Mechanisms of Vascular Disease:

A Reference Book for Vascular Specialists

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Edited by Robert Fitridge and Matthew Thompson

Completely Updated Edition 2011

Barr Smith Press

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A Reference Book for Vascular Specialists

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BARR SMITH PRESS
An imprint of
The University of Adelaide Press

Published in Adelaide by

The University of Adelaide, Barr Smith Press
Barr Smith Library
The University of Adelaide
South Australia 5005
press@adelaide.edu.au
www.adelaide.edu.au/press

The University of Adelaide Press publishes peer-reviewed scholarly works by staff via Open Access online editions and print editions.

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This CIP cataloguing for this work is as follows;

Mechanisms of vascular disease : a reference book for vascular surgeons / Robert Fitridge, Matthew Thompson, [editors].

1. Blood vessels, Diseases.
2. Blood vessels, Surgery.

I. Fitridge, Robert
II. Thompson, M. M.

For the full Cataloguing-in-Publication data please contact National Library of Australia:
cip@nla.gov.au

ISBN (paperback) 978-0-9871718-2-5

Book design: Midland Typesetters
Cover design: Emma Spoehr, based on a diagram by Dave Heinrich of the Medical Illustration and Media Unit, Flinders Medical Centre
Paperback edition printed by Griffin Press, South Australia

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Acknowledgements

The Editors gratefully acknowledge the outstanding contributions of each Author involved in this reference book. We would also like to acknowledge the invaluable efforts of Ms Sheona Page who has worked tirelessly on this project. We would also like to thank Prue Cowled PhD and Ms Cayley Wright for their assistance.

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

23 • Principles of Wound Healing

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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

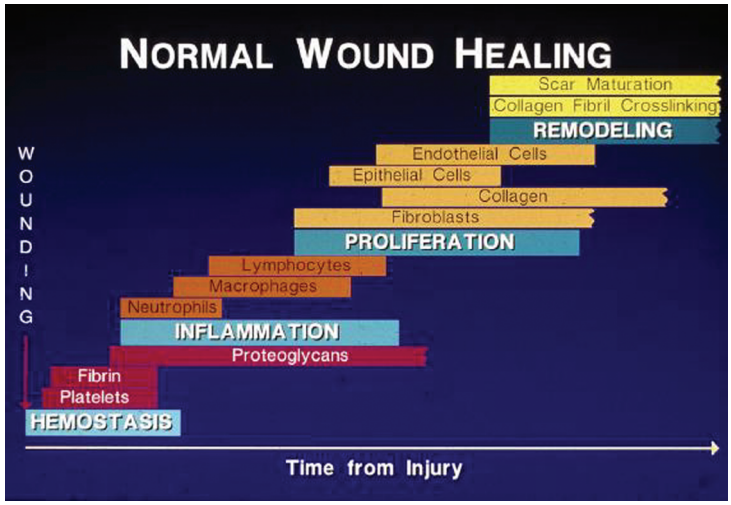

FIGURE 23.1: Phases of Normal Wound Healing. Cellular and molecular events during normal wound healing progress through four major, integrated, phases of haemostasis, inflammation, proliferation and remodelling.

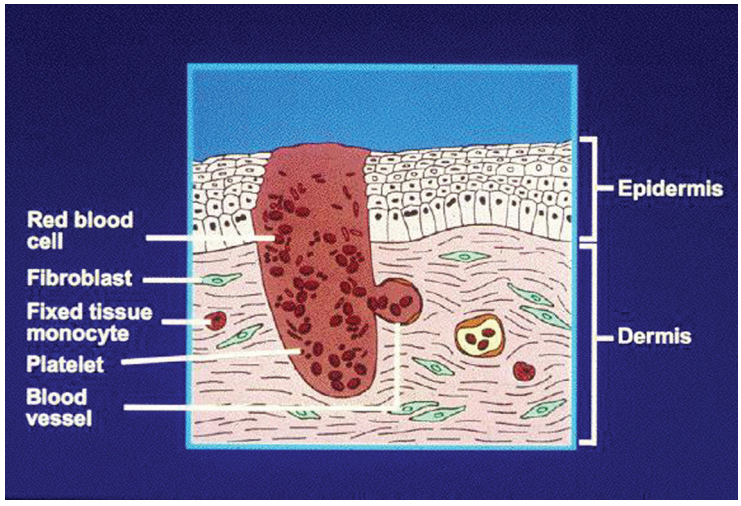

FIGURE 23.2: Haemostasis Phase. At the time of injury, the fibrin clot forms the provisional wound matrix and platelets release multiple growth factors initiating the repair process.

