microscopy, even though further immunohistochemical studies revealed optimal conditions for keratinocyte recruitment, proliferation, and differentiation. The extracellular matrix and the expression of integrin receptors by keratinocytes that allow them to translocate play an important regulatory role in epithelialization. After receiving the signal to migrate, epidermal cells begin by disassembling their attachments from basement membrane and neighboring cells. They then travel over a provisional matrix containing fibrinogen, fibronectin, vitronectin, and tenascin and stop when they encounter laminin. During this process, keratinocytes are producing fibronectin, and continue to do so until the epithelial cells contact, at which time they again begin manufacturing laminin to regenerate the basement membrane.

There is evidence that the interaction between the integrin receptors on keratinocytes with the ECM will transform resting cells to a migratory phenotype. Integral in this transformation is the alteration in the pattern of integrin receptors expressed. After epithelialization is completed, integrin expression reverts back to the resting pattern. To further complicate this process, growth factors are involved in mediating keratinocyte activation, integrin expression, and in alterations in the matrix. Growth factors are able to differentially affect these processes. For example, TGF-β is able to promote epithelial migration while inhibiting proliferation. Although TGF-β induces the necessary integrin expression for migration, the cells behind those at the leading edge have little proliferative ability and so epithelial coverage of the wound is inhibited. Some chronic wounds may be deficient in TGF-β and its receptor.[42]

Pressure ulcers

Chronic wounds have also been demonstrated to have elevated matrix degrading enzymes and decreased levels of inhibitors for these enzymes. Pressure ulcers, unlike chronic venous stasis ulcers, appear to have difficulty in healing related to impairment of ECM production. Studies have indicated that neutrophil elastase present in chronic wounds can degrade peptide growth factors and is responsible for degrading fibronectin. Pressure ulcers have also shown an increase in matrix metalloproteinases and in plasminogen activators in tissue. Chronic wound fluids demonstrate increased levels of gelatinases MMP-2 and MMP-9. Levels of MMP-1 and MMP-8 were also found to be higher in pressure ulcers and in venous stasis ulcers than in acute healing wounds. In addition, several of the endogenous proteinase inhibitors were shown to be decreased in chronic wounds. Proteinase inhibitors serve a regulatory role in matrix degradation by containing the matrix-degrading enzymes. Factors that promote MMP production or activation could counteract the effectiveness of proteinase inhibitors, for example the destruction of TIMP by neutrophil elastase.

The tissue inhibitor level to MMP ratio may indicate an imbalance which contributes to the wound chronicity.

FUTURE CONCEPTS FOR THE TREATMENT OF CHRONIC WOUNDS

Although the aetiologies and the physical characteristics for the various types of chronic wounds are different, there is a common trend in their biochemical profiles. The precise pattern of growth factor expression in the different types of chronic wounds is not yet known; but it has been determined that there is generally a decreased level of growth factors and their receptors in chronic wound fluids. The absolute levels of growth factors may not be as important as the relative concentrations necessary to replace the specific deficiencies in the tissue repair processes. For the treatment of chronic wounds, Robson[43] proposed that growth factor therapy be tailored to the deficiency in the repair process. Therefore, the effectiveness of the therapy is predicated on adequate growth factor levels and the expression of their receptors balanced against receptor degradation by proteases and the binding of growth factors by macromolecules such as macroglobulin and albumin.

Studies that evaluated topical growth factor treatment of chronic wounds, such as PDGF in diabetic foot ulcers and EGF in chronic venous stasis ulcers, have shown an improvement in healing. These findings have led to the hypothesis that altering the cytokine profile of chronic wounds through the use of MMP inhibitors, addition of growth factors, and the elimination of inflammatory tissue and proteases by debridement would shift the wound microenvironment towards that of an acute wound, thereby improve healing.

Current treatment strategies are being developed to address the deficiencies (growth factor and protease inhibitor levels) and excesses (MMPs, neutrophil elastase, and serine protease levels) in the chronic wound microenvironment. Although the more specific and sophisticated treatments remain in the lab at this time such as the new potent, synthetic inhibitors of MMPs and the naturally occurring protease inhibitors, TIMP-1 and 1-antitrypsin, available by recombinant DNA technology, the use of gene therapy in the treatment of chronic diabetic foot ulcers is currently being evaluated in a clinical trial. A phase III clinical trial is underway to determine the efficacy of keratinocyte growth factor-2 (KGF-2) in the treatment of chronic venous stasis ulcers. The treatment strategy to add growth factor to a chronic wound has been in place for the past several years. Regranex®, human recombinant platelet derived growth factor (PDGF-BB), has been available for the treatment of diabetic foot ulcers; demonstrated approximately 20% improvement in healing compared to controls.[44] In keeping with the strategy to restore a deficient wound environment, Dermagraph® and Apligrapf®, engineered tissue replacements, have been applied to chronic diabetic ulcers.[45,46] Although Apligrapf® is no longer available, both tissue replacements have proven to be effective in selected types of ulcers. Other approaches to the treatment of chronic wounds have been to remove the increased protease levels. This is in part the strategy of a vacuum-assisted negative pressure wound dressing[47] and in the recent development of dressings that bind and remove MMPs from the wound fluid, such as Promogran®.[48,49]

There have been some advances made in the development of new antimicrobial dressings and they have been summarized by Hamm in a recent publication (Antibacterial Dressings in Advances in Wound Care: Volume 1; Mary Anne Libert Inc. 2010, page 148).

Another strategy is to use synthetic protease inhibitors to decrease the activities of MMPs in the wound environment. Doxycycline, a member of the tetracycline family of antibiotics, is a moderately effective inhibitor of metalloproteinases, including MMPs and the TNFα converting enzyme (TACE). We have demonstrated a reduction in inflammatory cell infiltrate and extracellular matrix in chronic pressure ulcers treated with 100mg doxycycline twice daily. Low dose doxycycline 20mg, twice daily has been proven to be beneficial in other pathologic states such as periodontitis that are characterized by chronic, neutrophil-driven inflammation, and matrix destruction.[50] In the future, treatment of chronic wounds may require the use of specific growth factors or inhibitors unique to the type of ulcer or the use of combinations of selective inhibitors of proteases, growth factors and tissue replacements to act synergistically to promote healing.

As previously described, endocrine hormones, such as insulin, glucocorticoids, and oestrogen, play important roles in regulating wound healing. Although there is no current therapy that specifically addresses the molecular deficits created by type I or type II diabetes (inadequate insulin levels or insulin resistance), systemic insulin injections may improve the local wound microenvironment. For patients receiving long-term corticosteroids, the use of vitamin A seems to facilitate wound healing. Studies are underway to determine the efficacy of topical oestrogen applications on skin aging.

New technologies are being developed to help researchers better understand the complex microenvironment that exists in chronic wounds.[51] A technique called Polymerase Chain Reaction (PCR) can amplify the microbial DNA that is extracted from the wound bed and then be used to identify and quantify specific organisms. The test is highly sensitive and there is a rapid turn around time. The drawback is that PCR can only be used to identify known organisms and new unknown microbes will not be detected.

Bacterial biofilms in chronic wounds

Bacterial biofilms are well known in other medical specialities to cause a variety of chronic pathologies including periodontal disease, cystic fibrosis, chronic otitis media and osteomyelitis and prosthetic graft infection.[52] Biofilms are characterized by an exopolymeric matrix of polysaccharides, proteins and DNA synthesized by the multiple bacterial species (polymicrobial) comprising the biofilm community. Bacteria (and fungi) contained within the biofilm matrix are highly tolerant to killing phagocytic inflammatory cells (neutrophils and macrophages), antibodies, and exogenous antibiotics, antiseptics and disinfectants. Several factors contribute to the increased tolerance of bacteria in biofilms to these agents, including reduced penetration of large proteins (antibodies) into the dense exopolymeric matrix, binding of oppositely charged molecules like antibiotics or cationic heavy metal ions (silver ion) by negatively charged components of the exopolymeric matrix, or neutralization of highly reactive chemicals like hypochlorous acid (bleach) by reaction with molecules comprising the exopolymeric matrix. Also, some bacteria in mature biofilms become metabolically quiescent and these 'persister cells' are therefore highly resistant to antibiotics that disrupt bacterial metabolism. These factors contribute to make biofilms extremely difficult to kill and clear from chronic wounds. Furthermore, components of the biofilm matrix and products produced by bacteria in the biofilm stimulate chronic inflammation, which leads to persistently elevated levels of molecules like proteases and reactive oxygen species that kill wound

cells and damage proteins that are essential for healing.

Assessment of the 'bioburden' of wounds has traditionally relied upon relatively simple microbiology laboratory techniques that typically provide information on major bacterial and fungal species in swabs or biopsies that can grow under the nutritional and environmental conditions provided in the lab. These assessments of bacteria and fungi in wound samples have unquestionably generated important data that have been used for decades to help select therapeutic regimens for patients and their wounds. However, multiple publications have pointed out that, in many patients, measurements of total bacterial bioburden (expressed as colony forming units per gram of tissue biopsy or 0-4+ levels of bacterial growth) alone do not correlate well with the failure of wounds to heal. As shown in Figure 23.9, this led to the concept of 'critical colonization' or 'occult infection' to explain the discrepancy, because there was an apparent link between microbial bioburden in these wounds and the impaired healing in the wounds. However, it was not clear what aspect of the relatively low total bioburden was 'critical' to impairing healing. More thorough evaluation of these 'standard' clinical microbiology assays led to the realization that these assays are inherently limited by the rather poor ability to culture or identify most of the bacterial and fungal species that are actually present in an individual chronic wound. In other words, standard clinical microbiology assays only culture planktonic bacterial and fungal species that are able (capable) of growing on agar media plates supplemented with general nutrients in air at 37°C. Thus, it is reasonable to assume that a more complete picture of different bacterial species (aerobes, facultative anaerobes, and obligate anaerobes) and fungal species in a particular wound should improve the ability to assess the microbial bioburden on individual wounds and to indicate what therapeutic strategies would be optimal for each wound. Fortunately, in the last few years sophisticated laboratory research techniques have been developed that allow a more complete assessment of bacterial bioburden. Specifically, these techniques demonstrated that a high percentage (~60%) of chronic skin wounds have extensive bacterial biofilms.[53] Using sophisticated polymerase chain reaction (PCR) techniques Dowd *et al*[54] reported that the bacterial and fungal complexity of chronic wound samples

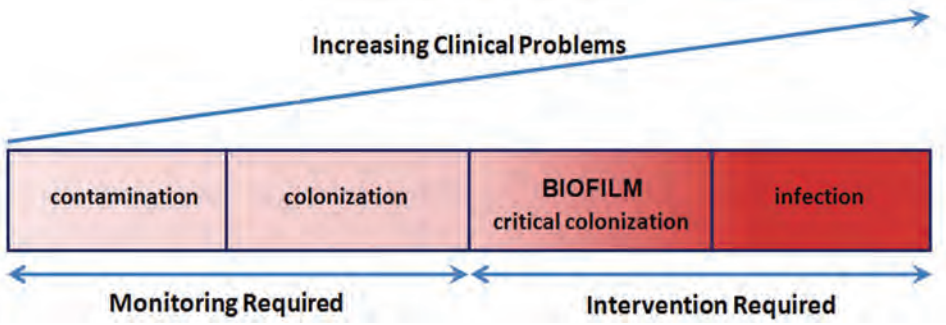

FIGURE 23.9: Spectrum of Bacterial Bioburden in Wounds. Contamination and colonization of bacteria usually do not substantially retard healing whereas infection clearly impairs healing. The concept of critical colonization evolved to describe a condition where levels of planktonic bacteria were not above 10^6 cfu/gm, but healing was impaired. Since biofilm bacteria are not detected by standard clinical microbiology assays, critical colonization probably represents a condition when biofilm bacteria are present in wounds and stimulate chronic inflammation that retards healing.

was much greater than previously thought. In fact, on average, approximately 60% of the bacterial species present in chronic pressure ulcers and around 30% of those present in diabetic ulcers were strict anaerobic bacteria, and many bacterial species were present that had never been reported in cultures of chronic wounds. These data suggest that many of the bacteria present in biofilms in a chronic wound may never be successfully cultured in the standard clinical microbiology laboratory due to obligate cooperation with other bacteria that create unique environmental conditions in a polymicrobial community of bacteria in biofilms. A second major concept recently reported by Wolcott and colleagues[55] showed that mature biofilms are rapidly re-established in chronic wounds following surgical debridement, on the time frame of 24 to 72 hours. This indicates that sharp debridement opens a time-dependent therapeutic window to prevent the re-establishment of mature biofilms that are highly tolerant to host inflammatory response or to exogenous antimicrobial agents.

The clinical principle that should guide 'biofilm-based wound care' is to reduce planktonic and biofilm bacterial burdens by the most appropriate and effective means (surgical debridement, curettage, irrigation, etc), then follow the debridement by covering the wound with an effective bacterial barrier dressing, of which there are many types, including dressings with microbicidal metal ions (silver), quaternary amines, or occlusive films.[56]

CONCLUSION

The molecular environment of chronic wounds contains elevated levels of inflammatory cytokines and proteases, low levels of mitogenic activity, and cells that often respond poorly to growth factors compared to acute healing wounds. As chronic wounds begin to heal, this molecular pattern shifts to one that resembles a healing wound. As more information is learned about the molecular and cellular profiles of healing and chronic wounds, new therapies will be developed that selectively correct the abnormal aspects of chronic wounds and promote healing of these costly clinical problems. With the aging of the population, wound care for the elderly is becoming a major issue[57] The Wound Healing Society has developed a series of guidelines for 'Acute Wound Care', 'Chronic Wound Care' and 'Prevention Guidelines' that are free as downloads on their web site (http://www.woundheal.org)

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24 • Pathophysiology and Principles of Management of Varicose Veins

ANDREW W BRADBURY

College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK

INTRODUCTION

The management of superficial and deep venous reflux and obstruction that leads to the development of varicose veins (VV)[1] and the post-thrombotic syndrome (PTS)[2] forms a large part of the workload for most vascular and endovascular specialists and is likely to increase as the population ages.[3] However, the epidemiology,[4,5] genetics[6] and pathophysiology of these conditions remains incompletely defined[7,8,9,10,11] and many clinicians lack a clear understanding of the underlying anatomy and vascular biology.[12] As a result, treatment outcomes are not infrequently sub-optimal.

ANATOMY

Venous blood from the lower limbs returns to the right heart against gravity through the superficial and deep venous systems. The superficial venous system comprises the great saphenous veins (GSV) and small saphenous veins (SSV) and their tributaries.[13] The GSV originates from the medial end of the dorsal venous arch, passes anterior to the medial malleolus, and continues up the medial aspect of the calf and then the thigh to enter the common femoral vein in the groin at the saphenofemoral junction (SFJ). The SSV originates from the lateral end of the dorsal venous arch, passes posterior to the lateral malleolus and then continues up the back of the calf between the heads of gastrocnemius to enter the popliteal fossa. It is joined variably by gastrocnemius veins and then usually enters the popliteal vein at the sapheno-popliteal junction (SPJ). The SPJ may be absent in which case the SSV continues up the postero-medial aspect of the thigh (Giacomini vein) and often joins the GSV. These two systems interconnect at many other (highly variable) points through an extensive network of tributaries. In the deep system, veins, which are often paired, accompany each named artery. The superficial and deep systems connect at numerous points at various non-junctional perforators in addition to the SFJ and SPJ. These systems and interconnections are interdependent, both anatomically and functionally in health and disease.

In health, the deep venous system transmits 90% of the venous return from the leg. The superficial system drains only the skin and subcutaneous tissues, with

most of that blood draining immediately into the deep system via perforators in the foot, calf and thigh. It also plays a role in thermoregulation.

HISTOLOGY

The vein wall comprises three layers but these are less well defined than in the arterial system. The intima is thin and surrounded by a fine elastic lamina. The media is made up of elastin and layers of muscular bundles that are arranged in different orientations. The relative amounts of muscle and elastin varies with the calibre and working pressure of the vein. Beyond this, the adventitia merges with the perivenous connective tissue, which contains nerve fibres and vasa vasorum and provides for vessel distension which is an important part of normal venous function. With increasing age, and particularly with the development of disease, abnormalities have been described in all three layers[14] and the structure of the vein wall becomes progressively more disorganised.[15] Typically, there is thickening of the intima with disorientation of the elastic fibres. The outer muscle layer of the media becomes hypertrophied with dystrophic elastic fibres and the adventitia is increasingly fibrous.

PHYSIOLOGY

Venous return against gravity is primarily dependent on muscle pumps located in the foot and the calf. Pressure on the sole of the foot, and muscular contraction (systole) in the fascial compartments of the calf compresses the sinusoidal intramuscular veins directing blood into the deep system and thence up the leg. Superficial veins collect blood from the superficial tissues, and during muscle relaxation (diastole) this blood enters the deep system through the perforating veins down a pressure gradient, filling the sinuses. Reverse flow (reflux) during muscle relaxation is prevented by the closure of valves. These are delicate but strong bicuspid leaflets at the base of a localized dilated sinus in the vein. In both superficial and deep systems the density of valves is greatest in the calf and reduces gradually up the lower limb, with the iliac and inferior vena cava (IVC) frequently lacking valves altogether. Valves are present in venules down to about 0.15mm diameter.

During systole, blood is prevented from re-entering the superficial system through the closure of junctional (SFJ, SPJ) and non-junctional perforators (NJP). This was originally thought to occur solely through the closure of valves but several studies have failed to demonstrate such valves in NJP. Instead, external pressure from the fascia and muscle through which the perforators pass is thought to be responsible for limiting outward blood flow; somewhat akin to the 'pinch-cock' mechanism that prevents reflux at the gastro-oesophageal junction. Importantly, this also protects the superficial veins, subcutaneous tissues and skin from the extremely high deep venous pressures (up to 250mmHg) generated by the calf muscle pump in systole.

When standing motionless, with venous valves in the neutral position, the pressure in the foot veins gradually increases as blood continues to enter the veins from the arterial side. As soon as the pressure in one venous segment exceeds that in the segment just above, the valve opens. Eventually the hydrostatic pressure in the veins of the foot is that developed by an unbroken column from the foot to the right atrium – perhaps 90mmHg in a person of average height. With active movement, deep veins and sinuses are compressed raising venous pressure and moving blood cranially and, initially, caudally (Figure 24.1A). However, valve closure

normally prevents retrograde flow within 0.5-1.0 seconds. At this point, these closed valves divide the high-pressure, single column of venous blood described above into a large number of low-pressure, shorter columns (Figure 24.1B). As a result, the pressure in the foot veins falls in health to less than 25mmHg on walking; the normal ambulatory venous pressure (AVP) (Figure 24.2). This reduces venous pooling and lowers capillary hydrostatic pressure, reducing the tendency for accumulation of interstitial fluid (oedema) in the feet.[16] Patients with muscle pump and/or venous valve failure and/or venous outflow obstruction, demonstrate raised AVP. It is this raised AVP that underlies all the symptoms and signs of chronic venous insufficiency (CVI).

VARICOSE VEINS

Varicose veins (VV) are dilated, tortuous subcutaneous veins that permit reverse flow. They are most commonly found in the lower limb and may be primary, or secondary to deep venous pathology. The GSV system is most frequently affected with the SSV being involved in about 20% of cases. The aetiology of VV at a microscopic level is still disputed but the essential defect macroscopically is generally agreed to be the failure of venous valve closure resulting in the superficial veins becoming dilated, elongated and tortuous.[17,18] The main factor contributing to the development and progression of varicose veins is sustained venous hypertension that increases the diameter of the superficial veins resulting in further valve incompetence.

VALVULAR ABNORMALITIES

Failure of valve closure leading to valve incompetence and reflux may affect the deep and/or superficial venous systems and may be primary or secondary. Primary valvular incompetence (PVI) is believed to be due to loss of mural elastin and collagen, which

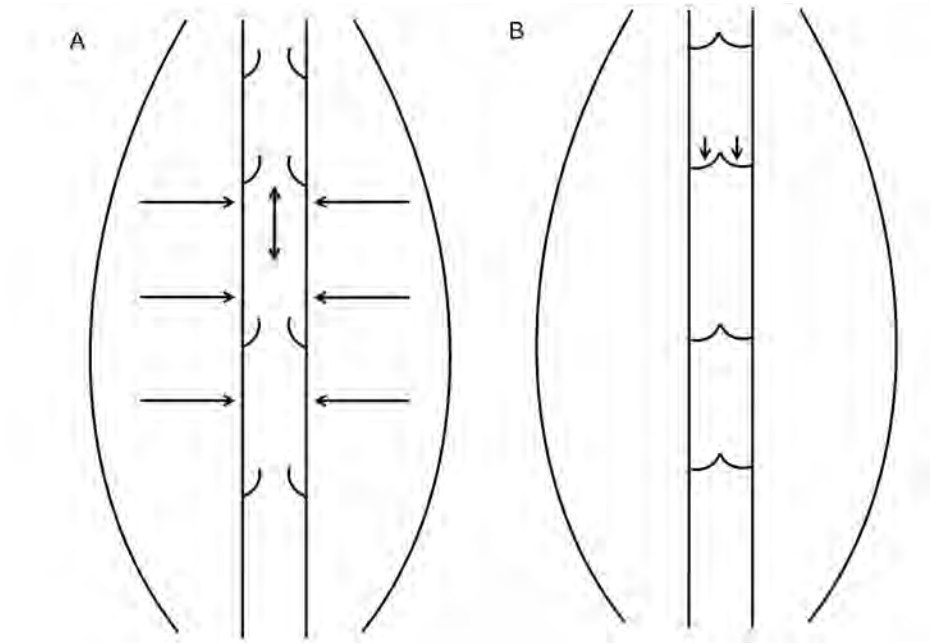

FIGURE 24.1: Influence of calf muscle pump and valves in venous return.

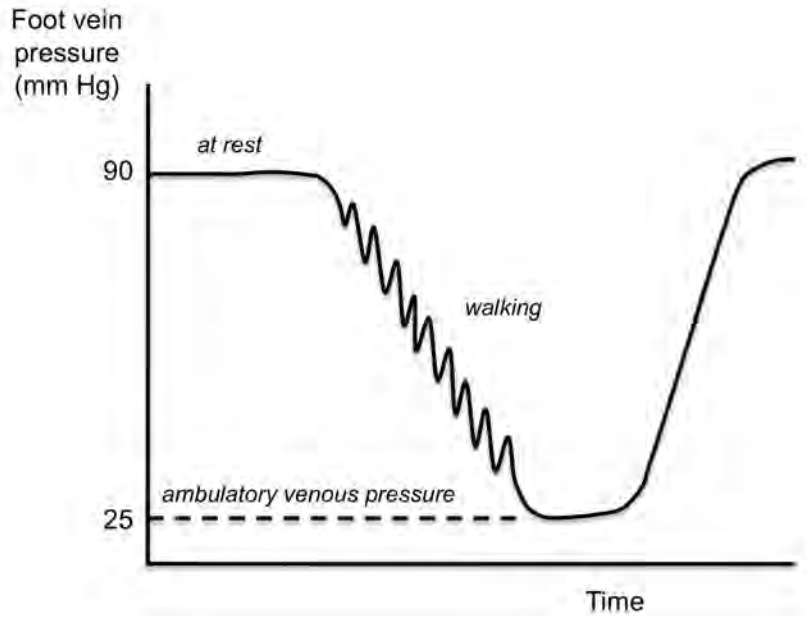

FIGURE 24.2: Resting and ambulatory venous pressure at the ankle in health.

leads to dilatation and separation of the valve leaflets. The commonest clinical consequence of this process is the development of VV. As an investing fascia often supports the main GSV trunk, it is often the tributaries that become varicose. PVI may also affect the deep venous system although because other tissues support the deep veins, the clinical consequences of PVI are less obvious and certain.

Secondary valvular incompetence may be due to a developmental weakness in the vein wall leading to secondary widening of the valve commissures, resulting in valvular incompetence and clinically, primary VV. It also follows thrombosis, most commonly in the deep venous system; deep venous thrombosis (DVT). Blood flowing within the lumen of the vein provides the vascular endothelium with its oxygen and nutrition. DVT prevents this, therefore leading to endothelial destruction and inflammation within and around the affected veins. Although most venous segments occluded by DVT recanalise over the subsequent 6–12 months the vein is often scarred and narrowed and, because the valves have been destroyed, incompetent. If recanalisation does not occur, blood is forced to find an alternative drainage route. For example, blood may be forced out of the deep venous system via the SFJ, SPJ and NJP leading to dilatation of the superficial veins (secondary VV). Obstruction of the iliac veins may lead to the development of groin and pelvic collaterals. Venous reflux and obstruction secondary to DVT leads to PTS which represents the most severe form of chronic venous insufficiency (CVI). The superficial venous system may also be affected by thrombosis, either in isolation or in combination with DVT, leading to superficial thrombophlebitis (SVT).

