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CYTOKINE SIGNALLING AND ITS ROLE IN WOUND HEALING.

Cytokine signaling is an important part of the human body regulation. Most cytokines are cell-secreted proteins from glial cells in the nervous system and are necessary for intracellular signaling. Most cytokines are local regulators that alert and activate lymphocytes. Some cytokine-signaling pathways involve hormones such as growth hormones and leptin, the hormone that controls fat storage. (Singer, A.J. & R.A. Clark 1999).

The immune system depends on cytokine signaling to keep the human body healthy. Macrophages and dendritic cells engulf foreign particles and send a cytokine signal to nearby dormant lymphocytes. The receptors on the lymphocytes recognize the signal and activate. Those cells are specialized to recognize certain antigens. The combination of the macrophages and activation of lymphocytes through cytokine signaling help keep the body in homeostasis — or the proper internal equilibrium. (Liekens, S. *et al* 2001).

Some cytokine signals are not local but rather travel a long distance throughout the body. These cytokines are sometimes classified as hormones. This classification is changing, however, because cytokines are not secreted from glands. Instead, they are secreted from glial cells of the nervous system (Khalil, N. 1999 Microbes Infect). These growth hormones are essential for embryonic development. Cytokines bind to receptors on target cells and activate a cascade of intercellular signals. The most common of these pathways is the protein kinase transduction cascade. After the cytokine binds to the receptor embedded in the membrane of the cell, inactive protein kinases are activated by a process known as phosphorylation.

Cytokine signaling pathway

Cytokine receptors contain one to three chains, one or more of which generally have limited similarity in the membrane-proximal region (often referred to as box1/box2 motifs). According to the nomenclature the ligand-binding subunit of a receptor is referred to as the alpha chain. Other signal transducing subunits are named beta chains, or gamma chains. All cytokine receptors are associated with one or more members of JAKs, which couple ligand binding to tyrosine phosphorylation of various signaling proteins (STATs) recruited to the receptor complex.

Molecular cloning of cytokine receptors and subsequent structure–function studies has revealed that unlike growth factor receptors, cytokine receptors are devoid of catalytic activity (Wahl, S.M. 1999). Nevertheless, interaction of a cytokine with its receptor rapidly induces tyrosine phosphorylation of the receptor and a variety of cellular proteins, suggesting that these receptors transmit their signals through cellular tyrosine kinases. During the past 10–15 years, a large amount of experimental data have accumulated to indicate that most

cytokines transmit their signals via a distinct family of tyrosine kinases termed *Janus* kinases or JAKs.

Cytokine receptors activate many signaling pathways generally by means of phosphotyrosine residues, which are recognized by SH2 domains on the signaling molecules (Wahl, S.M. *et al.* 1987). The STATs contain a carboxy-terminal SH2 domain, an SH3-like domain and several conserved amino-terminal regions, and a conserved region in the middle of the protein that binds DNA. Tyrosine phosphorylation of a carboxy-terminal site mediates homo- or heterodimerization through the SH2 domains, triggering movement to the nucleus and DNA binding.

A native un-liganded receptor in complex with a JAK is in a catalytically inactive latent state. Receptor dimerization/oligomerization due to ligand binding results in the juxtapositioning of the JAKs, which are in the vicinity through either homo- or heterodimeric interactions. The recruitment of JAKs appears to result in their phosphorylation, either via autophosphorylation and/or cross phosphorylation by other JAKs or via other families of tyrosine kinases. This activation is presumed to result in increased JAK activity. Activated JAKs then phosphorylate receptors on target tyrosine sites. The phosphotyrosine sites on the receptors can then serve as docking sites that allow the binding of other SH2-domain containing signaling molecules such as STATs, Src-kinases, protein phosphatases and other adaptor signaling proteins such as Shc, Grb2 and phosphatidylinositol 3-kinase (PI3K).

Cytokines in Wound Healing

The response to injury is a phylogenetically primitive, yet essential innate host immune response for restoration of tissue integrity. Tissue disruption in higher vertebrates, unlike lower vertebrates, results not in tissue regeneration, but in a rapid repair process leading to a fibrotic scar. Wound healing, whether initiated by trauma, microbes or foreign materials, proceeds via an overlapping pattern of events including coagulation, inflammation, epithelialization, formation of granulation tissue, matrix and tissue remodeling (Ashcroft, G.S. *et al.*1999). The process of repair is mediated in large part by interacting molecular signals, primarily cytokines, that motivate and orchestrate the manifold cellular activities which underscore inflammation and healing (Figure 1).

Response to injury is frequently modeled in the skin, but parallel coordinated and temporally regulated patterns of mediators and cellular events occur in most tissues subsequent to injury. The initial injury triggers coagulation and an acute local inflammatory response followed by mesenchymal cell recruitment, proliferation and matrix synthesis (McCartney-Francis, N.L. & S.M. Wahl, 2001). Failure to resolve the inflammation can lead to chronic nonhealing wounds, whereas uncontrolled matrix accumulation, often involving aberrant cytokine pathways, leads to excess scarring and fibrotic sequelae. Continuing progress in deciphering the essential and complex role of cytokines in wound healing provides opportunities to explore pathways to inhibit/enhance appropriate cytokines to control or modulate pathologic healing.

Platelet Activation and Cytokine Release

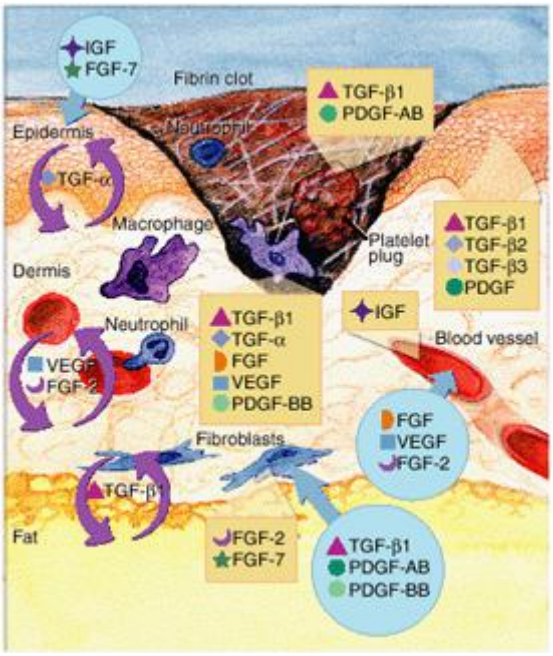

Figure 1. Wound healing is a complex process encompassing a number of overlapping phases, including inflammation, epithelialization, angiogenesis and matrix deposition. During inflammation, the formation of a blood clot re-establishes hemostasis and provides a provisional matrix for cell migration. Cytokines play an important role in the evolution of granulation tissue through recruitment of inflammatory leukocytes and stimulation of fibroblasts and epithelial cells. [Note: figure is adapted from reference 1.]