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

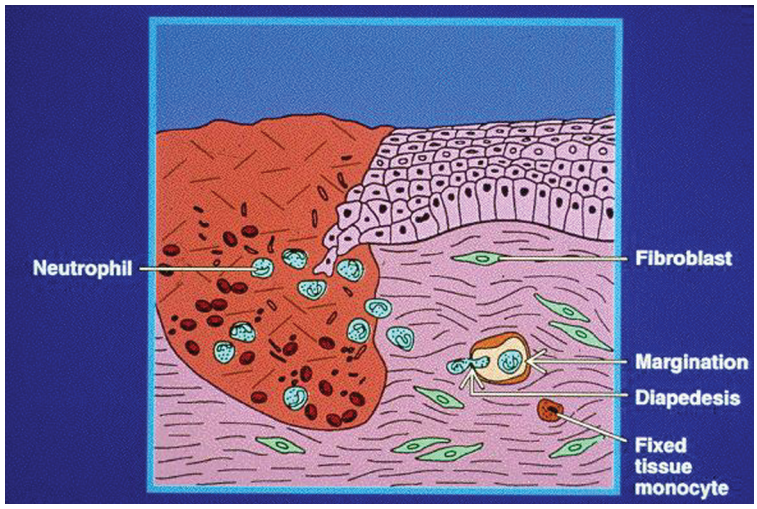

FIGURE 23.3: Inflammation Phase. Within a day following injury, the inflammatory phase is initiated by neutrophils that attach to endothelial cells in the vessel walls surrounding the wound (margination), change shape and move through the cell junctions (diapedesis), and migrate to the wound site (chemotaxis).

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.

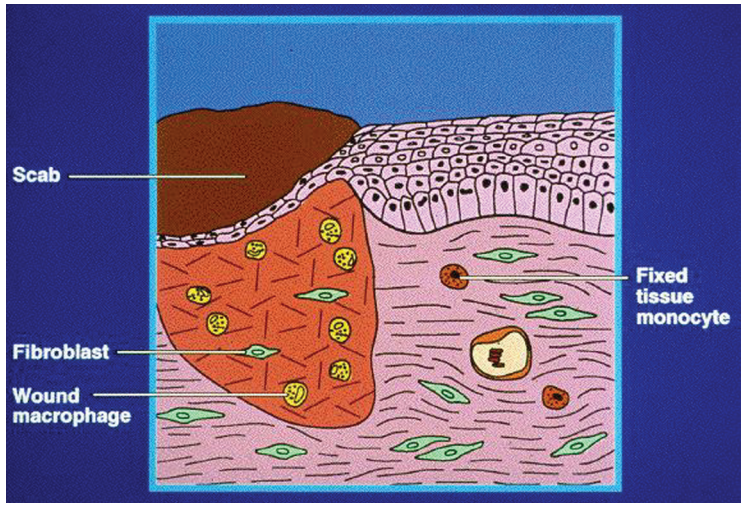

FIGURE 23.4: Proliferation Phase. Fixed tissue monocytes activate, move into the site of injury, transform into activated wound macrophages that kill bacteria, release proteases that remove denatured ECM, and secrete growth factors that stimulate fibroblasts, epidermal cells and endothelial cells to 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

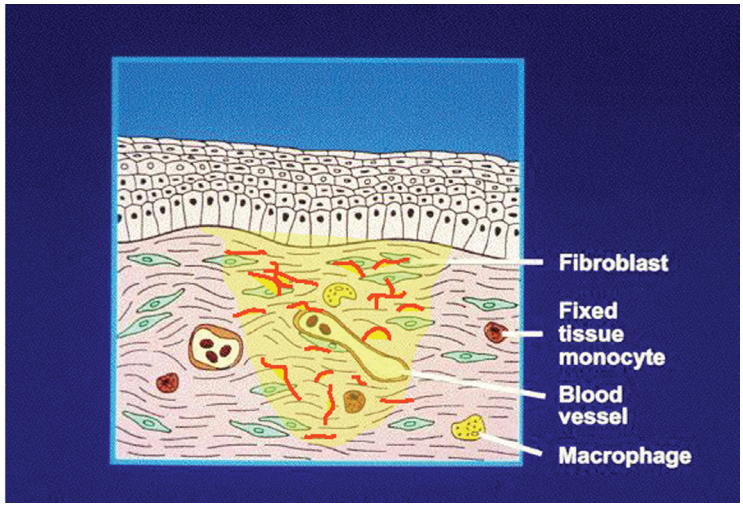

FIGURE 23.5: Remodelling Phase. The initial, disorganized scar tissue is slowly replaced by a matrix that more closely resembles the organized ECM of normal skin.

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.

