The Basic Science of Wound Healing

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Summary: Understanding wound healing today involves much more than simply stating that there are three phases: "inflammation, proliferation, and maturation." Wound healing is a complex series of reactions and interactions among cells and "mediators." Each year, new mediators are discovered and our understanding of inflammatory mediators and cellular interactions grows. This article will attempt to provide a concise report of the current literature on wound healing by first reviewing the phases of wound healing followed by "the players" of wound healing: inflammatory mediators (cytokines, growth factors, proteases, eicosanoids, kinins, and more), nitric oxide, and the cellular elements. The discussion will end with a pictorial essay summarizing the wound-healing process. (*Plast. Reconstr. Surg.* 117 (Suppl.): 12S, 2006.)

Wound healing has traditionally been divided into three distinct phases: inflammation, proliferation, and remodeling.[1] Within each phase, a myriad of orchestrated reactions and interactions between cells and chemicals are put into action. There is considerable overlap for each phase, and lines separating them are blurred. This article will first provide the reader with a general overview of the wound-healing process, followed by a more detailed discussion of the cells and inflammatory mediators involved in wound healing.

PHASES OF WOUND HEALING

Table 1 summarizes the process of wound healing. Readers are encouraged to review Table 1 while reading this article.

Hemostasis and Inflammation (Immediately upon Injury through Days 4 to 6)

Hemostasis serves as the initiating step and foundation for the healing process. Inflammation results in vasodilation and increased vascular permeability. However, the first action the body takes immediately after wounding is to control bleeding. The injured blood vessel vasoconstricts, and the endothelium and nearby platelets activate the intrinsic part of the coagulation cascade. The clot that forms is made of collagen, platelets, thrombin, and fibronectin, and these factors release cytokines and growth factors that initiate the inflammatory response.[2] The fibrin clot also serves as a scaffold for invading cells, such as neutrophils, monocytes, fibroblasts, and endothelial cells, to use.[3] The clot also serves to concentrate the elaborated cytokines and growth factors.[4] The importance of hemostasis is illustrated by conditions that cause inadequate clot formation. Deficiency of factor XIII (the fibrin-stabilizing factor) is associated with impaired wound healing[5] secondary to decreased chemotaxis or decreased adhesion of cells in the inflammatory area.[6,7] Table 2 highlights the important functions the hemostatic and platelet-derived factors have in wound healing.

Chemotaxis and Activation

Immediately as the clot is formed, cellular signals are generated that result in a neutrophil response. As the inflammatory mediators accumulate, prostaglandins are elaborated and the nearby blood vessels vasodilate to allow for the increased cellular traffic as neutrophils are drawn into the injured area by interleukin (IL)-1, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, PF4, and bacterial "products."[8,9] Monocytes in the nearby tissue and blood are attracted to the area and transform into macrophages, usually around 48 to 96 hours after injury. Activation of the inflammatory cells is critical, especially for the macrophage. An activated macrophage is important for the transition into the proliferative phase. An activated macrophage will mediate an-

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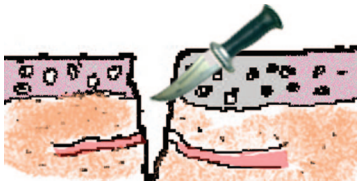
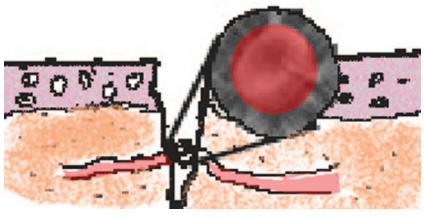
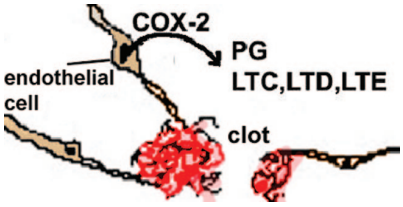
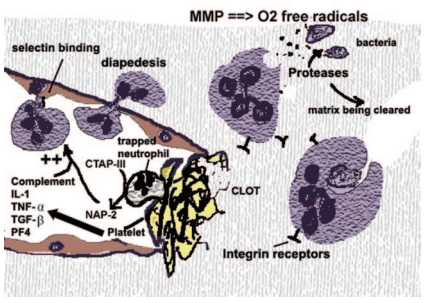
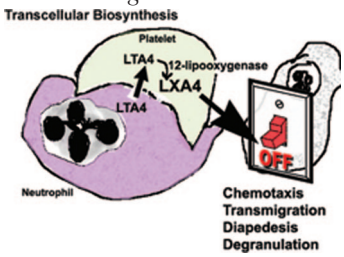
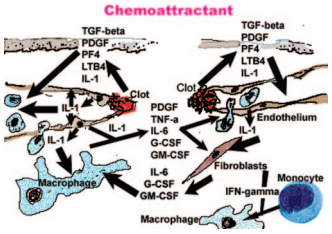
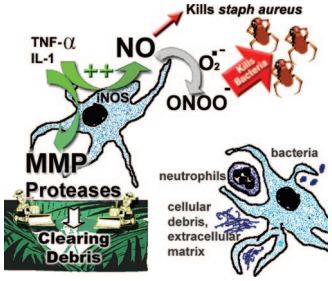
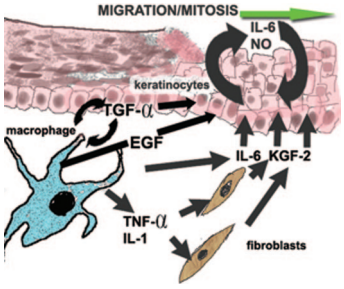
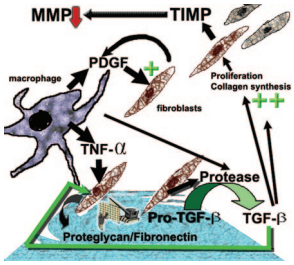
Table 1. Summary of Wound Healing

Event	Description
Hemostasis/Inflammatory Phase Wounding/hemostasis	Injured endothelial cells immediately vasoconstrict. Intrinsic coagulation pathway is activated and hemostasis is achieved. Platelets become involved and a clot is formed. Platelets in the immediate area of the wound aggregate to form a clot. They also release and synthesize several growth factors, cytokines, and other inflammatory proteins and activate the intrinsic coagulation pathway. Inflammatory mediators include thromboxane (to result in further vasoconstriction) and growth factors and cytokines to recruit more platelets and are chemoattractant for neutrophils and fibroblasts.
	
Hemostasis	Entrapped platelets within the thrombus release: Mediator: fibrin, plasma fibronectinResult: coagulation, chemoattraction, adhesion, scaffolding for cell migration Mediator: factor XIII (fibrin-stabilizing factor) Result: induces chemoattraction and adhesion Mediator: circulatory growth factors Result: regulation of chemoattraction, mitogenesis, fibroplasia Mediator: complement Result: antimicrobial activity, chemoattraction Mediator: cytokines, growth factorsResult: regulation of chemoattraction, mitogenesis, fibroplasia Mediator: fibronectin Result: early matrix, ligand for platelet aggregation Mediator: platelet-activating factor Result: platelet aggregation Mediator: thromboxane A_2 (via platelet COX-1) Result: vasoconstriction, platelet aggregation, chemotaxis Mediator: serotonin Result: induces vascular permeability, chemoattractant for neutrophils Mediator: platelet factor IV Result: chemotactic for fibroblasts and monocytes, neutralizes activity of heparin, inhibits collagenase
	
Inflammation	After a short time (1 hour) the endothelial cell's COX-2 enzyme is activated to synthesize prostaglandins, to cause vasodilation and platelet disaggregation, and leukotrienes, which results in increased vascular permeability, chemotaxis, and leukocyte adhesion (inflammation).
	
Role of neutrophil	Increased vascular permeability from endothelial prostaglandin and leukotriene synthesis allows neutrophils to adhere to activated endothelial cells via binding to the endothelial cell membrane receptor- selectin (which is expressed from LTB4 influence). The inflammatory effects from leukotrienes also cause the endothelium to form gaps (causing the capillary to "leak"), allow the neutrophils to slip through (diapedesis), and allow proteins to pass through, causing swelling. Neutrophils trapped in the clot converts CTAP-III to NAP2, a very potent and early neutrophil chemotactic protein. Platelets also release complement, IL-1, TNF-α, TGF-β, and PF4, all of which are chemoattractants for neutrophils. IL-1 and TNF stimulate adherence of neutrophils to endothelial cells by induction of intracellular adhesion molecules. Neutrophils attach themselves to the extracellular matrix by binding to the matrix with their integrin receptors. Other neutrophils are attracted to other sites and travel through the matrix easily, elaborating proteases and MMP to form oxygen free-radicals to kill bacteria and clear the extracellular matrix.
	

