Molecular and Translational Medicine
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Monte S. Willis · Cecelia C. Yates
Jonathan C. Schisler *Editors*

Fibrosis in Disease

An Organ-Based Guide to Disease Pathophysiology and Therapeutic Considerations

Humana Press

Molecular and Translational Medicine

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As we enter into this new era of molecular medicine with an expanding body of knowledge related to the molecular pathogenesis of human disease and an increasing recognition of the practical implications for improved diagnostics and treatment, there is a need for new resources to inform basic scientists and clinical practitioners of the emerging concepts, useful applications, and continuing challenges related to molecular medicine and personalized treatment of complex human diseases. This series of resource/reference books entitled Molecular and Translational Medicine is primarily concerned with the molecular pathogenesis of major human diseases and disease processes, presented in the context of molecular pathology, with implications for translational molecular medicine and personalized patient care.

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ISSN 2197-7852 ISSN 2197-7860 (electronic)
Molecular and Translational Medicine
ISBN 978-3-319-98142-0 ISBN 978-3-319-98143-7 (eBook)
https://doi.org/10.1007/978-3-319-98143-7

Library of Congress Control Number: 2018957985

This Humana Press imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

We dedicate this book to the vast number of patients struggling with the numerous diseases with primary fibrosis. While the therapeutic options are extraordinarily limited, in editing this book, it is clear that the future is bright, with new and exciting approaches already on the horizon. We thank our mentors for their tireless inspiration and guiding light in the face of uncertainties and risk – Charles Jennette, MD; Cynthia Lander, PhD; Lynda F. Bonewald, PhD; Marion E. Couch, MD, PhD, MBA; Chris Newgard, PhD; Cam Patterson, MD, MBA; Timothy Turner, PhD; Mark E. Sobel, MD, PhD; and Clayton C. Yates, PhD. It would be an honor to have passed any fraction of their enthusiasm, guidance, and unwavering encouragement they were so generous to share with us.

To Tina, Connor, and Declan,

thank you for the inspiration and distraction making it all possible.

Monte

To Frances Chisholm and Frances Freedman,

for consistently encouraging me to write often and write well, especially about matters that are most meaningful and impactful to mankind.

Cecelia

I dedicate this book to all of my science and English teachers throughout my education, most notably the late Father Ebenhoeh:

How many moles?

Jonathan

Acknowledgments

We are grateful for the support of our academic and research programs by the National Institutes of Health, the Leducq Foundation, the Moerae Matrix, the FibroKine™ peptide team, and the American Society of Investigative Pathology for their unwavering support during challenging times. We also wish to thank Saranya Ravi, Jun Heo, and Zariel Johnson for their editorial support.

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Introduction

Systemic and organ-specific human fibrotic disorders, the loss of physiological organ structure and function, are a worldwide leading cause of morbidity and mortality and can affect all tissues and organ systems. Fibrotic disorders or fibroproliferative diseases result from chronic tissue damage or out-of-control wound healing response, thereby resulting in a vast number of conditions in which it is the primary factor as well as the large number consequence of the disease. The spectrum of these disorders includes, but is not limited to, pulmonary fibrosis, systemic sclerosis, liver cirrhosis, cardiovascular disease, and progressive kidney disease. This range of diseases also encompass pathophysiologies not readily recognizably related, such as macular degeneration and cancer metastasis. Thereby approximately, 100 million patients have fibrosis-related tissue damage; in some cases, this causes progressive and life-threatening disease outcomes. Remarkable progress in elucidating the pathogenesis of these common diseases with fibrotic components as the scope of fibrotic disorders and their etiological manifestations is diverse, yet they share underlying factors. Most notably, these factors include the complex and intrinsically linked relationship between inflammation and extracellular matrix remodeling. As the importance of inflammation and fibrosis is apparent in the long-term recovery and treatment of diseases, effective anti-fibrotic therapies targeting the underlying ongoing disease processes are lacking.

Despite the advances in the discovery of organ-specific mechanisms of disease and therapeutic targets efforts, success remains indefinable for a multitude of reasons. Among these reasons is the lack of understanding or defining (i) early detection, (ii) reversibility mechanisms, and (iii) extracellular matrix and inflammation crosstalk. The complexity of discovering and applying therapies to fibroproliferative disease may be due to the diversity of the systems the pathogenesis of disease itself involves. By nature, fibroproliferative diseases are interdisciplinary, involving multiple cell types (organ-specific epithelial cells), immune cells, endothelial cells, and fibroblasts. Bone marrow, cytokines and chemokines, and organ-specific pathologies further speckle both the clinical and scientific disciplines in such a way that communication is often limited to the clinical or scientific tribes we live in, despite the most significant access to information known to man available today.

Therefore, the primary focus of this text is to bring together authors from diverse clinical, scientific, and therapeutic backgrounds for readers to more fully appreciate the current state of the science in organ fibrosis. We also hope the reader recognizes the impact and potential opportunities gained from identifying well-defined mechanisms in other organ systems. This book comprehensibly makes available the advances in the discovery of preclinical therapeutic targets (at least 20+ to date) involving TGF-beta (and other cytokines), transcription factors, and downstream kinases; it's important to recognize both the broader impact and potential opportunities that exist even today.