Rarely, VV and CVI may be due to congenital valve hypoplasia or agenesis, or due to arterio-venous malformations. In Klippel-Trenaunay syndrome, for example, there is deep venous hypoplasia and a laterally placed venous complex that acts as

the main venous outflow of the limb. All the symptoms and signs of chronic venous insufficiency are due to ambulatory venous hypertension resulting from these various pathological processes acting upon the microvasculature of the skin and subcutaneous tissues.

MUSCLE PUMP FAILURE

Any cause of chronic debility or immobility is associated with calf muscle pump dysfunction; for example, old age, stroke, neuromuscular conditions, arthritis and trauma. Injuries that limit or prevent ankle movement have a particularly adverse effect upon the calf muscle pump.

VENOUS RECIRCULATION

In patients with VV there is often a recirculation of venous blood within the leg. During calf relaxation abnormally large volumes of blood enter the muscle pump from the superficial varices (increased preload). During exercise the muscle pump expels blood from the leg only for it to re-enter the lower leg by refluxing down GSV and/or SSV VV (akin to an increase in afterload due to aortic regurgitation). This blood then re-enters the muscle pump through the perforating veins in the lower calf and so on. The effect is that the same blood can re-circulate up and down the leg several times before eventually finding its way up the iliac veins to the heart.

Patients with mild superficial reflux and/or an efficient calf pump are able to compensate for this by increasing their calf muscle pump 'stroke volume' and output. This allows them to still reduce their AVP to (near) normal levels on walking. However severe reflux and/or a weak muscle pump may overwhelm the deep system and lead to the development of sustained venous hypertension and skin changes of CVI. This accounts for two important clinical observations:

- CVI & ulceration can develop without primary deep venous pathology
- In a proportion of patients with VV and deep venous reflux the latter disappears following eradication of superficial disease.

RECURRENT VARICOSE VEINS

Recurrent VV after conventional surgical or endovenous intervention may be classified into three groups: new, persistent and true recurrent.

New varicose veins

This is the development of new VV, often in a second saphenous system, since the original operation.[19] This may be due to:

1) Inadequate assessment at the time of the initial treatment; however, now that most patients undergo full duplex ultrasound mapping prior to intervention for their VV this should be less common
2) Reflux developing at a site that was previously demonstrated to be competent; in other words, true disease progression

Persistent varicose veins

This is due to inadequate treatment of VV at the time of the original intervention. Again, with proper use of duplex ultrasound and modern techniques this should be a relatively uncommon scenario in current phlebological practice. The risk is perhaps greater with catheter based techniques such as radiofrequency ablation (RFA) and Laser ablation (EVLA) which, while being highly successful in eradicating truncal reflux,

do not deal with the varices themselves. A proportion of patients undergoing RFA and EVLA will, therefore, need further treatment, either with foam sclerotherapy or local anaesthetic phlebectomies.

True recurrent varicose veins

This is where further VV develop in the same, previously treated saphenous system. When surgery was the main treatment modality most were the result of failure to properly perform a 'flush' SFJ (SPJ) ligation and/or to 'strip' the GSV or SSV.

Neovascularisation (NV), defined as the 'development of new vessels connecting previously ligated superficial veins to the deep venous system', and the role it might play in the development of recurrent VV after surgery has received a lot of attention over the years. There is no doubt that in a proportion of patients with recurrent GSV (SSV) VV, duplex ultrasound clearly shows the presence of small venous channels within scar tissue apparently connecting the 'stump' of the GSV in the groin (SSV in the popliteal fossa) to recurrent VV in the thigh (calf). However, it seems unlikely that such small, therefore high resistance, veins will be capable of transmitting significant reflux and thus of constituting a significant cause of recurrence on their own. In an era where the vast majority of patients can have non-surgical treatment for their VV, the whole issue of NV becomes much less important. Going forward, most true recurrent VV are likely to be due to recanalisation of the trunk veins and/or their major tributaries that have previously been occluded by means of foam sclerotherapy, RFA or EVLA.[20,21] However, unlike redo surgery which is technically demanding and often associated with disappointing outcomes, such recanalisation can be successfully treated as an out-patient and so poses no real clinical difficulty.[19]

CELLULAR AND MOLECULAR BIOLOGY OF VARICOSE VEINS

The molecular biology of varicose veins has recently been reviewed. The aetiology of varicose veins is undoubtedly multifactorial. There are some genetic disorders and mutations that predispose to venous incompetence and development of varicosities (FOXC2, NOTCH3). However these diseases are rare whilst varicose veins are common.

In recent years there has been much research to define the structural and molecular events that accompany the formation of varicose veins, with an overall underlying hypothesis that varicose vein formation is most likely due to a structural, cellular or molecular abnormality within the vein wall. On a gross level, varicose veins exhibit intimal hyperplastic areas and underlying plaques with infiltration of leukocytes and mast cells. There is fragmentation of elastin fibres and the total content of elastin and Type III collagen is reduced. These extracellular matrix abnormalities may be regulated by disordered MMP and TIMP production.

Cell types within the varicose vein may show disordered function with endothelial activation leading to vasodilatation and a possible loss of venous tone. Many of the smooth muscle cells in the varicose vessel wall exhibit a synthetic rather than a contractile phenotype, and appear to have reduced rates of apoptosis. These cells may have a reduced capacity for contraction, which may exacerbate the vasodilatory tendency. The stimuli for the disordered function demonstrated by these intrinsic cells remains ill defined, but hypoxic stress and low shear stress may play a role.

There is certain to be further research in the next few years to further define the vascular biology of varicose veins. There has been some suggestion that this may lead to a medical therapy for varicose veins, although the practicality of this is not immediately apparent. Nevertheless research into the molecular aetiology of varicose veins will continue to define vascular pathways.[22]

CONCLUSION

Despite the very large numbers of patients affected by CVI and VV, research into venous disease is generally given low priority and so there are still significant gaps in our knowledge. Further work is needed if we are to improve our understanding of the aetiology of the disease and improve the results of treatment.

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25 • Chronic Venous Insufficiency and Leg Ulceration: Principles and Vascular Biology

MICHAEL STACEY

University Department of Surgery, Fremantle Hospital, Fremantle, Western Australia

DEFINITIONS

Chronic Venous Insufficiency

Chronic venous insufficiency (CVI) is a term that is used to describe changes in the leg that include a variety of different clinical problems, which are caused by several types of abnormalities in the veins, and which may occur at a number of different locations in the leg.[1] For these reasons it has been difficult to make accurate comparisons of reports of chronic venous insufficiency from different institutions. As a result attempts have been made to formulate systems of classification that enable accurate comparisons to be made.

The most recent classification, referred to as the CEAP classification, was devised by an international panel and encompasses features of some of the earlier classifications.[2] This classification has four categories which include – Clinical (C), Etiology (E), Anatomy (A) and Pathophysiology (P). Within each category the different levels are each given a number or a letter or both. The clinical classification has seven levels from no visible or palpable signs of venous disease through to skin changes with active ulceration (Table 25.1). In addition the *Clinical* categories are further characterized according to the presence or absence of symptoms. The *Etiological* classification recognizes the roles of congenital (E_c), primary (E_p) and secondary (E_s) causes in venous dysfunction. The *Anatomical* classification can be represented as a simple or more detailed form. The simple form refers to the site at which the veins are involved as superficial (A_s), deep (A_d) or perforating (A_p). The more detailed form identifies the specific veins that are involved and has 18 segments that can be identified. The *Pathophysiologic* classification identifies the cause of venous dysfunction being either reflux (P_R) or obstruction (P_O), or both (P_{RO}). This classification can be used in part or in whole when describing the patients in a published report.

Leg Ulceration

Leg ulceration may occur as a result of many different aetiological factors (Table 25.2). For patients presenting with an ulcer on the leg it is imperative to determine the aetiology since the treatment may differ according to the cause. For ulcers on the leg, not including

TABLE 25.1: Clinical classification of chronic venous disease of the lower extremity. The presence or absence of symptoms is denoted by the addition of 's' for symptomatic, or 'a' for asymptomatic.

Class	Definition
0	No visible or palpable signs of venous disease
1	Telangiectases or reticular veins
2	Varicose veins
3	Oedema
4	Skin changes ascribed to venous disease (e.g. pigmentation, eczema, lipodermatosclerosis)
5	Skin changes as above in conjunction with healed ulceration
6	Skin changes as above in conjunction with active ulceration

TABLE 25.2: Commonest causes of leg ulceration.

Venous disease
Arterial disease
Rheumatoid arthritis
Diabetes
Vasculitis
Scleroderma
Pyoderma grangrenosum
Trauma
Infective
Ulcerating skin cancer

the foot, the commonest aetiology is chronic venous disease either alone or in common with another cause of impaired healing such as arterial disease, diabetes or rheumatoid arthritis. The venous abnormality that leads to venous leg ulceration may involve abnormalities at different locations in the venous system, of different extent and different aetiologies.

Assessment of Cause of Leg Ulceration

In order to determine the aetiology of a leg ulcer, a standardized assessment is strongly recommended.[3] That assessment should consist of the following

1) History – to determine the presence of other known diseases that might result in an impairment of the healing process. These include disease processes listed in Table 25.2.
2) Examination –
   a. General examination to assess for evidence of diseases in Table 25.2
   b. Examination of the lower limbs to assess for
      i. Venous disease
      ii. Arterial disease
      iii. Examination of the ulcer, specifically documenting
         1. Location
            a. Gaiter region – likely venous
            b. More proximal on the leg – consider other aetiologies
            c. Foot – not commonly venous aetiology
         2. Skin surrounding the ulcer
            a. Lipodermatosclerosis – venous disease
            b. Atrophie blanche – venous disease or vasculitis
            c. Atrophic skin changes – arterial

   d. Normal skin – consider other aetiologies
3. Ulcer edge
   a. Raised – neoplastic
   b. Punched out – arterial
   c. Undermined – infective
   d. Sloping – venous
   e. Dusky – vascilitis, pyoderma grangenosum
4. Ulcer base
   a. Necrotic tissue – arterial; large or small vessel disease
   b. Granulating – multiple aetiologies including venous
   c. Fibrotic slough – venous, multiple aetiologies

3) Investigations
   a. Ankle: brachial Doppler arterial pressure index
   b. Confirmation of venous disease
      i. Venous plethysmography
      ii. Venous duplex scan to assess for sites of venous reflux
      iii. Blood tests – anemia, renal failure, liver failure, diabetes, vasculitis
      iv. Ulcer biopsy – if suspicious appearance or if not responding to adequate compression therapy

Epidemiology

A number of epidemiological studies of leg ulceration have been conducted in different Western countries and have found a similar prevalence of leg ulceration ranging from 0.11% to 0.18% of the population.[4-8] These studies have confirmed that chronic venous disease is the commonest cause, representing approximately 65% of ulcers on the leg. These occur most commonly in elderly people with a mean age in excess of 65 years. There are nearly twice as many women as men with leg ulcers, however, when these are related to age, the prevalence for males and females is similar because there are more women than men in the older age groups.[4]

An Australian epidemiological study found that venous ulcers are associated with delayed healing (with a median duration of 26 weeks) and also tend to recur in over 70% of patients.[4]

Pathophysiology

Venous Abnormality

The basic underlying physiological abnormality in chronic venous disease is altered return of blood in the veins of the leg, which results in ambulatory venous hypertension in the superficial veins.[9] When venous pressures are measured in the surface veins in the foot or ankle, the pressure is normally highest (approximately 100mmHg) when standing immobile, and drops to 30 to 40mmHg when walking (Figure 25.1). This occurs because the 'calf muscle pump' assists the return of blood from the leg by compression of the deeper veins during muscle contraction. When the muscles relax, the emptied deeper veins have a lower pressure which allows more blood to flow into them from the surface veins, thereby reducing the pressure in those veins (Figure 25.2).

In patients with chronic venous insufficiency the pressure in the surface veins drops only a small amount, hence the term 'ambulatory' venous hypertension (Figure 25.1). An increase in the pressure in the surface veins above that present on standing is very uncommon, and only occurs when there is extensive proximal venous occlusion. The failure to reduce superficial venous pressure on exercise occurs when there is reflux in either the deep veins or the surface veins. Reflux in the deep veins results in rapid refilling of the deep veins when the calf muscles relax (Figure 25.3). This results in only a small increase in the amount of blood that

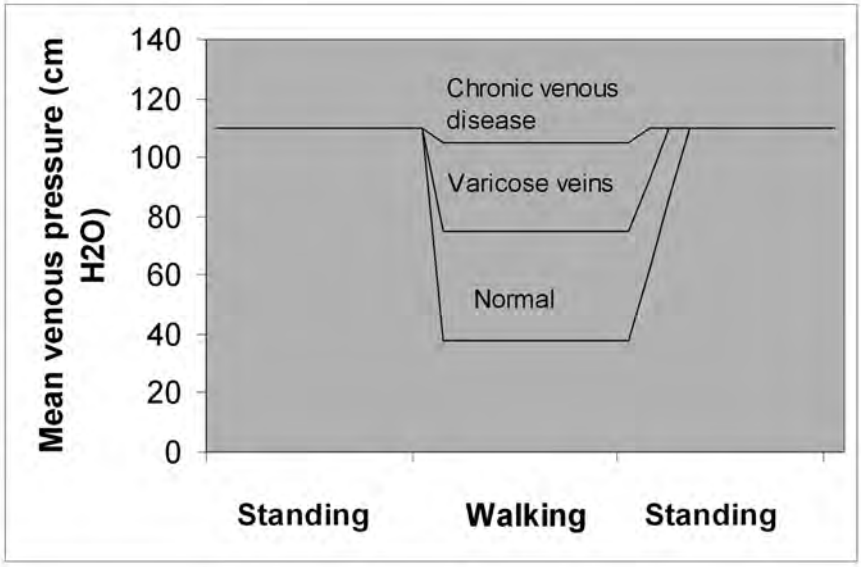

FIGURE 25.1: Superficial venous pressures in normal legs and legs with chronic venous disease.

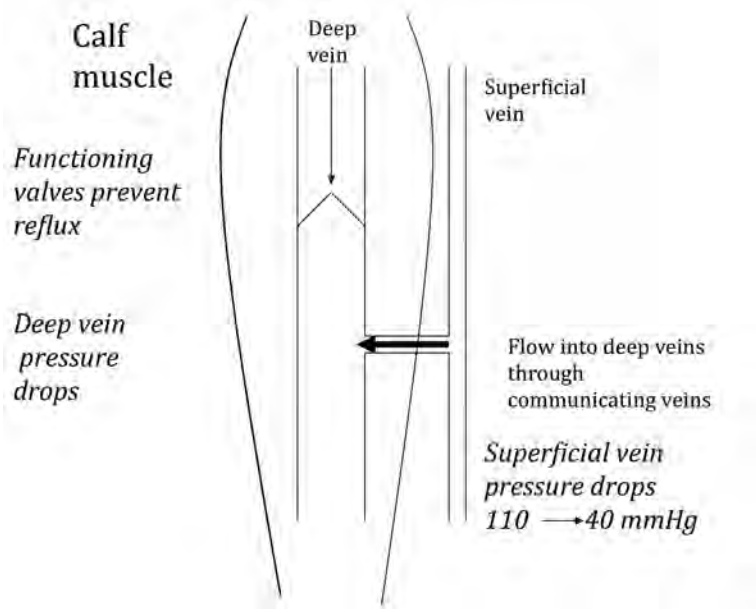

FIGURE 25.2: Pressure in the deep and superficial veins in a normal leg after calf muscle contraction.

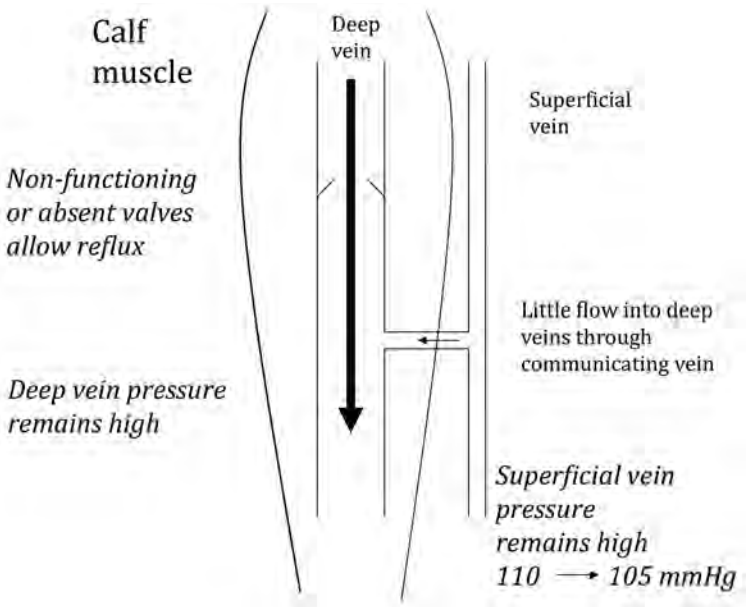

FIGURE 25.3: Pressure in the deep and superficial veins in a leg with chronic venous disease after contraction of the calf muscle.

flows from the surface veins into the deep veins. If the reflux is primarily in the superficial veins, the superficial veins refill quickly and the ambulatory venous pressure remains elevated even though more blood may be flowing into the deep veins.[10]

Reflux in veins is caused either by destruction of the valves when a venous thrombosis recanalizes, or by primary incompetence of the valves. The relative proportions of the two causes in the deep veins remain a point of debate. Definite evidence of previous deep vein thrombosis has been reported in 40 –50% of patients with venous ulceration.[10,11] In chronic venous insufficiency the venous reflux may involve the deep veins or may involve only the surface and perforating veins. Involvement of the deep veins has also been variously reported at between 40 and 90%.[11,12] The major reason for this variation in reporting is the use of different techniques for assessing the deep veins. The rate of deep vein involvement was reported at higher rates when the standard method of assessing veins was venography.[11] Duplex scanning has now become the major method for assessing veins, and the quoted rates of deep vein involvement have dropped.[12] This possibly relates to the difficulty in visualizing the calf veins with duplex scanning.

Effect of Ambulatory Venous Hypertension on the Tissues in the Leg

The obvious clinical finding in CVI is the pigmentation and fibrosis that occurs in the skin in the gaiter region of the leg, referred to as lipodermatosclerosis (Figure 25.4). Histologically there is also an increase in the extent of the capillary bed in the skin, although there is some debate as to whether the capillaries are all perfused.[13,14] This is particularly the case in areas of atrophie blanche (white atrophy) in which there are tufts of capillaries interspersed within

relatively avascular skin (Figure 25.5). This condition is common in venous ulceration, but may also be present in other conditions such as vasculitis.[15]

A number of hypotheses have been proposed over the years to try and explain how ambulatory venous hypertension affects the tissues and thereby leads to impaired healing. It has generally been acknowledged that there must be some impairment to the nutrition to the skin cells either due to reduced nutrients or reduced oxygenation. To date such reduced skin nutrition has not been conclusively demonstrated. Many studies have shown reduced transcutaneous oxygen measurements,[14] however because of the change to the structure of the skin this may not be an accurate reflection of tissue oxygenation.

Theories that have been proposed to support the concept of reduced nutrition to the skin are arteriovenous shunting,[16] the

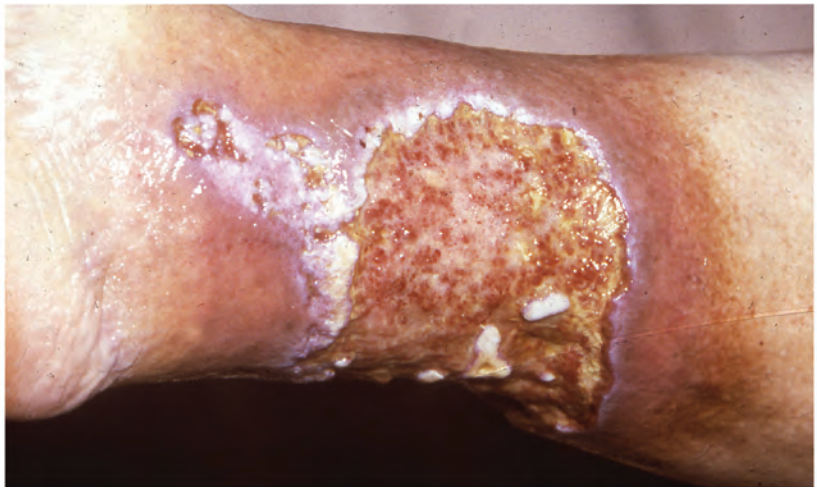

FIGURE 25.4: Lipodermatosclerosis and ulceration in a leg with chronic venous disease.

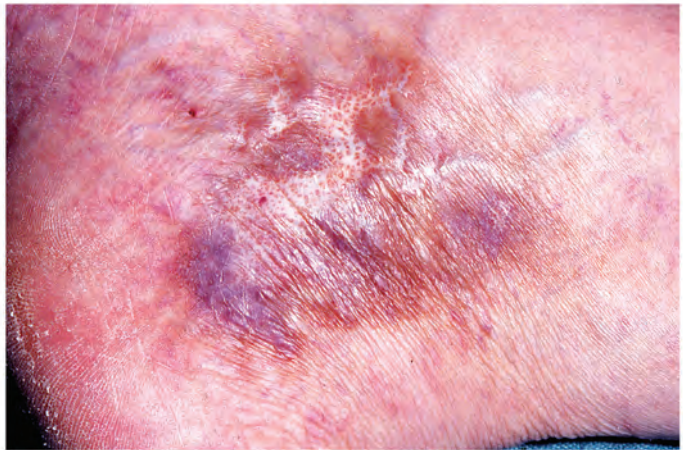

FIGURE 25.5: Atrophie blanche in a leg with chronic venous disease.

presence of a diffusion barrier to oxygen by fibrin and other proteins that deposit around skin capillaries,[17] and the occlusion of capillaries by activated white cells becoming 'trapped' in the capillary bed.[18] Other theories have hypothesized that growth factors are trapped in the pericapillary protein deposits which therefore impedes the healing process (Figure 25.6).[19] Localised reperfusion injury has also been hypothesized in association with intermittent periods of ambulatory venous hypertension and lower venous pressures.[20] Other hypotheses have focused on the presence of factors that directly damage the tissue by activation of white cells which subsequently release factors such as cytokines, proteases and oxygen free radicals that can impede healing.[21,22] To date there is no conclusive support for any given hypothesis, although the presence of an excessive inflammatory response has been repeatedly demonstrated in venous ulcers.

Influence of Venous Disease on the Wound Healing Process

The cause of impaired healing in venous ulceration remains uncertain. This is in spite of the clinical observation that non-healing venous ulcers begin to heal once patients are admitted to hospital for bed rest. Efforts are continuing to try and determine what is occurring at a cellular and molecular level to impede the healing process. These have demonstrated a highly inflammatory environment with high levels of inflammatory cytokines, proteases and large numbers of immune cells.[22-24] The proteases that are elevated in venous ulcers compared to acute wounds include matrix metalloprotease-2 and -9 (MMP-2 and 9) (Figure 25.7) and collagenases (Figure 25.8). It is possible that this highly inflammatory environment may result in destruction of factors that are important in the normal healing process. These factors may include growth factors, cell surface receptors, the matrix in the base of the wound and cellular adhesion molecules.

Bacteria that colonise open wounds may contribute to the increased inflammatory wound environment. No clear link has been shown between the presence of bacteria and healing ability in venous ulcers.[25,26] Bacteria are known to form biofilms which consist of a matrix that is secreted by the

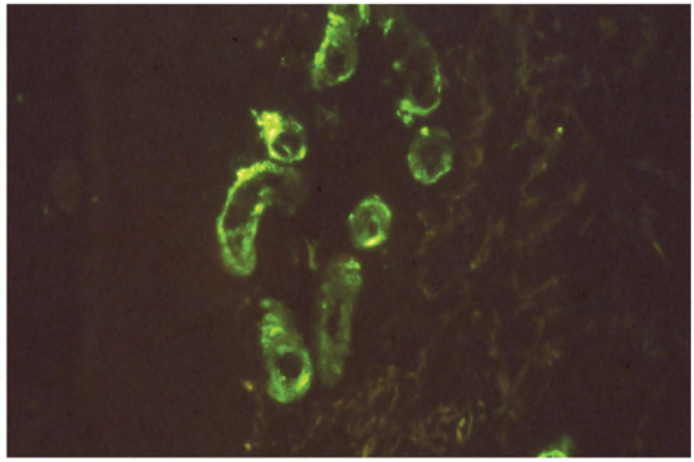

FIGURE 25.6: Pericapillary deposit of protein – fibrinogen.