Most types of injury damage blood vessels, and coagulation is a rapid-fire response to initiate hemostasis and protect the host from excessive blood loss. With the adhesion, aggregation and degranulation of circulating platelets within the forming fibrin clot, a plethora of mediators and cytokines are released, including transforming growth factor beta (TGF-beta), platelet derived growth factor (PDGF), and vascular endothelial growth factor (VEGF), that influence tissue edema and initiate inflammation. VEGF, a vascular permeability factor, influences the extravasation of plasma proteins to create a temporary support structure upon which not only activated endothelial cells, but also leukocytes and epithelial cells subsequently migrate (Heldin, C.H. *et al*. 2001). Angiopoietin-1 (Ang-1), the ligand for Tie-2 receptors, is a natural antagonist for VEGF's effects on permeability, a key regulatory checkpoint to avoid excessive plasma leakage.

Latent TGF-beta1, released in large quantities by degranulating platelets, is activated from its latent complex by proteolytic and non-proteolytic mechanisms to influence wound healing from the initial insult and clot formation to the final phase of matrix deposition and remodeling. Active TGF-beta1 elicits the rapid chemotaxis of neutrophils and monocytes to the wound site in a dose-dependent manner through cell surface TGF-beta serine/threonine type I and II receptors and engagement of a Smad3-dependent signal. Autocrine expression of TGF-beta 1 by leukocytes and fibroblasts, in turn, induces these cells to generate additional cytokines including tumor necrosis factor alpha (TNF-a), interleukin 1 beta (IL-1 beta) and PDGF, as well as chemokines, as components of a cytokine cascade (Braddock, M. 2001). Such factors act to perpetuate the inflammatory cell response, mediating recruitment and activation of neutrophils and monocytes. In response to TGF- beta and other cytokines, which engage their respective cell surface receptors, intracellular signaling pathways are mobilized to drive phenotypic and functional responses in target cell populations. Among the upstream signaling cascades engaged in acute tissue injury are NF-?B, Egr-1, Smads, and

MAP kinases, which result in activation of many cognate target genes, including adhesion molecules, coagulation factors, cytokines and growth factors (Chen, W. & S.M. Wahl 1999).

Inflammation

Of the myriad of cytokines that have been investigated in terms of wound healing, TGF- beta 1 has undoubtedly the broadest effects. Despite the vast number of reports documenting the actions of TGF-beta in this context, both *in vitro* and *in vivo*, controversy remains as to its endogenous role. The paradoxical actions of TGF-beta are best appreciated in inflammation, where dependent upon the state of differentiation of the cell and the context of action, TGF-beta acts in a bi-directional manner. Moreover, this understanding of the nature of TGF-beta has led to the hypothesis that it may act as a therapeutic tool in some circumstances, but also a target for therapeutic intervention in others (Sundy, J.S. & B.F. Haynes 2000). Recent studies, in particular those utilizing genetically manipulated animal models, have highlighted the impact of TGF-beta on various aspects of wound healing, and surprisingly, not all of its effects are conducive to optimal healing. Intriguingly, mutations within the TGF-beta1 gene, or in the cell signaling intermediate Smad3, lead to normal or even accelerated cutaneous wound healing responses. The rate of healing of full-thickness wounds in Smad3 null mice was significantly greater than in their wild-type counterparts, associated with enhanced epithelialization and keratinocyte proliferation, and a markedly diminished inflammatory response. These observations have broad implications for understanding the role of TGF-beta in the endogenous wound healing response, in that an excess of TGF-beta may be a normal constituent of the response for rapid and optimal protection of the host. In the absence of infection, however, reduction of this overexuberant recruitment, inflammation and keratinocyte suppression may result in a more cosmetically acceptable scar (Gillitzer, R. & M. Goebeler ,2001). This knowledge may allow us to optimize the response by modulating selective cell pathways and to tailor therapy to specific cellular defects in pathological conditions such as chronic ulcers and fibrotic processes.

With the initial barrage of mediators, including TGF-beta, a chain reaction is set in motion, with recruitment, proliferation and activation of the cellular participants. Among the first cells to respond are the vascular endothelial cells, which not only respond to cytokines, but release them as well. Cytokine-induced enhancement of adhesion molecules (VCAM-1, ELAM-1, ICAM-1) on the endothelium provides the platform upon which circulating leukocytes expressing counter-adhesion molecules (integrins, selectins, Ig superfamily members) tether, slowing them down to sense the microenvironment and respond to chemotactic signals at the site of tissue injury (Cacalano, G. *et al.* 1994). Adhesion molecule interactions between blood leukocytes and endothelium enables transmigration from inside to outside the vessel wall in response to multiple chemotactic signals. In addition to the powerful chemotactic activity of TGF-beta1 for neutrophils and monocytes, multiple chemokines are released to entice leukocytes into the site of tissue injury. Chemokines are represented by several families of related molecules based on the spatial location of the cysteine residues. Deletion of genes for chemokines leads to specific alterations in wound healing, underlying their role in this process (Morales, J. *et al*. 1999).