Fibrotic Signaling in the Skin

There is a broad spectrum of human fibrotic skin diseases: nephrogenic fibrosing dermopathy, mixed connective tissue diseases, scleromyxedema, eosinophilic fasciitis, and most notably systemic sclerosis (SSc). The initiating factors for this process in the early stages are believed to be driven by vascular injury, immune dysfunction, and an autoimmune response. The immune dysfunction, resulting in a dysregulated inflammatory response, and vascular injury result from pathologic wound healing. *Chapter* 1 introduces the chemokines and chemokine receptor signaling that influences cellular activities after tissue injury and alternation in the chemokine response that leads to fibrosis. *Chapter* 2 dissects the underlining mechanisms that drive the pathogenesis of systemic sclerosis while comprehensively analyzing human and animals to study this disease. *Chapter* 3 outlines cellular aspects of skin fibrosis, specifically the changing macrophage phenotypes in skin fibrosis and the influence that macrophage ontogeny, epigenetic factors, heterogeneity, and the microenvironment exert on these phenotypes.

Fibrotic Signaling in the Lung

Lung fibrosis is characterized as a progressive scarring of the lung parenchyma, which ultimately leads to impaired gas exchange, respiratory failure, and death. *Chapter* 4 describes the signaling mechanisms that occur in lung fibrosis while highlighting the three principal cellular actors in the fibrotic lung: the epithelial cell, the macrophage, and the fibroblast. *Chapter* 5 explores the extracellular matrix element of fibrosis and discusses the major matrikines identified in the mouse and human lung as well as proposes how targeting these molecules could be used in treating these diseases. *Chapter* 6 underlines the less well-studied cell type, mast cells, and their characteristic function that influences the progression of pulmonary fibrosis has not been pursued. *Chapter* 7 describes the advances in our understanding of the heterogenous pericyte phenotype, including its distribution and origin that contributes to their involvement in both ECM remodeling and inflammation features

of fibrosis. *Chapter* 8 circumscribes the emerging therapeutic targets and therapies in idiopathic pulmonary fibrosis. *Chapter* 9 describes the changes in the matrix microenvironment in chronic lung diseases and how these changes amplify and sustain pathological lung fibrosis, which has limited the effectiveness of current therapies.

Fibrotic Signaling in Heart

Most etiologies of heart disease involve pathological myocardial remodeling that produces excessive deposition of extracellular matrix (ECM) proteins by cardiac fibroblasts (CFs), which reduces tissue compliance and accelerates the progression to heart failure. *Chapter* 10 introduces fibrosis in the heart, specifically the signaling pathways that are associated with fibrosis and cardiomyopathies. Despite the numerous etiologies of cardiomyopathies, it is essentially a fibrotic disease that ultimately disrupts cardiac output. Several of these pathways are further explored in depth, including the WNT signaling pathway, in *Chapter* 11, and matrix metalloproteinases that modify the extracellular fibrotic matrix in the heart, in *Chapter* 12. The remodeling of the extracellular matrix in the heart produces peptides that may be used diagnostically or even therapeutically, concepts explored in *Chapter* 13.

Fibrotic Signaling in the Vasculature, Liver, Kidney, and Eye

The role of fibrosis in other organ systems reveals several commonalities to the pathophysiology of fibrosis observed in the skin, lung, and heart. In disease conditions, the plasticity of vascular cells results in the transformation of different cell type into fibroblast-like cells, contributing to vascular stiffness, as discussed in *Chapter* 14. Recent insights into how the ECM in the liver changes in the development and progression of cirrhosis revealed insight into the reversibility of liver fibrosis, profoundly impacting the development of new therapies for cirrhosis, as well as tumorigenesis, explored in *Chapter* 15. The balance between fibrosis and angiogenesis highlights the complex interplay between adaptive and maladaptive processes that occur in the pathology of chronic kidney disease, as detailed in *Chapter* 16. Macular degeneration is hallmarked by the accumulation of nonfunctional, fibrotic tissue, exemplifying the pathological effects of excessive and aberrant would repair. *Chapter* 17 highlights how the recent advances in our understanding the balance between angiogenesis and inflammation revealed new approaches in pharmacotherapies targeting fibrosis in macular degeneration.

Part I
Skin, Cell Injury, and Wound Healing

Chapter 1
The Role of Chemokines in Fibrotic Dermal Remodeling and Wound Healing

Zariel I. Johnson, Christopher Mahoney, Jun Heo, Erin Frankel, Dana R. Julian, and Cecelia C. Yates

Introduction

Skin is the largest organ in the adult body and serves many crucial roles in health and disease. It is part of the integumentary organ system, which also includes hair, nails, glands, and nerves. The skin is responsible for protecting from pathogens by acting as a physical barrier. The outer layer of skin prevents excessive water loss to enable homeostasis of electrolyte balance. Specialized neurons provide feedback to sense stimulation by temperature, pressure, and touch. The lower fatty layer of skin acts as an energy reserve and provides padding. Thus, the integrity of the skin and its composite layers is critical for normal function of the organism.