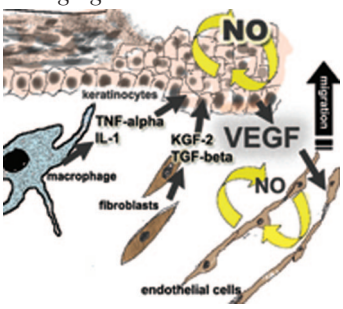
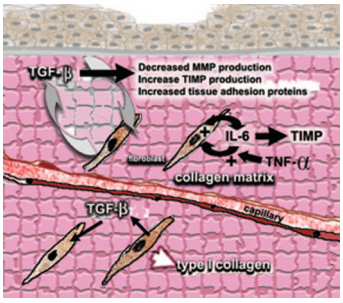
(Table continued)

Table 1. Continued

Event	Description
Turning inflammation off 	As the number of neutrophils and platelets increases, neutrophils and platelets bond. Increasing levels of LTA4 and LTB4 in the neutrophil pass through gaps between the cells and are acted on by the platelets' 12-lipoxygenase enzyme to form lipoxin A4 or lipoxin B5. The lipoxin is capable of turning off destructive inflammatory acts in a variety of cells.
Macrophages migrate into the wound 	Macrophages are attracted to the wound site by chemoattractants released by the platelets and the clot (TGF-β, PDGF, PF4, LTB4, and IL-1). Endothelial cells also help to bring macrophages and fibroblasts into the wound by synthesizing IL-1. IL-1 stimulates macrophages and fibroblasts to express more of their own chemoattractant cytokines and growth factors. Macrophages release the chemoattractants PDGF, TNF-α, IL-6, G-CSF, and GM-CSF to recruit more macrophages and fibroblasts. Fibroblasts elaborate IL-6, G-CSF, and GM-CSF to further enhance macrophage and fibroblast chemoattraction. Fibroblasts release IFN-γ, which causes monocytes to transform into macrophages.
Macrophages clear matrix of debris and bacteria 	iNOS is activated by rising concentrations of IL-1 and TNF-α, resulting in nitric oxide being synthesized and released. NO will kill pathogens (especially *Staphylococcus aureus*) and combine with free oxygen radicals to form the very toxic peroxynitrite ($ONOO^-$) and hydroxyl radicals. NO in the wound area also prevents viral DNA replication and serves as an immune regulator. Protease transcription is induced by several different inflammatory cytokines. IL-1 and TNF-α induce matrix metalloproteinase transcription in keratinocytes, fibroblasts, and macrophages (all the way through into the repair phase of wound healing). Proteases and matrix metalloproteinase clear the extracellular matrix from inflammatory debris and allow migration of cells through the matrix. Macrophages will also phagocytose apoptotic neutrophils, debris, and bacteria.
Proliferative phase Proliferation/epithelialization 	Epithelization is initiated by macrophages by stimulating fibroblasts with IL-1 and KGF-2. Fibroblasts, through the expression of KGF-2 and IL-6, cause keratinocytes to proliferate and migrate. Keratinocytes are then able to self-express IL-6 and NO to perpetuate the process. collagen replaces proteoglycan and fibronectin.
Matrix formation 	Macrophages initiate provisional matrix formation through the expression of PDGF and TNF-α. Fibroblasts perpetuate the process with autocrine and paracrine stimulation with more PDGF. Fibroblasts will initially synthesize proteoglycans and fibronectin to create the matrix. TGF-β exists in the matrix as an inactive pro-TGF-β. Macrophages and fibroblasts release proteases that activate the TGF-β, which in turn stimulates further fibroblast proliferation and collagen synthesis. It also prevents collagen degradation by causing TIMP secretion. TGF-β also causes increased fibronectin secretion and integrin receptor expression to allow cellular adhesion to the matrix.

(Table continued)

Table 1. Continued

Event	Description
Angiogenesis 	Macrophages stimulate keratinocytes to express VEGF with IL-1 and TNF-α. Fibroblasts stimulate keratinocytes to express VEGF with KGF-2 and TGF-β Keratinocytes direct angiogenesis at the wound edge by expressing VEGF. VEGF causes the proliferation of endothelial cells and the formation of capillaries. VEGF expression is upregulated by the presence of NO
Wound contraction 	(1) Fibroblasts already located within the injury site are triggered by macrophages (through TGF-β1 and PDGF) to transform phenotype into a myofibroblast (2). The fibroblast must be fully attached to the matrix by fibroblasts integrin receptors and fibronectin within the matrix. TGF-β1 and PDGF (3) both cause myofibroblasts to contract, closing the wound (4).
Maturation and remodeling phase Remodeling 	TGF-β directs collagen matrix construction. TGF-β levels peak in the wound 7 to 14 days in an incisional wound (hence the rationale for the timing of suture removal). The matrix becomes thicker and stronger as type I collagen replaces proteoglycan and fibronectin. TGF-β also upregulates expression of TIMP, decreases MMP production, and increases the expression of tissue adhesion proteins. TIMP production is also upregulated by IL-6. TNF-α stimulates the release of IL-6 by fibroblasts, further enhancing TIMP production.

giogenesis [by synthesizing vascular endothelial growth factor (VEGF), fibroblast growth factor, and TNF-α] and fibroplasia [by synthesizing TGF-β, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), IL-1, and TNF-α] and synthesize nitric oxide (NO) (from activation of inducible nitric oxide synthase by IL-1 and TNF-α).[10]

Neutrophils enter into the wound site and begin clearing it of invading bacteria and cellular debris. The neutrophil releases caustic proteolytic enzymes that will digest bacteria and nonviable tissue. The neutrophil has several different types of proteases grouped by their preferred target: proteins, amino acids, or the metal ion within the enzyme. Serine proteases have broad specificity (e.g., elastase), whereas metalloproteinase (which contains a zinc ion) specifically digests collagen. Both types of proteases will destroy the preexisting extracellular matrix in the wound area. The matrix in unwounded tissue is protected by an "armor" made of protease inhibitors.[11] The "antiprotease armor" can be overwhelmed and penetrated if the inflammatory response is extremely robust from a massive release of proteases. Neutrophils can also generate reactive oxygen free radicals (through a myeloperoxidase pathway) that combine with chlorine to help sterilize the wound of

Table 2. Hemostatic and Platelet-Derived Factors Associated with Wound Healing

Factor	Function
Hemostatic factors	
Fibrin, plasma fibronectin	Coagulation, chemoattraction, adhesion, scaffolding for cell migration
Factor XIII (fibrin-stabilizing factor)	Induces chemoattraction and adhesion
Circulatory growth factors	Regulation of chemoattraction, mitogenesis, fibroplasia
Complement	Antimicrobial activity, chemoattraction
Platelet-derived factors	
Cytokines, growth factors	Regulation of chemoattraction, mitogenesis, fibroplasia
Fibronectin	Early matrix, ligand for platelet aggregation
Platelet-activating factor	Platelet aggregation
Thromboxane A_2	Vasoconstriction, platelet aggregation, chemotaxis
Platelet factor IV	Chemotactic for fibroblasts and monocytes, neutralizes activity of heparin, inhibits collagenase
Serotonin	Induces vascular permeability, chemoattractant for neutrophils
Adenosine dinucleotide	Stimulates cell proliferation and migration, induces platelet aggregation

Adapted from Witte, M., and Barbul, A. General principles of wound healing. *Surg. Clin. North Am.* 77: 509, 1997.

bacteria.[11] Neutrophils shortly succumb to an unknown stimulus for apoptosis and are replaced by macrophages (which phagocytosize the dead neutrophils). Macrophages do not possess myeloperoxidase but do continue in pathogen killing by generating NO. The macrophages' iNOS is stimulated to synthesize very large quantities of NO by TNF and IL-1 that react with peroxide ion oxygen radicals to yield an even more toxic peroxynitrite and hydroxyl radicals.[12]

The damaged extracellular matrix is also cleared by matrix metalloproteinase (MMP), which is expressed by keratinocytes, fibroblasts, monocytes, and macrophages in response to TNF-α. MMP clears inflammatory debris and enables migration of individual wound cells through the extracellular matrix.[13]

For many years it was thought that, as with a fire, the inflammatory phase would just "burn itself out" when initiating exogenous stimuli or when signals were depleted.[14] There is now evidence that such a well-coordinated, elegant, and destructive process is organized as a series of reactions to produce "stop signals," referred to as "checkpoint controllers of inflammation."[14,15]