Migrating through the provisional matrix (scaffolding) provided by the fibrin-enriched clot, leukocytes release proteases and engage in essential functions including phagocytosis of debris, microbes and degraded matrix components. Proteolytic activity is not constitutive, but transcriptionally driven by the cytokines, TGF-beta, IL-1beta and TNF-&alhpa;, released from multiple cellular sources (Leibovich, S.J. & R. Ross 1975). Neutrophil recruitment

typically peaks around 24-48 hours post wounding, followed by an increasing representation of monocytes which are essential for optimal wound healing.[1] Activation of these cells in the context of the wound microenvironment results in enhanced release of chemokines, recruitment of reinforcements, and amplification of the response, with the further release of cytokines, TNF-a, IL-1 and IL-6, that act as paracrine, autocrine and potentially, endocrine mediators of host defense. Antigen stimulation drives lymphocytic recruitment and activation, but at a delayed pace compared to the rapid acute response essential to maintain tissue integrity. Beyond the neutrophil, monocyte/macrophage and lymphocyte participants, mast cells have become increasingly recognized as active participants with increased numbers noted at sites of cutaneous injury (Clarke, R.A.F. 1996). Mast cells respond to monocyte chemotactic protein (MCP-1) and TGF-beta1, -beta2 and -beta3, and within the lesion, release mediators (histamine, proteoglycans, proteases, platelet activating factor, arachidonate metabolites) and cytokines, including TGF-beta and IL-4 . Once the inflammatory cells are activated, they become susceptible to TGF-beta1 mediated suppression to reverse the inflammatory process (Huttunen, M. *et al.* 2000). Moreover, IL-4 may also dampen the inflammatory response as the inciting agent/trauma is dealt with and promote collagen synthesis during the repair phase.

Re-epithelialization

Clearance of debris, foreign agents, and/or infectious organisms promotes resolution of inflammation, apoptosis, and the ensuing repair response that encompasses overlapping events involved in granulation tissue, angiogenesis, and re-epithelialization. Within hours, epithelial cells begin to proliferate, migrate and cover the exposed area to restore the functional integrity of the tissue. Re-epithelialization is critical to optimal wound healing not only because of reformation of a cutaneous barrier, but because of its role in wound contraction. Immature keratinocytes produce matrix metalloproteases (MMPs) and plasmin to dissociate from the basement membrane and facilitate their migration across the open wound bed in response to chemoattractants. The migration of epithelial cells occurs independently of proliferation, and depends upon a number of possible processes including growth factors, loss of contact with adjacent cells, and guidance by active contact. TGF-beta1 stimulates migration of keratinocytes *in vitro*, possibly by integrin regulation and/or provisional matrix deposition (Hebda, P.A. 1998). Behind the motile epidermal cells, basal cell keratinocyte proliferation is mediated by the local release of growth factors, with a parallel up-regulation of growth factor receptors including TNF-a, heparin-binding epidermal growth factor (EGF) and keratinocyte growth factor (KGF or FGF-7). Such growth factors are released not only by keratinocytes themselves, acting in an autocrine fashion, but also by mesenchymal cells and macrophages, as paracrine mediators. Numerous animal models in which cytokine genes have been deleted or over-expressed have provided further evidence that such factors are involved in the process of epithelialization. TGF-beta1, and -beta2 are potent inhibitors of keratinocyte proliferation, with the Smad3 pathway implicated as the negative modulator (Wikner, N.E. *et al.* 1998). Since epithelialization is significantly accelerated in mice null for the Smad3 gene, with unchecked keratinocyte proliferation, but impaired migration in response to TGF-beta1, the implication is that the early proliferative event is critical to normal epithelialization. Once contact is established with opposing keratinocytes, mitosis and migration stop, and in the skin, the cells differentiate into a stratified squamous epithelium above a newly generated basement membrane. Other factors secreted by keratinocytes may exert paracrine effects on dermal fibroblasts and macrophages. One such factor is a keratinocyte-derived non-glycosylated protein termed secretory leukocyte protease inhibitor (SLPI), which inhibits elastase, mast cell chymase, NF-?B and TGF-beta1 activation. In

rodents, SLPI is a macrophage-derived cytokine with autocrine and paracrine activities, but production by human macrophages has not yet been demonstrated. In mice, an absence of this mediator of innate host defense (SLPI null) is associated with aberrant healing (Barrandon, Y. & H. Green ,1987).

Granulation Tissue and Angiogenesis

Granulation tissue forms below the epithelium and is composed of inflammatory cells, fibroblasts and newly formed and forming vessels (Figure 2). This initial restructuring of the damaged tissue serves as a temporary barrier against the hostile external environment. Within granulation tissue, angiogenesis (*i.e.* the generation of new capillary blood vessels from pre-existing vasculature to provide nutrients and oxygen) is potentiated by hypoxia, nitric oxide (NO), VEGF and fibroblast growth factor 2 (FGF-2) and by the chemokines, MCP-1 and macrophage inflammatory protein (MIP-1a). VEGF, released from wound epithelium and from the extracellular matrix by endothelial-derived proteases, stimulates endothelial cell proliferation and increases vascular permeability.[2,30,31] VEGF may be transcriptionally up-regulated in response to NO which also influences vasodilatation, an early step in angiogenesis (Higashiyama, S. *et al.* 1991). In a cyclic fashion, VEGF also drives nitric oxide synthase (NOS) in endothelial cells. Endothelial cells express high affinity receptors for VEGF, VEGF R1 (Flt-1) and VEGF R2 (Flk-1), and represent a primary target of this angiogenic and vascular permeability factor. Mice heterozygous for targeted inactivation of VEGF or homozygous for inactivation of its receptors are embryonically lethal, confirming the essentiality of VEGF in angiogenesis. Besides VEGF, FGFs transduce signals via four protein tyrosine kinase receptors[34] to mediate key events involved in angiogenesis. FGFs recruit endothelial cells, and also direct their proliferation, differentiation and plasminogen activator synthesis. Clearly a multifactorial process, the cellular events underlying neovascularization are also impacted by TGF-beta1, EGF, TGF-a, endothelin 1, leptin, and indirectly, TNF-a and IL-1beta.