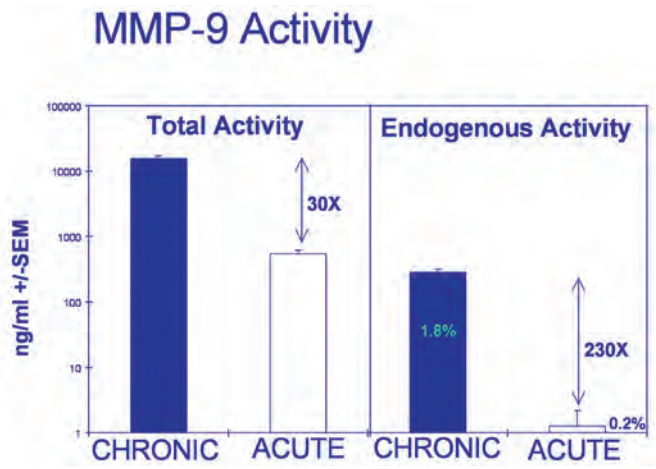

FIGURE 25.7: Matrix metalloprotease levels in wound fluid from acute and chronic wounds.

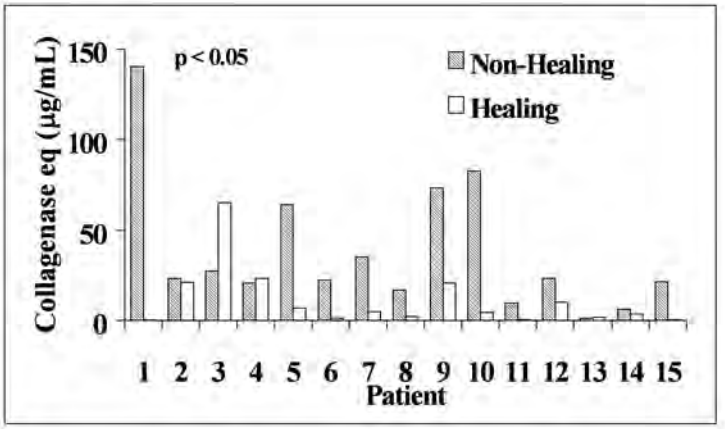

FIGURE 25.8: Collagenase levels in wound fluid from chronic venous ulcers.

bacteria which enables the bacteria to persist in an environment in which they are protected from the body's defense mechanisms.

The underlying causes of venous ulceration may affect both the ability to develop ulcers and the ability to heal ulcers; however, it is likely that additional factors will contribute to the impaired healing process once an ulcer has occurred. Further understanding of the factors that impede the healing process may lead to better treatments to improve ulcer healing.

Genetic Associations with Venous Ulceration

A number of research groups have now demonstrated that genetic polymorphisms are associated with the development of venous ulceration. Working independently, groups have demonstrated that the following polymorphisms to be associated with venous ulceration – tumour necrosis factor alpha 308 (a regulatory polymorphism),[27] fibroblast growth factor receptor type 2,[28] oestrogen receptor beta[29] and haemochromatosis

factor.[30] There is also a suggestion that a polymorphism in coagulation factor XIII may be associated with delayed healing of venous ulceration following venous surgery.[31] All of these studies have been performed on relatively small samples sizes and do require further confirmation in larger samples sizes. It is likely that a number of genetic factors will ultimately be shown to be associated with the ability to heal wounds.

ASSESSMENT OF VENOUS FUNCTION

In patients with venous ulceration, the history and clinical signs on the leg will give a strong indication of the presence of venous disease. When surgical treatment is to be considered it is imperative to have a clear outline of the veins with reflux and the presence of venous obstruction. If the deep veins are involved, the benefits of operating on surface and or perforating veins are limited and appear to be dependent on the extent of the venous reflux.[32,33] The commonest current method that is used to assess for sites of incompetence is Duplex scanning. Venography and imaging with contrast CT scan or MR venography are used infrequently and usually only when there is uncertainty about the information from the Duplex scan. Other methods of diagnosing venous disease such as plethysmography and hand held Doppler do not give a good indication of the sites of venous incompetence.[34,35] When performing a Duplex scan for chronic venous disease, the deep veins, superficial veins, and the communicating veins should all be evaluated.

TREATMENT OF VENOUS ULCERATION

The objective of the treatment of venous ulceration is to improve the physiological abnormality of ambulatory venous hypertension. The two methods available are compression therapy applied to the leg or direct treatment of the veins by surgery or other ablative techniques such as sclerotherapy, laser or radiofrequency ablation.[36] There are also ongoing studies to identify topical and systemic therapies that will have a direct influence on improve the healing process in the ulcers. The only such therapy that has been used in clinical practice in some parts of the world is topical platelet derived growth factor.[37]

Compression Therapy

Compression applied to the leg improves the venous return in the leg, primarily by reducing venous reflux into the leg and also by reducing the leg volume.[38] Compression bandages have also been shown to significantly improve the time taken to heal venous ulcers.[39] This reduces oedema in the leg that is considered to impede the healing process. The compression is applied to the leg below the knee from the base of the toes to just below the knee, and the level of compression that is recommended is to achieve a pressure of between 25 and 45mmHg at the ankle with a graduated reduction in pressure up the leg. The compression may be applied by either bandages or by compression stockings. In patients with an open ulcer, bandages are normally preferred because the exudate damages the stockings and shortens their lifespan.

There are many different types of bandage systems that have been employed for venous ulcers. Most benefit has been shown to occur with systems that are multilayered.[39,40]

The first layer consists of orthopaedic wool or similar padding that is placed beneath a plaster cast. This is to help protect the skin overlying bony prominences from excessive pressure. The next one or preferably two layers are the compression bandages that may be elastic or inelastic or a combination of the two. The top layer is one to help prevent slippage of the bandage. This may be a bandage that adheres to itself or a tubular stockingette. To date no difference has been shown in the efficacy of inelastic (short stretch) or elastic bandages (long stretch), as long as these are used as part of a multilayered system.

If a compression stocking is used this should be either class 2 (25–35mmHg at the ankle) or class 3 (35–45mmHg), and should aim to provide graduated compression. These come in a variety of sizes and should be fitted to the individual's leg. They come with and without zippers on one side that help with applying and removing the stocking. Patients with small or very large legs may need to have stockings custom made to fit their legs. Stockings can be difficult to both apply and to remove, particularly in patients with arthritis or in frail patients. To assist with application there are frames onto which the stockings can be placed and into which the patient then places their foot. Stockings are often useful in younger patients who have active jobs and for whom wearing bulky bandages is difficult.

Dressings

The dressing applied to the ulcer may be chosen from any that are available. No dressing, including those dressings containing silver and other topical antibacterial agents, have been shown to have a direct effect on improving the healing process.[41] Dressings are chosen according the needs of the patient and their wound. Different dressings may be chosen to absorb exudate, reduce pain, help liquefy slough, reduce bacterial contamination, reduce odour from the wound, or to protect the surrounding skin from maceration. The cost of the dressing also needs to be considered when making a selection. For patients who are using stockings a dressing that adheres to the skin is preferable as this aids with applying the stocking over it.

The dressings and compression may be left on the leg and for up to one week and even longer in cooler climates. Commonly however, the bandages and dressings may be changed anything from daily to weekly. The main determinants of the duration of application are the amount of exudate, the state of the wound and odour from the bandages.

Surgery

Surgery to the veins in the lower leg may include procedures on the superficial varicose veins, incompetent communicating veins or the deep veins in the leg. Surgery to the superficial veins includes surgery to the long and/or short saphenous vein by ligation and stripping, together with avulsion of varicosities.[33,42] Incompetent communicating veins can be accurately located by Duplex scanning and may be approached directly by a small incision over the site and subfascial ligation. In order to avoid making incisions through areas of lipodermatosclerosis or ulceration, the perforators in the lower leg may be treated by subfascial endoscopic perforator surgery (SEPS) (Figure 25.9).[43] The deep veins may be treated by direct repair of intact but incompetent valves. The repair may be directly by suture[44,45] or may involve applying a cuff around the valve to reduce the diameter of the vein and to restore competence.[46] Veins that have had previous venous thrombosis and which have recanalised with destruction of the valve can

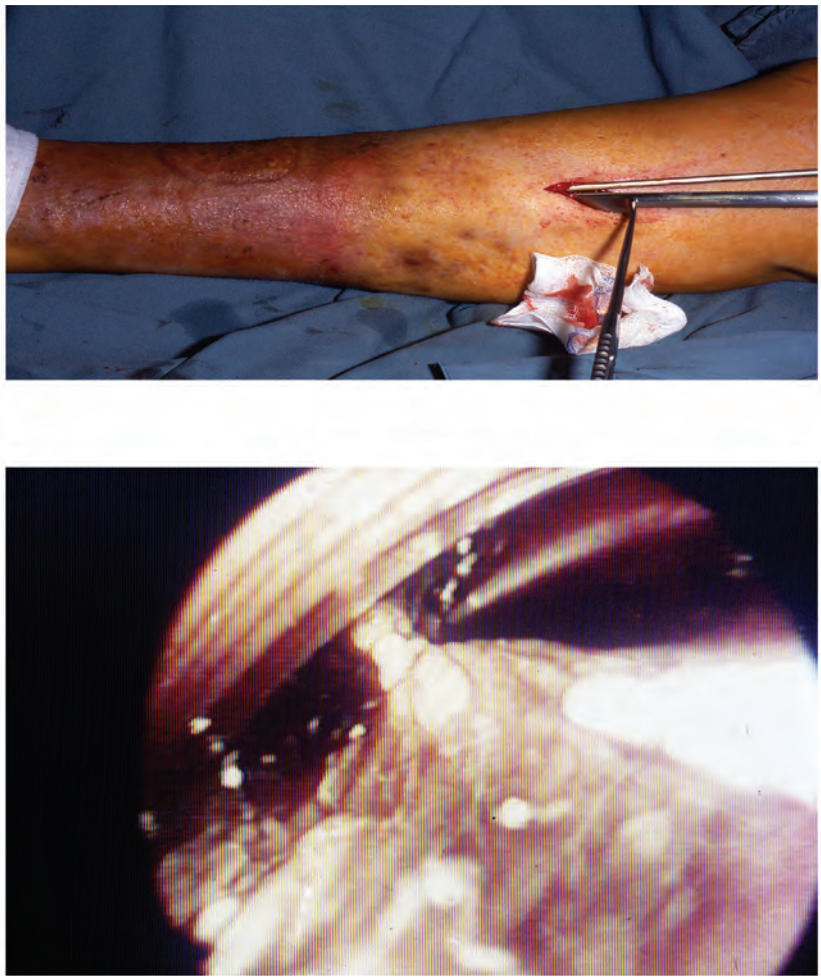

FIGURE 25.9: Subfascial endoscopic perforator surgery – instruments in position and clip on a perforator.

have a segment of vein containing competent valves taken from the arm and inserted to replace a segment of the femoral or popliteal vein.[47]

The roles of a number of these operations in patients with venous ulcers has in part been clarified by recent clinical trials and reviews of published data.[32,33] The benefits of surgery to the deep veins for patients with open or healed venous ulcers remain unproven and should be confined to studies assessing their efficacy rather than be used in routine clinical practice. Surgery to the superficial veins in combination with compression does not result in any improvement in venous ulcer healing compared to compression therapy alone.[32] However, surgery to the superficial veins in combination with compression does result in a significant reduction in venous ulcer recurrence. Individuals who are most likely to benefit from surgery to the superficial venous system are those with superficial venous incompetence and no deep incompetence or in patients with superficial incompetence and segmental deep incompetence. In both groups of patients there is a significant reduction in ulcer recurrence compared to compression

therapy alone. For patients with extensive deep vein incompetence, there was not a significant reduction in the rate of ulcer recurrence.[32,33,42]

The need to treat incompetent communicating veins at the same time as treating superficial veins remains uncertain.[42] Studies on patients with superficial venous incompetence and incompetent communicating veins, but without deep vein abnormalities, have indicated that in a majority of patients, the communicating veins cease to be incompetent after the superficial veins are ablated.[48,49] Another study has indicated that in the presence of deep vein reflux, ablation of superficial veins does not result in a return to competence in incompetent communicating veins.[50]

Anecdotal reports have suggested that operating on the superficial and perforating veins does improve the healing of venous ulcers. Randomised controlled trials have however, shown no improvement in venous ulcer healing when surgery is combined with compression bandaging compared to compression bandaging alone.[32,51] However, in patients who are not responding to optimal compression therapy and who have no or minimal reflux in the deep veins, the author's anecdotal experience is that surgery to the superficial and perforating veins does result in an active healing process. In addition, in patients with very painful ulcers, this surgery appears to reduce their level of pain. It is still important to continue the compression after the surgery.

Surgery to correct venous obstruction has included vein bypass, transposition of a vein to bypass an obstruction,[52] or balloon dilatation with stenting.[53] The efficacy of these procedures remains uncertain due to the infrequency with which they are performed, and the consequent anecdotal nature of reports.

Prevention of Venous Ulcer Recurrence

Once an ulcer has healed continued treatment should be implemented to help reduce the risk of ulcer recurrence. The simplest method is to use compression stockings.[54] These have been shown to significantly prolong the time before venous ulcers recur, however, they do not remove the risk completely. The stockings that are used are class 3, however, it is generally considered that class 2 stockings will have a similar benefit.

Surgery to the leg veins in patients with venous ulcers is most commonly used to reduce the risk of ulcer recurrence. This surgery is usually performed after ulcers have healed, to prevent the potential for wound contamination and infection from an open ulcer. As indicated above the only form of surgery that is used in routine practice is surgery to the surface and/or perforating veins. The benefits of this surgery are greatest in patients who have no evidence of post-thrombotic damage to the deep veins.

Sclerotherapy and Other Techniques to Obliterate Surface and Perforating Veins

Techniques other than surgery have been used to treat varicose veins and incompetent perforating veins. These include sclerotherapy,[55] ultrasound guided sclerotherapy to incompetent perforating veins and saphenous veins,[56] and laser or radiofrequency ablation of the long saphenous vein.[57] These techniques have not been specifically evaluated in the treatment of venous ulceration or the prevention of ulcer recurrence. It is likely that the benefit with these treatments would be commensurate with their efficacy in obliterating the appropriate veins compared to that achieved with the surgical techniques.

Other Therapies

The use of other systemic or topical therapies is an area in which there is ongoing research. Many different therapies have been evaluated; however, to date no single therapy has been shown to be of sufficient benefit to be used in routine clinical practice. Therapies that have or are being assessed include aspirin, oxpentifylline, platelet lysate or releasate, a number of different recombinant growth factors, growth factors derived from bovine whey, protease inhibitors, topical antibacterial preparations or dressings, systemic or topical antibacterials, and various vasoactive preparations.[58] There is some evidence that oxpentifylline does improve ulcer healing.[59] Other topical methods include ultrasound, magnetic therapy, ultraviolet light, electrical stimulation, and topical laser therapy.[60] To date there is no convincing evidence to support the efficacy of any of these therapies. Many therapies that have been proposed to aid venous ulcer healing have been selected on theoretical grounds rather than detailed knowledge of the cellular and molecular abnormalities that result in venous ulcers forming and that impede their healing. There is a need to better understand these processes so that this knowledge can be used to help identify improved methods for treating venous ulcers.

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26 • Pathophysiology and Principles of Management of the Diabetic Foot

[1]DAVID G. ARMSTRONG, [2]TIMOTHY K. FISHER, [1]BRIAN LEPOW, [4]MATTHEW L. WHITE, [1,4]JOSEPH L. MILLS.

[1]The University of Arizona, Southern Arizona Limb Salvage Alliance (SALSA), Tucson, Arizona, USA

[2]Rashid Centre for Diabetes and Research, Sheikh Khalifa Hospital, Ajman, UAE

[3]Vascular and Endovascular Surgery, University of Arizona, Tucson, Arizona, USA

INTRODUCTION

The incidence of diabetes continues to grow at a staggering pace. The United States' Centers for Disease Control and Prevention estimate that 23.6 million people or 7.8% of the U.S. population has diabetes, with 1.6 million new cases being diagnosed each year.[1,2] These figures are even more astonishing when one considers worldwide estimates. Close to 4 million deaths in the 20–79 year old age group may be attributed to diabetes in 2010, accounting for 6.8% of global all-cause mortality in this age group.[3] The number of people with diabetes worldwide is expected to reach 366 million people by 2030, more than double the estimated 177 million people affected with the disease in 2000.[2] The increased disease prevalence is accompanied by an increase in associated comorbidities. The literature estimates that patients with diabetes have nearly a 25% lifetime risk of developing a foot ulcer with more than 50% of these ulcers becoming infected and requiring hospitalization.[4] In fact, at least 20% of all diabetes-related hospital admissions are due to diabetic foot ulcers. Associated with foot ulcers and infection is the incidence of amputation. It has been conservatively reported that, worldwide, a major amputation takes place every 30 seconds with over 2500 limbs lost per day.[5] At least 60% of all non-traumatic lower extremity amputations are related to complications of diabetes.[1] People with diabetes who have had one amputation have a 68% risk of having another in the next 5 years and have a 50% mortality rate in the 5 years following the initial amputation.[6] It is estimated that up to 85% of diabetic foot-related amputations could be prevented through prompt intervention and with centers directed at educating individuals about proper foot care.

The economic impacts on the patient, national healthcare system and economy also impose a great burden. Healthcare expenditures on diabetes are expected to account for 11.6% of the total healthcare

expenditure in the world in 2010.[3] The estimated global costs to treat and prevent diabetes and its complications are expected to total at least 376 billion USD in 2010, with some projections exceeding 490 billion USD. Between 22 and 27% of the total diabetes costs are attributable to lower extremity disease.[3,7]

PATHOPHYSIOLOGY OF THE DIABETIC FOOT

Neuropathy

Neuropathy, or the loss of protective sensation (LOPS) of the lower extremity, is the predominant etiology for diabetic foot ulceration. The absence of this most basic nociceptive mechanism results in the patient's inability to perceive local foot trauma both from intrinsic factors such as abnormal or faulty foot mechanics or deformity, as well as extrinsic factors such as foreign objects or improper footwear. These factors place the foot at increased risk for developing an ulcer, which can lead to amputation.

Neuropathy can be subdivided into sensory, motor, and autonomic categories.

Sensory neuropathy typically begins distally, moving proximally, and is symmetrical. It is often described as a 'stocking-glove' distribution.

Motor neuropathy results in intrinsic muscle wasting, causing a progressive deformity such as hammertoe, claw toe, and plantar flexion deformities of the metatarsals. These deformities can cause an increase in focal pressure at the areas of the interphalangeal joints of the digits and beneath the metatarsal heads, respectively, increasing the probability of ulcer formation.

Autonomic neuropathy is associated with pathology of the sympathetic nervous system. In the lower extremity, this usually results in the absence of sweat production causing drying and scaling of the skin, increasing the likelihood of cracking and fissuring. These cracks or fissures, especially in the heel, may then serve as a portal of entry for bacteria, creating an increased chance of infection.

Other factors precipitating diabetic foot ulcers have been identified and are listed in Table 26.1. A risk factor classification scheme has been developed based on LOPS and other comorbidities (i.e. vascular disease) (Table 26.2). This classification system provides treatment recommendations and suggested follow-up time.

Structural abnormalities/gait abnormalities

Structural deformities of the foot and ankle can be a potential cause of increased pressure and subsequent ulceration. The development of foot ulceration is often based on a biomechanical abnormality.[8,9] It is important to evaluate both feet of a patient for any potential problem areas. As described earlier, motor neuropathy can lead to the loss of intrinsic foot musculature causing a deformity (and subsequent overpowering of the intrinsics by the larger extrinsic muscles). Identification and management of any of the following will assist in prevention of potential ulceration: hammertoe, claw toe, bunion, tailor's bunion (bunionette), hallux limitus/rigidus, flat feet, high arched feet, Charcot deformities, or any postsurgical deformities such as amputations. Limited joint mobility should also be evaluated as it can cause an increase in vertical and shear force in certain areas, particularly to the plantar hallux, and lead to tissue breakdown. The Achilles tendon should be evaluated for any type of functional shortening causing equinus deformity. It is common to find an increase in glycosylation of soft tissues and tendons causing contracture of the muscles in the

TABLE 26.1: Risk Factors For Ulceration

General or Systemic Factors	Local Factors
• Uncontrolled Hyperglycemia • Duration of Diabetes • Peripheral Vascular Disease • Older Age • Chronic Renal Disease • Blindness or visual loss	• Peripheral Neuropathy • Structural Foot Deformity • Limited Joint Range of Motion • Trauma • Improperly fitted shoes • Callus • Prolonged Elevated Focal Pressure • History of Previous Ulcer or Amputation

TABLE 26.2a: American Diabetes Association Risk Classification for The Diabetic Foot.

Risk Category	Definition	Treatment Recommendations	Suggested Follow-Up
0	No LOPS, no PAD, no deformity	– Patient education including advice on appropriate footwear	Annually (by podiatrist)
1	LOPS ± deformity	– Consider prescriptive or accommodative footwear – Consider prophylactic surgery if deformity cannot be safely accommodated in shoes. Continue patient education	Every 3–6 months (by podiatrist +/– specialist)
2	PAD ± LOPS	– Consider prescriptive or accommodative footwear – Consider vascular consultation for combined follow-up	Every 2–3 months (by podiatrist +/– vascular surgeon)
3	History of ulcer or amputation	– Same as category 1 – Consider vascular consultation for combined follow-up if PAD present	Every 1–2 months –(by podiatrist)

TABLE 26.2b: The University of Texas (UT) Wound Classification System

UT System		
Grade	**Description**	**Stage**
1	Pre- or post ulcerative lesion	A–D
2	Superficial	A–D
3	Penetrated to tendon or capsule	A–D
4	Penetrates to bone	A–D

Stages: A = no infection or ischemia; B = infection; C = ischemia; D = infection and ischemia

posterior compartment of the leg.[10-12] This will cause an increase in plantar pressures in the forefoot making this area more prone to breakdown.

Gait evaluation and muscle testing should also be conducted to evaluate any potential abnormality with ambulation and muscle strength. Testing of the muscles in the lower extremity should be done both actively and passively, weight bearing and non-weight bearing. Plantar foot pressures can also be assessed with the use of a Harris ink mat or pressure sensitive foot mat.

Angiopathy

In combination with the risk factors for ulceration discussed above, the presence of vascular occlusive disease increases the risk of potential amputation. Vascular disease is a common finding in individuals with long-standing diabetes. While vascular insufficiency alone is not usually the primary cause of ulceration, inadequate perfusion can inhibit ulcer healing, leading to further tissue necrosis and the inability to clear infection. Table 26.3 provides a list of other potential risk factors for amputation. Most cases of vascular disease in individuals with diabetes, interestingly, affect the infrapopliteal vessels with relative sparing of the pedal vessels. In many instances, this allows for a distal bypass to a pedal target artery in order to increase the blood flow to the foot (Figure 26.1). The vascular examination is addressed in further detail later in this chapter.

DIAGNOSIS

History and rapid visual screening

A thorough history and physical examination of each patient presenting with diabetic foot pathology should include a history of pedal wounds, history of prior amputations, and lower extremity vascular interventions.[13,14] Physical examination should note the types of deformities present, neurological status, vascular status, and dermatological presentation. The patient should be instructed to remove both shoes and socks for their examination. A systematic approach should be taken in order to avoid missing any

TABLE 26.3: Risk Factors for Amputation:

- Peripheral Neuropathy (LOPS)
- Vascular Insufficiency (PAD)
- Infection
- Structural Foot Deformity
- Trauma
- History of prior foot ulcer or amputation
- Charcot foot
- Poor glycaemic control
- Older age
- Male gender
- Ethnicity (higher in Hispanics and African-Americans)

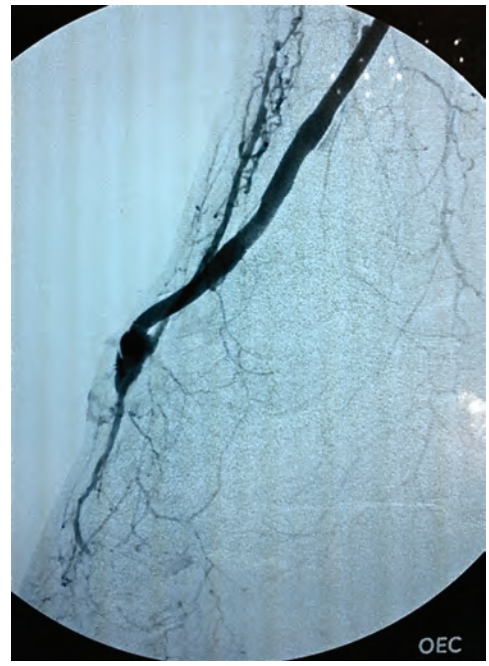

FIGURE 26.1: Dorsalis Pedis Bypass

important aspects of the examination. All surfaces of the foot and ankle should be evaluated including the nails, digits, interdigital webspaces, the soles, and the heels, inspecting for cracks, blisters or bullae, hyper/hypopigmentation, fissuring, calluses, and ulcers. The footwear should also be inspected for signs of wear patterns, foreign bodies, and any irregularities. Any gross deformities as described above can be identified during this rapid visual screening.[14]

Neurological examination

As stated earlier, peripheral sensory neuropathy is the major risk factor for the development of a diabetic foot ulcer. There are many simple, noninvasive examinations that can be performed to test and monitor sensation. A simple history of neuropathic symptoms such as tingling, burning, numbness, the feeling of insects crawling on the feet (formication) can help to identify those patients at risk for developing foot ulceration.[15]

Monofilament testing

One of the most common methods used to assess neuropathy is the use of the Semmes-Weinstein monofilament (10-g) wire.[14,15] The nylon monofilament is placed on ten different pre-determined locations on the foot and pressed down manually until there is a slight bend in the wire (Figure 26.2). The patient is instructed to say 'yes' if he or she thinks they feel slight pressure or sensation. If the patient is unable to feel the sensation at two or more locations then it is safe to assume that protective sensation is lost.