One may recall the synthesis of the eicosanoid inflammatory mediators: prostaglandin, prostacyclin, thromboxane, and leukotrienes. The lipoxygenase enzyme also synthesizes another lipid mediator: lipoxins (LXA_4 and LXB_4). Lipoxins and aspirin-triggered lipoxins are the stop signal for inflammation. Aspirin acetylates COX-2 enzyme (the inducible form of COX) and triggers the formation of 15-epimeric lipoxins.[14,16] Serhan's laboratory[14] has also identified autacoids synthesized by aspirin-acetylated COX-2 from omega-3 polyunsaturated fatty acids that display potent anti-inflammatory and "proresolving actions," which they termed "resolvins." Lipoxins are formed by platelets and leukocytes through a transcellular biosynthesis. Platelets cannot produce lipoxins on their own, but when platelets and neutrophils adhere to one another, the leukocyte produces leukotriene A_4 (via 5-lipoxygenase), which is transferred to the platelet; the platelet's 12-lipoxygenase converts it to lipoxin A_4 and B_4.[14] Neutrophils alone have also been shown to synthesize lipoxins. Clinical and experimental wound exudate studies have shown that the early appearance of leukotrienes and prostaglandins coincides with neutrophil infiltration to the site of inflammation. This is shortly followed by lipoxin biosynthesis, which is concurrent with spontaneous resolution of the inflammation.[14] Human neutrophils in peripheral blood were exposed to prostaglandin E_2 (PGE_2), which resulted in a switch in eicosanoid biosynthesis from predominantly LTB_4 (a 5-lipoxygenase-initiated pathway) to LXA_4, a 15-lipoxygenase product that "stops" polymorphonuclear neutrophil infiltration. In addition, PGE_2 initiates 15-lipooxygnease gene expression and RNA processing in vitro in a temporal frame that is consistent with the "switching on" of lipoxin production in vivo.[14] As Serhan and Chiang[14] point out, "these results indicate that functionally distinct lipid mediator profiles switch during acute exudate formation to 'reprogram' the exudate (polymorphonuclear neutrophil) to promote resolution." In addition, inhibition of prostaglandin products might alter the duration of resolution.

Aspirin-triggered lipoxins are the result of aspirin's inhibition of the COX-2 enzyme. Aspirin's ability to regulate neutrophil-mediated inflammation or cell proliferation continues to be a topic of interest, with new and alternative therapeutic uses for aspirin (e.g., decreasing the incidence of lung, colon, and breast cancer and preventing cardiovascular diseases).[17] Serhan's laboratory[14] has uncovered a new action

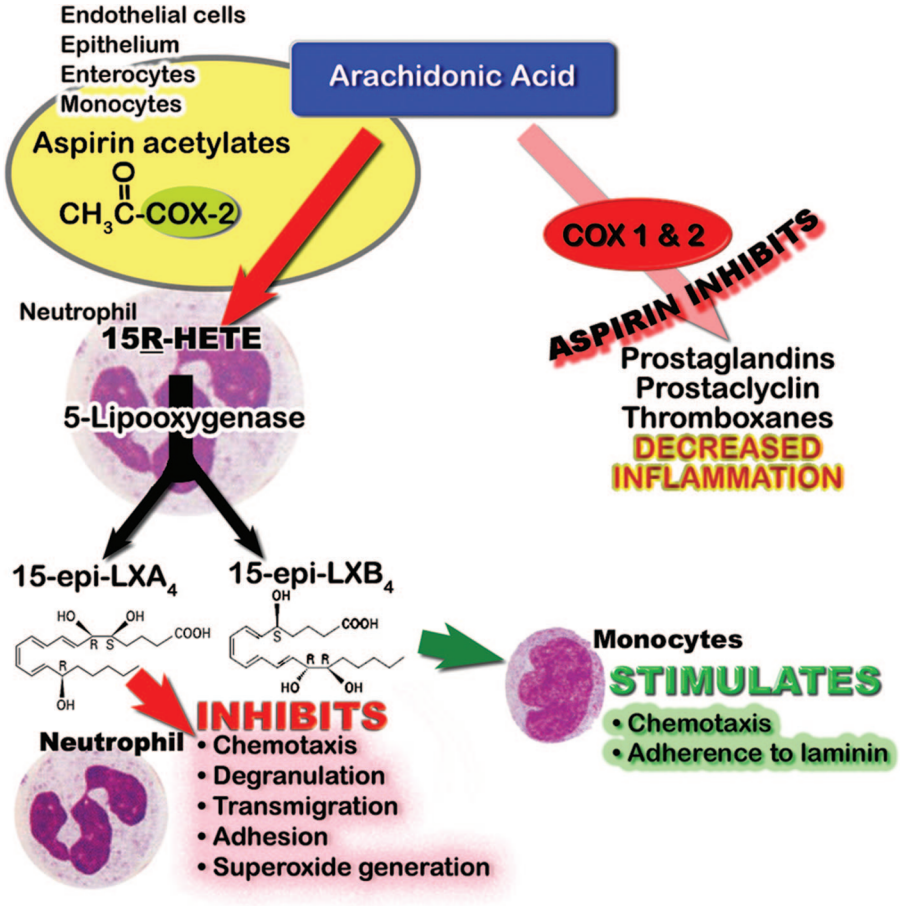


Fig. 1. Effect of aspirin of lipoxin synthesis.

of aspirin that involves COX-2–bearing cells, such as vascular endothelial cells or epithelial cells, and their co-activation with polymorphonuclear neutrophils. Inflammatory stimuli (e.g., TNF, lipopolysaccharide) induce COX-2 to generate 15R-HETE when aspirin is administered.[16] This intermediate carries a carbon-15 alcohol in the "R" configuration that is converted rapidly by 5-lipooxygenase in activated neutrophils to 15 epimeric-LX or aspirin-triggered lipoxins that carry their 15 position alcohol in the "R" configuration rather than 15S native LX. This lipoxin epimer may provide alternate explanations for aspirin's new therapeutic actions. The cellular effects of lipoxins and aspirin-triggered lipoxins are summarized in Figure 1.

Proliferative Phase: Epithelization, Angiogenesis, and Provisional Matrix Formation (Day 4 through 14)

Epithelial cells located on the skin edge begin proliferating and sending out projections to re-establish a protective barrier against fluid losses and further bacterial invasion. The stimulus for epithelial proliferation and chemotaxis is EGF and TGF-α produced by activated platelets and macrophages (fibroblasts do not appear to synthesize TGF-α).[18,19] Epithelization begins shortly after wounding and is first stimulated by inflammatory cytokines (IL-1 and TNF-α upregulate KGF gene expression in fibroblasts). In turn, fibroblasts synthesize and secrete keratinocyte growth factor (KGF)-1, KGF-2, and IL-6, which simulate neighboring keratinocytes to migrate in the wound area, proliferate, and differentiate in the epidermis.[20,21] In humans, it seems that KGF-2 is most important for directing this process.[22]

Fibroblasts and endothelial cells are the predominant cells proliferating during this phase. Endothelial cells located at intact venules are seduced by VEGF (secreted predominantly by keratinocytes on the wound edge, but also by macrophages, fibroblasts, platelets, and other endothelial cells) to begin forming new capillary tubes. Recall that keratinocytes can be stimulated

Table 3. Effect of Growth Factors on Fibroblast Proliferation

Factor	Effect	Comment
PDGF	Increases	
IFN-γ	Increases (low concentration)/decreases (high concentration)	
TGF-β	Increases (low concentration)/decreases (high concentration)	Via release of PDGF
FGF	Increases	
EGF	Increases	
IL-1	Increases	Via release of PDGF
TNF-α	Increases (low concentration)/decreases (high concentration)	Via increase of PDGF

to express VEGF by IL-1, TNF-α, TGF-β1, and KGF. NO is made by endothelial cells (from endothelial nitric oxide synthase eNOS) in response to hypoxia, and this in turn stimulates more VEGF production. The increased concentrations of NO also protect the new tissue from the toxic effects of ischemia and reperfusion injury[12] and cause endothelium to vasodilate.[10]

Fibroblasts migrate into the wound site from the surrounding tissue, become activated and begin synthesizing collagen, and proliferate. PDGF and EGF are the main signals to fibroblasts and are derived from platelets and macrophages (Table 3). PDGF expression by fibroblasts is amplified by autocrine and paracrine signaling. Fibroblasts already located in the wound site (termed "wound fibroblasts") begin synthesizing collagen and transform into myofibroblasts for wound contraction (induced by macrophage-secreted TGF-β1). They have less proliferation compared with the fibroblasts coming in from the wound periphery.[23–25] In response to PDGF, fibroblasts begin synthesizing a provisional matrix composed of collagen type III, glycosaminoglycans, and fibronectin.[26] Integrins are a matrix component that serves to anchor cells to the provisional matrix and is upregulated by TNF-α.[27] In a normal incisional wound, TGF-β peaks around day 7 to 14 and directs extracellular matrix production and a decrease in its degradation. TGF-β causes fibroblasts to synthesize type I collagen, decrease production of MMP, enhance production of tissue inhibitors of metalloproteinase, and increase production of cell adhesion proteins.[12] The signal to turn off activity seems to come from interferon-in-