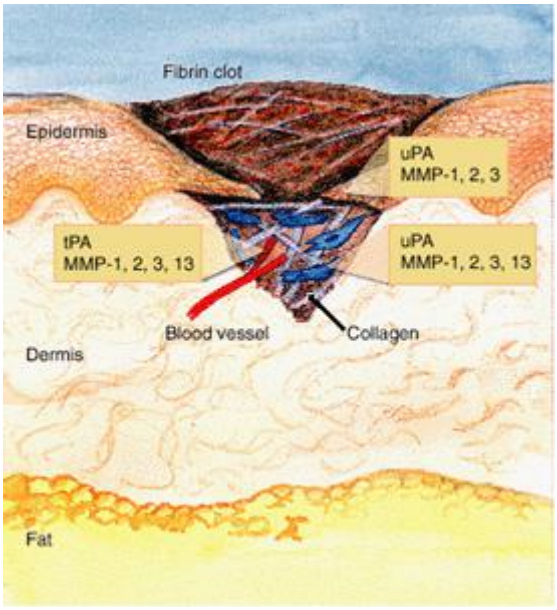

Figure 2. The remodeling phase (*i.e.* re-epithelialization and neovascularization) of wound healing is also cytokine-mediated. Degradation of fibrillar collagen and other matrix proteins is driven by serine proteases and MMPs under the control of the cytokine network. Granulation tissue forms below the epithelium and is composed of inflammatory cells, fibroblasts and newly formed and forming vessels. [Note: figure is adapted from reference 1.]

Of necessity, angiogenesis is a tightly controlled process. It is characterized not only by the presence of endogenous inducers, but also inhibitors which mediate a decline in the process as the granulation tissue, named for the granular appearance of the blood vessels in the wound, matures and scar remodeling continues. Among the identified endogenous inhibitors of re-vascularization are thrombospondin (TSP-1), IFN-?, IP-10, IL-12, IL-4 and the tissue

inhibitors of MMPs (TIMPs), in addition to the recently recognized activities of angiostatin and endostatin (reviewed in reference 2). Since loss of angiogenic control may have negative consequences as evident in tumors, rheumatoid arthritis, and endometriosis, identification of potential endogenous and therapeutic modulators continues.

Matrix Production and Scar Formation

With the generation of new vasculature, matrix-generating cells move into the granulation tissue. These fibroblasts degrade the provisional matrix via MMPs and respond to cytokine/growth factors by proliferating and synthesizing new extracellular matrix (ECM) to replace the injured tissue with a connective tissue scar. Although the stage is being set for tissue repair from the beginning (provisional matrix, platelet release of PDGF and TGF-beta, cytokine reservoir), fibroblasts migrate into the wound and matrix synthesis begins in earnest within a couple of days, continuing for several weeks to months. TGF-beta contributes to the fibrotic process by recruiting fibroblasts and stimulating their synthesis of collagens I, III, and V, proteoglycans, fibronectin and other ECM components. TGF-beta concurrently inhibits proteases while enhancing protease inhibitors, favoring matrix accumulation. *In vivo* studies have confirmed that exogenous TGF-beta1 increases granulation tissue, collagen formation, and wound tensile strength when applied locally or given systemically in animal models. Increased levels of TGF-beta are routinely associated with both normal reparative processes, as well as fibropathology. In Smad3 null mouse wounds, matrix deposition (fibronectin) could be restored by exogenous TGF-beta, implying a Smad3-independent pathway, whereas collagen deposition was not restored, suggesting a dichotomous Smad3-dependent regulation. The progressive increase in TGF-beta3 over time and its association with scarless fetal healing have implicated this member of the TGF-beta family in the cessation of matrix deposition. Other members of the TGF-beta superfamily may also contribute to the wound healing response. Activin A when over-expressed in basal keratinocytes stimulates mesenchymal matrix deposition, whereas BMP-6 over-expression inhibits epithelial proliferation.

PDGF, released at the outset by degranulating platelets, represents a family of cytokines consisting of two polypeptide chains (A and B) which form the dimers PDGF-AA, AB and B. In addition to platelets, PDGF is released by activated macrophages, endothelial cells, fibroblasts and smooth muscle cells and is a major player in regulating fibroblast and smooth muscle cell recruitment and proliferation through PDGF specific receptor-ligand interactions. Beyond its role in fibroblast migration and matrix deposition, PDGF-A and -B also up-regulate protease production, in contrast to the anti-protease activity of TGF-beta. PDGF represents the only FDA approved cytokine/growth factor for the clinical enhancement of delayed wound healing. Also central to repair are the FGFs, which signal mitogenesis and chemotaxis, underlying granulation tissue formation, and the production of MMPs. FGF-1 (acidic FGF) and FGF-2 (basic FGF) have been the most intensely studied, but the additional members of this family may also support tissue repair and/or have clinical application. The role of FGF-2 has been confirmed in the FGF-2 null mouse which shows not only retarded epithelialization but also reduced collagen production.

With many overlapping functional properties with FGFs, epidermal growth factor (EGF) orchestrates recruitment and growth of fibroblasts and epithelial cells in the evolution of granulation tissue. EGF and TGF-a, which share sequence homology, enhance epidermal regeneration and tensile strength in experimental models of chronic wounds. TNF-a and IL-1beta, key mediators of the inflammatory process, also contribute to the reparative phase

either directly by influencing endothelial and fibroblast functions or indirectly, by inducing additional cytokines and growth factors. IL-6 has also been shown to be crucial to epithelialization and influences granulation tissue formation, as shown in the wound healing studies of mice null for the IL-6 gene. As repair progresses, fibroblasts display increased expression levels of adhesion molecules and assume a myofibroblast phenotype, mediated in part by TGF-beta and PDGF-A and -B, to facilitate wound contraction.[48]

Remodeling Phase

The remodeling phase, during which collagen is synthesized, degraded and dramatically reorganized (as it is stabilized via molecular crosslinking into a scar), is also cytokine-mediated. Although repaired tissue seldom achieves its original strength, it provides an acceptable alternative. Degradation of fibrillar collagen and other matrix proteins is driven by serine proteases and MMPs under the control of the cytokine network. MMPs not only degrade matrix components, but also function as regulatory molecules by driving enzyme cascades and processing cytokines, matrix and adhesion molecules to generate biologically active fragments. TIMPs provide a natural counterbalance to the MMPs and disruption of this orderly balance can lead to excess or insufficient matrix degradation and ensuing tissue pathology. Similarly, there exists a naturally occurring inhibitor of elastase and other serine proteases (*i.e*. SLPI). The coordinated regulation of enzymes and their inhibitors ensures tight control of local proteolytic activity. In physiologic circumstances, these molecular brakes limit tissue degradation and facilitate accumulation of matrix and repair.