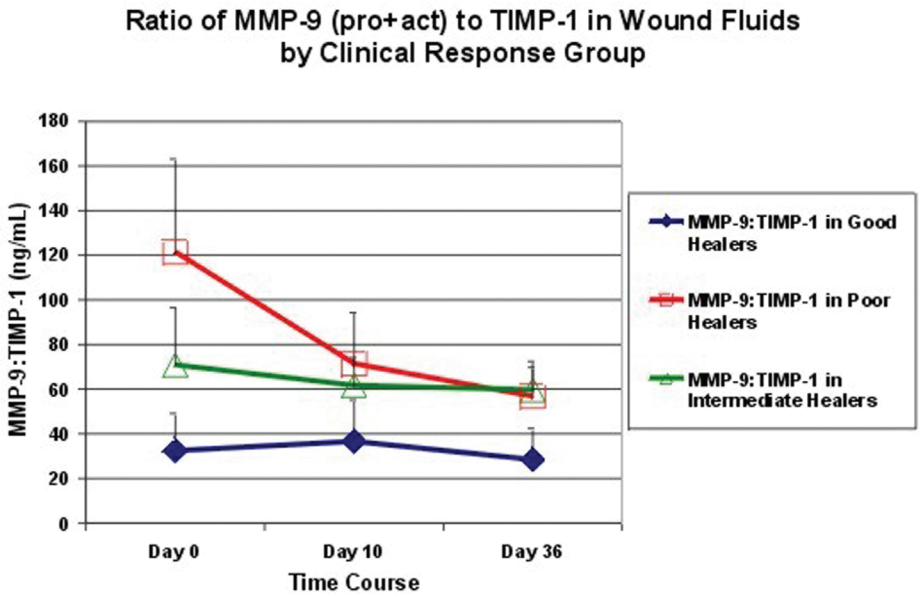

FIGURE 23.6: Low Protease/Inhibitor Ratios Correlate with Healing. Low values of the ratio of MMP-9/TIMP-1 in wound fluids from patients with chronic pressure ulcers correlate with healing of chronic pressure ulcers over 36 days of treatment, supporting the concept that high protease/inhibitor ratios 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.

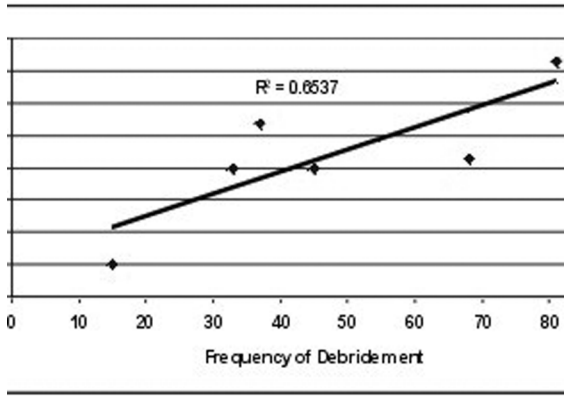

FIGURE 23.7: Frequency of Wound Debridement Correlates with Improved Healing. There was a strong correlation between the frequency of debridement and healing of chronic diabetic foot ulcers, supporting the concept that the abnormal cellular and molecular environment of chronic wounds impairs healing.

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

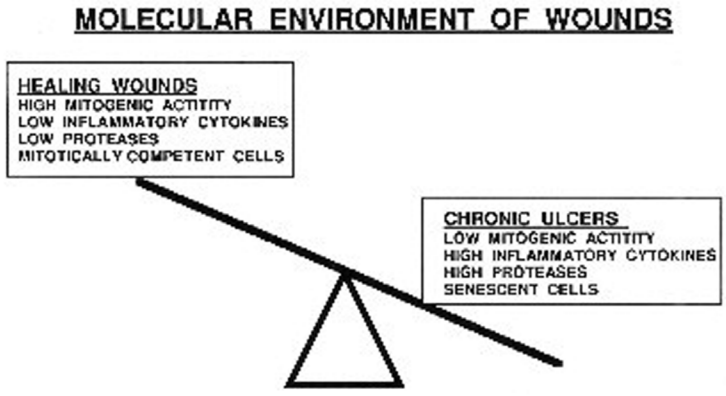

FIGURE 23.8: Comparison of the Molecular and Cellular Environments of Healing and Chronic Wounds. Elevated levels of cytokines and proteases in chronic wounds reduce mitogenic activities and response of wound cells, impairing healing.

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

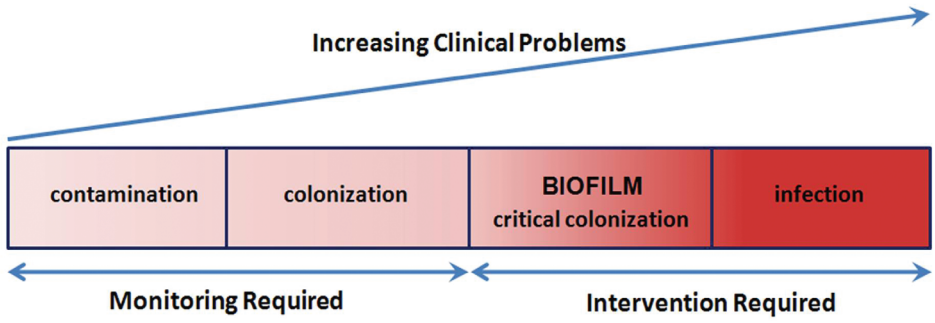

FIGURE 23.9: Spectrum of Bacterial Bioburden in Wounds. Contamination and colonization of bacteria usually do not substantially retard healing whereas infection clearly impairs healing. The concept of critical colonization evolved to describe a condition where levels of planktonic bacteria were not above 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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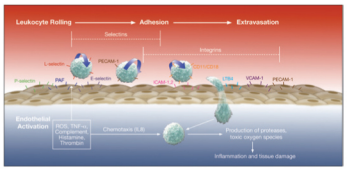

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