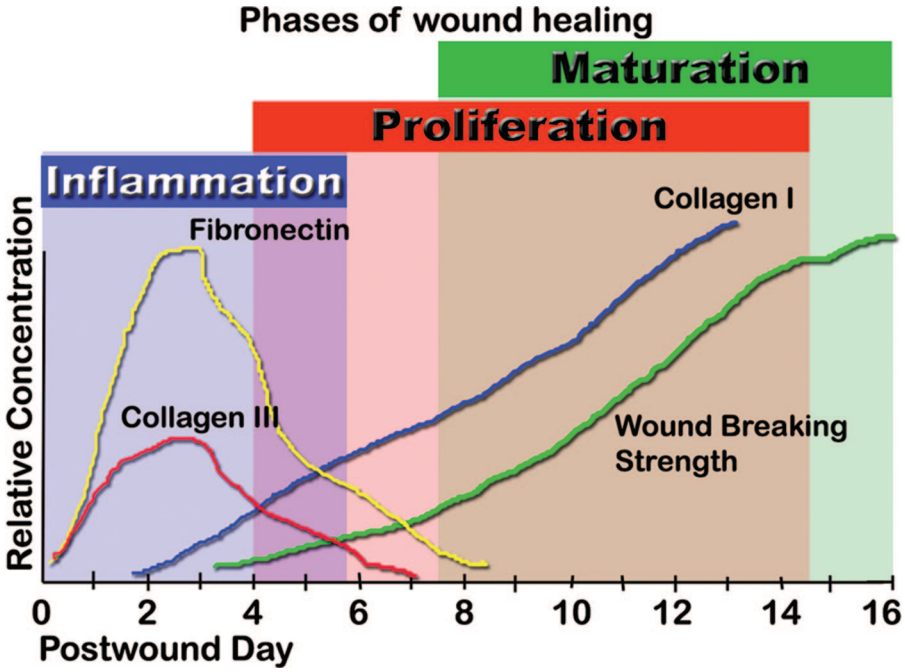


Fig. 2. The deposition of wound matrix components over time. Although fibronectin and collagen type III constitute the early matrix, collagen type I accumulates later, corresponding to the increase in wound-breaking strength. Adapted from Witte, M., and Barbul, A. General principles of wound healing. *Surg. Clin. North Am.* 77: 509, 1997.

ducible protein (IP-10), which inhibits EGF-induced fibroblast motility and thereby limits fibroblast recruitment, interferons themselves, and PF4, which has a negative mitogenic effect on fibroblasts.[28]

Larger wounds healing by secondary intention are still directed, in part, by TGF-β, which causes wound contracture (transforming "wound fibroblasts" into myofibroblasts) and epithelization.[29] Components of the wound matrix at different points are summarized in Figure 2.

Maturation and Remodeling (Day 8 through 1 Year)

Clinically, the maturation and remodeling phase is perhaps the most important. The main feature of this phase is the deposition of collagen in an organized and well-mannered network. If patients have matrix deposition problems (from diet or disease), then the wound's strength will be greatly compromised. If there is excessive collagen synthesis, then a hypertrophic scar or keloid can result.

The building of the wound matrix follows a pattern. Initially, the matrix is composed mainly of fibrin and fibronectin (arising from the efforts for hemostasis and by macrophages).[3] Glycosaminoglycans, proteoglycans, and other proteins (such as secreted protein acidic rich in cysteine, or SPARC) are synthesized next by the fibroblasts.[2] This haphazard and disorganized collection of glycans provides a preliminary framework for the new matrix. This temporary matrix is replaced by a stronger and organized matrix made of collagen. The collagen in uninjured skin is 80 to 90 percent type I and 10 to 20 percent type III. In granulation tissue, collagen type III comprises 30 percent, and in the mature scar, it is back down to 10 percent.[24] The appearance of collagen type III also coincides with the presence of fibronectin. It has been proposed that the coating of denatured collagen with fibronectin facilitates its phagocytosis.[30] The role for the early and increased deposition of type III collagen (which does not significantly contribute to the strength of the wound) is unclear. The matrix remodeling proteinases, MMPs (there are several different ones, each specific for a type(s) of collagen and under the influence and control of different cytokines), are influenced by changing concentrations of TGF-β, PDGF, IL-1, and EGF. MMP activity is further suppressed by tissue inhibitors of metalloproteinase, whose production by fibroblasts is upregulated by TGF-β and IL-6; TNF-α stimulates the release of IL-6 by fibroblasts.[28]

Grinnell[7] wrote an elegant discussion on the relationship of fibroblasts and other dermal cells to the matrix as a three-dimensional structure. Early in wound healing, the matrix is thin and compliant and allows fibroblasts, neutrophils, lymphocytes, and macrophages to easily maneuver through it. As the matrix becomes denser with thicker, stronger collagen fibrils, it becomes stiff and less compliant. The fibroblasts are capable of "adaptive response" to the changing mechanical loading on the matrix as it matures. Before isometric tension develops, remodeling of the compliant matrix depends on the cell migration throughout the matrix and proteolysis of the matrix proteins. Isometric tension is defined as a situation in which internal and external mechanical forces are balanced such that cell contraction occurs without cell shortening or lengthening. At an early point, cellular adhesion to the matrix is not possible. As the matrix stiffness increases and isometric tension develops, lysophosphatidic acid–stimulated remodeling switches to a Rho-kinase-dependent myosin-light-chain phosphorylation mechanism of contraction.

PDGF stimulates the fibroblast dendritic network to swell and reach out, which it can do when the matrix is compliant. Lysophosphatidic acid, the simplest of all glycerol phospholipids, causes the dendritic branches of the fibroblasts to retract. Lysophosphatidic acid is widely distributed in mammalian tissues and serum and is generated by cleavage from membranes of stimulated cells (most likely from platelets for wound healing).[31] Cellular effects of lysophosphatidic acid can be categorized as "growth-related" or "cytoskeleton-dependent," resulting in the modulation of adhesion, chemotaxis, contraction, or aggregation. As a mitogen for fibroblasts and with additional effects on endothelial cells, macrophages, and vascular smooth muscle cells, lysophosphatidic acid has been implicated in wound healing,[32] because it activates its associated G-protein–coupled receptors, three of which have been identified. Lysophosphatidic acid receptors couple to at least three distinct G-proteins and thereby activate multiple signal transduction pathways, particularly those initiated by the small GTPase Ras, Rho, and Rac.

To increase contractility more, fibroblasts differentiate into myofibroblasts under the influence of TGF-β. Differentiation is signaled by cell interaction with an alternatively spliced form of fibronectin that causes the fibroblast to increase its expression of α-smooth muscle actin isotype, which has been shown to be linked to cell

contractility.[7] Because the fibroblasts are anchored and not free floating, they can organize to form focal adhesions that give the myofibroblasts the mechanical leverage to contract. TGF-β only stimulates the differentiation of fibroblasts in a restrained matrix; therefore, switching between mechanically loaded and unloaded conditions regulates the differentiation and regression of myofibroblasts. Unloading of mechanical contraction results in apoptosis and decreased collagen synthesis, with the net effect of an improved scar. Persistent mechanical loading creates the pathological condition of contracture and results in hypertrophic or widened scars caused by the persistence of fibroblasts and collagen synthesis. Interestingly, fibroblast-collagen matrix remodeling results in plasma membrane tears from mechanical changes that accompany rapid remodeling upon release of restrained collagen. These membrane tears result in activation of phospholipid and mitogen-activated protein-kinase signaling pathways. The significance of this is not known.[7]

Net collagen synthesis will continue for at least 4 to 5 weeks after wounding. The increased rate of collagen synthesis during wound healing is from not only an increase in the number of fibroblasts but also a net increase in the collagen production per cell.[33,34] The collagen that is initially laid down is thinner than collagen in uninjured skin and is orientated parallel to the skin (instead of the basket weave pattern seen in uninjured skin). Over time, the initial collagen threads are reabsorbed and deposited thicker and organized along the stress lines. These changes are also accompanied by a wound with an increased tensile strength, indicating a positive correlation between collagen fiber thickness/orientation and tensile strength.[2] The collagen found in granulation tissue is biochemically different from collagen from uninjured skin. Granulation tissue collagen has a greater hydroxylation and glycosylation of lysine residues, and this increase of glycosylation correlates with the thinner fiber size.[35] The collagen in the scar (even after a year of maturing) will never become as organized the collagen found in uninjured skin. Wound strength also never returns to 100 percent. At 1 week, the wound only has 3 percent of its final strength; at 3 weeks, it is 30 percent; and at 3 months (and beyond), it is approximately 80 percent.[36]

THE ELEMENTS OF WOUND HEALING

Inflammatory Mediators

"Inflammatory mediator" is an all-encompassing and confusing label given to a collection of soluble factors released by damaged and nearby cells, platelets, and leukocytes in an attempt to control the damage and begin healing. Inflammatory mediators include, but are not limited to, cytokines, growth factors, proteases, eicosanoids, kinins, and cellular metabolites.