Aberrant Healing

Rapid clearance of the inciting agent and resolution of inflammation during healing minimizes scar formation, whereas persistence of the primary insult results in continued inflammation and chronic attempts at healing. Prolonged inflammation and proteolytic activity prevent healing as evident in ulcerative lesions. On the other hand, continued fibrosis in the skin leads to scarring and potentially, disfigurement, whereas progressive deposition of matrix in internal organs such as lungs, liver, kidney or brain compromises not only their structure, but also function, causing disease and death. Inhibitors of TGF-beta (*e.g*. antibodies, decorin, Smad 7, antisense oligonucleotides)[50-52] reduce scarring, as does local administration of exogenous TGF-beta3[36] or systemic delivery of TGF-beta1.[53] IFN-? is a natural antagonist of fibrogenesis through its ability to inhibit fibroblast proliferation and matrix production and has been shown to have clinical efficacy.[54,55] IL-10 may be considered anti-fibrotic via its anti-inflammatory activities,[56] as are inhibitors of TNF-a.[57]

Wound healing is a complex process encompassing a number of overlapping phases, including inflammation, epithelialization, angiogenesis and matrix deposition. Ultimately these processes are resolved or dampened leading to a mature wound and macroscopic scar formation. Although inflammation and repair mostly occur along a proscribed course, the sensitivity of the process is underscored by the consequences of disruption of the balance of regulatory cytokines. Consequently, cytokines, which are central to this constellation of events, have become targets for therapeutic intervention to modulate the wound healing process. Depending on the cytokine and its role, it may be appropriate to either enhance (recombinant cytokine, gene transfer) or inhibit (cytokine or receptor antibodies, soluble receptors, signal transduction inhibitors, antisense) the cytokine to achieve the desired outcome.

Wound healing is an evolutionarily conserved, complex, multicellular process that, in skin, aims at barrier restoration. This process involves the coordinated efforts of several cell types including keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets. The migration, infiltration, proliferation, and differentiation of these cells will culminate in an inflammatory response, the formation of new tissue and ultimately wound closure. This complex process is executed and regulated by an equally complex signaling network involving numerous growth factors, cytokines and chemokines. Of particular importance is the epidermal growth factor (EGF) family, transforming growth factor beta (TGF-beta) family, fibroblast growth factor (FGF) family, vascular endothelial growth factor (VEGF), granulocyte macrophage colony stimulating factor (GM-CSF), platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), interleukin (IL) family, and tumor necrosis factor-alpha family. Currently, patients are treated by three growth factors: PDGF-BB, bFGF, and GM-CSF. Only PDGF-BB has successfully completed randomized clinical trials in the Unites States. With gene therapy now in clinical trial and the discovery of biodegradable polymers, fibrin mesh, and human collagen serving as potential delivery systems other growth factors may soon be available to patients. This review will focus on the specific roles of these growth factors and cytokines during the wound healing process.

IMPAIRED WOUND HEALING

In wounds where oxygenation is not restored, healing is impaired. Temporary hypoxia after injury triggers wound healing, but prolonged or chronic hypoxia delays wound healing. In acute wounds, hypoxia serves as a signal that stimulates many aspects of the wound-healing process (Werner, S. *et al*. 1994).

Nonhealing wounds represent a significant cause of morbidity and mortality for a large portion of the population. One of the underlying mechanisms responsible for the failure of chronic wounds to heal is an out-of-control inflammatory response that is self-sustaining. Underappreciation of the inherent complexity of the healing wound has led to the failure of monotherapies, with no significant reduction in wound healing times. A model of the inflammatory profile of a nonhealing wound is one in which the equilibrium between synthesis and degradation has been shifted toward degradation. This review summarizes the current information regarding acute wound healing responses as contrasted to the delayed response characteristic of chronic wounds. In addition, some initial complexity theoretical models are proposed to define and explain the underlying pathophysiology.

Chronic wounds are, by definition, wounds that have failed to progress through the normal stages of healing and therefore enter a state of pathologic inflammation. As a result, the healing process is delayed, incomplete, and does not proceed in a coordinated manner, subsequently resulting in poor anatomical and functional outcome.1 These wounds cause a

major disability and are characterized by chronicity and frequent relapse. The differential diagnosis of the underlying etiology of a nonhealing wound is large , but most (~70%) ulcers are caused by ischemia, secondary to diabetes mellitus, venous stasis, and pressure.2 There are no large-scale, population-based studies that examine the prevalence and economic cost of chronic wounds in the United States. The prevalence of the 3 major types of nonhealing wounds is estimated to be between 3 and 6 million in the United States,3 with patients 65 years and older accounting for 85%.4 Nonhealing wounds result in enormous health care expenditures with the total cost being estimated at more than $3 billion per year.2

the financial estimates take into account the amount of lost work time, decreased productivity, disability payments, nor the cost of rehabilitation. In addition, the resultant psychosocial damage incurred by patients and their significant others, friends, and families is incalculable. Unfortunately, nonhealing wounds are prone to complications that not only effect the time to healing completion but also have a negative impact on the patients themselves. The complications of chronic wounds include functional limitations, infections, and malignant transfor- mation. Functional limitations include gait changes and difficulty ambulating. Many patients have chronic pain that decreases their quality of life. Another large category of complications is related to infections. Cellulitis, abscess formation, osteomyelitis, gangrene, and even sepsis all may occur as a result of an infected wound. Furthermore, chronic wounds have the potential for malignant transformation (ie, Marjolin's ulcer).5,6 Lastly, foot ulcers are one of the most common causes of nontraumatic amputation.7