Injury to the skin comes in many forms and is repaired through a sequence of overlapping, ordered phases that restore barrier function and, in cases of complete healing, biomechanical properties. Healing involves sealing the wound through hemostasis, preventing infection and attracting cells through inflammation, regenerating lost tissue and permanently closing the wound, and remodeling the initial

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M. S. Willis et al. (eds.), *Fibrosis in Disease*, Molecular and Translational Medicine,
https://doi.org/10.1007/978-3-319-98143-7_1

replacement tissue. Alterations in this healing cascade can lead to the development of fibrotic or scar tissue that lacks biological functions and/or mechanical properties of healthy skin.

Chemokines are a family of small cytokines initially identified as chemoattractant molecules. Over time, further study has shown these molecules have essential roles in many areas of physiology ranging from promoting differentiation to influencing metastasis. Ligands may signal through cognate receptors or exert their actions through interaction with the extracellular matrix. Promiscuous binding of ligands paired with the ability of receptors to form oligomers results in a vast diversity of signaling outputs.

At the beginning of wound healing, the pro-inflammatory and pro-angiogenic chemokines play an essential role by attracting immune and endothelial cells. This process allows for the growth of the vascular supply needed to support extracellular matrix synthesis by fibroblasts and migration of keratinocytes to re-epithelialize the wound area. Following the formation of this granulation tissue, there is a switch to angiostatic chemokines, which halt angiogenesis and cause the regression of the overabundant blood supply. These same chemokines have proved essential to stop the migration of keratinocytes and promote maturation of the dermis and epidermis.

Normal Dermal Architecture and Function

Skin is the largest organ in the body and comprises about 15% of the adult body weight [1]. The skin is the body's primary physical barrier with the outside world and plays essential roles in immunity and homeostasis. It must continuously sense and respond to environmental changes across the entire body.

The skin is composed of three layers: the epidermis, dermis, and hypodermis (Fig. 1.1). Within each layer, there exists a multitude of individual structures, including: hair follicles, sweat glands, nerves, connective tissue, adipose tissue, and blood vessels [2]. The relative proportion of layer depth varies with the body area to match form to function. For example, the epidermis is much thicker on surfaces that experience friction, such as the soles of the feet, than other areas. The epidermis is the outermost layer of skin and is a stratified, squamous epithelium layer made up primarily of keratinocytes, which produce keratin, and dendritic cells. Other cell types present in this layer include melanocytes, Langerhans cells, and Merkel cells, each with specialized functions. Specific structures found in the epidermis include sweat glands, pilosebaceous follicles, and, on the distal phalanges, nails. The epidermis can be further separated into two anatomical layers: the regenerative layer and the outer stratum corneum. The dermis is the next deepest layer of skin and is made up of relatively few cells that reside in an abundant extracellular matrix. Present in this layer are cells such as fibroblasts, monocytes, Langerhans cells, lymphocytes, and vascular-related cells and structures including sweat glands, nerve endings, hair follicles, and blood vessels. These cells account for only about 10% of the tissue con-

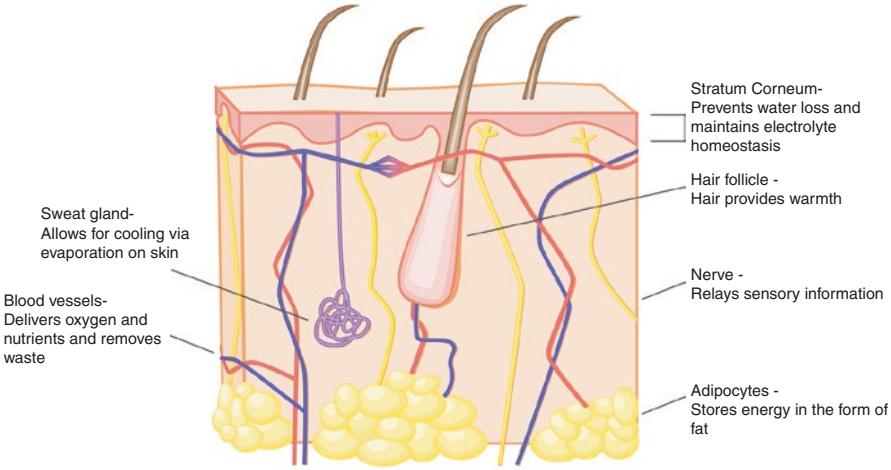

Fig. 1.1 Normal skin architecture is critical to meet functional needs of the human body. Each layer of skin has biomechanical properties and structures that allow it to perform specific functions, including immune defense, homeostasis, and sensory functions. Chemokines, through interaction with their receptors and the extracellular matrix, play a vital role in skin repair following injury and regeneration of the complex structures depicted here

tent, while the remaining 90% is mostly collagen type I and elastin. The rich vascular supply of the dermis is essential to deliver nutrients and other circulatory products to the epidermis, which does not have its own blood supply. Finally, the deepest layer of skin is the subcutaneous layer, also known as the fatty layer. The most abundant cell type in this layer is the adipocyte, which stores energy in the form of fat, followed by the fibroblasts and macrophages.