Vibration testing

Vibratory perception testing can be assessed utilizing several different modalities. The more traditional method of testing is by the use of a 128-Hz tuning fork. The tuning fork is struck and then placed on a prominent bony surface of the foot, such as the great toe or metatarsal head. The patient is instructed to identify when the vibration stops.

As an alternative, a vibratory perception threshold monitor (VPT – Diabetica Solutions, San Antonio, Texas, USA) (Figure 26.3) can be used to test for vibration perception threshold and is useful in identifying those at high risk for development of an ulcer. The instrument consists of a hand piece with a testing probe on the end, a motor, rheostat, and voltmeter. The probe is held gently on the distal aspect of each hallux, or distal most prominent area. The rheostat is slowly increased until the patient senses the vibration. Once the patient identifies the sensation, the rheostat is then decreased until the sensation is no longer felt. The average value of the two numerical readings is taken and the level of sensation is documented to the location where the numbers were obtained. Average values above 25 Volts

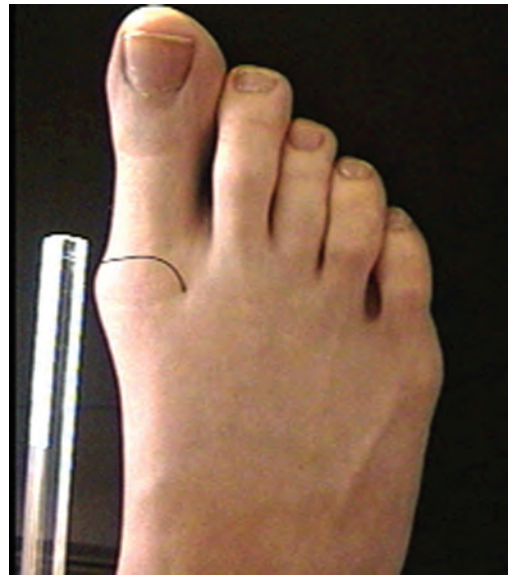

FIGURE 26.2: Proper use of S-W Monofilament when applied to the foot. Slight bend of the filament noted.

FIGURE 26.3: Vibratory Perception Threshold Monitor (VBT)

are considered positive for neuropathic changes.[14]

Dermatologic examination

Following assessment for any loss of sensation, evaluation of the integumen is a critical part of the foot screening. The skin can be evaluated for color, texture, turgor, quality, and presence of any areas of dryness or fissures. Calluses, if present, can be problematic as they indicate areas of increased pressure and an ulcer can form under the hyperkeratotic lesion, which may cause hemorrhage beneath the callus. Debridement of these areas is recommended in order to reduce a potential focus of pressure.[16, 17]

Appearance of the nails should also be noted. If there are nails that are incurvated or ingrown, these could be a potential area for skin breakdown and possible infection. Other nail issues to be aware of are onychomycosis (fungal nails), dystrophic nails, atrophy or hypertrophy, or paronychia (infected ingrown nail), as all can be potential problems.

Evaluation for any ulceration to the foot or lower leg should be assessed. Important characteristics of ulcers are depth, size, presence of fibrotic or granulation tissue, location, and whether or not the area appears to be infected.

Anatomy of occlusive disease – vascular examination

For decades, clinicians incorrectly believed that ischemia in diabetes was due to microvascular occlusion of arterioles, so-called small vessel disease.[18] However, this assumption has been disproven by subsequent study.[19,20] Diabetic vascular disease is a macrovascular phenomenon, commonly affecting infrapopliteal arteries with calcified stenoses and occlusions. More than 90% of patients have sparing of at least one major artery at the ankle level. The peroneal artery is often the last infrapopliteal artery to occlude; it provides blood flow to the foot through anterior and posterior perforating branches to the tibial arteries. Bypass to an infrapopliteal artery usually provides adequate blood flow to the foot, although some patients appear to have more compartmentalized pedal flow. Some patients with heel ulcers remain slow to heal

after dorsalis pedis artery bypass, suggesting inadequate pedal circulation.[21] Thus, if possible, patients with ischemic ulcers should preferentially receive a bypass to the arterial bed directly supplying the ulcer; this axiom is especially pertinent to patients with large heel ulcers.

Prediction of wound healing: assessment of perfusion

Pedal blood flow should be assessed before any surgical intervention on the diabetic foot. Absence of pedal pulses, dependent rubor, pallor on elevation, and loss of hair are clinical signs of advanced peripheral artery disease. If there is concern for ischemia, noninvasive testing is an appropriate initial diagnostic choice. The ankle-brachial index (ABI), while nearly 100% specific for PAD in non-diabetics, can be falsely elevated (>1.3) in diabetic patients due to medial calcinosis of the affected arteries. More useful are the systolic toe pressure and the toe-brachial index, since digital arteries are seldom calcified in those with diabetes.[22] Additional tests to assess foot perfusion include skin perfusion pressure,[23,24] and transcutaneous oxygen pressure (TcP_{O2}).[25-27]

Currently there is no single test or combination of tests that can always predict wound healing. Apelqvist and colleagues prospectively studied 314 consecutive patients with diabetic foot ulcers.[22] Primary healing occurred in 85% of patients with a toe pressure greater than 45 mmHg, while only 36% of patients with a toe pressure of 45 mmHg or less healed without an amputation. No patients with an ankle pressure less than 40 mmHg healed primarily. Kalani and colleagues also prospectively studied 50 patients with diabetic foot ulcers over a 12-month period.[26] A toe pressure of at least 30 mmHg had a 15% sensitivity, 97% specificity, and 67% positive predictive value for wound healing. In that same study, TcP_{O2} was also evaluated; a TcP_{O2} of at least 25 mmHg had an 85% sensitivity, 92% specificity, and 79% positive predictive value for wound healing. Likewise, Carter and colleagues reported that TcP_{O2} measurements correlated significantly with the risk of major amputation, with a relative risk of 2.55 for a TcP_{O2} of 20 mmHg of less and 2.22 for a TcP_{O2} of 30 mmHg or less.[27] In addition, an ankle pressure of 50 mmHg or less had a 5.83 relative risk of major amputation. Skin perfusion pressure (SPP) has also been used to predict wound healing. Faris evaluated 61 diabetic subjects with wounds and found that only 5% healed with an SPP less than 40 mmHg, while 88% of patients healed with an SPP higher than 40 mmHg.[23] Yamada and colleagues studied 211 subjects, half of who were diabetic, and compared SPP to other noninvasive methods to predict wound healing. They found that SPP was superior to ankle pressure, toe pressure, or TcP_{O2} for predicting wound healing. An SPP of 40 mmHg had a 72% sensitivity and 88% specificity for predicting wound healing.[24] Furthermore, the accuracy of prediction increased when a toe pressure greater than 30 mmHg was combined with SPP.

Arterial imaging

A number of techniques of arterial imaging are currently in use: arterial duplex scanning, computed tomographic angiography (CTA), magnetic resonance angiography (MRA), and digital subtraction arteriography (DSA). Duplex scanning is non-invasive and does not require administration of contrast. However calcification of the infrageniculate arteries can be problematic in many patients. Both CTA and MRA have the advantage of being noninvasive; however, both these studies are limited

in diabetic patients with impaired renal function. CTA requires a large bolus of iodinated contrast, and the gadolinium-based contrast used in MRA has been associated with nephrogenic systemic fibrosis.[28] In addition, MRA tends to overestimate the degree of arterial stenosis. Using selective catheterization techniques, DSA can produce high-quality images with low volumes of iso-osmolar contrast, thereby minimizing contrast-induced nephropathy. In addition, carbon dioxide can be selectively utilized as a 'contrast' agent; it is not nephrotoxic and quickly dissolves in blood before travelling to the lungs where it is readily expelled. DSA also has the advantage that angioplasty and stenting, if appropriate, may be performed at the time of diagnostic arteriography (Figure 26.4).

Soft tissue imaging

Imaging studies should be conducted to evaluate any underlying bone or soft tissue deformity. The diabetic foot is prone to many common and uncommon infectious and non-infectious processes due to the combination of vascular and neurological impairments. Imaging studies may be difficult to interpret and may lack specificity; therefore, a 'shotgun' approach to imaging studies should be avoided. Appropriate imaging studies should be ordered to establish

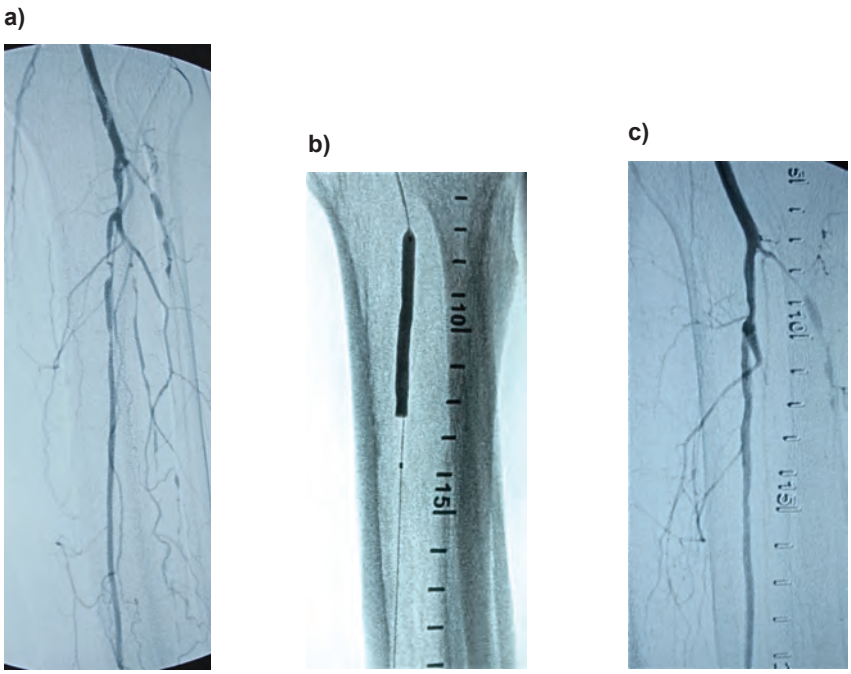

FIGURE 26.4: 56 year-old man with diabetes, renal insufficiency (serum creatinine 0.180mmol/L), absent foot pulses and non-healing dorsal foot ulcer for 6 weeks. Angiography showed normal arteries to the trifurcation and severe tibial artery occlusive disease with a long stenosis in dominant posterior tibial artery. Angioplasty markedly improved circulation and the wound healed with debridement, negative pressure wound therapy, and subsequent split-thickness skin graft placement.

a) long segment, proximal posterior tibial artery stenosis

b) 8 cm long, 4 mm percutaneous transluminal angioplasty (PTA) of proximal posterior tibial artery stenosis

c) excellent angiographic result after balloon angioplasty

or confirm a suspected diagnosis in order to properly manage the patient. For example, it is very difficult to differentiate acute Charcot arthropathy from osteomyelitis on plain radiographs. The reliability of imaging studies is diminished in the presence of arterial occlusive disease, Charcot arthropathy or after recent surgery or trauma.[29] A thorough history and physical examination and clinical overview by the treating physician must be correlated with the imaging studies in order to interpret them correctly.

Plain radiographs should be the initial study considered for patients that present with any signs or symptoms of foot problems. These studies help to identify osteomyelitis, fracture, dislocation, foreign bodies, soft tissue gas, arterial calcification as well as any other biomechanical or structural deformity. It should be noted that it might take time for acute osteomyelitis to be detected on plain film radiographs and serial radiographs or magnetic resonance imaging (MRI) should be considered if osteomyelitis is strongly suspected.

Computed tomography (CT) scans may be indicated if plain films fail to depict a suspected bone or joint deformity. CT offers higher anatomic detail and resolution in regards to bone and joints. 3-D CT reconstruction can be done to provide a 360-degree view and increased visualization of any suspected abnormality.

MRI is usually preferred over a CT scan for suspected osteomyelitis due to the increased resolution of the image. MRI scans also allow the physician to visualize the extent of the infection- osteomyelitis, deep abscess and septic joints, but also other soft tissue pathology such as tendon rupture.

Bone scans can also provide some useful data when evaluating the diabetic foot. Technetium-99 methylene diphosphonate (Tc-99 MDP) as well as indium-111 bone scans can be useful in determining the presence of osteomyelitis. However, while bone scans and other scintigraphic techniques are certainly possible tools to employ, their lack of specificity often makes their specific utility problematic, particularly if used in isolation. Advanced imaging studies beyond plain radiographs should not be routine. Rather, they should only be obtained to answer specific clinical questions when the results will alter management (e.g. concern over deep space infection with equivocal clinical picture).

CLASSIFICATION SYSTEMS

Diabetes mellitus foot risk classification

Based on a thorough history and physical examination, each patient should then be classified and assigned to a specific foot risk category as outlined in Table 26.2a. These categories were designed to direct and expedite the referral process to the necessary specialist and should also serve as a guide for subsequent follow-up visits. Increased categorical levels are associated with increasing risk for ulceration, hospitalization, and amputation.

University of Texas wound classification system

While many wound classification systems exist, The University of Texas (UT) ulcer classification system was developed to provide a more uniform evaluation of diabetic foot wounds. Like other classification systems the UT wound classification system builds on the depth-ischemia classification however, the UT system also considers infection.[30] The presence of infection and ischemia has been found to be more strongly predictive of outcome than the wound depth alone.[31] The UT system uses a 4 by 4 matrix (classes

A to D, wound depths 0 to 3) and evaluates 3 factors of ulceration, which include depth, infection, and peripheral arterial disease (PAD).[32] The frequency and level of amputation increases in deeper wounds and in the presence of infection and PAD.[30] Regardless of which specific classification is used, the key factors of depth, infection and ischemia should generally be communicated in some form. This classification is listed in (Table 26.2b).

CLINICAL PROBLEMS AND PRINCIPLES OF MANAGEMENT

Ulceration

Initial presentation of the diabetic foot usually entails the presence of an open ulcer, located on the plantar aspect of a patient's foot. Most patients will not suspect an ulcer on the bottom of their foot until they, or a loved one, notices blood either on the floor after walking or on a sock. Advising the patient that ambulation is contraindicated with an open ulcer is often challenging especially if the patient is neuropathic, as they have lost the 'gift of pain.'[33] However, reduction of pressure to the foot is essential for treatment. By effectively off-loading the area, the risk of continued trauma and resultant infection is decreased.

Epidemiology and risk factors

Ulceration of the diabetic foot is one of the single most common problems for which medical assistance is sought in a diabetic individual. Up to 25% of patients with diabetes will have a foot ulceration during their lifetime.[4] Remarkably, at least 50% of those ulcerations will become infected, and in 20% of those cases an amputation is required.[34] There are 81,000 major amputations performed on individuals with diabetes in the United States annually. Conservatively, 60% of all non-traumatic amputations occur in the diabetic patient.[35] Studies have shown that after a major amputation the contralateral limb develops a serious lesion in 50% of cases.[36] The 5 year adjusted mortality rate after a major amputation of a limb is 46%;[6] this is alarming and is higher than the mortality rate for many forms of cancer. It has also been shown in more than 85% of lower extremity amputations a wound was a critical aspect of the causal pathway.[8,34,37,38] The cost of treating diabetic foot ulcers is also growing at a staggering pace, reaching nearly $30 billion in the United States in 2007.[39]

Offloading

Numerous products are available to assist in redistributing pressure over a larger unit area (off-loading) on the foot. Some examples include removable cast walkers, non-weight bearing casts with crutch/walker assist, wedge sandals (either forefoot or heel wedge), healing sandals with a multilaminar, multidurometer foot bed. However, the choice of modality should be chosen with respect to the patient's functional status. For example, if a patient presents with a plantar forefoot ulcer, a wedge shoe that does not allow the forefoot to bear weight would be an appropriate device. However, if the patient is not properly educated on use of the device and/or has issues with balance, the offloading modality could cause the patient be unstable when walking. In regards to diabetic shoes, whether custom-made or off the shelf, it is important to keep in mind one key fact. While diabetic shoes are effective in preventing ulcerations, they are (in general) not very effective in healing them.

For many years the gold standard in off-loading an ulceration on the foot has been the total contact cast (TCC).[40] This fully weight-bearing cast consists of a multiple

layers of both fiberglass and plaster with minimal padding. When applied properly, the TCC allows the patient to be ambulatory. The TCC redirects pressure from the bottom of the foot, which is then redistributed over the bottom of the entire foot and up the cast. Some disadvantages of using a TCC is the time it takes to apply, the bulkiness for the patient, and the fact that the patient must wear the cast for 1 week intervals requiring weekly visits to the clinic. Newer TCC variants have reduced the time required for application of a traditional device whilst retaining many of the desirable therapeutic characteristics. An example of newer TCC variants can be seen in Figure 26.5.

Recent studies have evaluated the use of a removable cast walker rendered irremovable. This technique has been termed the 'instant total contact cast (iTCC).' This can be fabricated by simply adding a layer of fiberglass, cohesive bandage, or plaster around the leg portion (Figure 26.6). The iTCC has proven to be an effective and easy offloading device that ensures patient adherence.[41]

In many instances, proper off-loading may be all that is necessary to achieve wound healing. However, if wound healing fails in the midst of proper off-loading, adequate blood flow, and satisfactory nutritional status, surgical intervention may be warranted.

Non-vascular surgical treatment

Surgical management of the diabetic lower extremity can be a challenging and frustrating task, but can be ultimately rewarding both to the physician and patient upon healing of an ulcer and correction of the underlying cause of the deformity. Surgery in the absence of critical limb ischemia is based on three fundamental principles: presence or absence of neuropathy (LOPS),

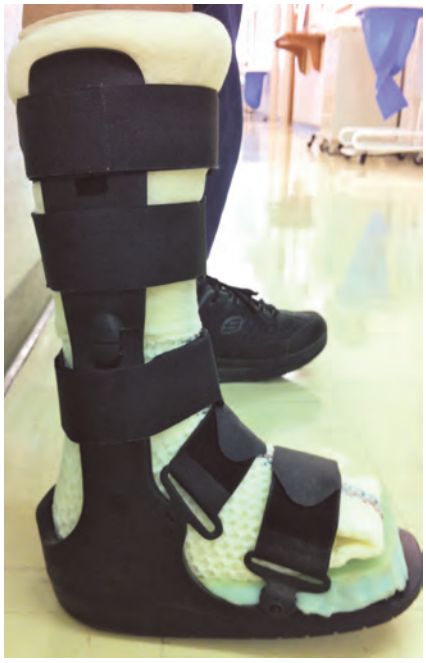

FIGURE 26.5: Anterior and lateral view of cast boot, which allows ambulation.

FIGURE 26.6: Example of an instant total contact cast (iTCC) with fiberglass wrapped around the leg portion, of an off-loading walking boot, to make it irremovable.

presence or absence of an open wound, and presence or absence of acute limb-threatening infection.[42]

A classification system has been developed outlining non-vascular surgical treatment of patients with diabetes and has been divided into four categories and highlighted in Table 26.4 and is based on indications and perceived risk.[42,43]

Class I: Elective

The goal of elective surgery is to reduce or eliminate any pain associated with a particular deformity and improve function.[42] Examples of these deformities include bunions, hammertoes and bone spurs, all in patients without peripheral neuropathy and with a low chance for ulceration. Basically any type of reconstructive surgical procedure can fall into this category with the exception of an amputation. Rarely are amputations done as an elective procedure. Only in the case of severe deformity or instability from a previous injury or surgical procedure, will an amputation be considered. Assuming good glycemic control, patients grouped to this class are not at any increased risk for the development of complications than corresponding patients without diabetes.[43]

Class II: Prophylactic

Procedures in this class are indicated to alleviate a deformity in a patient who is neuropathic however does not have the presence of an ulcer.[42] The goal of prophylactic surgery is to reduce the plantar sheer and vertical stresses. Many procedures for prophylactic surgery would be considered elective if the patient did not suffer from neuropathy. Several examples of procedures in this class include a Charcot foot reconstruction, a metatarsal head resection, a Keller arthroplasty, an Achilles tendon lengthening or a hammertoe repair.

Class III: Curative

Curative surgery often is identical to prophylactic surgery with the exception that procedures in this class are designed to speed the healing rate of an open wound but also to eliminate any potential recurrence of the ulceration.[42] Surgical procedures frequently utilized in this category may include exostectomy, digital arthroplasty, sesamoidectomy, single or multiple metatarsal head resection, joint resection or partial calcanectomy as well as Achilles tendon lengthening. Some surgeons may also elect to combine a plastic surgical flap and/or skin graft to help expedite healing.

Class IV: Emergency (Urgent)

Urgent procedures are performed to limit the spread of acute, limb and potentially life-threatening infection. Most often these procedures are done in the presence of significant ischemia. They require the removal of all infected and necrotic tissue to the level of viable soft tissue and bone and usually involve some level of amputation. When at all possible these procedures should be performed to allow for maximum amount of function to the extremity to be maintained.

With respect to any of the above classifications it is best to evaluate the vascular status of the patient to consider the necessity of any prior or subsequent arterial procedure that may be needed.[42]

Post-operative management

The management of the diabetic foot patient in the post-operative setting is much the same as a patient without diabetes. Adequate pain control, rest, elevation of the extremity and proper dietary intake are all recommended. One key difference is the timeframe for protected weightbearing or complete non-weightbearing. It is often

stated that diabetics require twice the healing time as a non-diabetic. That means that if a simple bunionectomy in a non-diabetic requires 4 weeks of protected weightbearing, the same procedure in a diabetic may require 6-8 weeks of protected weightbearing. The same can be said for a procedure requiring non-weightbearing. Most surgical procedures done for class 1–3 can be managed with protected weightbearing.[43] Most class 4 procedures will require some period of non-weightbearing or protection. The same can be said for any type of surgical implant or skin closure technique. For example, leaving skin sutures in place for an additional 1–2 weeks is advantageous over early removal to allow for the tissue to heal.

Surgical and non-surgical complications in this patient population are to be expected. Joint dislocation, bone fracture, surgical incision dehiscence, new ulceration or re-ulceration, and infection are all commonplace, not infrequently leading to re-hospitalization. Proper management by use of off-loading, local wound care, antibiotics and patient education will all be beneficial when dealing with any potential complication. It should also be kept in mind that not all diabetic foot complications can be prevented, but all can be adequately managed.

Infections

The Infectious Diseases Society of America (IDSA) has produced a classification system for diabetic foot infections, which is based on clinical signs and symptoms (Table 26.5). The IDSA divides infections into four categories, uninfected, mild, moderate, and severe.[44] These guidelines and classification system has become a reliable clinical predictor of the outcome of a diabetic foot infection.[45] It is important that initial antibiotic therapy cover the broad range of potential aetiological organisms in the diabetic foot until targeted therapy can be instituted once appropriate cultures have been obtained.

Charcot arthropathy

Charcot arthropathy is defined as a progressive condition characterized by joint dislocation, pathologic fractures, and severe destruction of the pedal framework (Figure 26.7). It is typically seen individuals with neuropathy and can be easily confused with acute infection or osteomyelitis. A

TABLE 26.4: Classification of non-vascular Diabetic Foot Surgery

Class	Type	Definition
1	Elective	Procedure performed on patient ***with protective sensation intact*** to eliminate pain or to improve function
2	Prophylactic	Procedure performed on patient ***with protective sensation absent but no open wound*** to reduce deformity and reduce occurrence/ recurrence
3	Curative	Procedure performed on patient **with an open wound** with the goal of promoting healing and reducing risk for recurrence
4	Emergency	Procedure performed with goal of limiting the spread of limb- or life-threatening infection

patient will often present with a red, hot, swollen extremity with no sign or history of ulcer or break in the skin (Figure 26.8). Usually all other constitutional signs of infection are absent and laboratory values are within normal limits.