Acute vs chronic inflammation

The inflammatory reaction of a chronic wound differs markedly from that of an acute healing wound. The normal function of inflammation in an acute wound is to prepare the wound bed for healing by removing necrotic tissue, debris, and bacterial contaminates as well as recruiting and activating fibroblasts (Coffey, Jr., R.J. *et al.* 1987). Under normal conditions, inflammation is a self-limiting process. In contrast, the inflammation in a chronic wound serves only to cause further injury and promote inflammation. In an acute wound, activated neutrophils are virtually nonexistent after the first 72 hours,

whereas in a chronic wound, neutrophils are present throughout the healing process. The possible reasons for the persistent presence of neutrophils include continued recruitment and activation due to tissue trauma by pressure, bacterial overgrowth, leukocyte trapping, or ischemic- reperfusion injury. The result of continued up-regulation of the inflammatory cascade leads to a markedly abnormal inflammatory profile for chronic wounds. The large

number of activated neutro- phils leads to excessive amounts of degradative matrix metallic proteinases (MMPs), especially MMP-8 and neutrophil-derived elastase. In a normal wound, all of the MMPs can be inhibited by the nonspecific proteinase inhibitor a2-macroglobulin and, specifically, by a small group of proteins called the b-tissue inhibitors of matrix metalloproteinases. Q In the non-healing wound, the MMPs are not balanced by an equal amount of tissue inhibitors of matrix metalloproteinases. As a result, an abnormal ratio of degradative vs protective enzymes ensues, which favors wound degradation. The excessive number of inflammatory cells also affects the cytokine profile in the wound. The inflammatory cytokines predominate, such as tumor necrosis factor a, and there is reduced concentration of factors that promote proliferation, such as platelet-derived growth factor (PDGF) and matrix deposition such as transforming growth factor b.19 Hence, the mitogenic activity of cells is sup- pressed in chronic wounds. The end result of the pathologic inflammation in a non- healing wound is more inflammation. The normal feedback mechanisms that end the inflammatory response are short- circuited, leading to an uncontrolled inflammatory positive feedback loop (Fig. 1). Neutrophils are activated, resulting in enzyme release and tissue degradation that further recruits neutrophils and continuing the cycle. As a result, fibroblasts are unable to make progress in depositing extracellular matrix because degradation of collagen occurs more rapidly than its synthesis. An uncontrolled inflammatory response therefore prevents rather than promotes wound healing. Not only is the inflammatory profile altered, but the fibroblasts in a chronic wound are also altered, as compared

Differential diagnosis of a nonhealing wound

Vascular Arterial Atherosclerosis, arteriovenous malformation Lymphatic Lymphedema Mixed venous-arterial Vasculitis Systemic lupus erythematosis, rheumatoid arthritis, scleroderma, polyarteritis nodosa, Wegener’s granulomatosis Venous Venous stasis Pressure Spinal cord injury, bedbound, elderly Neuropathic Diabetes, peripheral neuropathy Hematologic Polycythemia rubra vera, sickle cell disease Traumatic Burns, cold injury, radiation, factitious Neoplastic Basal carcinoma, squamous cell carcinoma, melanoma, Marjolin’s ulcer, Bowen’s disease Others Sarcoidosis, obesity, tropical ulcer, pyoderma gangrenosum, necrobiosis lipoidica diabetecorum

When a wound is subjected to continuous inflammation, it is unable to progress through the normal stages of wound healing. 20 N.B. Menke et al. with a normal wound. Studies have demonstrated that some fibroblasts have premature senescence that disrupts their normal functioning.22 These fibroblasts have impaired migration capacity as well as reduced

response to growth factors. Unpublished data from the authors has demonstrat- ed another subset of fibroblasts in chronic wounds that enter a hypersynthetic mode in which large amounts of collagen is being synthesized but not deposited in an efficient manner. We hypothesize that excessive inflammation from the large number of activated neutrophils results in an up-regulation of the synthetic function of a subset of fibroblasts. The excessive amount of proteases degrades the collagen, thereby preventing mature scar formation; the resultant breakdown products fuel further inflammation. Ultimately, the major difference in acute and chronic wounds is the rate of healing. The time to chronic wound healing completion is significantly delayed, and in some cases, the wounds, in fact, enlarge rather than improve because of the high protease microenvironment. Despite the differing mechanism for chronic ulcer formation, it has been shown that ulcers heal at a remarkably similar rate. The implication is that despite the different underlying patho- physiology of the various ulcer types, all ulcers have a final common pathway that leads to similar behaviors.

Treatment modalities

Because excessive inflammation is the ultimate cause of the poor healing found in chronic wounds, most treatments are aimed at reducing inflammation. Surgical debridement and wound care methods are aimed at decreasing the necrotic tissue and protease burden, thus providing a virtual resetting of the wound back into the acute healing phase. If the inflammation level is subsequently kept low, the wound is then able to progress forward and begin to heal. Another method for altering the inflammatory cascade involves using exogenous cytokines and growth factors to shift the degradative disequilibrium found in a chronic wound towards a more synthetic mode (Fig.2). Theoretically, alteration of the molecular environment of the wound may disrupt the inflammatory cycle and allow normal progression of the wound healing process. The only growth factor to have been shown to be clinically efficacious is recombinant PDGF; PDGF therapy, however, has been of limited clinical value because many chronic wounds do not respond. A likely cause is the persistent presence of excessive amount of proteases that have been shown to be capable of destroying PDGF and transforming growth factor b.25 Such growth factor therapy can only be successful once the inflammatory and protease microenvironment are under control. The degradative products of neutrophils represent a potential therapeutic target for modifying the excessive inflammation found in a chronic wound. Although more than 50 different products have been identified in the various neutrophil granules, only a few have been impli- cated as having important roles in neutrophil-mediated tissue injury.26 Neutrophil-derived elastase is an abundant primary granule serine proteinase with the ability to degrade a wide range of

connective tissue macromolecules. In addition, the collagenase MMP-8 is a major constituent of neutrophil secondary granules. The collagenases are critical enzymes in the breakdown of the extracellular matrix because they are unique in their ability to initiate degrada- tion of collagen. There is growing evidence suggesting that these enzymes may also play a role in the pathophysiology of chronic wounds by not only degrading the synthetic products of fibroblasts and peptide growth factors such as transforming growth factor b and PDGF but also important wound components such as fibronectin, a-1 antiprotease, and a-2 macroglobin.27,28 In addition to the tissue inhibitors of matrix metal- loproteinases, a number of other naturally occurring and synthetic inhibitors also exist. Included in this group of synthetic inhibitors are the tetracyclines. The inhibitory concentration 50 for doxycycline is approximately 25 mmol/L for MMP-8 and 300 mmol/L for MMP-1.29 There are growing numbers of studies suggesting that tetracycline derivatives can reduce the activity of MMPs as well as elastase, although the latter may be an indirect action.30-32 Once the proteolytic environment of the nonhealing wound is brought under control, then the positive production of matrix components can proceed with the development of granulation tissue, contraction, epithelization, and healing.