Eicosanoids

Eicosanoids comprise a family of biologically active, oxygenated arachidonic acid metabolites, including prostaglandins, prostacyclin, thromboxanes, leukotrienes, and lipoxins. A phospholipase (usually phospholipase A_2, but others exist) in lysosomes or bound to cell membranes is released in response to specific and nonspecific stimuli (e.g., cellular trauma, including ischemia and hypoxia,[37,38] oxygen free radicals,[39] or osmotic stress).[40,41] Phospholipase A_2 (PLA_2) moves to the cell membrane and hydrolyzes arachidonic acid off of a cellular membrane lysophospholipid (usually phosphatidylcholine). This cleavage step is rate-limiting in the production of biologically relevant arachidonate metabolites. Other hormones and growth factors, including EGF and PDGF, activate PLA_2 directly through tyrosine residue kinase activity. After deesterification, arachidonic acid is rapidly reesterified into membrane lipids or avidly bound by intracellular proteins,[42] in which case it becomes unavailable for further metabolism. Should it escape reesterification and protein binding, free arachidonic acid becomes available as a substrate for one of three major enzymatic transformations, the common result of which is the incorporation of oxygen atoms at various sites of the fatty acid backbone and ring formation[42–44] to result in the formation of biologically active molecules, or "eicosanoids." Arachidonic acid can be converted into biologically active compounds by cyclooxygenase-, lipoxygenase-, or cytochrome P-450–mediated metabolism. The cytochrome P-450–dependent oxygenation of arachidonic acid mediates the formation of epoxyeicosatrienoic acids, their corresponding diols, mono-, di-, and tri-hydroxyeicosatetraenoic acids, and monooxygenated arachidonic acid derivatives.[45] No further discussion will follow on the cytochrome P-450–dependent oxygenation of arachidonic acid. The cyclooxygenase and lipoxygenase pathways are discussed in more detail below.

Cyclooxygenase pathway. The generation of prostaglandins is mediated by two different enzymes, COX-1 and COX-2. Prostaglandins are divided into series based on structural features, as coded by a letter (PGD, PGE, PGF, PGG, and PGH) and a subscript numeral (e.g., 1, 2) that indicate the number of double bonds in the compound.[46] The

most important ones in inflammation are PGE_2, PGD_2, PGF_2-α, PGI_2 (prostacyclin), and TxA_2 (thromboxane), each of which is derived by the action of a specific enzyme. Some of these enzymes have restricted tissue distribution. For example, platelets contain the enzyme thromboxane synthetase, and hence TxA_2 is the major product in these cells. TxA_2, a potent platelet-aggregating agent and vasoconstrictor, is itself unstable and rapidly converted to its inactive form, TxB_2. Vascular endothelium lacks thromboxane synthetase but possesses prostacyclin synthetase, which leads to the formation of prostacyclin (PGI_2) and its stable endproduct, $PGF_1\alpha$. Prostacyclin is a vasodilator and a potent inhibitor of platelet aggregation. It also markedly potentiates the permeability-increasing and chemotactic effects of other mediators.[47]

Cyclooxygenase has received a lot of attention recently because of select COX-2 inhibitors. COX-1 is a constitutive form and is considered a "housekeeping enzyme" involved in physiological reactions such as regulating renal and vascular homeostasis and gastric mucosa protection.[29] The COX-2 enzyme is considered an "immediate early" gene that can be synthesized rapidly in response to a wide variety of growth factors, cytokines, and hormones, particularly in the course of the inflammatory process.[48,49] COX-1 is thought to be involved in normal skin homeostasis and does not respond to inflammatory mediators.[48] However, COX-1 can respond and trigger an inflammatory response to generate prostaglandins if the concentration of arachidonic acid is high.[50] COX-2, on the other hand, can generate prostaglandin when arachidonic acid concentrations are low. In response to injury, COX-2 is induced in keratinocytes, macrophages, and endothelium in the granulation tissue[50] (Fig. 3).

The prostaglandins are also involved in the pathogenesis of pain and fever in inflammation. PGE_2 is hyperalgesic, causing the skin to become hypersensitive to painful stimuli.[51] It causes a marked increase in pain from suboptimal concentrations of intradermal histamine and bradykinin and interacts with cytokines to cause fever during infection. PGD_2 is the major metabolite of the cyclooxygenase pathway in mast cells; along with PGE_2 and $PGF_2\alpha$ (which are more widely distributed), it causes vasodilation and potentiates edema formation.

Lipoxygenase pathway. The initial products are generated by three different lipoxygenases, which are present in only a few types of cells. 5-Lipoxygenase (5-LO) is the predominant enzyme in neutrophils.[52] On cell activation, it translocates to the nuclear membrane and interacts with a membrane-associated regulatory protein (5-lipoxygenase–activating protein) to form the active enzyme complex. The main product, 5-HETE, which is chemotactic for neutrophils, is converted into a family of compounds collectively called leukotrienes. LTB_4 is a potent chemotactic agent and activator of neutrophil functional responses, such as

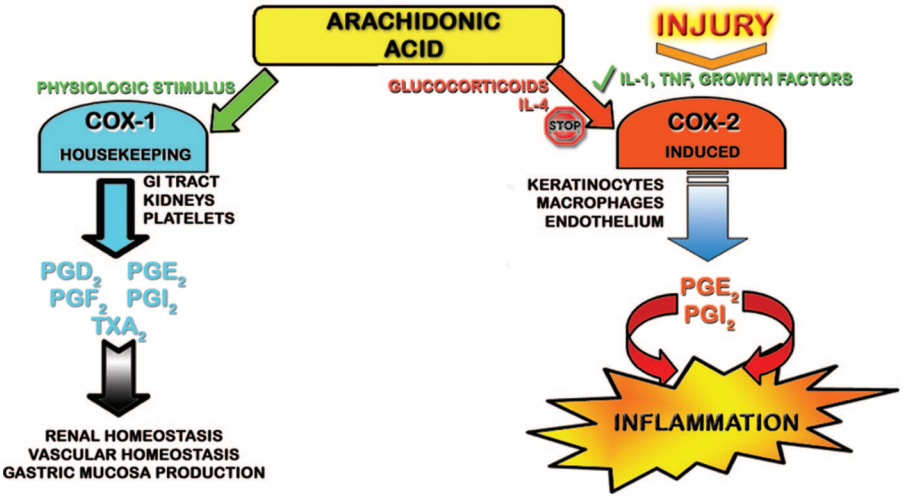


Fig. 3. COX-1 and COX-2 enyzmes. The COX-1 enzyme is stimulated by physiologic stimuli and is a housekeeping enzyme involved in maintaining hemostasis and gastric mucosa production. COX-2 enzyme is found in keratinocytes, macrophages, and endothelium and is induced by growth factors and cytokines released in response to injury. It also initiates inflammation.

aggregation and adhesion of leukocytes to venular endothelium, generation of oxygen free radicals, and release of lysosomal enzymes. The cysteinyl-containing leukotrienes C_4, D_4, and E_4 (LTC_4, LTD_4, and LTE_4) cause intense vasoconstriction, bronchospasm, and increased vascular permeability. The vascular leakage, as with histamine, is restricted to venules. Cell-cell interactions are important in the biosynthesis of leukotrienes.[53] Arachidonic acid products can pass from one cell type to another, and different cell types can cooperate with each other to generate eicosanoids (termed *transcellular biosynthesis*).[54] In this way, cells that are not capable of generating a particular class of eicosanoid can produce these mediators from intermediates generated in other cells, thus expanding the array and quantities of eicosanoids produced at sites of inflammation. One example of transcellular biosynthesis is the generation of lipoxins.

Lipoxins are the most recent addition to the family of bioactive products generated from arachidonic acid, and transcellular biosynthesis is key to their production. Platelets alone cannot form lipoxins, but when they interact with leukocytes they can form the metabolites from neutrophil-derived intermediates. Lipoxins A_4 and B_4 (LXA_4 and LXB_4) are generated by the action of platelet 12-lipoxygenase on neutrophil LTA_4.[55] Cell-cell contact enhances transcellular metabolism, and blocking adhesion inhibits lipoxin production. Lipoxins have a number of proinflammatory and anti-inflammatory actions. They inhibit neutrophil chemotaxis and adhesion but stimulate monocyte adhesion.[56] LXA_4 stimulates vasodilation and attenuates the actions of LTC_4-stimulated vasoconstriction. There is an inverse relationship between the amount of lipoxin and the amount of leukotriene formed, suggesting that the lipoxins may be endogenous negative regulators of leukotriene action. The inflammatory synthesis and actions of eicosanoids are shown Figure 4.

Cytokines

Cytokines are extremely potent small regulatory peptides or glycoproteins with a molecular weight of 5 to 30 kDa that are released by nucleated cells. They act to modulate immune or repair processes by controlling cellular growth, differentiation, metabolism, and protein synthesis. Cytokines are related more directly to the control of cell immune responses and have hematopoietic cells for targets (*growth factors*, which are often confused with cytokines, have *nonhematopoietic* cells as targets). They can be subcategorized as chemokines, lymphokines, monokines, interleukins, colony-stimulating factors, and interferons.