Pedal manifestations of Charcot foot result in a debilitating deformity frequently leading to an amputation. While not well understood, several theories regarding the development of the disorder exist with many authors believing it is caused by a combination of the neurovascular and neurotraumatic theories. Neuropathy and trauma seem to be the common event preceding the development of an acute Charcot foot. As a result of the trauma and associated autonomic neuropathy, blood flow to the foot increases causing osteopenia and weakening of the bony structure. Fracture is often associated with unrecognized injury or minor trauma that might otherwise appear innocuous.[46,47] An unfortunate cycle continues as the patient ambulates, without pain, causing further destruction of the foot.

The initial diagnosis of acute Charcot is often clinically based on the characteristics listed above. It can become complicated as

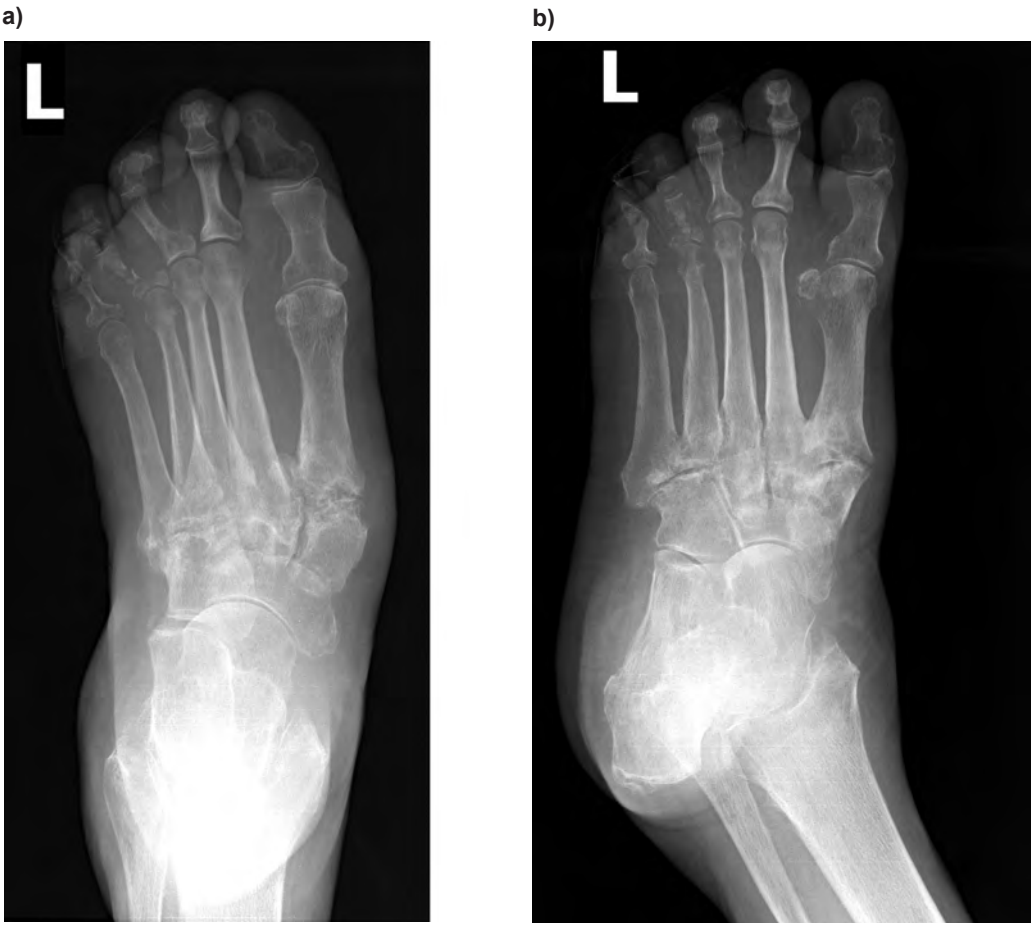

FIGURE 26.7a & b: X-ray of acute Charcot arthropathy. This plain x-ray demonstrates degenerative changes in the tarso-metatarsal joints (Lis-franc joints) with joint space narrowing and subchondrial sclerosis, consistent with Charcot arthropathy. Also noted is bone destruction and pathological fracture of the proximal phalanx of the 4th toe, indicative of osteomyelitis of this bone.

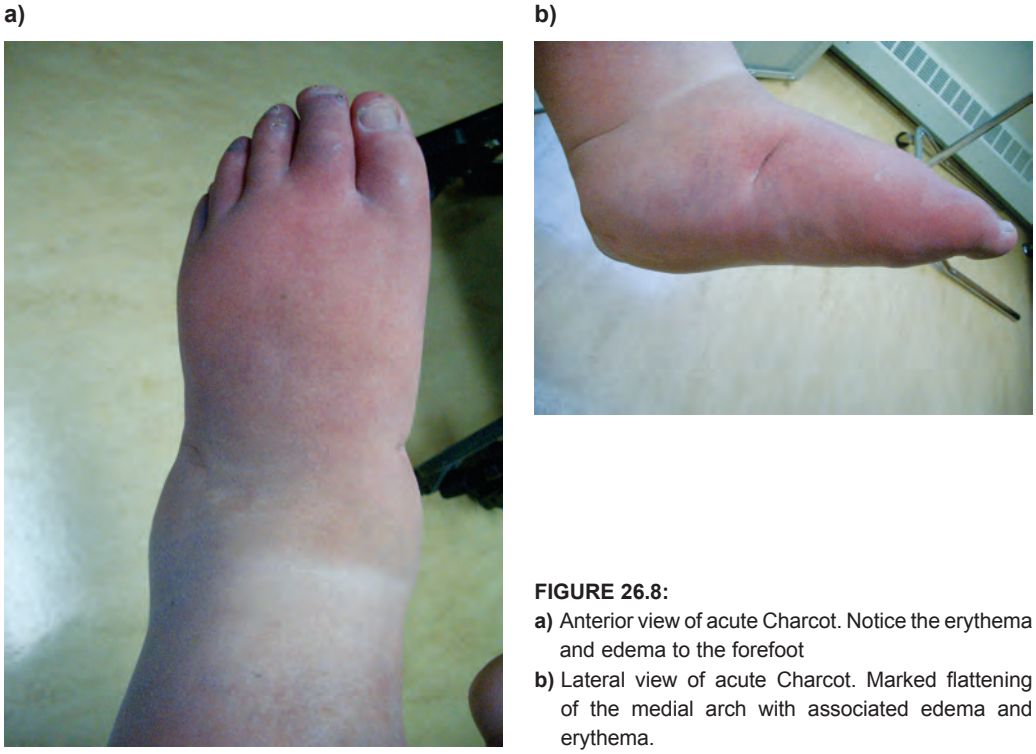

FIGURE 26.8:
a) Anterior view of acute Charcot. Notice the erythema and edema to the forefoot
b) Lateral view of acute Charcot. Marked flattening of the medial arch with associated edema and erythema.

patients often present with a concomitant ulcer, which raises questions of possible osteomyelitis. Someone presenting with the previously mentioned characteristics and lack of an open ulcer are more pathognomonic of acute Charcot arthropathy. Routine laboratory values are often of little use in diagnosing Charcot. They are valuable, however, with respect to excluding potential infection. An elevated WCC with a left shift is often seen with acute infection and not with acute Charcot. Elevated erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) level may also be elevated in acute infection but they often respond to any inflammatory process and thus are nonspecific. The most important diagnostic aid in determination of acute Charcot is the high index of clinical suspicion when a neuropathic patient presents with a deformed or swollen foot.

Treatment of Charcot consists of immediate immobilization and stress reduction. Most physicians would recommend strict non-weight bearing to the affected limb. However, this then subjects the contralateral extremity to increased pressure thereby predisposing it to repetitive stress and the potential for ulceration or even acute Charcot.[48] Nonetheless, non-weight bearing by means of a short leg plaster or fiberglass cast, or TCC remains the gold standard in the acute phase of the disease.[40]

Following the initial period of off-loading, skin temperatures and edema of the affected extremity will decrease. At this point protected weight bearing is permitted, usually with some sort of assistive device. This can be achieved through use of a Charcot restraint orthotic walker (CROW) (Figure 26.9), total contact cast, fixed ankle walker, bivalved casts or patellar tendon-

bearing braces, to name a few. The average time of rest and immobilization in a patient that has undergone an acute Charcot event is 4 to 6 months before returning to permanent and proper footwear.

If offloading is unsuccessful or if the deformity is unstable, reconstructive surgery may be considered. The goal of surgical intervention in the Charcot foot is to provide a stable, plantigrade foot that may be appropriately accommodated with proper shoe gear.[49] Most surgical interventions consist of an exostectomy for prominent plantar bony prominences or 'rocker-bottom' deformities that cause ulcerations and could lead to potential infection.[50] Other, more complex procedures such as arthrodesis of the midfoot and hindfoot, use of external fixation, and intramedullary nail have also been used in the latent stage of the Charcot disease process.

FIGURE 26.9: Charcot Restraint Orthotic Walker (CROW) boot. A patient is able to ambulate with this device once the acute phase has passed.

PREVENTION

Diabetic foot care requires an interdisciplinary team approach given the progressive nature of the disease in the foot. It is unlikely that one individual medical or surgical specialty is able to manage all aspects of diabetic lower extremity disease and appropriately manage these patients. Recent evidence suggests a reduction in major amputation rates following the development of an interdisciplinary approach to limb salvage.[51] The components of a limb salvage team are predicated on the pathology at presentation. The core of the team typically starts with clinicians caring for the structural aspects of the foot (Podiatrists), along with clinicians caring for the vascular integrity of the lower extremity (Vascular Surgeons). Other specialties of the team, to add a more comprehensive care model may include Endocrinology, Infectious Diseases, Orthopaedic surgery, Physiotherapy, Plastic Surgery, Nursing and Orthotics/ Prosthetics.

Vasculopathy and neuropathy are two major contributors to diabetic foot disease and subsequent ulceration. Therefore, appropriate utilization of an interdisciplinary team approach, as stated above, will help to address the varying factors associated with lower extremity ulceration and reduce amputation. A diabetic rapid response acute foot team has been proposed in an effort to combine the knowledge of certain specialties to promote limb salvage.[52] This is an interdisciplinary team model whose core involves the ability to rapidly diagnose and provide treatment to patients with lower-extremity complications of diabetes, utilizing seven basic skill sets (Table 26.6). At the forefront of the team are members from podiatry (Toe) and members from vascular surgery (Flow). These specialties, with adjunctive teams added as necessary,

combine to collectively posses the ability to perform the 7 essential skills, stated above, to be effective in promoting limb salvage. This model has been effective at the University of Arizona's Southern Arizona Limb Salvage Alliance (SALSA) in which the Vascular Surgery/Podiatry team approach has been able to significantly reduce the number of major diabetes-associated lower-extremity amputations as well as provide a framework for prevention of diabetic ulcers. In fact, abundant data suggest that establishing such comprehensive diabetic foot care teams can significantly reduce the incidence of major amputation in both community and academic hospital settings.[53]

TABLE 26.5: Infectious Diseases Society of America (IDSA) Diabetic Foot Infection Classification

Clinical Presentation	IDSA Infection Severity	Threatened Limb Class
Wound without inflammation or purulence	Uninfected	Not applicable
Presence of >2 manifestations of inflammation, cellulitis <2cm surrounding ulcer, no systemic symptoms of infection	Mild Infection	Non-limb threatening
Presence of infection with >2 cm of surrounding cellulitis; any infection with presence of gangrene, abscess, deep tissue involvement or gas in tissue; no systemic signs/ symptoms of infection	Moderate Infection	Limb threatening
Presence of infection as above with the presence of systemic signs or symptoms	Severe Infection	Life and limb threatening

TABLE 26.6: Seven Essential Skills of a Diabetic Rapid Response Acute Foot Care Team (DRRAFT).

1. Ability to perform hemodynamic and anatomic vascular assessment with revascularization
2. Ability to perform a neurologic assessment
3. Ability to perform site-appropriate culture technique
4. Ability to perform wound assessment and staging/grading of infection and ischemia
5. Ability to perform site-specific bedside and intraoperative incision and debridement
6. Ability to initiate and modify culture-specific and patient-appropriate antibiotic therapy
7. Ability to perform appropriate postoperative monitoring to reduce risks of reulceration and infection

CONCLUSION

Treating the diabetic foot, but moreover the patient with diabetes, is an extremely challenging yet rewarding experience. As the incidence continues to grow worldwide the likelihood of people with diabetes constituting a significant proportion of a vascular and podiatric surgical practice is quite high. Common, seemingly trivial foot problems such as calluses, corns, ingrown nails, and dry scaly skin, may provide sufficient trauma to develop into limb-threatening problems. Early recognition and diagnosis of these factors combined with aggressive preventive measures and a multidisciplinary team approach will all be beneficial in the treatment of the diabetic foot with the ultimate goal of amputation reduction and prevention.

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27 • Lymphoedema – Principles, Genetics and Pathophysiology

MATT WALTHAM

Academic Department of Surgery, St Thomas' Hospital, Westminster Bridge Road, London

INTRODUCTION

The lymphatic circulation consists of a network of blind-ended capillaries lined with endothelial cells that drain into larger vascular trunks and eventually empty into the blood circulation. It is otherwise totally separate from the blood circulation although lymphatics are often anatomically related to arteries and veins. Lymphatic vessels are found in nearly all tissues and have several important functions including transportation of fluids, plasma macromolecules, and cells back to the blood circulation. The lymphatics also form a major transport route for lipids absorbed from the intestinal tract, and are a critical component of the immune system transporting leucocytes and antigens from the tissues to the lymphoid organs.

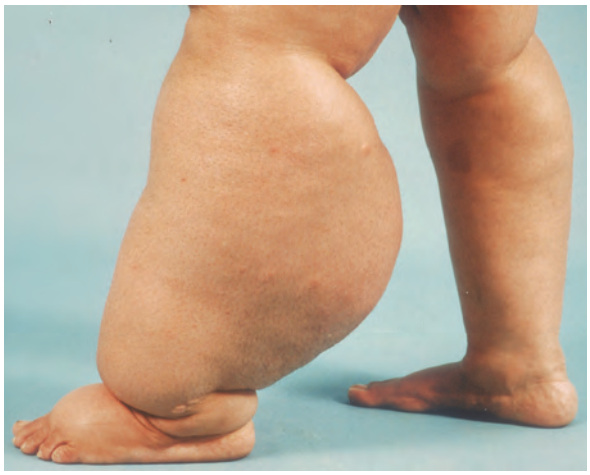
Lymphoedema is an accumulation of tissue fluid in the interstitial space as a result of failure of the lymphatic circulation. This can be severe and disfiguring. Defects in the lymphatic system can be primary or acquired. Lymphoedema most frequently affects the legs (80%) although can present as swelling of the arms, face or external genitalia. (Figure 27.1)

CLASSIFICATION OF LYMPHOEDEMA

The diagnosis of lymphoedema should be reserved for those patients in whom a secondary cause of oedematous swelling has been excluded. (Table 27.1). Chronic venous disease is a common cause of unilateral swelling and there are often other characteristic skin changes. Sub-clinical lymphoedema sometimes becomes apparent when other conditions such as venous hypertension cause an increase in fluid and protein forced into the interstitial space that overwhelms a poorly functioning lymphatic system.

Lymphoedema is classified as primary when there is an intrinsic defect in the lymphatic vessels or nodes that leads to failure to drain lymph from the tissues. It has an incidence of 1:6000 and is three times more common in women than men.[1]

Secondary lymphoedema is more common and occurs when the lymphatics are damaged by a defined external cause. The commonest cause worldwide with approximately 120 million cases is filarial infection (*Wuchereria bancrofti, Brugia malayi*



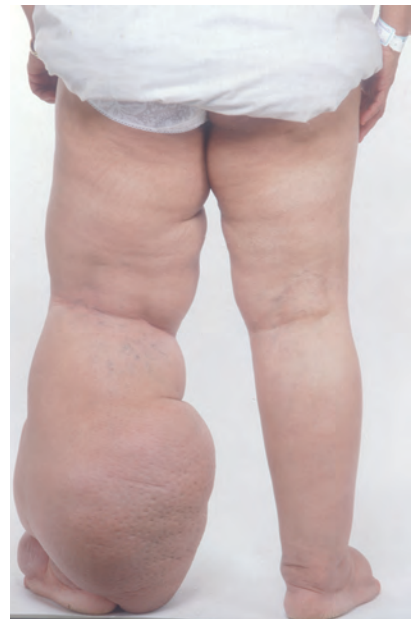


FIGURE 27.1 (a,b): Lower limb lymphoedema

or Brugia timori) leading to inflammation and fibrosis of lymph nodes or the adjacent lymphatics.[2] This often presents as hydrocele (in men), massive lymphoedema and elephantiasis. It is common in tropical and sub-tropical regions of Africa, the Far East and South America. Another common cause in the tropics is podoconiosis (endemic non-filarial elephantiasis), a geochemical disease that occurs in individuals exposed to red clay soil derived from alkalic volcanic rock.[3] Ultra fine silica particles are absorbed through the skin of barefoot agricultural workers and cause a progressive obliterative lymphangitis.

In Europe and North America secondary lymphoedema is usually related to trauma, surgery, and radiotherapy, often associated with the treatment of malignancy.

Classification of primary lymphoedema

The original classification of primary lymphoedema according to age of onset into congenital (present at birth), praecox (appearing before 35 years of age) and tarda is of little use in differentiating the underlying disease processes. In the 1950s Kinmonth proposed both the clinical distinction of primary and secondary lymphoedema and a classification system based on lymphangiographic appearance.[4] Browse later combined these into a system that reflected clinical and aetiological factors known at the time.[5]

1) Primary lymphoedema: Lymphoedema caused by a primary abnormality or disease of the lymph conducting elements of the lymph vessels or lymph nodes. Those in which the functional abnormality and its cause are known are divided into three groups:
   a. large vessel abnormalities such as congenital aplasia of the thoracic duct or cysterna chyli
   b. congenital lymphatic valvular incompetence or congenital aplasia
   c. lymph node fibrosis.

TABLE 27.1:

Secondary causes of swelling that must be excluded before making a diagnosis of lymphoedema
Cardiac failure
Renal failure
Hepatic failure
Hypoproteinaemia
Allergic disorders
Vasculitis
Hereditary angio-oedema
Idiopathic cyclic oedema
Venous insufficiency (Obstruction or reflux)
Vascular malformations
Lipoedema / lipodystrophy
Functional (disuse)
Factitious
Gigantism (overgrowth syndromes)
Investigations to exclude other causes of swelling
ECG
Echocardiography
FBC
U+E / Creatinine
LFT including albumin
CRP / ESR
Autoimmune screen
Complement tests
Venous duplex
Contrast venography
MRI for soft tissue swelling / vascular malformation
CT abdomen and pelvis
Lymphoscintigraphy
Lymphography

The remainder are characterised by a reduced number of lymphatics on lymphography

2) Secondary lymphoedema: Oedema caused by disease in the nodes or vessels that began elsewhere (e.g., neoplasia or filariasis), or lymphocytic proliferative disorders such as Hodgkin's disease or following surgical extirpation of lymph nodes or vessels.

More recently genetic abnormalities have been discovered in both congenital (present at birth) and delayed onset forms of lymphoedema and this has lead to a modified view of this classification. There is also a distinction between 'lymphangio-obstructive' and 'lymphangio-obliterative' to indicate underlying pathology.

1) Genetically determined abnormalities
   a. Aplasia, malformation and valvular incompetence of the central lymphatic ducts, namely the cisterna chyli and thoracic duct
   b. Aplasia, hypoplasia or dilatation and valvular incompetence of the collecting ducts in the subcutaneous tissues of the limb and trunk. This group therefore includes the familial conditions such as Milroy's, Meige's and lymphoedema-distichiasis syndromes. This group also includes sporadic congenital lymphoedema associated with some recognised congenital abnormalities. (Table 27.2).
2) Acquired abnormalities
   a. Lymphangio-obliterative lymphoedema
      i. Distal
      ii. Proximal
      iii. Combined
   b. Intra-glandular (hilar) fibrosis; representing the lymphangio-obliterative process in the lymph conducting parts of the lymph gland

3) Kinmonth's numerical hyperplasia; the lymphangiographical abnormality is of increased numbers of normally sized lymphatic channels associated with excessive numbers of small lymph glands.

THE GENETICS OF LYMPHANGIOGENESIS IN PRIMARY LYMPHOEDEMA

Milroy's disease

Milroy first described a syndrome of inherited, painless, non-progressive swelling of the legs present at birth in 1892.[6] The family genealogy of the affected clergyman was followed across six generations and 22 out of 97 descendants were thought to have limb swelling indicative of lymphoedema.

Milroy's disease is an autosomal dominantly inherited condition. Linkage studies have mapped the condition to a locus on chromosome 5q35.3. More than 30 mutations in vascular endothelial growth factor receptor-3 (VEGFR-3), which maps to this region, have now been identified.[7–13] De novo mutations in the VEGFR-3 have also now been reported in patients with sporadic congenital lymphoedema.[14,15]

VEGFR-3 is the receptor for VEGF-C and VEGF-D. VEGF-C acts through VEGFR-2 and VEGFR-3 during formation of the vascular system, with expression of VEGFR-3 becoming restricted to the lymphatic endothelium.[16,17,18] The ability to specifically target lymphatic endothelium has allowed the visualisation of channels in mouse and human lymphatics with markers such as lymphatic vessel endothelial hyaluronan receptor (LYVE-1)[19] (Figure 27.2).

TABLE 27.2:

Disorders and syndromes involving primary lymphoedema
Milroy disease
Lymphoedema-distichiasis syndrome
Hypotrichosis-lymphoedema-telangiectasia syndrome
Meige disease (Primary non-syndromic lymphoedema)
Lymphoedema and yellow nails
Lymphoedema with ptosis
Hennekam syndrome (Lymphoedema-lymphangiectasia-mental retardation)
Aagenaes syndrome (Hereditary intrahepatic cholestasis with lymphoedema)
Microcephaly-lymphoedema-chorioretinal dysplasia (MLCD)
Noonan syndrome
Turner syndrome (45, X karyotype)
Prader-Willi syndrome
Klippel-Trenaunay syndrome
Maffucci syndrome
Proteus syndrome

Transfection of adenoviral VEGF-C into the skin of mice causes massive dermal lymphangiogenesis,[20,21,22] and transgenic expression of VEGF-C in mice increases lymphatic endothelial cell proliferation and causes lymphatic channel hyperplasia.[23] In contrast, targeted deletion of VEGFR-3 in mice causes defective vasculogenesis and embryonic death.[24] Transgenic mice expressing a soluble form of VEGFR-3 that is a potent inhibitor of VEGF-C and –D signalling survive into adulthood if a keratinocyte promoter is used to deliver the genetic mutation selectively to the dermis.[25] These animals have a normal blood vasculature but develop a lymphoedema phenotype with swollen limbs. These studies show that VEGFR-3 has an essential role in lymphangiogenesis. Further study of patients with Milroy's disease show that the lymphatics in the upper limb are completely normal, and in the lower limb they are present in the skin but there is no functional uptake.[13]

Lymphoedema-distichiasis syndrome

Lymphoedema-distichiasis syndrome is an autosomal dominantly inherited condition caused by mutations in the FOXC2 (forkhead transcription factor) gene at 16q24 locus.[26] Distichiasis described an extra growth of eyelashes from the Meibomian glands, and 30% also have ptosis. Distichiasis often causes corneal irritation, recurrent conjunctivitis and photophobia (Figure 27.3). It can be treated in a number a ways, including lubrication, plucking, electrolysis and surgery. The condition is associated with other congenital abnormalities including congenital heart defects (tetralogy of Fallot), cleft lip and palate, varicose veins and spinal extradural cysts.[27]

In this condition distichiasis is present from birth and lymphoedema appears from puberty. It is often bilateral and is usually below the knee. Duplex ultrasound and lymphoscintigraphy reveal that patients have both lymph and venous reflux in lower

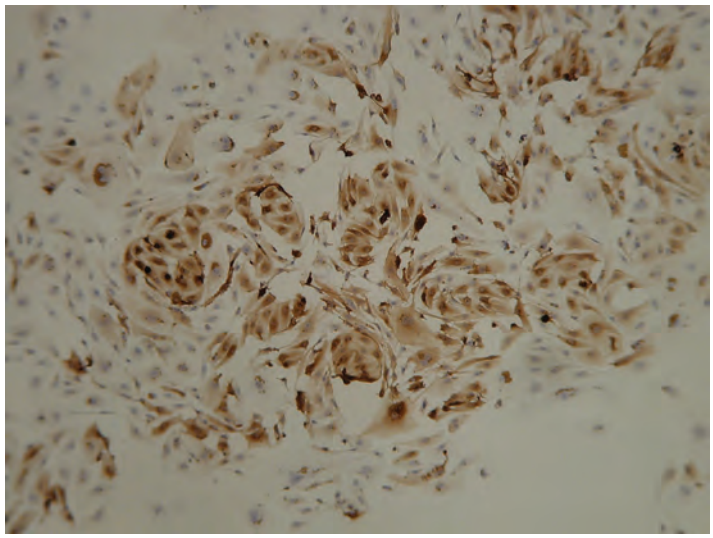


FIGURE 27.2: Human dermal tissue stained with marker for LYVE-1 expression on lymphatic endothelium

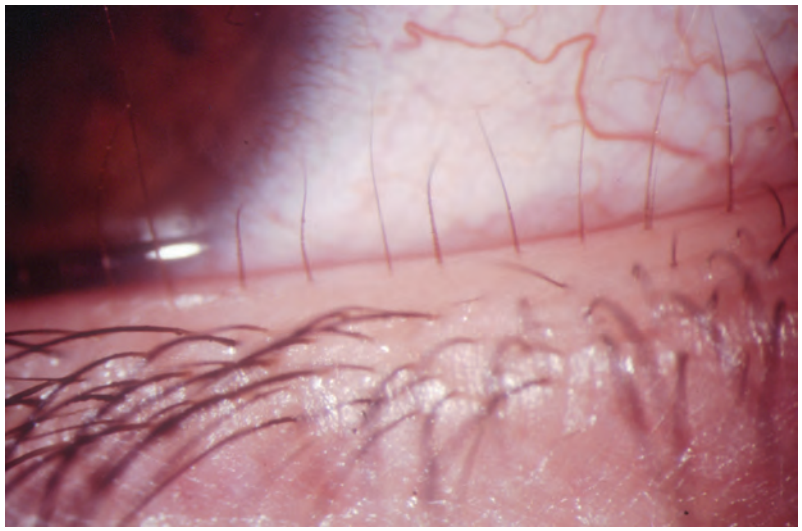


FIGURE 27.3: Distichiasis with accessory eyelashes along the posterior border of the lid margin in the position of the Meibomian glands

limbs, suggesting primary valve failure.[28] Skin biopsies in individuals with FOXC2 mutations demonstrate an abnormally large proportion of lymphatic vessels which are covered with smooth muscle cells, compared to family members without the mutation. Similar findings in FOXC2 knockout mice indicates that FOXC2 is both essential for valve morphogenesis as well as normal interactions between lymphatic endothelial cells and pericytes.[29] Venous reflux in lymphoedema-distichiasis syndrome could be a significant factor in the onset and progression of swelling.