Complexity constructs

The complex, multiscale, multitemporal, hierarchical nature of the chronic wound has, thus far, been underap- preciated. Historically, research scientists and clinicians have focused on the study of individual cytokines and growth factors and how they affect the individual cells in vitro, out of context of the injury and the organism. The

Acute vs chronic disequilibrium.

Impaired wound healing 21 relationships among the various cell types and how they affect the dynamics of the wound healing process has not been properly studied to date. As a result, therapies to improve wound healing have failed or shown minimal improvements in outcomes. To account for these relation- ships, a more encompassing systems biologic approach is required—one that draws upon the nuanced methodologies of complexity theory. Fueled by the ongoing identification of numerous inflammatory and immune mediators produced in the normal response to acute soft tissue injury, ample evidence of this oversimplification can be found in the failure of single mediator-targeted therapies. By definition, a complex system is one that satisfies certain critical properties. First, it is composed of many parts that are coupled in a nonlinear fashion with no overlying controller that determines the behavior of the system (the system must be self-organizing). Second, the

network must show emergence. That is, the whole network must contain properties that are unpredictable from the parts/component pieces of the network or system. Typically, in such systems, as the number of components and their relationships increase, the complexity also increases. As a result of the spatiotemporal relationships of the system, the system may exhibit a variety of dynamic behaviors from stable flows to seemingly random oscillations. Complex systems may demonstrate many other dynamic behaviors. Possible behaviors include sensitivity to initial conditions where a small change in a variable may oftentimes cause a large effect in the dynamical outcome of the system (butterfly effect). Other properties include hysteresis, a property in which model parameter sets may generate nonunique solutions with the same initial parameter conditions and bifurcation dynamics where slight changes in parameter values can cause the system to exhibit radically different behaviors such as going from stable to unstable or stable to oscillatory over very small parametric ranges. Spatial and temporal patterns can exhibit properties of self- similarity or fractality in which changes in scale visualization of the pattern show no alteration in the basic structure of the pattern itself.38,39 Systems can exhibit self-organization and coalescence, evolution, and adaptation.40 Finally, system dynamics may contain a historical component in which the temporal dynamics of the system, at a particular time point, is functionally dependent upon some component of or all of the prior history (dynamical behaviors) of the system or its components. Reductionist approaches would argue that we should dissect the system in order to better understand its nature. One of the fundamental properties of synergistic complex systems, however, is that subfractionation can and does cause a loss of information (structural, relational, temporal, and hierarchical). Subsequently, destruction of emergent properties that are not contained within the subfraction pieces occurs. Despite that upper-level hierarchical structures may contain a super emergent dynamics set, sublevels may contain their own emergent dynamics that are not necessarily seen until the sublevel is separated from the whole system. One must be clear, however, that sublevel emergence is potentially artifactual because of the breaking of links to the whole system. These emergent properties are always present in the whole system but are invisible and cannot be appreciated from a reductionist approach. Thus, we cannot dissect a complex system in the parts, study them, and expect to fully understand the system. Dissection of the overall system destroys the important ingredient of semantics-syntax, context dependence, and self-reference; properties that are vital in the production of emergent behaviors. In short, a complex nonlinear system may be viewed as a system consisting of an extremely large and variable number of components. These components are capable of displaying significant temporal and spatial variability but, at the same time, can retain a high degree of interdependence between each other. What we learn from this is that topology and dynamics interact to produce system

behaviors. We find that relationships define dynamics, and dynamics can define relationships. Whether form follows function or function follows form becomes irrelevant as form and function are no longer separate entities but, rather, are intimately tied to each other. Thus, it is the combination of the dynamics of the various components themselves and the relationship or connections (nature and degree) between the components, which emerge as being of utmost importance. The components and their relationships are best expressed as dynamic evolutionary networks the topological and dynamic properties of which make it possible to quantify the system complexity and to predict potential behavior patterns and scenarios.41,42 We are now able to see how these concepts apply to the problem of understanding and modeling the dynamics of a healing wound. The wound healing system is composed of multiple levels of organizational scale including multiple cell types (fibroblasts, neutrophils, macrophages, etc), intercellular messengers (cytokines, chemokines, hormones, growth factors, etc), synthetic products (collagen, proteoglycans, etc), and enzymes (MMPs and tissue inhibitors of matrix metalloproteinases). The different cell types produce and are affected by the same cytokines in an autocrine and paracrine manner (multiple pathways of interaction). As a result, a signal may be quickly amplified in the system or dampened. The feedback loops that control the release and resulting effect of the cell signals result in nonlinear behavior. The behavior of the cells is determined not only by the current state of the wound but also the individual receptors that have been activated at earlier time points (state-dependent and history-dependant behaviors). The normal process of wound healing contains many separate but interacting events and host responses, including microcirculatory oxygen transport, immune and inflamma- tory responses, metabolic changes, and the neuroendocrine system modulation. An unknown degree of these and secondary responses will occur because complex changes in genome expression. Instead of viewing each of these (22 N.B. Menke et al)responses as separate and independent mechanisms involved in the development of a healed wound, it must be argued that events leading to healing should be viewed through the complexity theoretical lens and that wound healing is most effectively studied as a complex nonlinear system. In addition, the intracellular signaling pathways also consist of a complex system in itself (subsystem hierarchies). Within each cell, there are multiple pathways that, when activated by a receptor, amplify a signal. Many times, there are pathways that directly oppose each other that lead to measured responses (multiscale interaction). Cross-talk and divergent pathways lead to many complex relationships among the various signaling pathways. Therefore, a wound must be considered a complex system composed of components that are themselves complex systems (modular hierarchies of complex systems). Based on the previous discussion, it is clear that the study of the dynamics of wound healing cannot be approached solely through traditional reductionist methodologies.