The skin serves many vital functions for organism survival. Perhaps the most critical function is acting as a barrier to infection. The outermost layer of the epidermis, the stratum corneum, consists of several layers of squamous epithelial cells. This cell layer is continuously undergoing cell desquamation, which sloughs bacteria with it before being replaced by fresh cells. The oil glands of the skin also secrete fatty acids and produce an acidic pH, making it difficult for many bacteria to colonize the skin. However, a person can become prone to infection if an injury is present, leaving gaps for microbes to penetrate deep into tissue. Skin is also critical for maintenance of water and electrolyte balance. It retains fluids along with electrolytes, but also can secrete them through sweat; this balance between retention and secretion of water and electrolytes allows for proper osmotic balance of tissue. The skin plays a crucial role in maintaining the temperature of the body. When the body gets overheated, the sweat glands release perspiration, which then absorbs heat and evaporates from the surface. The blood vessels will also dilate to increase surface area and dissipate heat outward. When the body becomes cold, shivering occurs to generate heat from the muscles. In addition, blood vessels constrict, and more blood flow is directed towards vital organs and away from face and apendages. The skin is crucial not only for regulating but also for providing insulation, sensing tempera-

ture, and other stimuli, such as pain and pressure. The skin is loaded with sensory receptors including the Meissner's corpuscle, the Pacinian corpuscle, the Ruffini corpuscle, the hair root plexus, and free nerve endings. These sensory receptors allow for the sensation of touch, heat, and pain. Along with acting as an energy reserve, the subcutaneous layer of skin is essential for insulating the body to maintain temperature homeostasis and provides mechanical padding. Finally, the skin is critical for the synthesis of Vitamin D. Vitamin D is produced via the stimulation of provitamin D3 from ultraviolet radiation from the sun. Provitamin D3 photolyzes into provitamin D3, which then isomerizes to Vitamin D3 (Holick Pincus 1987).

Dermal Wounds

A wound is a disturbance in the normal anatomic structure and function of the tissue. Wounds come in many forms and may include non-penetrating wounds that do not break the skin (abrasions and contusions), penetrating wounds, or other wounds (burns, frostbite, electrical wounds). For the remainder of this chapter, we will focus on features of dermal wounds such as penetrating wounds and burn injury. These wounds may result from pathologic processes beginning internally or externally, and they may also be acute or chronic [3]. Acute wounds usually proceed through an orderly and relatively quick reparative process that results in, at least to some extent, restoration of the native anatomy and functional integrity. On the other hand, chronic wounds often fail to heal through such an ordered healing process and often do not allow the tissue to regain its original features and integrity [4, 5]. Many environmental and genetic factors influence the rate and quality of dermal healing. These include, but are not limited to, age, nutrient availability at the wound site, lifestyle factors, use of medications, comorbidities, and infection [6–8].

Dermal wounds can heal through primary, secondary, or tertiary intention. The primary intention, also known as primary closure, generally takes place within the first few hours following wounding and involves bringing the two edges of the wound together also known as approximation. This process typically requires mechanical means such as adhesive strips, sutures, staples, or clips. Wounds that heal through primary intention usually close rapidly and have minimal scarring. In cases where the wound edges cannot be brought together, wounds are left open and allowed to close by secondary intention. In this process, wound contraction and re-epithelialization lead to closure, albeit at a slower rate than in primary intention. Often, wounds that require healing by secondary intention are large, traumatic wounds. Finally, tertiary intention occurs in wounds that cannot approximate the edges within 3–6 days, often in cases of inadequate blood supply or infection.

The process of wound healing requires precise coordination of signaling, both temporally and spatially. On one end of the spectrum, too little healing results in non-healing, open wounds that are susceptible to infection. At the other end, over-activation of the healing response results in a buildup of scar tissue and have detrimental effects on adjacent heathly functional tissue.

Dermal Wound Healing Is a Complex, Multistep Process that Involves Many Cell Types and Secreted Factors

Dermal wound healing encompasses three critical, overlapping phases that include activities of various cell types and secreted factors. The stages are essential to seal the wound and prevent water loss and infection (hemostasis/inflammation), regenerate lost tissue (tissue replacement/proliferation), and remodel the provisional extracellular matrix to restore native tissue function (resolution). The specific roles of chemokine signaling in these stages of healing will be discussed in more detail later in this chapter.