Chemokines. Chemokines are a subset of cytokines that are soluble proinflammatory factors

Action	Metabolite
Vasoconstriction	Thromboxane A2 , leukotrienes C4, D4 , E4
Vasodilation	PGI2 , PGE1 , PGE2 , PGD2
Increased vascular permeability	Leukotrienes C4 , D4 , E4
Chemotaxis, leukocyte adhesion	Leukotriene B4 , HETE, lipoxins

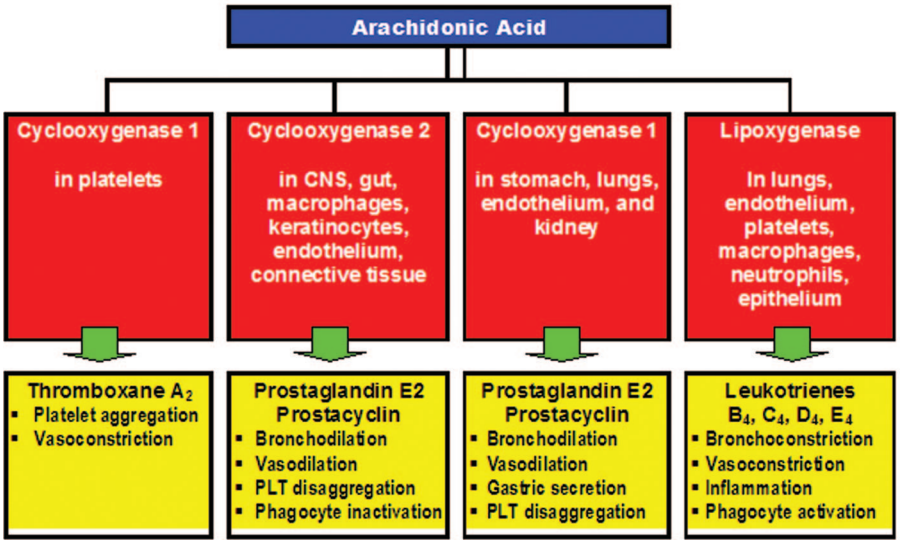


Fig. 4. Inflammatory actions of eicosanoids and their synthesis.

that attract and activate leukocytes. Chemokines are further subdivided into four families characterized by a conserved amino acid pattern at the first two cysteine residues near the *N* terminus.[57] This pattern is important in that different cysteine patterns are chemoattractive to different types of leukocytes. The nomenclature for these families is descriptive: C represents cysteine and X represents a nonconserved residue. The four families and their significance are as follows: CXC chemokines, C-C chemokines, C chemokines, and Cxxxc chemokines.

The CXC family of chemokines is further divided into two subtypes separated by the presence or absence of a glutamic acid-leucine-arginine sequence near the *N* terminus (this is referred to as the ELR motif).[57] In the first subtype, CXC chemokines with the ELR motif attract neutrophils only. This subtype includes the chemokines important for wound healing [IL-8, (GRO)-α, (GRO)-β, (GRO)-γ, NAP-2, and ENA-78). In the second subtype, CKC chemokines without the ELR motif attract activated lymphocytes. The important chemokines in this group include IP-10 and MIG.

C-C chemokines are chemoattractant for lymphocytes, monocytes, eosinophils, and basophils, but not neutrophils. The important chemokines belonging to this family include MCP-1 through -5, RANTES, MIP, and MDC.

The C chemokines are known to stimulate neutrophils.

The Cxxxc chemokines are associated with natural killer cell activation.

Lymphokines, Monokines, and Interleukins. Lymphokines are a subset of cytokines that are produced by activated T lymphocytes.

Monokines are a subset of cytokines that are produced by mononuclear phagocytes.

Interleukins are a subset of cytokines originally thought to be secreted by one type of leukocyte that acts on another type of leukocyte. Now it is known that these mediators are also released from nonhematopoietic cells and have a myriad of effects. The progressive numbering system for interleukins was started in 1978 and ranges from IL-1 through IL-23.[58] Many of the interleukins belong to other cytokine families. Interleukins with numbers higher than 10 seem to have no recognizable role in wound healing but are involved in immunity. A summary of important interleukins and their affects on wound healing is given in Table 4.

Colony-Stimulating Factors. Colony-stimulating factors are a subset of cytokines that have a stimulatory wound-healing effect. CSF-1 is secreted by macrophages, is an autocrine mediator, and "aids in self-preservation."[28] Once activated, the macrophage releases granulocyte-macrophage CSF which has generalized chemotactic, cellular proliferation, and activation properties.

Interferons. There are three members of the interferon (IFN) family (α, β, and γ), and their nomenclature is based on their ability to "inter-

Table 4. The Role of Interleukins during Wound Healing

	Description	Source
IL-1	Induces fever, adrenocorticotrophic hormone release, enhances TNF-α and IFN-γ, activates granulocytes and endothelial cells, and stimulates hematopoiesis	Macrophages, mast cells, keratinocytes, lymphocytes
IL-2	Activates macrophages, T cells, natural killer cells, and lymphokine-activated killer cells; stimulates differentiation of activated B cells; stimulates proliferation of activated B and T cells; and induces fever	Macrophages, mast cells, keratinocytes, lymphocytes
IL-6	Is released in response to IL-1; induces fever; enhances release of acute-phase reactants by the liver; and is important in inhibiting extracellular matrix breakdown during proliferation	Macrophages, mast cells, keratinocytes, lymphocytes
IL-8	Enhances neutrophil adherence, chemotaxis, and granule release; and enhances epithelization	Macrophages, mast cells, keratinocytes, lymphocytes
IL-4	Early: stimulates fibroblast proliferation; later (72 hours): downregulates cytokine expression	Mast cells
IL-10	Early: unknown; later (72 hours): downregulates cytokine expression	Unknown

Sources: Henry, G., and Garner, W. Inflammatory mediators in wound healing. *Surg. Clin. North Am.* 83: 483, 2003; Lawrence, W., and Diegelmann, R. Growth factors in wound healing. *Clin. Dermatol.* 12: 157, 1994; Cross, K., and Mustoe, T. Growth factors in wound healing. *Surg. Clin. North Am.* 83: 531, 2003; and Bennet, N., and Schultz, G. Growth factors and wound healing: Biochemical properties of growth factors and their receptors. *Am. J. Surg.* 165: 728, 1993.

fere" with viral growth. IFN-α is primarily derived from monocytes, macrophages, B lymphocytes, and natural killer cells. It has significant antiviral activity mediated through its ability to inhibit viral replication within virus-infected cells, protect uninfected cells from infection, and stimulate antiviral immunity by cytotoxic lymphocytes and natural killer cells. The most important interferon for wound healing is IFN-γ. It is primarily produced by T-helper lymphocytes but is also derived from cytotoxic T cells and natural killer cells. IFN-γ stimulates antigen presentation and cytokine production by monocytes and also stimulates monocyte effector functions, including adherence, phagocytosis, secretion, the respiratory burst, and nitric oxide production. The net result is the accumulation of macrophages and destruction of intracellular pathogens. In addition to its effects on mononuclear phagocytes, IFN-γ stimulates killing by natural killer cells and neutrophils. It stimulates adherence of granulocytes to endothelial cells through the induction of intracellular adhesion molecules, an activity shared with IL-1 and TNF.[59]

Growth Factors

Growth factors are constitutively present mediators that act on *nonhematopoietic* cells to modulate wound healing by stimulating protein production, extracellular matrix synthesis, matrix turnover, and cellular death. Growth factors are proteins weighing between 4000 and 60,000 kDa. They can affect cellular function through endocrine (released from a distant organ or cluster of cells through the blood stream), paracrine (affecting a neighboring cell), autocrine (affecting itself by stimulating a membrane receptor), or intracrine (affecting itself intracellularly) mechanisms. Their actions are enhanced by their ability to act on target cells in an autocrine fashion.[60] Growth factors typically have the words "growth factor" in their name; the only notable exception is TGF-β, which is more like a cytokine owing to its smaller molecular weight and its selective effect on multiple inflammatory processes.[28] It is the only known growth factor to use a serine/threonine kinase instead of a tyrosine kinase transduction system.[61]

There are five known superfamilies of growth factors.[62] Most growth factors originate from large proteins that have undergone posttranslational modification before being released in an active state. Growth factor receptors are transmembrane glycoproteins that exert their effect through a tyrosine kinase enzyme and phosphorylation reactions.[63]

Table 5 lists the five growth factor superfamilies and includes examples pertinent to this discussion on wound healing.[18,19,64–82] A summary of the growth factors and cytokines is shown in Tables 5 and 6.