We have established that the experimental understanding currently demonstrates that wound healing is a highly complex process that involves multiple cell types, all of which are interacting in a nonlinear fashion. These interactions take place over different time scales, across different organismal hierarchies, and generate relationships through which unpredictable emergent properties can occur. For example, there are many feedback loops and redundancies in the wound healing network that make

wound healing difficult to study. Feedback loops give rise to the potential for oscillatory behavior, chaotic dynamics, and other nonlinear phenomena. Topological structure of the network can give rise to unpredicted properties that are defined, not by dynamical relations but, rather, by spatio-structural ones. Thus, it is not sufficient to understand healing dynamics in terms of classic chemical reaction mechanisms; rather, we must examine the integration of topological (spatio structural and hierarchical) and temporal (multiscale) perspectives in order to truly understand the underlying mechanisms of wound healing.

Modeling

The previous description of complex nonlinear systems, as applied to wound healing, is highly attractive in attempting to explain our failure in creating successful mono target therapies. New understandings tell us that the cells involved in wound healing and the cytokines and growth factors used to transmit signals could be better categorized as highly complex, layered, modular networks with stochastic dynamics at risk for dysfunction. The extent to which acute and chronic soft tissue wounds can be modeled in terms of complex nonlinear systems, based on the preceding discussions and assumptions, is not at all clear. Examining the wound healing process as a complex system may allow for discovery and for analysis of possible important emergent properties of the system. These emergent properties which, again, can only be appreciated from the whole of the interactions within the system, are what hold the potential promise of breakthroughs in treatments and diagnostics. Understanding that wound healing is most realistically represented through complexity theoretical viewpoints now allows the traditional reductionist philosophy to be poten- tially complemented by the increasingly powerful new concepts of complexity theory.41,44,45 Effective studies of complex systems must be made by analysis of the parts, followed by a sophisticated reconstitution or synthesis in order to model as much of the system as is known at the given time. The resulting model must allow for almost an infinite number of input variables and provide a means to interact with it. In essence, the resulting model is itself desired to exhibit its own emergent properties, allowing science to catch the necessary glimpses that provide for the creation of new hypotheses. Manipulation of such a model will

provide direction as to the subsequent approach needed to take the next generation of steps in discovery. An important concept while discussing normal wound healing is the concept of feedback and feed forward loops. For wound healing to progress in a coordinated manner, each stage in the healing process must not only self- perpetuate but also initiate the next stage. Furthermore, each progressive stage must have the ability to turn off the previous stage once it has reached the critical point at which it is no longer dependant on the previous stage for positive feedback to continue. The interlocking feedback/feed for- ward loops allow for an orderly progression of wound healing and prevent any one stage from inhibiting and/or preventing progress into the next stage. The mechanism by which the different phases inhibit and promote each other is through the expression of soluble mediators such as growth factors and cytokines. Each cell type involved is capable of responding to and releasing a multitude of factors that allows the formation of feedback/forward loops to control cell concentrations as well as activity. In chronic wounds, failure of the normal wound healing process prevents normal wound closure. There are multiple etiologies for the formation of chronic wounds that include diabetic ulcers, venous stasis ulcers, and pressure ulcers, as discussed earlier. Although ischemia is an underlying cause, each of these wounds results from different pathologic mechanisms; however all non-healing wounds have remark- ably similar wound healing trajectories as well as similar inflammatory profiles. In the chronic wound, neutrophils are the predominant cell type with large amounts of proteases and inflammatory cytokines. Because of the excessive amount of inflammation, it is likely that these wounds have failed to progress from the predominantly inflammatory phase to a predominantly proliferative phase. The wound is not able to mature, and the disorganized extracellular matrix degrades liberating breakdown products, thus causing further inflammation. As a result, the chronic pressure ulcer becomes enmeshed in an inflammatory phase that self-perpetuates without providing enough forward momentum to propel the process to the next stage of wound healing. The effect of chronic inflammation is further tissue destruction and worsening of the chronic wound, thereby causing more inflammation and preventing any progress toward a healed wound. If the feedback/feed forward model is correct, not only is it imperative to slow down the self- perpetuating inflammatory cycle, but also, the wound must be matured into the proliferative phase. The inhibition of neutrophils and their products is not sufficient to restore the normal course of wound healing; the wound must enter a self-sustaining mode of tissue repair. Therefore, a milieu must be created in which the wound is forced to leave the chronic inflammatory phase, enter a proliferative phase, and subsequently move forward to the remodeling stage. The advantages of developing a successful mathematical/ computational model cannot be overstated. A successful model will allow the targeting of promising novel therapeutic approaches, thereby improving the success rates of

clinical studies. It will also promote the discovery of new therapeutic approaches that come directly out of the modeling process. The time from bench to bedside will be decreased as millions of experiments may be run on a model in a very short period, allowing the investigator to choose the best possible interventions before any clinical work being done. Refinement to the model can eventually include individual genetic data that could assist in personalizing treatment development.

Oxidative stress in normal and impaired wound repair

A large percentage of the population suffers from wound healing abnormalities, in particular aged individuals, patients with diabetes, and those treated with immunosuppressive drugs, chemo- or radiotherapy. The mechanisms underlying the impaired healing response are still poorly understood. Recent studies provided strong evidence for a role of oxidative stress in the pathogenesis of non-healing ulcers. Therefore, it is of major importance to identify and functionally characterize the factors involved in the generation and detoxification of reactive oxygen species (ROS). This will provide the basis for the development of new strategies for therapeutic intervention. In this review we summarize the current information about the roles of low molecular weight antioxidants and ROS-detoxifying enzymes in normal and impaired wound repair, and we report on the consequences of their modulation at the wound site.

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