The primary objective of the hemostasis and inflammation phase is to halt blood loss and begin the process of cell migration to the wounded area. The inflammatory/hemostasis phase takes place in the initial 24 hours and can last up to 2 days. Signs of inflammation such as local edema and redness characterize this phase. In the inflammatory/hemostasis phase, blood that leaked during the initial injury coagulates through the aggregation of thrombocytes and platelets in a fibrin network, which is the product of thrombin cleaving fibrinogen. This network serves three primary roles: to act as a temporary barrier to infection, to form a provisional matrix for the migration of incoming cells, and to stimulate fibroblast proliferation. Soon after injury, neutrophils and other inflammatory cells traffic to the site. These cells release lysosomal enzymes that facilitate the breakdown and engulfment of damaged tissue. As this phase continues, activation of the complement system and platelet degranulation attract neutrophils, which produce reactive oxygen species and clean up cell debris. With time, monocytes are also attracted to the wound site where they become polarized to various phenotypes. They can then contribute to phagocytosis and release multiple secreted factors to promote proliferation and angiogenesis [9]. These activated macrophages play an essential role in initiating the second step of healing through the secretion of cytokines, pro-angiogenic factors, and fibrogenic factors.

In the tissue replacement/proliferation phase, the wound must begin to close, usually through a combination of contraction and proliferation of fibroblasts. This phase spans from roughly 48 h to 2 weeks post-injury [10]. An essential component of this process includes angiogenesis, which allows for the transport of oxygen, nutrients, and immune cells (via blood) to the wound bed [11]. The proliferation of local and recruited fibroblasts and the production of extracellular matrix components result in granulation tissue. These fibroblasts are highly synthetic producers of collagen and other extracellular matrix components. At the same time, keratinocytes migrate to re-epithelialize the wound. Contraction will bring the edges closer together, thus decreasing the area that will require re-epithelization.

In the final resolution phase, the provisional extracellular matrix (ECM) that has been produced by fibroblasts is remodeled to match the characteristics of the native tissue. This phase can begin weeks after the injury and last for more than a year afterward. The constituents of the provisional ECM, mainly composed of type 3 collagen, are broken down, reorganized, and resynthesized, and the content of type

1 collagen is increased. Compared to the granulation tissue, the resulting ECM is less abundant in cellular material and blood supply but better matches the biological and mechanical attributes of unwounded skin. Inflammation that took place during the early phases of injury is resolved during this phase as well. The migration of keratinocytes that were previously essential to re-epithelialize the wound is halted.

While dermal wounds sustained at the fetal stage can heal perfectly, adult dermal wounds often result in scarred or fibrotic tissue [12, 13]. In cases of large initial wounds, the resulting scars can be painful and disfiguring [14]. Scarring results when the wound does not correctly move from the proliferative phase to the resolution phase [15]. In skin, scarring may be classified by clinical phenotypes that include atrophic, hypertrophic, and keloid scars. Atrophic scars are flat areas that are depressed compared to the area of skin surrounding the scar [16]. On the other hand, raised scars include hypertrophic and keloid scars. While hypertrophic scars remain within the area bounded by the original wound, keloid scars are characterized by the fact that they extend beyond the border of the wound margin. Similar to other scars, keloids demonstrate increased collagen content and proliferation of fibroblasts compared to normal tissue [17, 18]. Scar tissue is in many ways inferior to healthy skin. While it can regain the barrier functions imperative for preventing infection and water loss, this scar tissue is mechanically less suitable than its healthy counterpart. Traditionally, biomechanics of skin tissue are evaluated based on the tensile failure, which is a measurement of the skin's ability to oppose rupturing. Recently, investigators have begun to assess more physiologically relevant parameters of skin biomechanics. Scar tissue is much less failure resistant and less compliant and has slower relaxation time [19].

The Discovery of Chemokines as Soluble Signaling Molecules

Historically, chemokines have been referred to by many names. These include small inducible secreted (SIS) family of cytokines, small inducible gene (SIG) family of cytokines, small cytokine (SCY) family of cytokines, platelet factor 4 (PF4) superfamily, and intracrine cytokine family [20–24]. Advancements in protein purification and molecular cloning in the early 1980s set the stage for the birth of chemokine research. In late 1986, Teizo Yoshimura and Kouji Matsushima isolated and purified a chemoattractant protein from the supernatant of media used to culture peripheral blood mononuclear cells (PMBCs) that had been activated by lipopolysaccharide (LPS) [25]. The protein was named monocyte-derived neutrophil chemotactic factor (MDNCF) for its activity and source. Shortly after, Ettore Appella's laboratory identified the N-terminal amino acid sequence of MDNCF. This discovery led to the observation that the N-terminus of MDNCF was highly similar to inflammation-related proteins that would later be classified as chemokines, including β-hemoglobin, platelet factor 4 (PF4), and interferon-gamma-inducible protein (IP) 10 [26].

In 1987, Yoshimura and Matsushima published their findings simultaneously along with the laboratories of Marco Baggiolini and Jo Van Damme, who had

purified the same protein [27, 28]. Because MDNCF acted as a chemoattractant for a subset of T lymphocytes, it was at this point renamed as interleukin-8 (IL-8) [29]. The Leonard and Oppenheim laboratories purified additional chemokines from the supernatant of LPS-activated PBMCs [30, 31] as other groups began to discover several chemokine family members. Oppenheim began to call these proteins chemokines, an abbreviated form of "chemoattractant cytokines."