Hypotrichosis-lymphoedema-telangiectasia syndrome

Hypotrichosis-lymphedema-telangiectasia syndrome is caused by mutations in the transcription factor gene SOX18.[30] This extremely rare syndrome is characterized by the association of childhood-onset lymphoedema in the legs, loss of hair, and telangiectasia, particularly in the palms. Inheritance is either autosomal dominant or autosomal recessive. Studies of the naturally occurring SOX18-mutant mouse strain suggest abnormal expression of a number of downstream gene targets required for structural integrity during microvascular maturation.[31] SOX18 directly activates transcription of the Prox1 gene which controls lymphatic vessel development from endothelial precursor cells.[32]

Meige disease (primary non-syndromic lymphoedema)

In 1898, Henri Meige described the most common variety of primary lymphoedema.[33] Meige disease is a familial lymphoedema developing at or soon after puberty in which no other congenital abnormality is identified. The lymphoedema is often symmetrical, rarely extends above the knee, and is clinically indistinguishable from that found in lymphoedema-distichiasis syndrome. It occurs three times more commonly in females than males and has a genetic predisposition in about a third of cases. Lymphography demonstrates peripheral

lymphatic hypoplasia with more proximal lymphatic channels remaining patent. The genetic abnormality in this syndrome has not been discovered but it has been shown not to involve the FOXC2 gene.[34]

Other primary lymphoedema disorders

Two other very rare forms of primary lymphoedema have been proposed to exist; lymphoedema associated with discoloured, slow growing and excessively curved nails (lymphoedema and yellow nail syndrome), and lymphoedema with ptosis. These may both represent poorly phenotyped cases rather than truly exist as separate entities, as yellow nails can be found in Milroy's disease, Meige disease and lymphoedema-distichiasis, and ptosis occurs in lymphoedema-distichiasis.[33]

Many other syndromes are known to have lymphoedema as a clinical feature (Table 27.2). Lymphoedema may affect the whole body or can affect arms, legs, face, conjunctiva, and the genitalia in a segmental pattern. In primary lymphoedema, facial, conjunctival or genital lymphoedema is often associated with limb involvement. Systemic disorders of the lymphatics include intestinal lymphangiectasia, chylous ascites, pleural effusions, pericardial effusions and pulmonary lymphangiectasia. The surgical treatment of these disorders is complex and may in involve ligation or excision of refluxing lymphatics, or drainage procedures.

STRUCTURE AND DEVELOPMENT OF THE LYMPHATIC CIRCULATION

The lymphatic system consists of a vascular network of thin-walled, blind ended capillaries made up of a single-cell layer of endothelial cells joined by discontinuous button-like junctions that open in response to increased interstitial fluid pressure.[35] Lymphatic capillaries have no basement membrane or supporting smooth muscle cells or pericytes, and so are highly permeable to protein-rich lymph fluid. They do, however, possess specialised anchoring filaments that link the endothelial cells to surrounding matrix and tissues; these help keep the capillaries open and increase their permeability as interstitial pressure rises.[36-38] The lymphatic capillaries converge into precollecting lymphatic vessels and these carry lymph to the main collecting trunks (e.g. the thoracic duct) for return to the venous circulation. Unlike lymphatic capillaries, precollecting and collecting trunks contain smooth muscle cells and pericytes. Collecting lymphatics also have internal valves to prevent retrograde flow of lymph fluid.

Early research into the origin of the lymphatics relied on either injection of substances (dyes or resins) into the circulation or serial sectioning to visualise early lymphatic vessel and sac development. This resulted in two opposing models: the first proposed by anatomists and embryologists using injection techniques that the lymphatic vessels bud off the primitive veins and grow out by lymphangiogenesis;[39] and the second that lymphatic vessels arise from the mesenchymal spaces with lymphatic sacs coalescing to form vessels.[40]

More recently molecular techniques have better characterised the origin of lymphatics in several models.[41] Studies using VEGFR-3 expression as a marker of lymphatic endothelial cells (LECs) in an avian model have suggested a dual origin from mesodermal lymphangioblasts and adjacent veins[42] and similar conclusions have been drawn in an amphibian model examining staged expression of the prospero-related homeobox gene, Prox1.[43] A number of other studies, both in mice and a zebrafish model, have concluded

that the majority of cells contributing to LEC arise from primitive veins. If there is a haematopoietic contribution to LEC this occurs relatively late and peripherally in their development, and may also contribute to postnatal physiological or pathological lymphangiogenesis.

The homeobox transcription factor, Prox1 is required for lymphatic cell differentiation. Prox1 is exclusively expressed in a subpopulation of endothelial cells in the anterior cardinal vein that emerge from the vein and form lymph sacs.[44] Prox1 knockout mouse embryos do not develop lymphatic vessels[44] with the budding embryonic venous endothelial cells defaulting to a blood vascular rather than lymphatic phenotype.[45] Lineage tracing studies have provided further evidence that LECs sprout, proliferate and migrate from venous-derived lymph sacs and haematopoietic cells do not contribute to the mammalian lymphatic system.[46] Temporal inactivation of Prox1 during embryonic and postnatal lymphangiogenesis causes loss of LEC phenotype and reversion to a blood vessel endothelial phenotype.[47]

Recent studies suggest that the transcription factor SOX18 controls Prox1 expression. Mutations in SOX18 were identified in patients with hypotrichosis-lymphoedema-telangiectasia syndrome.[30] SOX18 directly controls Prox1 expression by binding to its promoter; SOX18 knockout mice do not express Prox1 in cardinal vein endothelial cells and develop gross oedema.[32]

Further proliferation and migration of LECs from embryonic veins is controlled by VEGFR-3. Initially expressed in both blood and LECs, expression becomes restricted during embryogenesis. As discussed above, mutations in VEGFR-3 cause Milroy's disease in humans. VEGFC is the principal ligand of VEGFR-3; in VEGFC knockout mice LECS are correctly specified, as defined by the normal expression of LYVE-1, Prox1 and VEGFR-3, but fail to proliferate and migrate.[21] Neuropilin-2 is a non-signalling transmembrane receptor that acts as a co-receptor for VEGFR-3; Neuropilin-2 knockout mice have lymphatic hypoplasia with normal development of arterial and venous vasculature.[48] The transcription factor Tbx1 activates VEGFR-3 expression in endothelial cells and is the major gene for DiGeorge syndrome in humans. Tbx1 does not seem to be required for LEC differentiation but is needed for further growth and maintenance of lymphatic vessels; deletions in the gene cause widespread disruption of lymphangiogenesis.[49]

A number of other genes have been implicated in further lymphatic maturation and remodelling. Mutations in the forkhead transcription factor FOXC2 have been found in patients with lymphoedema-distichiasis syndrome, as discussed above. Lymphatic vessels are correctly differentiated in FOXC2 knockout mice[50] but there is abnormal recruitment of smooth muscle cells to lymphatic capillaries as well as agenesis of lymphatic valves.[29] Recently FOXC2 has been shown to play an important role in the formation of mature lymphatic collectors, including the formation of valves, recruitment of mural cells and smooth muscle, and pruning of branches.[51] In addition the transcription factor NFATc1 interacts with FOXC2 binding enhancers during valve formation.

Many other genes that have been implicated in abnormal lymphatic maturation including Angiopoietin-2,[52] EphrinB2, Aspp51, Emilin1, Slp76 and Syk.[41] For example Ang-2 knockout mice have subcutaneous oedema and chylous ascites; their lymphatics have a disorganised appearance, with poorly developed and disorganised circumferential smooth muscle coat. The relationship between these genes and human conditions has yet to be defined. Platelets may also play

a role in separating blood and lymphatic vessels during embryogenesis.[53] Understanding the mechanisms of lymphangiogenesis may lead to pro-lymphangiogenic treatments for lymphoedema.[54]

CLINICAL ASPECTS OF LYMPHOEDEMA

Most patients present with unilateral leg swelling. At an early stage the swelling will easily pit if pressure is applied, but chronic lymphoedema is associated with fibrosis and the subcutaneous tissues become thickened and hard. At a microscopic level the perilymphatic space becomes chronically thickened with a granulofilamentous material containing degenerate elastic fibres and collagen. In the presence of poorly functioning lymphatics, interstitial fluid becomes stagnant and can become infected; sometimes an infection may be an initial event and the subsequent swelling is blamed on this, but it is more likely to be a consequence of pre-existing abnormal lymphatic drainage. *Streptococcus pyogenes* is the most common pathogen. Patients may present with recurrent episodes of cellulitis, and each episode of infection predisposes to fibrosis and further lymphatic damage. Acute inflammation induces hyperaemia and increased hydrostatic pressure as well as increased vascular permeability and so increases accumulation of protein rich interstitial fluid. Endothelial derived nitrous oxide and oxygen free radicals are released; these are vasodilators and also inhibit the spontaneous tonic and phasic contractions of the lymphatic vessel wall smooth muscle, further reducing lymphatic flow.

Inguinal lymph nodes may be enlarged, especially if there is pelvic obstruction. Cutaneous lymphatic vesicles or a capillary naevus are signs of underlying megalymphatics with reflux. Release of cytokines causes thickening of dermal keratinocytes and an acanthotic appearance of the dermis. Inflammatory cells migrate from the papillary dermal layers into the epidermal cell layer. Microfilament deposition in the dermo-epidermal junction leads to a thickened epidermal basal lamina. This hyperplasia and hypertrophy of the dermal vascular endothelial and epidermal cells is responsible for the abnormal papillomatosis that develops in the skin of many patients with chronic lymphoedema.

Patients with megalymphatics may have chylous ascites, chyluria, chylometrorrhoea, chylothorax or other manifestations of lymphatic fistulae.

SUMMARY

Primary lymphoedema and other syndromes associated with lymphoedema cause significant morbidity. Molecular techniques have greatly improved our understanding of lymphatic specification, lymphangiogenesis, and lymphatic maturation. The relevance of genetic abnormalities in the development of different types of primary lymphoedema is now being elucidated. Increased understanding of these mechanisms will increase the number of candidate genes for genetic testing in both idiopathic inherited and sporadic forms of lymphoedema. Understanding of each of these processes will eventually lead to more effective treatments for disorders of the lymphatic system.

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28 • Graft Materials Past and Future

MITAL DESAI, GEORGE HAMILTON

Department of Vascular Surgery, Royal Free Hospital, University College, London, UK

THE PATHOPHYSIOLOGY OF GRAFT HEALING

The mechanisms of graft healing are of central importance in understanding the successes and failures of current bypass grafts. The tissue response to implantation of a prosthetic graft is complex with many variable factors involved such as the material used, its construction, its porosity, and its length. Further important factors relate to the interaction between the graft and the host artery at the anastomotic areas. Until recently graft design focused on simple conduits for blood flow which were strong (resistant to pressure), biologically inert (resistant to biodegradation) and non-leaking. Each of the major causes of graft failure, luminal thrombogenicity, compliance mismatch and anastomotic intimal hyperplasia, have the potential to be modulated if their aetiology could be better understood. A further stimulus to study of this area is the still unresolved puzzle of man's inability to endothelialise a prosthetic graft beyond the immediate 2 cm or so from the anastomosis.

The peri-anastomotic area

Intimal or neointimal hyperplasia is a characteristic healing reaction to vascular injury.[1] In prosthetic grafting the injury typically involves the direct trauma of implantation, and subsequent exposure of the anastomotic areas to haemodynamic stress (compliance mismatch, turbulent flow and altered shear stress). This results in injury which is transmural with endothelial removal, variable disruption of the internal elastic lamina and medial smooth muscle cells (SMC).

The three phases of intimal hyperplasia will develop quite rapidly with the first being proliferation of medial smooth muscle cells as soon as 24 hours after injury and lasting up to 4 weeks. The second phase of SMC migration into the intima starts as early as 4 days after injury and continues for about a month. The final phase is of intimal expansion by the dual action of SMC migration and intimal proliferation by deposition of matrix proteins such as collagen, elastin and proteoglycan. This phase is complex and is mostly self-limiting but may continue unabated if certain factors are present, (refer to Chapter 7 for a more detailed discussion of intimal hyperplasia).

The endothelial cell plays a pivotal role via its mechanoreceptors which will be sensitive to changes in flow and shear stress. High shear stress, as found in laminar flow, promotes endothelial cell survival and quiescence,

and secretion of nitric oxide (NO). Low or changing shear stress direction (turbulent flow), promotes endothelial proliferation and apoptosis, shape change, and reduced secretion of NO. If by a process of flow change towards high shear stress and endothelialisation by regrowth in the injured area, a balance between stimulatory and inhibitory factors is achieved, the drive towards intimal hyperplasia will cease. If this balance is not achieved because of ongoing factors such as lack of endothelial cover, major haemodynamic disturbance such as severe compliance mismatch or turbulent flow with areas of stagnation and low shear stress, then the drive towards intimal hyperplasia will continue unabated leading to severe narrowing at the anastomosis and graft failure. In prosthetic grafting therefore several factors will persist which have the potential to promote intimal hyperplasia.

Healing of prosthetic grafts

Healing of prosthetic grafts takes place by two main mechanisms, capillary in-growth through the graft wall, and growth of endothelial cells along the luminal surface of the graft from each anastomosis.[2] Studies of prosthetic graft healing in various animal models used short lengths of graft, typically 10cm or less, which readily developed a full lining of endothelial cells. In man however, endothelialization is restricted to the first centimetre or two of the anastomotic regions with no evidence of healing having taken place beyond this area. This observation based on a few individual explants has led to the conviction that man is different from other species in his inability to endothelialise a graft.

The healing process at the anastomosis

Endothelialisation along the graft from the host artery occurs more aggressively in animals compared to man. A review of all animal studies found that the average graft length was 10 cm with 89% being only 5.5 cm. In all of these studies therefore it is very likely that anastomotic ingrowth was the sole avenue for endothelialization.[4]

The speed of trans-anastomotic endothelialization differs between species. Many of the models used young animals with rapid endothelialization but in low porosity grafts, endothelialization stopped 2 cm from the anastomosis. To set this species difference in context, trans-anastomotic endothelialization is 7–8 times more pronounced in any animal compared to man.[4] Two factors are foremost among the possible explanations. The first is the exclusive clinical use of low or zero porosity grafts. The second is the clinical reality of grafting performed in the sick and elderly in whom vascular cells are known from tissue culture studies to be less vigorous.

Graft porosity and permeability

The terms porosity and permeability are used interchangeably but have separate meanings. Permeability is the property of material to allow passage of substances through its interstices and classically is measured by the volume of water traversing a given area and pressure. Porosity refers to the spaces or pores that exist within the graft material, which depending on the material, may not traverse its entire thickness but end blindly. Zilla suggests that to facilitate transmural healing and endothelialization, graft spaces should be wide enough to allow ingrowth of a capillary tuft with accompanying fibroblasts or pericytes requiring minimum pore diameters of 60–80μm.[4,9] Currently available grafts, even those described as having high porosity fail in this regard. (Table 28.1)

Macrophages are the predominant inflammatory cells found in large numbers after implantation and later as part of

TABLE 28.1: The effects of graft porosity

Low porosity ePTFE grafts: **(<45μm of internodal distance)**

- Low porosity ePTFE grafts (<30mm) no difference in healing between animal and human.
- Within two weeks surface is covered with fibrin and platelet thrombus 15μm thick which over following months increases to between 80–300μm.
- Pannus persists for years and is actively thrombogenic.
- Ingrowth of connective tissue is limited to the outer graft wall.

High porosity ePTFE grafts: **(>45μm internodal distance)**

First layering similar to that of low porosity ePTFE grafts.

- In older animals very little ingrowth – luminal thrombus without any cellular component.
- Early and spontaneous endothelialization is found in young animals.
- These changes happen as early as 1–2 weeks.
  - patches of endothelial cells and capillary orifices found approximately 100-500μm apart which proceed to confluence.[3]
  - These endothelial cells lie over a layer of arterial smooth muscle cells probably derived from pericytes.
  - Stable neo-intima evenly distributed along the surface, as compared to the limited peri-anastomotic coverage in low porosity grafts.
  - This extensive endothelialization arises from cells reaching the luminal surface by transmural ingrowth.
  - In older primate models and also in the dog these developments take longer but only with sprouting capillaries reaching the outer third to one half of the graft wall.[4]

Low porosity Dacron grafts (woven)

- Immediately after implantation thin pannus of fibrin and platelets deposited on the surface.
- Thrombus compacts over time and in man stabilises after one year.[5]
- Endothelialization does not happen either in animals or in humans.
  - small islands of endothelial cells found after many years in explants in man.[6,7]
- Narrow graft interstices filled with fibrin.
- Foreign body giant cell reaction present.
- Variable spread of some capillaries and fibroblasts into interstitial spaces never breaks through the compacted fibrin of the inner lining.[8]

High porosity Dacron grafts (knitted)

- Initial pannus same as woven Dacron but develops to a thickness of 100–120μm increasing to 500μm by six months.
- In dogs and other animals this inner lining replaced with a confluent layer of smooth muscle cells resting directly on the graft surface, covered by endothelium.
- These come from anastomotic ingrowth but in longer grafts endothelialization in the midgraft region fails to occur despite partial ingrowth of capillary fibroblasts from the adventitia.

Prosthetic grafts made of PTFE and Dacron can show a degree of healing by endothelialization related to the porosity of the graft. High porosity PTFE grafts promised the best endothelialization but were not marketed because of concerns regarding long-term strength and the practical difficulties of haemorrhage and serum leakage through the graft wall at implantation.

a chronic inflammatory process. Soon after implantation, the interstices become filled with fibrin and matrix similar to any early wound. Macrophages form part of a normal inflammatory response releasing cytokines to stimulate migration and proliferation of fibroblasts and endothelial cells. In the later stages however, macrophages persisting in large number may have an adverse effect on healing and ingrowth. Consistently the outer portion of the graft has high concentrations of macrophages and foreign body giant cells while the deeper layers lose these cells, probably due to the dense impenetrable nature of the fibrinous pannus. Dacron seems to be more inflammatory than polytetrafluoroethylene (PTFE) where less giant cells develop.

PHYSICAL PROPERTIES OF PROSTHETIC MATERIALS

Arterial wall pulsatility is due to a combination of elastic and viscous components inherent in the structure of the artery which can therefore be described as being viscoelastic. Most commonly this property is measured as compliance, defined as the ratio of change in diameter over change in blood pressure (percentage/mmHg $\times 10^{-2}$).

Arterial compliance is complex, having both longitudinal and circumferential components but only this latter measurement is commonly quoted when the elasticity of different materials is compared. Compliance mismatch has been implicated as an important factor in the performance of vascular grafts since 1976.[10] This mismatch should be considered to have two major components, tubular and anastomotic.

Tubular compliance

Mismatch of tubular compliance is present when there is a significant difference in elasticity between the prosthetic graft and native artery. A compliant vessel acts as an elastic reservoir absorbing energy during systole which is released during diastole giving an extra push to pulsatile blood flow. A rigid conduit will consequently diminish this secondary pulsatile energy and reduce distal perfusion. At the interface between a compliant artery and a non-compliant graft, changes in impedance (defined as the resistance to pulsatile flow) will diminish pulsatile energy by as much as 60%.[11] Furthermore, optimal organ perfusion depends on pulsatile blood flow with a change from pulsatile to static perfusion shown to increase peripheral resistance by 10%.[12] Finally, at the graft to artery interface there is wave reflection of pulsatile energy which can lead to increased velocity gradients and turbulence. As a result of these increased vibratory movements and mechanical stresses, endothelial damage and intimal hyperplasia occurs.

Anastomotic compliance mismatch

A sutured anastomosis generates a decrease in diameter and drop in compliance determined primarily by the lack of elasticity of the suture material. Interrupted sutures give a more compliant anastomosis, while a continuous technique results in a ring of non-compliant suture material – both prolene and PTFE sutures are profoundly non-elastic. Within a few millimetres on either side of the suture line, there is a paradoxical increase of compliance which is known as the para-anastomotic hyper-compliant zone (PHZ)[13] (Figure 28.1). Intimal hyperplasia develops typically in these areas of hyper-compliance.

The compliance hypothesis of graft failure

Compliance mismatch will lead to a region of excessive mechanical stress which can give

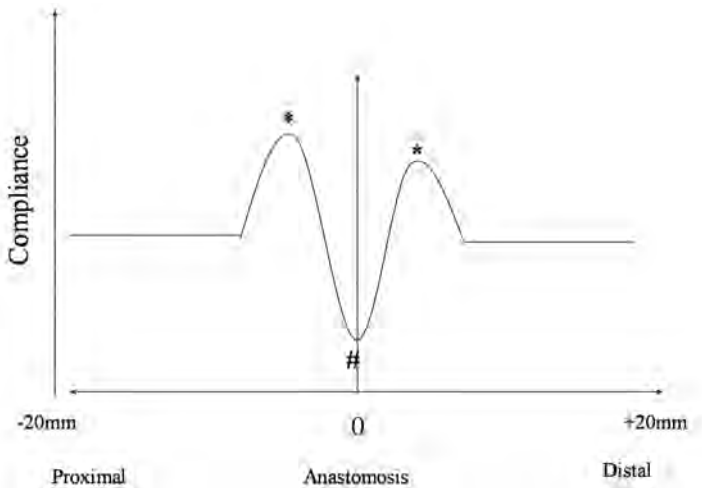

FIGURE 28.1: The peri-anastomotic hypercompliant zones (PHZ); compliance at the anastomosis is lower due to the suture (**#**) while compliance is increased compared to the vessel wall several mms from the anastomosis (*). This effect further aggravates compliance mismatch in bypass grafting.

rise to subtle arterial wall injuries and initiate the first phase of intimal hyperplasia. Cyclical stretching is known to have a positive influence on proliferation of vascular smooth muscle cells and production of extracellular matrix. This increased cyclical stretch at the zones of PHZ, will cause proliferation of the smooth muscle cells. Finally changes in compliance are known to affect flow and shear stress. Where there is turbulent flow, there will be areas of low shear stress and this is known to promote endothelial proliferation, apoptosis and reduce production of nitric oxide.