Nitric Oxide

NO is a small radical formed from the amino acid L-arginine by three distinct isoforms of nitric oxide synthase (NOS). Two of the isoforms are called cNOS because they are *constitutively* expressed.[83] Neuronal NOS (nNOS, ncNOS, NOS1), the first to be discovered, is found in neurons, skeletal muscle, the pancreas, and the kidneys.[84,85] The other constitutive enzyme, endothelial NOS (eNOS, ecNOS, NOS3), is predominantly membrane-bound in endothelial cells, but it can also be found in other cell types (e.g., neurons and cardiac myocytes).[86] Intracellular calcium concentrations are the dominant mechanisms for activation, leading to low-level NO production in the span of just a few minutes.[87,88] The third isoform, inducible NOS (iNOS, NOS2), is not typically expressed in cells in the basal state.[89] First isolated from activated macrophages, this enzyme can be expressed in virtually all tissues under the appropriate conditions.[15,90] iNOS is synthesized in the early phase of wound healing de novo in response to cytokines, microbes, microbial products, and hypoxia, resulting in the sustained production of NO.[89,90] Once formed, iNOS is maintained in an active state by calmodulin bound to the enzyme, allowing it to operate independent of calcium concentrations.[91] This leads to a much larger release of NO, limited only by substrate and cofactor availability and enzyme concentration.

Although the in vitro signals of iNOS induction are well described, little is known of the in vivo signals during wound healing. Among the numerous cytokines and growth factors secreted and released into the wound environment, IL-1, TNF-α, and IFN-γ are the most likely inducers of iNOS. Wound fluid, as a biological reflection of the wound environment, induces NO synthesis in a variety of cells.[92] Although iNOS expression is high during the early phases of wound healing, little is known about the downregulation of iNOS activity at the wound site during the later phases of healing. Presumably, iNOS activity can be downregulated by resolution of the inflammatory response or by cytokine signaling. It is likely that colonized or infected wounds with continued inflammatory responses would continue to generate large amounts of NO, although this has not been studied directly. TGF-β1 is one of the strongest iNOS inhibitors during wound healing.[93] However, even

Table 5. Growth Factor Superfamilies and Their Role in Wound Healing

Superfamily	Members	Discussion
Platelet-derived growth factor	PDGF	PDGF affects cells of mesodermal origin and VEGF has its primary effect on endothelial cells.[64] PDGF was originally isolated from platelets, but it is now known that it is secreted by many different cells types, including macrophages, monocytes, fibroblasts, smooth muscle cells, and endothelial cells. PDGF secretion results in chemotaxis, proliferation, and new gene expression in these cells.[26]
	VEGF	VEGF receptors are found almost exclusively on endothelial cells and act as an effective mitogen; once stimulated, they result in angiogenesis. VEGF does not act on macrophages, fibroblasts, or smooth muscle cells.[65] Although VEGF has little direct effect on most cells of the skin, many cells either produce it or release factors that regulate its expression. FGF-4, PDGF, TNF-α, IGF, and some interleukins stimulate VEGF production and others inhibit it.[66]
Epidermal growth factor	EGF	EGF is found in saliva, urine, milk, plasma, and platelets. Platelets release it when they degranulate. Most cells have receptors for it, but epithelial cells have the largest number of receptors. Significant numbers of receptors are found on endothelial cells, fibroblasts, and smooth muscle cells. EGF is chemotactic and a potent mitogenic stimulant for epithelial cells, endothelial cells, and fibroblasts. It also stimulates angiogenesis and collagenase activity.[18,19]
	TGF-α	TGF-α has 30% structural homology with EGF and may represent a variant of EGF that functions more in an autocrine fashion.[67] It is produced by activated macrophages, platelets, keratinocytes, and other tissues. It stimulates mesenchymal, epithelial, and endothelial cell growth and endothelial cell chemotaxis. [18,19]
Fibroblast growth factor	aFGF, bFGF	FGF has two different forms: an acidic FGF (aFGF) and a basic FGF (bFGF); both have 50% homology, although bFGF is 10 times more potent as an angiogenic stimulant. Both aFGF and bFGF stimulate endothelial cell proliferation and motility and contribute to wound angiogenesis. bFGF stimulates collagen synthesis, wound contraction, epithelialization, and fibronectin and proteoglycan synthesis.[18,68]
	KGF-1, KGF-2	KGF is found at very low levels in normal (undamaged) skin; however, after tissue damage, fibroblasts produce high quantities. KGF-1 is the most potent mediator of keratinocyte proliferation and motility. It also results in production of glutathione peroxidase, a DNA repair enzyme that helps to protect the keratinocyte from damaging reactive oxygen species released into the wound by neutrophils to sterilize the wound.[69] KGF-2 shares 57% homology with KGF-1 and has been shown to increase granulation tissue formation by directly stimulating the migration of fibroblasts into wounds.[60]
Transforming growth factor	TGF-β1, β2, β3	TGF-β got its name because of the initial and now erroneous belief that it was capable of transforming normal cells into malignant ones. Several subtypes have been identified, but there are no known major differences in terms of function.[70] TGF-β has been isolated from platelets, macrophages, lymphocytes, bone, and kidneys.[18] Like PDGF, it is released by platelets during degranulation[71] (in case you were wondering, they are found in the alpha granules). It stimulates monocytes to secrete other growth factors (FGF, PDGF, TNF-α, and IL-1)[72] and is chemotactic for macrophages and regulates its own production within macrophages in an autocrine fashion.[18] TGF-β stimulates fibroblast chemotaxis and proliferation. At different concentrations, it can either stimulate or inhibit cellular proliferation, and this effect may be regulated or driven by what other growth factors are present.[18,73] It may be the most potent stimulant for collagen synthesis,[73] but it also decreases the stimulatory effect of other factors on collagenase activity.[74] TGF-β also stimulates fibronectin and proteoglycan synthesis by fibroblasts[75,76] and fibronectin synthesis by keratinocytes.[77] It also has the ability to organize the extracellular matrix and may be involved in scar remodeling and wound contracture.[78] It stimulates epithelial cell proliferation and inhibits endothelial cell proliferation, but with a cofactor it will stimulate angiogenesis.[18]
Insulin growth factor	IGF-I and IGF-II	IGF-II is most prominent during fetal development, whereas IGF-I persists throughout life and is synthesized in the liver, heart, lung, kidney, pancreas, cartilage, brain, and muscle. IGF-I (also known as somatomedin C) is stimulated by human growth hormone (especially in the liver) and the two together stimulate skeletal cartilage and bone growth.[79] Platelets will release IGF-I during degranulation and fibroblasts will also produce it when stimulated. IGF-I is a potent chemotactic agent for endothelial cells, resulting in neovascularization.[80] It also stimulates mitosis of fibroblasts, osteocytes, and chondrocytes and may act with PDGF to enhance epidermal and dermal growth.[81] It reversibly binds to an IGF-binding protein in the plasma.[82] When IGF is bound it is inactive; therefore, the affect IGF-I has on wound healing depends on the amount of available free IGF-I.

Table 6. Cytokine and Growth Factor Summary by Source and Function

		Source								Function				
	Class	Platelet	Neutrophil	Macrophage	Lymphocyte	Fibroblast	Endothelium	Keratinocyte	Mast cell	Leukocyte chemo-attractant	Leukocyte activator	Epithelialization	Angiogenesis	Wound fibroplasia
IL-1	CY/IL			X	X			X	X	+++		+		++
IL-2	CY/IL			X	X			X	X		+			
IL-4	CY/IL								X	−	−			++
IL-6	CY/IL			X	X			X	X					++
IL-8	CY/CK			X	X			X	X			++	++	
IL-10	CY/IL								?	−	−			
TNF-α	CY/GF			X	X		X		X	+++				−
TGF-α	GF	X		X				X				+	+	
TGF-β	CY/GF	X		X	X							++	+	
(GM)-CSF	CY/CSF			X							+++			
(G)-CSF	CY/CSF			X							+++			
(M)-CSF	CY/CSF			X							+++			
MDC	CY/CK									++				
MIP	CY/CK			X						+++				
MCP 1-5	CY/CK							X		+++				
IFN-γ	CY/IFN				X	X				+	+			—
PDGF	GF	X		X		X	X			+		++	++	+++
VEGF	GF	X		X		X	X						+++	
EGF	GF	X		X							+	+++	+	+
FGF	GF			X	X		X					+	+++	+
KGF	GF					X						+++		+
IGF-I	GF	X				X							++	
PF4		X												−

+, positive effect; −, negative effect; GF, growth factor; CY, cytokine; CY/CK, chemokine cytokine; CY/CSF, colony-stimulating factor cytokine; CY/IFN, interferon cytokine; CY/IL, interleukin cytokine.