Early on, chemokines were most recognized for their action on the innate and adaptive immune systems through modulating leukocyte communication and migration. Over time, various studies uncovered additional functions including hematopoiesis, angiogenesis, and oncogenesis. Some chemokines can co-stimulate T-cell differentiation, enhance survival of immune cells, or even act as antimicrobial peptides in peripheral epithelial tissues [32, 33].

Chemokines may be constitutively produced or induced rapidly under certain conditions. Some chemokines with homeostatic roles are produced and secreted at all times. These may be involved in immune surveillance or localization of immune cells. Those that promote inflammation are typically induced in response to some stimulus. These chemokines usually induce trafficking of leukocytes to the site of inflammation or infection and activate cells that will be critical for wound healing.

The Chemokine Family Tree

Chemokines are a large family of cytokines characterized by their ability to bind heparin, their small size, and the presence of four highly conserved cysteine amino acids. Although considerable homology between chemokines exists at the amino acid and overall structural level, they are required to transmit various signaling cues needed for a myriad of downstream functions. The 50 chemokines identified to date fall into four categories (C, CC, CXC, and CX3C), and with variability in splicing and enzymatic cleavage of the N- and C-termini results in hundreds of different molecules. In general, these chemokines signal through activation of G-protein-coupled receptors (GPCRs), atypical/alternative chemokine receptors (ACRs), or interaction with GAGs. In most cases, a single chemokine is recognized by multiple GPCRs, contributing to the essential diversity of the chemokine signaling system. Chemokine GPCRs can function as dimers or even higher-order oligomers, further adding to this complexity [34].

The more than 50 chemokines identified are divided into four categories (C, CC, CXC, and CX3C), based on the characteristic pattern of cysteine residues in proximity to the amino terminus of the mature proteins [35]. Chemokines in the CC family have the first two cysteine amino acids adjacent to one another. Those in the CXC family have a single, variable amino acid dividing these two cysteines. The CX3C group (CX3CL1) has three amino acids between these cysteines. Finally, the C family (XCL1 and XCL2) only has two of the usual four cysteines.

The tertiary structure of chemokines is well conserved. A flexible unfolded N-terminal acts as the signaling domain. Next, the core domain is made up of an

N-loop, a three-stranded β-sheet, and a C-terminal helix. For many of the CXC chemokines, the β-sheets act as an interface for dimerization. This results in a functional dimer consisting of six β-sheets and two α-helices. For CC family chemokines, dimerization uses residues near the N-terminus and overlapping with the CC cysteines [35]. CX3CL1 most commonly exists as a monomer and XCL1 forms a head-to-tail dimer [36, 37]. Interestingly, monomer forms of chemokines can bind their cognate receptors and activate downstream signaling [38, 39]. However, the body of evidence supports that chemokine oligomerization required interaction with GAGs.

Several amino acid motifs are critical for chemokine function. CXC chemokines can be categorized based on containing a three-amino-acid motif known as the ELR motif (Glu-Leu-Arg) at the N-terminus [40]. Classically, those lacking the ELR motif (CXCL4, CXCL9, and CXCL10) have been considered angiostatic, and those containing the motif (CXCL5, CXCL6, CXCL8, and others) have been considered angiogenic [41]. Chemokines containing the ELR motif bind to CXCR2, while those lacking the motif bind to CXCR3. Specific aspartic acid residues of chemokines can also be necessary for receptor activation. For example, Asp2.63, Asp4.50, and Asp6.58 are key for ligand binding with the CXCR4 receptor [42].

Chemokine Receptors and Signaling

Chemokine receptors, which are mainly seven transmembrane G-protein-coupled receptors (GPCRs) that belong to the class A rhodopsin-like family, primarily mediate chemokine signaling [43]. GPCRs make up approximately 3% of genes in the human genome and are vital for signaling ranging from the sensation of light and taste to the regulation of blood pressure [44]. GPCRs contain three extracellular loops (ECLs) and three intracellular loops (ICLs) that link α-helices. A disulfide bond between ECLs 1 and 2 restricts movement of the α-helices. When activated by agonist binding, GPCRs undergo structural changes on the cytoplasmic face, which catalyze the exchange of GDP on the alpha subunit of the heterotrimeric G protein [45]. In turn, the alpha and β-gamma subunits are free to dissociate and bind to downstream effector molecules, commonly cAMP, IP3, and others. Chemokine-stimulated GPCRs can trigger several downstream effects that eventually lead to actin polarization, changes in cell shape, and directed cell locomotion. Stimulation of the Gαi subunit causes the activation of calcium channels and inhibition of adenyl cyclases and cyclic adenosine monophosphate (cAMP) production. The G beta-gamma subunits are required for chemotaxis. The activation of these subunits triggers signaling effectors including GPCR kinases (GRKs), ion channels, and phospholipase C-β (PLC-B). PLC-B catalyzes PIP3 to inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 initiates a release of calcium from endoplasmic reticulum (ER) stores, while DAG can activate protein kinase C (PKC), a kinase involved in receptor regulation. Furthermore, both Gα and Gβγ subunits can

independently activate phosphoinositide 3-kinase (PI3K), resulting in the activation of the kinases Akt and the mitogen-activated proteins kinases (MAPKs).