The clinical evidence for the compliance hypothesis is largely speculative but analysis of the clinical performance of grafts of differing compliance reveals a positive correlation between compliance and patency rates (Figure 28.2). The most commonly used prosthetic grafts, namely PTFE and Dacron are profoundly rigid over the physiological pressure range. A feature of the visco-elastic nature of human artery is compliance which diminishes with increasing pressure but which increases exponentially as the mean pressure falls below 80mmHg (Figure 28.3). The ideal prosthetic graft should share this property.

SYNTHETIC GRAFTS

The history of prosthetic grafts began in 1952 with successful placement of Vinyon –N tubes into the abdominal aorta of dogs, and subsequent human implantation in 1954 in 18 patients.[14] An explosion of interest followed with synthetic grafts being made from various textiles but their major problem was loss of tensile strength. Two materials proved to be resistant namely Dacron and PTFE, and because of their bio-durability have dominated graft development to this day.

Newer developments of dacron grafts

Heparin coating has been utilised for improving biocompatibility of Dacron. Besides enhancing the function of heparin-binding proteins, immobilised heparin also potentially reduces Dacron hydrophobicity.

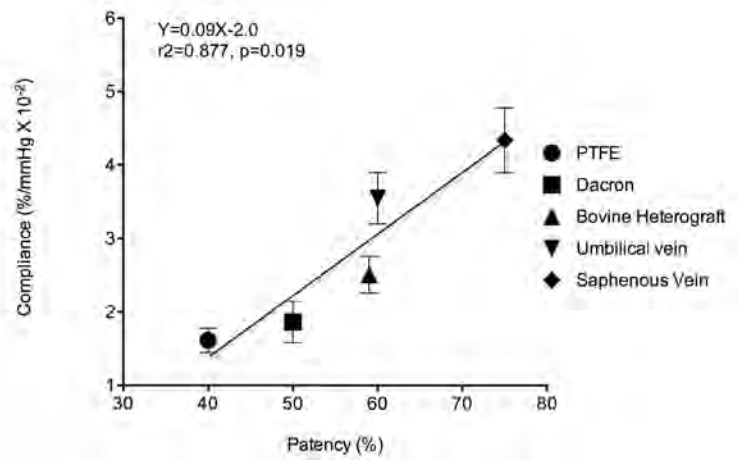

FIGURE 28.2: Correlation between typical compliance and 2 year patency of several graft materials in clinical use.

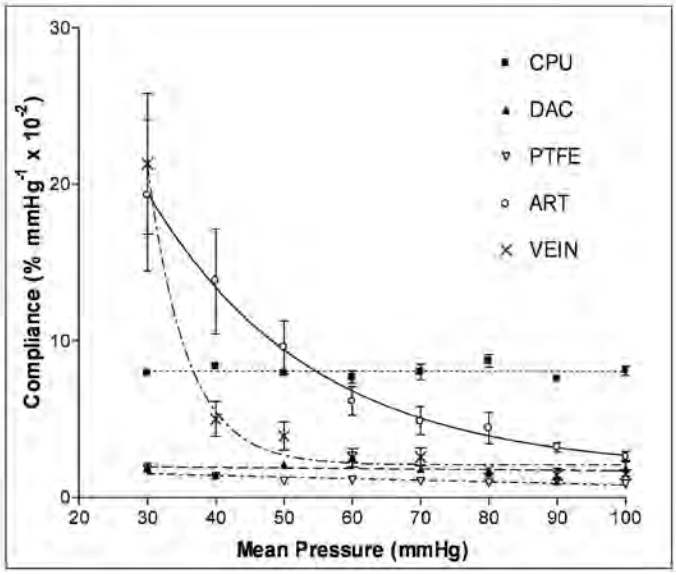

FIGURE 28.3: Compliance / Pressure curve for compliant polyurethane (CPU), Dacron (DAC), ePTFE (PTFE), human femoral artery (ART) and saphenous vein (VEIN). None of the prosthetic materials possess the visco-elastic properties of artery and vein which give higher compliance at lower pressures. CPU maintains higher compliance at all pressures compared to Dacron or ePTFE.

This change in surface chemistry might alter the proteins present at the interface, thereby influencing biocompatibility independent of the biological action of heparin. It has been shown that this is associated with exposure of the fibrinogen P2 epitope as well as the adhesion of monocytes.[18] Independent of the inflammatory response, the hydrophilic nature of the heparin coating may affect tissue interaction (reduction in cell adhesion,

growth and mobility). Overall, compared to human umbilical vein (HUV) or PTFE, heparin-bonded Dacron shows significantly better primary patency up to 2 years but not at 5 years of follow-up.[19,20]

Modifications and newer developments of PTFE grafts

The ePTFE graft has been modified in various ways. Thin wall ePTFE grafts have improved handling characteristics but still have an outer wrap to provide strength. Stretch ePTFE grafts have improved longitudinal rather than circumferential elasticity with improved handling characteristics but no other benefit has been demonstrated in clinical studies. External support, either rings or spirals, is thought to be beneficial in extra-anatomic (axillo-femoral or femoro-femoral) or below knee grafts.

A further valuable adjunct shown in prospective studies to improve below knee PTFE graft patency is an interposition vein cuff or patch at the distal anastomosis.[24] This appears to improve the haemodynamic situation at the distal anastomosis perhaps acting through minimising compliance mismatch and improving blood flow.[25]

Several reports indicate potential benefit with ePTFE aortic grafts including reduced bleeding and a lower risk of infection. The only prospective randomised comparison of ePTFE and Dacron aortic grafts, however, failed to show any difference.[26] The supremacy of ePTFE in lower limb bypass grafting has been challenged in a randomised trial which showed no difference between ePTFE and gelatin sealed Dacron.[27,28]

Heparin bonded PTFE is being widely utilised in contemporary practice. Two year primary patency and limb salvage rates were similar to autologous saphenous vein in lower limb bypass including below-knee locations.[29] While there are case series data implying that this is an effective material, results from randomised trials are awaited.[20]

Other ePTFE coating materials evaluated include citric-acid based biodegradable elastomers. In porcine carotid artery circulation, they were found to be biocompatible without causing increased risk of thrombosis, restenosis or inflammation.[31] These findings are important as this may serve as the foundation for a drug eluting vascular graft.

Polyurethane grafts

Polyurethanes are segmented polymers initially formulated in the early 60's to provide elasticity in garment materials (Lycra). These are a very large family of which the most important component is the urethane group present in repeating sequences on the main chain of the polymer. This forms the hard segment providing strength with the soft segment being the other main component (macromonomers ranging from hundreds to over a thousand Daltons). These hard and soft components have a degree of incompatibility which allows microphase separation delivering superior visco-elastic and compliant properties. Polyurethanes also possess excellent blood and tissue compatibility and are in extensive use in access catheters and linings of various prosthetic devices. Clinical experience of conventional polyurethane grafts has confirmed their superior thrombo-resistance, rapid ingrowth of living tissue and reduced anastomotic hyperplasia.[32]

Polyurethane vascular access grafts for haemodialysis have several advantages including easy cannulation, rapid compression haemostasis and early use after implantation. Disadvantages with polyurethane grafts include poor patency rates when compared with PTFE and most problematically hydrolytic degradation leading to aneurysm formation. It is this complication that has

limited their clinical use despite the advantages of good compliance[33-39] (Table 28.1).

Newer developments of polyurethane vascular grafts

Conventional polyurethanes are biodegradable at the soft segment of the polymer particularly at the ester and ether groups in poly(ester)urethane and poly(ether)urethane. Recent interest has focused on replacing these susceptible groups with other moieties in particular polycarbonate, which are more hydrolytically and oxidatively stable. One polycarbonate polyurethane is currently available for clinical use, Corvita (Corvita Inc) and also a renal access graft composed of polyether polyurethane, the Vectra graft (Bard Inc.).

Development of a compliant small calibre vascular graft has been a major goal of our unit. The focus has been on a poly (carbonate) polyurethane with a honeycomb structure (Figure 28.4) composed of an inner and outer skin enclosing a spongy middle wall thus maintaining pulsatile flow even after peri-graft tissue incorporation. Because this polymer lacks ether and ester compounds it resists biodegradation as proven both in vitro, and in long term implantation study. Comparison of this graft with artery, vein, Dacron and PTFE shows compliance similar to artery at mean pressures of 30–60 mm Hg, with very significantly superior compliance compared to Dacron or PTFE at all pressures (Figure 28.3).[42,43] Soldani et al have developed a new compliant small diameter graft with a poly (ether) urethane–polydimethylsiloxane semi-interpenetrating polymeric network and featuring two different porous wall layers; this showed superior compliance and patency rates in comparison with standard ePTFE, with the ability of remodeling in vivo being gradually replaced by natural tissue with no sign of calcification.[44]

In addition, small-diameter poly (epsilon-caprolactone) grafts represent a promising alternative polyurethane with better healing characteristics compared with ePTFE giving

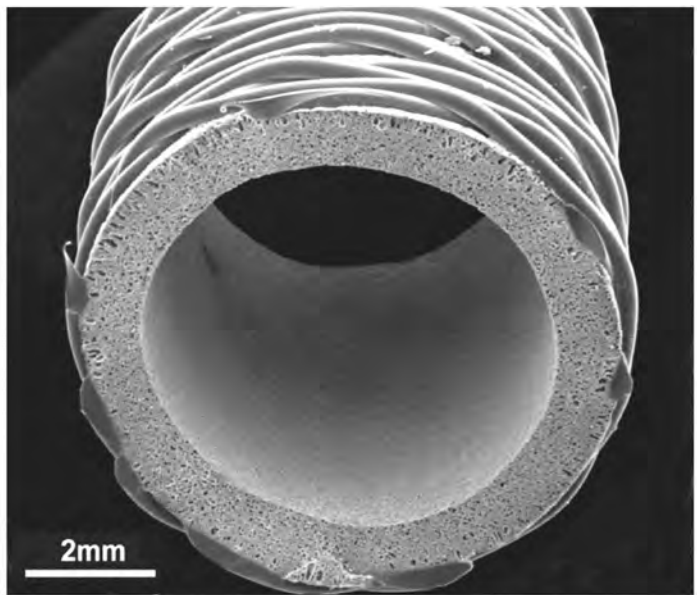

FIGURE 28.4: Compliant polyurethane graft with external support; The sponge-like structure of the wall allows pulsatile elastic recoil even with external support and after perigraft tissue incorporation has taken place.

faster endothelialisation and extracellular matrix formation, accompanied by resistance to structural deterioration during remodelling.[45,46]

Reinforced polyurethane grafts using polyester filament yarns knitted into tubular fabrics to form a composite vascular graft have been demonstrated to be 5–10 times stronger than pure polyurethane grafts.[47] A bioengineered microporous polycarbonate-siloxane polyurethane graft has been developed for coronary artery bypass grafting. Biological agents including heparin and sirolimus can be impregnated into its absorbable collagen and hyaluronan microstructure giving a unique drug-eluting graft with endothelialisation without excessive intimal hyperplasia.[48] Biodegradable polymer systems provide the opportunity for release of various growth factors to promote vascular wall regeneration. For example, fibroblast growth factor-2 (FGF-2) release from poly (ester urethane) urea scaffolds amalgamates the favourable mechanical properties of polyurethanes with the bioactivity of an angiogenic protein.[49]

Nitric oxide releasing polyurethanes reduce platelet adhesion and vascular smooth muscle cell growth, while stimulating endothelial cell growth.[50] Furthermore, the elastomeric copolymer, poly(1,8-octanediol citrate), with mechanical and degradation properties suitable for vascular tissue engineering, decreases platelet adhesion.[51] In vitro studies evaluating the biocompatibility of these materials confirm their potential for vascular graft coatings.[52]

Although tissue-engineered vascular grafts based on biodegradable polymers have yielded promising results, some drawbacks exist. Challenges of cell sourcing are compounded by long culture periods that range between 2 and 6 months, and the proliferative capacity of cells isolated from elderly patients is limited.

Biological vascular grafts

Biografts, vascular grafts made from biological sources, have been used over many years. Allografts (sourced from the same species) in current use are primarily umbilical and saphenous vein. Xenografts (derived from other species) have a long history with disappointing results and there is no xenograft currently in clinical use.

The major problems with biografts are biodegradation and immunogenicity which can be counteracted by chemical treatment and cryopreservation. The first clinical use of an allograft was in 1948 in the treatment of aortic coarctation.[53] Arterial allografts harvested from cadavers were first used in the 1960s to perform lower limb bypass but these were prone to significant degeneration, aneurysm formation and wall calcification.[54]

Improved cryo-preservation with protectant solutions to prevent intra-cellular ice crystals on thawing, allowed the development of tissue banks to provide a ready source of allografts. Clinical use of cryopreserved allografts in the 1960s showed good short term function and the attractive possibility that cryopreservation might reduce immunogenicity.[55] Further clinical experience however revealed disappointing one year patency rates of less than 50%.[56] The stable functioning of arterial and venous grafts in human liver transplantation suggests that immuno-suppressive therapy will improve the function of these grafts. However the associated complications probably make this approach unacceptable.

Xenografts were introduced in the 1970s, most commonly the bovine carotid artery. Various chemicals including glutaraldehyde were used to cross link collagen to provide stability and reduced immunogenicity. Clinical success rates of these xenografts were poor with biodegradation after 6 months due to progressive breakdown of the collagen cross linkages.

The human umbilical cord vein was developed as a bypass graft by Drs Irving and Dardik.[57] This was stabilised using glutaraldehyde supported by an external Dacron mesh and used specifically in lower limb bypass grafting but were prone to aneurysmal degeneration. Deficiencies in the manufacturing process were corrected in the late eighties with apparent significant reduction of this problem. The graft, however, never regained popularity despite impressive results in a large series of 1,275 cases (five year secondary patency rates of 71% for femoro-popliteal and 56% for femoro-crural bypass).[58]

Newer developments of biological vascular grafts

Bacterial cellulose is a novel vascular material with the potential to reduce surface thrombogenicity. In vitro it had the slowest activation of coagulation cascade as compared to standard synthetic graft materials.[59] Bacterial cellulose has the added advantage of promoting in situ vascular tissue regeneration,[60] so it has potential as a scaffold for small bore vascular grafts.

A fibrin scaffold supported by a poly lactide mesh, and seeded with autologous arterial-derived cells prior to dynamic conditioning has been used to develop conditioned grafts with good mid-term patency and no evidence of thrombosis, aneurysm formation or calcification in vivo.[61] They also show a confluent monolayer of endothelial cells lining the inner surface of the graft. The integrated biodegradable polylactide mesh has also been used to provide temporary mechanical support during the initial period of tissue development, while an autologous fibrin cell carrier system acts as the basis of remodeling the entire graft into a viable tissue structure.[62]

The in-vivo evaluation of cryopreserved human umbilical arteries treated with poly (styrene sulfonate)/ poly (allylamine hydrochloride) has demonstrated a high graft patency after 3 months of implantation.[63] An allogenic vascular graft has also been developed from a decellularised scaffold prepared from canine carotid arteries and modified through heparin immobilisation and vascular endothelial growth factor (VEGF) coating.[64]

L'Heureux et al. have demonstrated the feasibility of assembling arterial bypass grafts exclusively from autologous cells in primate models.[65] No synthetic or exogenous materials were used; instead, the vessels were created with the use of autologous fibroblasts and endothelial cells harvested from a small biopsy specimen of skin and superficial vein. In vivo results indicated that the grafts were antithrombogenic and mechanically stable for 8 months, with histology and microscopy displaying complete tissue integration, regeneration of a vascular media, as well as elastogenesis and a collagen fibre network.

PROSTHETIC GRAFT MODIFICATIONS

Modifications to reduce graft infection

Graft infection is a devastating complication particularly in the modern era of increasing methicillin-resistant *Staphlococcus aureus* (MRSA) infection. Several different strategies have been employed to reduce the risk of infection. The simplest approach is soaking grafts coated with albumin, collagen or gelatin with antibiotics, in particular rifampicin.[66] Gelatin sealed grafts prebonded with two antibiotics have shown resistance to infection by *Staphylococcus aureus* in a dog model.[67] In vitro studies show that antibacterial levels of rifampicin will remain present for 48 to 72 hours with reduced risk of graft infection to bacterial challenge.[68]

The clinical experience of rifampicin bonded Dacron grafts relates to two randomised controlled trials the first from Italy in

aorto-femoral grafts and the second from the United Kingdom in extra-anatomical bypass grafts. There was no long term benefit in terms of reduced graft infection rate found although early wound infection rates were found to be significantly reduced.[69,70] However, these grafts should be used with caution because it has been noted that in approximately 30% of cases, microbial organisms isolated from infected grafts are resistant to rifampicin.[71]

A further approach to reducing infection is the binding of Triclosan (Irgason) to grafts. This is an antimicrobial with broad spectrum activity which in experimental studies appears to bind effectively to dacron grafts for four weeks.[72] Silver bonded PTFE grafts have been shown experimentally to reduce the risk of infection, and are currently available for clinical use[73] (Interguard Silver Graft. InterVascular, France). However, in vivo comparison in a dog model between rifampicin /gelatin sealed and silver/collagen coated Dacron grafts, revealed significantly greater resistance to infection for rifampicin bonding.[74] Silver-coated grafts did not differ from standard grafts and had no effect on reducing graft infection in a recent retrospective study.[75]

The other experimental strategies proposed include direct pre-treatment with soaking prosthetic grafts in antibiotic solution (Daptomycin) which has been dissolved in a fibrin sealant.[76] At present, all graft modifications intended to reduce the risk of infection in arterial reconstruction, although promising, lack evidence of effectiveness.[77]

Modifications to improve patency

Carbon has been used because of its lack of reactivity and potential reduction of luminal thrombogenicity with flowing blood. Experimental studies have suggested improved primary and secondary patency rates.[78] Prospective randomised comparison of carbon impregnated PTFE grafts with standard PTFE found no significant difference at 2 years but with a trend for improved patency in the carbon graft.[79]

As shown earlier heparin bonded Dacron shows only short-term advantage in improving primary patency. A commercially available graft is the Fluoropassiv (Terumo-Vascutek), a Dacron graft coated with a fluoropolymer which has been shown in experimental studies to cause less tissue reaction and to have reduced thrombogenicity.[80] There are no clinical data available to confirm any beneficial effect of this graft.

Nanocomposite Grafts

Recent developments in the field of nanotechnology have facilitated vascular tissue-engineering mimicking the nanostructure of native vessels. One such application is electrospinning of synthetic polymers into nanofibers.[81-84] The advantages of forming scaffolds with high porosity as well as high surface area-to-volume ratio, thus simulating the dimensions and structure of native collagen and elastin fibrils holds great promise for future off-the-shelf-grafts.[85,86]

Our group has developed a family of nanocomposite polymers-based on polyhedral oligomeric silsesquioxanes (POSS) and poly (carbonate-urea) urethane (PCU). POSS-PCU has been used to develop a small diameter bypass graft which shows matching viscoelastic properties to human arteries.[87] Furthermore, a biofunctionalised small diameter graft based on this nanocomposite polymer demonstrates the potential for relatively rapid endothelialisation from progenitor cells extracted from peripheral blood in an in vitro model.[87] An extrusion-phase-inversion technique is used to make uniform walled porous conduits from POSS-PCU. These elastic microporous grafts demonstrate

favourable mechanical integrity and are currently undergoing in-vivo evaluation of durability and healing properties.[88]

Other groups have utilised the strength and flexibility of carbon nanotubes as fillers to enhance base polymer properties but although these composite polymers decrease thrombogenicity, toxicity of carbon nanotubes remains a concern.[89,90]

ENDOTHELIAL CELL SEEDING

Achieving endothelial cell coverage is important in improving graft performance. Endothelial cells have been extracted from three main sources – vein, subcutaneous fat and omentum.[91] Further potentially promising sources are from bone marrow, circulating blood and mesenchymal stem cells. There is good experimental evidence to support the benefit of endothelial cell seeding of bypass grafts. These have better patency rates, are less thrombogenic, will tolerate low flow states and have been shown to have normal endothelial cell activity.[92] In addition seeded grafts have been shown to resist bacteraemic infection in animal models.[93-95]

Single stage seeding

Single stage seeding requires sourcing of larger numbers of endothelial cells to allow immediate seeding of the graft at implantation. With this method seeding is not expected to be fully confluent but rather is achieved over the early post-implantation period by endothelial cell replication. Herring and his colleagues in 1978 were the first to report the seeding of Dacron grafts in a dog model and showed that PTFE seeded more rapidly and completely than Dacron.[9,96] This group confirmed in an explant from a patient that endothelium was present in the mid-portion of the graft some months after implantation.[97,98] A further clinical study demonstrated reduced thrombogenicity in the endothelialised limb compared to the non-seeded contralateral limb of aorto-bi-femoral grafts.[99] The major disadvantage of one stage seeding is a lack of sources of sufficient cells to allow immediate seeding.

Two stage seeding

Two stage seeding involves harvesting a modest quantity of endothelial cells typically from a vein, and culturing sufficient numbers for confluent seeding. A group in Vienna have performed the largest and the most detailed study in man using two stage seeding with endothelial cells harvested from cephalic or jugular veins.[100] The ePTFE grafts were pre-coated with fibrin glue and then seeded with the patient's own cultured endothelial cells. This group's experience is of 213 patients with patency for below knee reconstructions of 68% at 5 and 7 years, and 65% at nine years.[101] Endothelial cell seeding has been successful in coronary artery bypass with a recent trial using two stage seeding of ePTFE reporting 90.5% patency rate at 28 months.[102] These early clinical results are very promising but two stage cell seeding is cumbersome, and not easily applicable particularly in emergency revascularisation.

VASCULAR TISSUE ENGINEERING

There are three major approaches to creating blood vessels. The first is in the addition of vascular cells to synthetic polymers of which seeding of existing graft materials forms the most basic example. The second approach is in the development of bioresorbable or biodegradable grafts made of polymers which will be absorbed to varying speeds and degrees with eventual replacement by host tissue. The third approach is that of growing

new grafts in tissue culture made from endothelial cell, vascular smooth muscle cell, collagen and matrix.

Non-degradable polymer and cell seeding

Deutsch and colleagues in Vienna showed in explants of endothelial cell seeded PTFE grafts that a neo-media develops between the prosthesis and the endothelium throughout the entire length of the graft.[103,104] The cells in the neo-media contained actin filaments and a true internal elastic membrane had developed to separate them from the endothelial layer. Probably the original inoculums of endothelial cells obtained from cephalic or jugular vein had been contaminated with some vascular smooth muscle cells or pericytes. There has been much debate in the past as to whether endothelial cells for seeding should be pure or whether there would be benefit from inclusion of vascular smooth muscle cells or pericytes. This finding of a neo-media with a well developed internal elastic membrane providing an inner structure very similar to that of a normal artery lends support to the argument that co-culturing of cells of vascular origin would be beneficial.

The reintroduction of high porosity prosthetic grafts (i.e. pores >90μm) merits further study. Impermeability at the time of implantation using established impregnation methodology avoids the risk of haemorrhage. Once the sealant is absorbed, capillary tuft ingrowth with development of a media and intima may result. An alternative approach would be to develop highly porous prosthetic grafts pre-seeded with vascular smooth muscle cells, collagen, and with a seeded inner layer of endothelial cells. The newer bio-resistant polyurethane polymers are promising materials for development of such hybrid grafts.

Bioresorbable and biodegradable polymers

The concept of degradable or absorbable graft materials providing initial vessel integrity but in time replaced by the host's own tissues has been under development for some time.[105,106]

Polyglycolic and polylactic acid are the two bioresorbable polymers which have been most fully investigated. In addition to polydioxanone, these are the polymers which have FDA approval and for this reason are the preferred materials.[107] Polyglycolic acid is susceptible to in-vivo hydrolysis after 2–4 weeks. Polylactic acid is more resistant to hydrolysis in-vivo and in the form of, L-polylactic acid, has high mechanical strength. Copolymers of these two substances are in wide use as absorbable sutures and are better known as vicryl and polyglactin 910 (PG910). Polydioxanone, otherwise know as PDS, is a much more slowly reabsorbed compound. The first fully bioresorbable vascular graft was made from sheets of vicryl but became aneurysmal.[108]

Greisler's group in Chicago has contributed significantly in this field initially making grafts from woven polyglycolic acid for a rabbit model.[109,110] Four weeks after implantation a confluent layer of endothelial cells with a medial layer of myofibroblasts surrounded by dense collagen fibres was found. Ten percent became aneurysmal early on probably because of reabsorption before adequate ingrowth of host tissue. Grafts made of polydioxanone (PDS) were more slowly reabsorbed for up to six months. Similar tissue ingrowth as in the previous experiments with full endothelialisation over a neo-media was found. These were strong grafts able to withstand very high static bursting pressures (600-2000 mmHg).[111]

Greisler's group then reported a composite bioresorbable graft of 74% PG910 and

26% PDS which at one year in a rabbit aorta model had 100% patency with no aneurysmal degeneration. Complete reabsorption of PG910 took place within 2 months and PDS within 6 months. These arteries withstood up to 800mmHg of pulsatile pressure.[112] Composite partially resorbable grafts were next looked at in two grafts, the first constituted of 69% PG910 and 31% polypropylene, and the second of 70% PDS and 30% polypropylene. In a dog aorto-iliac interposition model, one year patency rates of 90% for the former graft and 86% for the latter graft were found.[113] Despite these experimental successes, so far no bioresorbable small diameter graft has been produced for human implantation.

Combined bioresorbable and tissue engineered grafts

Later work focused on the concept of a graft composed of autologous vascular cells with a bioresorbable scaffold providing sufficient strength during tissue ingrowth and replacement.[114] The first report was of smooth muscle cell seeding onto polylactic acid scaffold in a rat model where a neomedia with vascular orientation of cells was found.[115] Langer and Vacanti reported the successful development of a tubular scaffold made of woven polyglactin as an outer layer and an inner layer of non-woven polyglactin onto which autologous cells were seeded. After seven days of culture the vessels were implanted into sheep pulmonary artery with 7 grafts remaining patent for up to 3 months. The polymer scaffold was found to be replaced as expected by host cells and matrix, but these grafts dilated.

A more robust graft was produced using a composite scaffold of polyglycolic acid as an inner layer designed to degrade by two months, and polyhydroxy alkanoate as an outer non-porous layer, designed to degrade much more slowly. This graft was implanted into sheep abdominal aorta with full patency and no dilatation being found at up to 150 days and complete replacement by host tissue. A normal endothelial layer and a vascular media containing collagen and elastin were found.[116,117]

Surface modulation of polyglycolic acid polymer with 1N NaOH increases absorption of seeded smooth muscle cells.[118] The RGD peptide, a component of fibronectin, is known to promote endothelial cell attachment and also influence cell differentiation.[119] Much work is now focused on the incorporation of RGD peptide sequence onto polymer surfaces to enhance endothelial attachment. A further promising approach is the incorporation of biologically active substances, for example vascular endothelial growth factor and basic fibroblast growth factor in order to stimulate and modulate the differentiation of the seeded cells into functional phenotypes.[120]

Mechanical conditioning of seeded vascular cells

The exposure of smooth muscle cell seeded scaffolds to physiological and pulsatile pressures results in orientation into multilayers with collagen fibrils in the extracellular matrix.[121] Elastin and proteoglycans are also released into the extra-cellular matrix after 8 to 16 weeks of exposure to arterial circulation.[122] Furthermore conditioning of seeded endothelial cells by exposure to pulsatile flow and shear stress has been shown to improve proliferation and adhesion.[123]

Alternative scaffolds

Biocompatible and biodegradable synthetic polymers made by recombinant DNA technology are under development. Examples include the elastic protein-based polymers

such as poly (GVGP), a repeating sequence in the elastin molecule. Carboxy-amides are chemical moieties which will hydrolyse at varying times depending on the amino acid sequence. By selection of carboxy-amides for inclusion in the structure of the poly (GVGPV) polymer a planned degradation rate can potentially be incorporated. Differential degradation, resorption and replacement by host tissue of several layers of a graft can therefore be achieved while maintaining its structural integrity.[124] These polymers have excellent elasticity and proven optimised cell attachment due to incorporation of RGD sequences.[125]

Decellularised vessels have well preserved collagen fibres theoretically ideal for ingrowth. Good long term results from allogenic decellularised biological scaffolds have been reported with minimal immunoreactivity[126] but this initial promise was not maintained in further experimental studies. Xenografts would be practical for human implantation but unfortunately even decellularised scaffolds maintain a significant degree of immunogenicity and inflammatory response sufficient to destroy elastin.[127] Endothelial cell seeding with autologous cells has not been successful in reducing their immunogenicity and thrombogenecity.[128] Although a very promising concept the continuing problems of antigenicity make their clinical application unlikely.

A poly-L-lactide/poly-epsilon-caprolactone scaffold releasing heparin by a combination of electrospinning and fused deposition modeling technique has been used. This particular scaffold design allowed the generation of both a drug delivery system amenable to surmount thrombogenic issues and a microenvironment able to induce endothelial differentiation.[129] Silk-based fibroin grafts have been developed and they provide excellent patency when implanted in smaller vessels.[130] The fibroin graft gradually degraded with formation of an artery-like structure by endogenous endothelial cells and smooth muscle cells. Fibroin may hold the promise to generate vascular prostheses for smaller-diameter arteries.

Genetically-modified cells have also been considered for the construction of vascular replacements. For example, genetically-modified endothelial cells over-expressing tissue plasminogen activator (t-PA) and urokinase-type PA, or bone marrow mesenchymal stem cells transduced to express endothelial nitric oxide synthase (eNOS), would promote cell repopulation of the graft and help to eliminate thrombotic events.[131] Growth-regulating substances, growth factors or antimigratory and antiproliferative drugs have been incorporated directly into prosthesis wall or delivered through drug-eluting stents, catheters and perivascular collars.[132,133] Artificial materials releasing nitric oxide (NO) are also being developed, consisting of synthetic polymers incorporated with NO donors such as diazeniumdiolates and S-nitrosothiols.[134]

Tissue-engineered grafts

Blood vessels made purely from biological materials and vascular cells have the major potential advantage of a vasoactive biological conduit which can both heal and remodel according to changing environment. In Japan Hiraj and Matsuda developed a graft from canine vascular cells and collagen which proved resistant to physiological pressures only with a dacron backbone.[135] In 1998 the Quebec group reported the first successful totally biological graft made from cultured human umbilical vein cells which withstood physiological pressures.[136] The addition of a period of pulsatile culture following an initial static culture of smooth muscle cells and collagen reliably produces grafts

which are strong and resistant to supra-physiological burst pressures.[137,138]

All work in this field has been based on young cells. The successful translation of these promising developments to clinical application requires proof that adult or senile vascular cells will behave similarly. Such cells will have to come from each individual vascular patient until such time as pluri-potential, non-immunogenic cells can be sourced.

GRAFT MATERIALS FOR AORTIC ENDOGRAFTS

The endografts in current clinical use are mainly made from either thin woven polyester (Dacron) or ePTFE. Sac enlargement after endovascular aneurysm repair (EVAR), without evidence of endoleak, has been attributed largely to endotension or material porosity. The first-generation Gore Excluder graft allowed serous transudate contributing to continued sac pressurization. AneuRx grafts had a higher incidence of microleaks, or persistent transgraft blood flow, occurring through the thin graft material. The Excluder and AneuRx devices modified their graft material in 2004 with subsequent reduced permeability.[139] Stent and graft materials have different mechanical properties and any repetitive movement between them may damage the fabric. Stronger sutures and tighter weaves have made current designs more stable, but none is yet free from fabric graft failure.[140]

Research has also been targeted to improve the delivery profile of endografts, which is a main limiting factor in utilisation of these grafts for thoracic aneurysms. One such approach is to use thin-film Nitinol (NiTi) and early in vitro results have confirmed its feasibility.[141] This device is presently being tested in animal models.

With the availability of new materials, reducing mismatch in aortic stiffness and compliance may become important in future EVAR grafts. Indeed, some differences in presently used materials have already been observed by van Herwaarden and colleagues finding differences in compliance between Gore Excluder and Medtronic Talent stent-grafts at the level of aneurysm neck.[142] In the next decade, we can expect continuing improvements in device design. Plasmid-loaded cationized gelatin (CG) hydrogel-coated stent grafts offer transduction of therapeutic genes into the vascular wall facilitating the biologic healing between the aorta and graft.[143] Novel graft materials such as POSS-PCU have the potential to deliver compliance, antithrombogenicity, biocompatibility and spontaneous endothelialisation to provide better configurations and reduce the risk of complications.

THE FUTURE

Over the next 5 years improved prosthetic grafts will become available with the introduction of biodurable and compliant materials. Lumen modulation by anticoagulant molecules, cell ligands and growth factors will further enhance performance thus adding thromboresistance to compliance. Attachment technology will allow Dacron and PTFE to be similarly modified although these can never be sufficiently compliant to abolish compliance mismatch.

New compliant graft materials will be developed using novel spinning technologies to incorporate collagen and elastin polymers resistant to degradation. The technology for totally bioresorbable grafts is already in clinical use for paediatric cardiovascular reconstruction and its applicability to adult use is under study. Similarly the development of totally autologous tissue engineered grafts is in its infancy. Endothelial cell seeding is clinically proven but cumbersome. With ongoing development to match as closely as

possible the mechanical characteristics and functions of normal human arteries there is real potential for new graft development within the next decade.

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29 • Pathophysiology of Vascular Graft Infections

MAURO VICARETTI

Department of Vascular Surgery, Westmead Hospital, Sydney, NSW, Australia.

INTRODUCTION

The introduction of prosthetic grafts has revolutionised the management of vascular disease but graft infection although uncommon, remains a dreaded complication with associated significant morbidity and mortality. Mortality occurs in approximately one third of all vascular graft infections,[1] with mortality highest when an aortic prosthesis is involved.[2,3] As many as 75% of survivors of an infected aortic prosthesis require amputation of a limb,[3] with the incidence of amputation highest when the infection involves more distal prosthetic grafts.[4] The incidence of graft infections is difficult to quantify as infection may manifest many years after implantation[1] with many reports being isolated or as part of case series. Nevertheless, the reported incidence is in the order of 5%, varying according to the site of operation, being higher when a groin incision is used, or if the procedure is an emergency or a redo procedure. Infection following endovascular stent deployment has been reported although its incidence is considered to be very low.

NATURAL HISTORY OF PROSTHETIC VASCULAR GRAFT INFECTIONS

Early prosthetic vascular graft infections typically occurring in the first four months following placement are relatively uncommon (approximately 1%) and are usually caused by the more virulent micro-organisms, such as *S. aureus, E. Coli, Pseudomonas, Klebsiella, Proteus and enterobacter*.[1] Late prosthetic vascular graft infections are the result of two possible mechanisms. Firstly, by haematogenous seeding from a septic focus elsewhere[5] or by the prosthetic graft becoming infected with enteric contents following a graft-enteric erosion.[6] In both the haematogenous and graft-enteric erosion situations the usual causative organisms are those with high virulence and clinical manifestations are signs and symptoms of sepsis. The second mode of presentation is insidious, caused by the less virulent coagulase negative staphylococci such as *S. epidermidis* with contamination likely occurring at the time of implantation.[1]

MECHANISMS OF GRAFT CONTAMINATION AT OPERATION

Prosthetic grafts most commonly become infected at the time of implantation either by contamination from the surgical team or by colonised microorganisms on the patient. It has been demonstrated that the majority of patients undergoing arterial revascularisation are colonised with coagulase negative staphylococci[7] and colonisation of patients with nosocomial bacteria is enhanced when the preoperative hospitalisation is lengthy.[8]

The incidence of infection following emergency aneurysmorrhapy has been reported to be increased to 7.5%.[9] The evidence of other potential mechanisms such as division of lymph nodes,[10,11,12] infected transudated fluid during aortic surgery[13,14,15] and infected laminated thrombus[4,14,16,17] is conflicting.

PATHOGENESIS OF GRAFT INFECTIONS

The exact aetiology of vascular graft infections is not completely understood but is likely to be multifactorial. According to Bandyk and Esses[18] the risk of vascular graft infection as demonstrated by animal models can be predicted by the formula:

$$\text{Risk of biomaterial infection} = \frac{\text{Dose of bacterial contamination X virulence}}{\text{Host resistance}}$$

The dose of bacterial contamination is dependent on the infecting microorganism. Experimentation in a canine aortic model has demonstrated that the infective threshold for bacteria to cause graft infection in over 50% of grafts was 10^7, 10^9, and 10^2 for *S. aureus, S. epidermidis and P. aeruginosa* respectively.[19] Virulence of microorganisms is often associated with the production of secreted toxins and enzymes with a resultant decline in structural integrity of the artery wall[18] and the release of toxins and enzymes to control the perigraft environment and cause graft infection.[19,20] Many bacterial strains, including *S. epidermidis, S. aureus and P. aeruginosa* are known to produce extracellular polymer substances (slime), forming a capsule incorporating the bacteria. This is referred to as a biofilm and protects the micro-organism against host defences and antibiotic therapy.[21] Biofilms allow greater adherence of the microorganism to the biomaterial[22, 23] and contribute to bacterial virulence. Multiple species of microorganisms may co-exist in a biofilm and unless the biofilm is disrupted and or the microorganism/s become planktonic the microorganism/s identification is limited. Different graft materials have varying susceptibility to infection. Dacron grafts are more likely to become infected than grafts made of PTFE (polytetrafluoroethylene).[24] The use of vein grafts instead of prosthetic material greatly reduces the risk of infection.

BACTERIOLOGY OF VASCULAR GRAFT INFECTIONS

Gram-positive, Gram-negative, anaerobic and fungal micro-organisms all have the potential to infect a vascular prostheses but in general the majority of infections are the result of a small number of micro-organisms. Staphylococci are the most prevalent organism associated with prosthetic graft infection.[2,25,26,27] Of the staphylococci, *S. aureus* is generally regarded as the most common causative bacteria,[2,26,28,29] particularly MRSA.[27] *S. epidermidis* is now being recognised as the leading cause of vascular graft infection, particularly chronic and late onset infections.[17,29,30,31,32]

The Gram-negative organisms, *E. Coli, Pseudomonas, Klebsiella, Enterobacter* and *Proteus,* although relatively uncommon

causative organisms for graft infections are of particular interest and concern because of their high virulence and their tendency to destroy the vessel wall.[18,33,34]

Candida mycobacterium, and Aspergillus infections are uncommon but pose a significant risk to patients who are immunocompromised.[2] Although uncommon they are all expected to increase in frequency because of their increasing resistance to standard prophylactic antibiotics.[35]

There is an association between the type of infecting organism, the type of vascular complication and the arteries that are involved in the anastomosis to the prosthetic graft. Bandyk and Bergamini[2] in a collective survey of 1258 patients who had a vascular graft infection found that the majority of aortoenteric fistulas were the result of either *Streptococci or E. Coli* and if the anastomosis involved the femoral artery, the thoracic aorta, the subclavian, carotid or innominate arteries *S. epidermidis or S aureus* was the likely causative organism. *E. Coli, Enterococci and Enterobacter* were the more likely organisms to be involved in aortoiliac anastomoses.

INVESTIGATIONS FOR DETECTION OF PROSTHETIC GRAFT INFECTIONS

The diagnosis of vascular prosthetic infections can be difficult as the presentation may be subtle especially if it is a late onset infection, the prosthesis is intra-abdominal and the micro-organism is one of low virulence. Presentation is thus very dependent on the location of infection and the causative microorganism/s. The diagnosis is aided by multiple available microbiological investigations and imaging but in general is directed more at proving the absence of infection rather its presence. Not only are investigations imperative in the diagnosis of vascular graft infection but they may assist in the planned therapy including vascular reconstruction when required. At times the only means of confirming graft infection is the surgical excision of the graft and further microbiological assessment.

History and physical examination

The clinical clues suggesting graft infection especially those placed superficially include an inflammatory perigraft mass, overlying cellulitus, presence of exposed prosthetic graft, a sinus tract with persistent purulent drainage and/or bleeding and/or a palpable anastomotic pseudoaneurysm, graft thrombosis and distal septic embolisation.[2-4,36,37] The presence of intra-abdominal prosthetic graft infection may be non-specific, such as fever of unknown origin, septicaemia, or abdominal pain.[3] Upper or lower gastrointestinal haemorrhage either of an acute or chronic nature may indicate a graft-enteric fistula[17,37,38] and can only be excluded when another source of gastrointestinal haemorrhage has been identified.

Laboratory investigations

Routine laboratory studies such as white cell count and differential, erythrocyte sedimentation rate (ESR), C-Reactive Protein (CRP), and blood cultures are routinely obtained but the results may be non-specific and even normal if the organism is *S.epidermidis.*[2] Wherever possible pus, exudates, tissue specimens, blood and wound cultures should be analysed microbiologically to aid in microorganism identification and to allow the commencement of appropriate and specific chemotherapy.[39] To aid in the diagnosis of *S.epidermidis* all solid material should be mechanically or ultrasonically disrupted.[40-42]

Diagnostic imaging

Various diagnostic modalities (Computerised Tomography (CT), ultrasonography, Magnetic Resonance Imaging, Leucocyte or immunoglobulin labelled scanning, Positron Emission Tomography (PET) scanning +/- CT, angiography and/or endoscopy) may assist the vascular surgeon in determining the presence and extent of prosthetic graft infection. Not infrequently, a combination of the diagnostic modalities to improve sensitivity and specificity are utilised to confirm the presence or absence of a vascular prosthetic graft infection.[43] These modalities are also helpful in planning definitive surgery. The utility of CT angiography with the capability of vascular three dimensional reconstructions has largely replaced digital subtraction angiography as the method of diagnosis and therapeutic planning. CT guided aspiration is also of benefit in diagnosis. In general the features suggestive of graft infection include perigraft fluid and/or gas, graft disruption, absence of graft incorporation, pseudoaneurysm formation. The presence of periprosthetic gas more than six weeks following graft implantation is an abnormal finding and should alert the physician to the likelihood of a graft infection.[44]

MANAGEMENT OF PROSTHETIC GRAFT INFECTIONS

The general principles in the management of prosthetic graft infections are initially preventative, but in the event of a vascular graft infection, therapy needs to be individualised accounting for clinical findings, graft material (prosthetic versus autogenous graft material), site of infection, microorganism/s involved and patient co-morbidities. It is imperative that not only is graft infection eradicated but recurrent infection be minimised with avoidance of significant morbidity and/or mortality.

Prevention

Preventive measures such as the routine use of skin preparations,[45] the use of a depilatory agent,[46] limiting the length of preoperative hospitalisation,[8] operating time and intensive care stay all contribute to the reduction in wound infection and more importantly the chance of developing resistant multiple nosocomial infections.[45] Antimicrobial prophylaxis has been shown to reduce wound infections in vascular surgery[47] and ideally should be given as close to the time of incision and repeated in the event of haemorrhage and lengthy operations every four hours. Prophylatic antibiotics are also indicated with percutaneous punctures of existing prosthetic grafts and the implantation of stents. Decolonisation of nasal carriers of *S. aureus* has been shown to significantly reduce the number of surgical site *S. aureus* infections especially deep surgical-site infections.[48] Institutional prevalence of resistant organism may also dictate antibiotic prophylaxis especially when prosthetic grafts are to be implanted.

As a preventive measure host resistance may be enhanced by the antimicrobial impregnation of grafts. A number of novel combinations of grafts and antibiotic with or without various forms of treatment have been trialled at both the *in-vitro* and *in-vivo* levels.

Rifampicin, a known anti-staphylococcal agent, particularly methicillin resistant,[49] is a hydrophobic semisynthetic substance with a high affinity for gelatin.[50] It inhibits DNA dependent RNA polymerase activity in bacterial cells without affecting mammalian cells[51] and has been passively incorporated into gelatin sealed Dacron grafts as a mode of staphylococcal protection at the time of implantation. It has been shown to be resistant to experimental bacterial contamination[52-55] with in-vivo bioactivity

to 22 days,[56] and *in-vitro* bioactivity to 4 days.[57-59] It is these qualities plus its excellent tissue and intracellular penetration[59] that make rifampicin an ideal antibiotic to be bonded to prosthetic grafts in order to prevent subsequent graft infection.

Reduction of prosthetic vascular graft infection with rifampicin bonded gelatin sealed Dacron

Using an established sheep model[60] we replaced a segment of sheep carotid artery with a rifampicin soaked Gelsoft graft. At the time of graft removal microscopic assessment (perigraft abscess formation, presence of anastamotic disruption and graft thrombosis) and microbiological assessments (cultures of perigraft tissues, graft external and internal wall and total graft cultures) were recorded. We showed that, following direct inoculation of the rifampicin (1.2mg/ml or 10mg/ml) soaked graft with 10^8 colony forming units of either methicillin resistant *Staphylococcal aureus* (MRSA) or methicillin resistant *Staphylococcal epidermidis* (MRSE), the rifampicin soaked graft offered significant prophylaxis.[61-63]

For the MRSE arm, in the 10mg/ml rifampicin group there was a significant reduction in graft infection when compared to both the control group ($p < 0.05$) and the 1.2mg/ml group ($p < 0.05$).[63] Similarly, for the MRSA group, in the 10mg/ml treatment group there was a significant reduction in the total number of positive cultures when compared to the control group ($p < 0.05$) and the 1.2mg/ml group ($p < 0.05$).[63]

ESTABLISHED INFECTION

Antibiotic therapy

Once the diagnosis or suspicion of prosthetic vascular graft infection is made then broad spectrum antimicrobial therapy is initiated and subsequently converted to organism specific antibiotics.[3] The length of antibiotic therapy following excision of the infected graft is unclear but Bergamini and Bandyk[2] advocate parenteral antibiotics for two weeks and oral for six months.

Operative management

The 'gold standard' treatment although technically challenging is the removal of all infected tissue and revascularisation extra-anatomically.[64] A number of more conservative approaches have been advocated depending on the site of the infection and the microorganism involved. The most conservative of treatments is aggressive local wound care with graft preservation (prosthetic/autologous) providing that the graft and anastomoses are intact and the patient has no systemic features of sepsis.[65] Calligaro *et al*[34] in a report of a series of patients who had graft preservation concluded that with the exception of *Pseudomonas*, vascular graft infections could be managed with debridement, antibiotic therapy and wound closure. The skeletonized prosthetic graft can be covered using viable regional rotational flaps.[66] Others have proposed graft excision and replacement with cadaveric arterial allografts,[67] venous autografts,[68] cryopreserved saphenous vein homografts,[69] autogenous arteries and/or veins[70] or prosthesis.[71] The major drawback with in-situ reconstruction is recurrent graft sepsis[72] with potential limb and/or life threatening graft and/or anastomotic disruption.

Schmitt, *et al*.[22] in an *in- vitro* model comparing the bacterial adherence of four strains of bacteria (*S. aureus*, 'mucin' and 'non-mucin' producing *S. epidermidis* and *E. coli*) to ePTFE, woven Dacron and velour knitted Dacron found that bacterial adherence was greatest to velour knitted Dacron

and least compared to ePTFE. In addition Schmitt, *et al*.[73] found that 'mucin' producing *S. epidermidis* adhered to Dacron in 10 to 100 fold greater numbers compared to PTFE. Bandyk and Bergamini[2] have postulated that the differential adherence of staphylococci relates to capsular adhesins.

Using the established sheep model[60] we have set out to determine if the replacement of a staphylococcal infected vascular graft with a graft impregnated with rifampicin would be considered appropriate surgical management in preventing early recurrent infection. Gelsoft grafts without any antibiotic treatment were infected with overwhelming concentrations of either MRSA or MRSE. The grafts were removed at three weeks and replaced with either control (no rifampicin) grafts or grafts soaked in either 1.2mg/ml or 10mg/ml of rifampicin. The replacement grafts were removed 3 weeks following placement.

For MRSA[74] there were no statistical significant differences between the groups for any of the macroscopic or microbiological parameters recorded.

For *S.epidermidis*[74] there were no statistical differences between the rifampicin concentrations for macroscopic findings. There were however, statistically significant reductions in the number of total infected specimens in the 10mg/ml group when compared to both the control, ($p<0.001$) and the 1.2 mg/ml groups ($p<0.005$).[74]

The conclusions from the studies[74] were that established *S. epidermidis* bacterial biofilm graft infections model can be treated by the in-situ replacement of the infected prosthesis with a 10 mg/ml rifampicin impregnated Gelsoft graft. However, such management for MRSA established infections cannot be recommended from the results obtained in this particular animal model.

To date a number of groups[75,76] have successfully managed prosthetic graft infections with rifampicin impregnated grafts with zero mortality, no requirement for limb amputation and to date no recurrence of infection.

CONCLUSION

The future management of vascular graft infections will be reliant on a better understanding of the interaction between the micro-organism, the prosthesis and the immune system. This will allow a more directed approach towards prevention and treatment. Possibilities would include more powerful antibiotics either administered parenterally or incorporated into the prosthesis, acting as a local delivery system for prolonged periods of time. The role of the biofilm in the pathogenesis of graft infection needs further understanding from both a molecular and an immune level.

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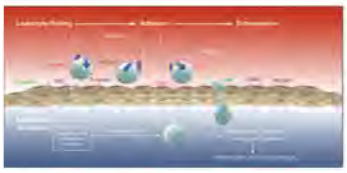

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