In addition to GPCRs, alternative/atypical chemokine receptors (ACRs) can bind chemokines, leading to various outcomes. These receptors, while structurally similar to GPCRs, utilize different downstream signaling cascades [46]. These receptors do not contain the canonical DRYLAIV motif in the second intracellular loop, preventing them from binding G proteins [47]. However, ACRs internalize their chemokine ligands, a process termed "interceptors" [48]. Cognate chemokine-ACR binding can lead to various outcomes. While some leads to chemokine degradation [49], others lead to chemokine transport [50]. Finally, these ACRs can act in concert with GPCRs through dimerization. There are at least five members of this group including the Duffy antigen receptor for chemokines (DARC), D6, CXCR7, and CC chemokine receptor-like 1 and 2 (CCRL1 and CCRL2) [46].

In addition to transmitting signals through membrane-spanning receptors, there is growing evidence that chemokines influence cellular activities via interaction with extracellular matrix components, particularly glycosaminoglycans (GAGs) [51]. GAGs are long, unbranched polysaccharide structures composed of repeated disaccharide units. They are found attached to protein cores of proteoglycans or can be shed into the extracellular matrix (ECM). GAGs can influence chemokine signaling through the immobilization of chemokines, which results in the formation of "haptotactic" chemokine gradients. These concentration gradients then direct the migration of receptor-bearing cells [35]. GAGs immobilize chemokines on the endothelial surface using chemokine ligand sugar moieties. This ensures the directionality of chemotactic signals for leukocytes [52]. Chemokines that cannot attach to endothelial GAGs are either dispersed from the production site or diluted to concentrations below the receptor-activating threshold, consequently reducing leukocyte extravasation into tissues such as skin which has very high GAG content.

Chemokines play essential roles in both health and disease. They are known to regulate leukocyte migration, inflammation, angiogenesis, organ development, autoimmunity, and tumorigenesis. Many of these activities have particular relevance to normal dermal healing and are crucial to dysfunctional repair that results in the persistence of chronic wounds or the development of fibrotic outcomes. Specifically, chemokine signaling orchestrates the recruitment of specialized leukocytes to the wound, epithelization, angiogenesis, and ultimate tissue remodeling (Fig. 1.2).

Chemokines in the Hemostasis and Inflammation Stages of Wound Healing

Chemokines are crucial during this stage for inducing the recruitment of various cell types to the wound site. These chemokines induce migration and proliferation along with promoting the cellular debris clearance.

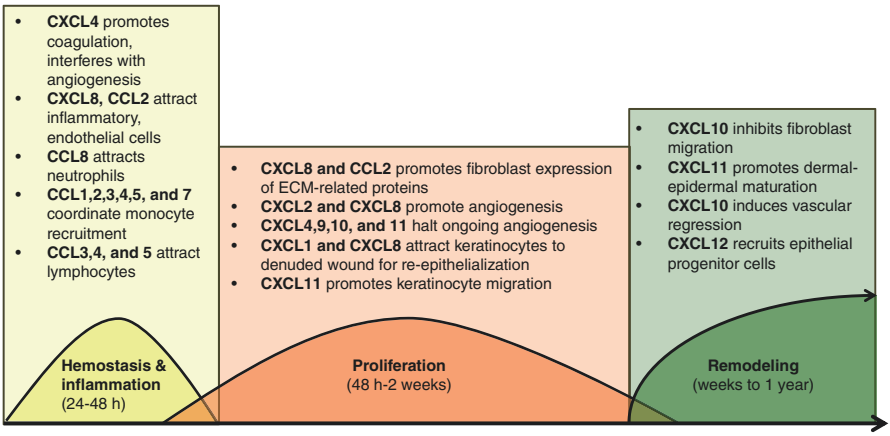

Fig. 1.2 Predominant chemokine and chemokine receptor activities vary with wound healing phase. In the immediate period following wounding, CXCL4, CXCL8, and CCL2 regulate the actions of endothelial cells while CCL1, 2, 3, 4, 5, 7, and 8 attract various immune cells to the site of tissue injury, promoting the inflammatory response. In the proliferative phase, CXCL8 and CCL2 promote the synthesis of extracellular matrix (ECM) components by fibroblasts, while CXCL1, CXCL8, and CXCL11 direct the migration of keratinocytes to close the wound. ELR motif-containing chemokines CXCL2 and CXCL8 promote angiogenesis early in the proliferative phase, and a slightly later increase in the angiostatic chemokines CXCL4, CXCL9, CXCL10, and CXCL11 prevents ongoing angiogenesis. Finally, in the remodeling phase, the new tissue must be reorganized to meet the biomechanical needs of the tissue. The CXCR3 ligands CXCL10 and CXCL11 act on multiple cell types to signal the end of the remodeling phase, while CXCL12 induces the migration of epithelial progenitor cells to the newly formed tissue. CXCL1 (GROa) C-X-C motif-containing ligand 1, CXCL2 (GRO2) C-X-C motif-containing ligand 2, CXCL4 (PF4) C-X-C motif-containing ligand 4, CXCL8 (IL8) C-X-C motif-containing ligand 8, CXCL9 (MIG) C-X-C motif-containing ligand 9, CXCL10 (IP-10) C-X-C motif-containing ligand 10, CXCL11 (I-TAC) C-X-C motif-containing ligand 11, CXCL12 (SDF-1) C-X-C motif-containing ligand 12, CCL1 CC motif chemokine ligand 1, CCL2 (MCP-1) CC motif chemokine ligand 2, CCL3 (MIP-1α) CC motif chemokine ligand 3, CCL4 (LAG1) CC motif chemokine ligand 4, CCL5 (RANTES) CC motif chemokine ligand 5, CCL7 (MCP-3) CC motif chemokine ligand 7, CCL8 (MCP-2) CC motif chemokine ligand 8

The chemokine CXCL4 plays an important role in the initial process of coagulation and inhibits the formation of blood vessels from occurring too early in the healing process. CXCL4 weakly attracts immune cells, but strongly impacts hemostasis through multiple mechanisms. In the normal coagulation cascade, heparin binds to and activates antithrombin III through a conformational change. Activated antithrombin III then inactivates thrombin and other proteases. Thrombin promotes platelet activation and coagulation through the conversion of fibrinogen to fibrin. CXCL4 can prevent the formation of this heparin/antithrombin III complex, thus slowing the process of thrombin inactivation and ultimately promoting coagulation [53]. At the same time, CXCL4 acts to suppress coagulation through inhibition of Hageman factor [54, 55] and stimulation of activated protein C, an inhibitor of clotting factors [56]. Angiogenesis is the formation of new blood vessels from existing

microvessels. This process is critical for wound healing. CXCL4 acts against angiogenesis through inhibiting both proliferation and migration of endothelial cells [57]. CXCL4 interferes with the key pro-angiogenic factors VEGF and bFGF [58, 59]. In another mode of action, CXCL4 binds and activates the CXCR3B receptor, leading to endothelial cell apoptosis [60]. Finally, CXCL4 can alter the course of angiogenesis through interaction with integrin proteins, which act as adhesion receptors on endothelial cells. CXCL4 has been shown to inhibit endothelial cell adhesion and migration on fibronectin and vitronectin through interaction with avb3, avb5, and a5b1 integrins [61]. CXCL4 also acts by attracting and activating granulocytes by promoting the release of multiple lysosomal enzymes by neutrophils [62]. CXCL4 suppresses hematopoiesis through inhibition of both the proliferation and maturation of megakaryocyte progenitor cells [63, 64]. Also, CXCL4 promotes the adhesion and quiescence of progenitor cells through crosstalk with chondroitin sulfate molecules and other pro-hematopoiesis chemokines [65]. Other chemokines play a role in controlling the migration of incoming cells. Platelet degranulation during this phase results in the release of cytokines including TGF-β, PDGF, and VEGF that act on macrophages, keratinocytes, and fibroblasts. In response, these cells produce chemokines CXCL8 and CCL2 to induce migration of inflammatory and endothelial cells to the wound area.

Various growth factors, cytokines, and chemokines coordinate inflammation that takes place in this phase, which together attract multiple cell types to begin the process of healing. It is mostly the job of CXC chemokines that are stored in blood platelets and released upon injury to initiate this response. Early on, activated platelets release pro-migratory factors that attract leukocytes, mainly neutrophils, to the wound to eliminate pathogens [66]. Neutrophil expression of CXCR2 primarily coordinates this process, which attracts them in response to CXCL8 [67]. To potentiate these effects, arriving neutrophils secrete CXCL8, resulting in the recruitment of more neutrophils. Along with pro-migratory functions, CXCL8 inhibits apoptosis of neutrophils that have been attracted [68] and increases endothelial permeability to allow leukocyte migration [69].

Following the initial neutrophil response, the primary leukocyte cell type switches to monocyte lineages, partially due to the effects of IL-6-mediated signaling [66, 70]. Several CC family chemokines, including CCL1, CCL2, CCL3, CCL4, CCL5, and CCL7, coordinate the recruitment of monocytes [71]. Monocytes express CCR2, the receptor for CCL2, a chemokine, which is produced by both neutrophils and macrophages themselves [72]. Triggered by surrounding chemokines and cytokines, monocytes polarize into different macrophage phenotypes, which act to remove cell debris, present antigens, and produce various secreted factors. Similar to these monocytes, chemokines regulate the migration of mast cells, which express multiple receptors [73]. Mast cells produce proinflammatory factors upon degranulation and can release chemokines to stimulate fibroblast proliferation.

Finally, lymphocytes are recruited to the wound to modulate the immune response. CCL3, CCL4, and CCL5 attract lymphocytes to the wound [67]. B lymphocytes produce antibodies, and T lymphocytes produce vital cytokines and interact with antigen-presenting cells to provide host defense.