Sources: Henry, G., and Garner, W. Inflammatory mediators in wound healing. *Surg. Clin. North Am.* 83: 483, 2003; Lawrence, W., and Diegelmann, R. Growth factors in wound healing. *Clin. Dermatol.* 12: 157, 1994; Cross, K., and Mustoe, T. Growth factors in wound healing. *Surg. Clin. North Am.* 83: 531, 2003; and Bennet, N., and Schultz, G. Growth factors and wound healing: Biochemical properties of growth factors and their receptors. *Am. J. Surg.* 165: 728, 1993.

during the inflammatory phase of wound healing, there is counterregulation of NO synthesis, possibly by the presence of an unknown factor that reduces iNOS activity but not by substrate depletion.[94]

Role of NO in Wound Healing

NO exerts itself in a variety of mechanisms. Some of its effects are due to its chemical reaction with oxygen, leading to formation of reactive radical species, or its interaction with heme- or metal-containing enzymes. A complete review of NO chemistry is beyond the scope of this discussion, but highlights of its role in wound healing are reviewed in Figure 5.

The First 72 Hours

Expression of iNOS may peak as early 48 hours (Fig. 17). During this time, many of the primary effects of NO are especially relevant to wound healing: vasodilation, antimicrobial activity, antiplatelet aggregation activity, and induction of vascular permeability.[95] As NO concentrations rise, they cause the downregulation of RANTES (a monocyte-attracting chemotactic cytokine)[96] and MCP-1 (a macrophage chemoattractant expressed by hyperproliferating keratinocytes located on the wound edge).[97] The net effect of this is to move the wound from an inflammatory state toward one of regeneration and repair.[95]

Cell Proliferation

The capacity of NO to regulate proliferation is dependent on the level of NO and the sensitivity of the cell to NO. Proliferation of fibroblasts and smooth muscle cells is inhibited by low does of NO[95]; low doses of NO stimulate endothelial cells and keratinocytes to proliferate, but higher levels (in vitro) are inhibitory.[98] NO has also been shown to protect endothelials cells from apoptosis[99] and to arbitrate VEGF-induced endothelial cell proliferation.[100]

Angiogenesis

Neovascularization is critical for successful wound healing, and NO plays a pivotal role. VEGF is the most potent angiogenic factor and it appears to be dependent on upon NO. VEGF helps itself by increasing NO production by upregulating eNOS.[101] VEGF's other effects—endothelial cell migration, decreased adhesion, and organization—are also dependent on NO. These effects may also rely on NO produced from iNOS in addition to eNOS.[95] NO also increases VEGF expression in stimulated keratinocytes,[102] resulting in a rapid accumulation of VEGF and NO.

Matrix Deposition and Remodeling

In animal studies and in vitro, the link between NO and collagen deposition has been well described. In most studies, treatment with NO donors, dietary arginine, or iNOS overexpression via gene therapy increases the collagen content of experimental wounds.[95,103] Likewise, NOS inhibition has been found to decrease collagen and granulation tissue formation in experimental burn wounds.[104] One study had the opposite result, that is, decreased wound collagen content following a topical NO donor treatment or arginine and improved wound collagen with NOS

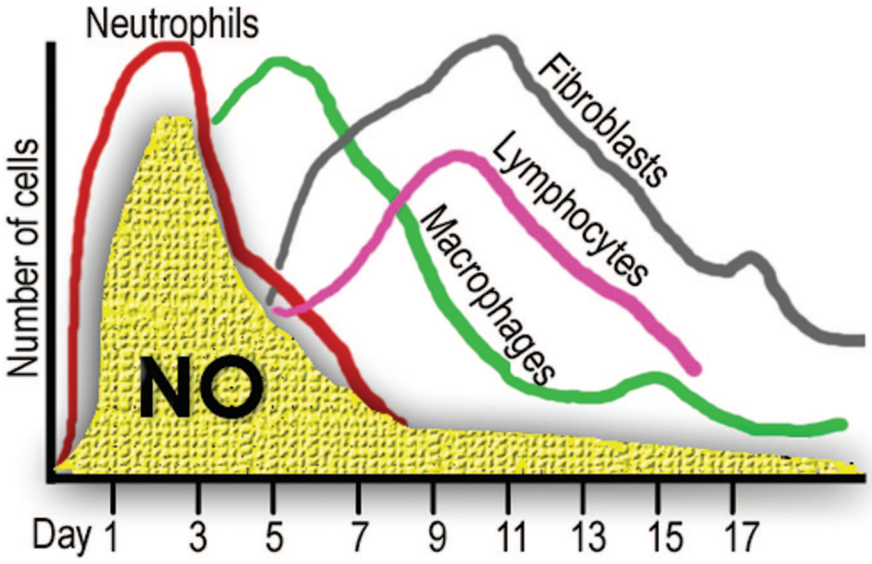


Fig. 5. Phases of wound healing and the generation of wound nitric oxide. Adapted from Witte, M., and Barbul, A. Role of nitric oxide in wound repair. *Am. J. Surg.* 183: 406, 2002.

inhibition.[105] It is likely that the timing and level of NO production in the healing wound must be carefully balanced to ensure a beneficial effect.[95] In vitro, both wound-derived and normal skin-derived fibroblasts produce increased collagen after NO donor treatment and decreased collagen after NOS inhibition.[95,106] This appears to be primarily due to posttranslational enhancement of collagen synthesis and not to increased transcription of relevant collagen genes.[95]

Cells

Platelets

Platelets play a pivotal role in wound healing. To achieve wound hemostasis, the coagulation cascade is initiated and platelet α-granules are opened, releasing large quantities of TGF-β and more (see below). This early concentration of TGF-β stimulates the chemotaxis of macrophages and lymphocytes and enhances their proliferation.[107] Lymphocytes and monocytes are also attracted to the wound by other platelet-derived inflammatory products (i.e., PDGF, TGF-β, PF4, C5a complement, PAF, and leukotriene B4).[18,107,108] The thrombus itself also has a role to play, as the fibrin provisional matrix of the resolving thrombus serves as a protein reservoir by binding cytokines and growth factors, locally concentrating and magnifying their affects.[4] MCP-1 is closely associated with newly formed thrombus and levels increase even more with thrombus resolution.[109]

The following factors are found in the platelet's α-granules: PDGF, TGF-β, FGF, EGF, β-thromboglobulin, PF4, platelet-derived angiogenesis factor, histamine, serotonin, bradykinin, prostaglandins, prostacyclin, and thromboxane.

Neutrophils

Neutrophils are the first immune cells to arrive at the wound site, peaking at 24 hours. Increased vascular permeability due to inflammation and release of prostaglandins, together with a concentration gradient of chemotactic substances released by platelets such as complement factors, IL-1, TNF-α, TGF-β, PF4, and bacterial products,[2,8] stimulate neutrophil migration into the injured area. Neutrophils attached or caught up in the thrombus at the wound site transform chemokine connective tissue–activating peptide III into neutrophil-activating peptide-2; this is one of the first potent signals for neutrophil chemotaxis.[28] Neutrophils will adhere to the endothelium at the wounded site by binding to selectins (receptors on the endothelial cell surface that preferentially help neutrophils to adhere to the endothelium) in a process called margination. Neutrophils next move through the vessel wall (diapedesis) to reside at the wound site. The neutrophil attaches itself to the extracellular matrix with integrin receptors found on the neutrophil cell surface.[2] The neutrophil's role is phagocytosis and wound débridement. During the inflammatory stage, neutrophils phagocytize invading organisms and debris and release proteolytic enzymes to destroy the invading organisms and digest nonviable tissue. There are several protease classes, depending on preferred target protein. All the protease types destroy preexisting extracellular matrix. Matrix in unwounded tissue is protected by protease inhibitors.[11] This protection can be overwhelmed by the massive release of proteases by neutrophils sometimes seen in the inflammatory phase of wound healing. Neutrophils also generate (via the myeloperoxidase pathway) reactive oxygen free radicals that combine with chloride and assist in bacterial killing within acute wounds.[11] As time passes, neutrophils are replaced by macrophages. Neutrophils *are not* required for wound healing or collagen synthesis.[110] Through a mechanism that is not yet fully understood, neutrophils receive a signal to end their destructive débridement of the wound, undergo apoptosis, and are ingested by macrophages.[2]

Macrophages

Macrophages migrate into the wound 48 to 96 hours after injury and are the predominant cell type before the fibroblasts migrate and begin replicating. Macrophages are important and necessary for wound healing. Macrophages are the "orchestra leader"[18] of wound healing because of their important role directing the wound-healing process (Fig. 6). Macrophages complete the neutrophil's job of débridement and conclude the inflammatory response with the release of cytokines and growth factors. Macrophages use phagocytosis and reactive radicals (nitric oxide, oxygen, and peroxide) to sterilize the wound and enzymes (collagenase and elastase) to débride the wound.[2]

Macrophages, unlike neutrophils, lack myeloperoxidase but do assist in proteolysis and pathogen killing. Their major contribution to wound healing is the secretion of cytokines and growth factors. These cytokines act in a paracrine manner to activate and recruit other cells involved in wound healing, such as other macrophages or lymphocytes. Macrophages secrete many different types of metalloproteinases that degrade the collagen matrix.[28] The cytokines TNF-α and IL-1 may activate iNOS in macrophages to synthesize large

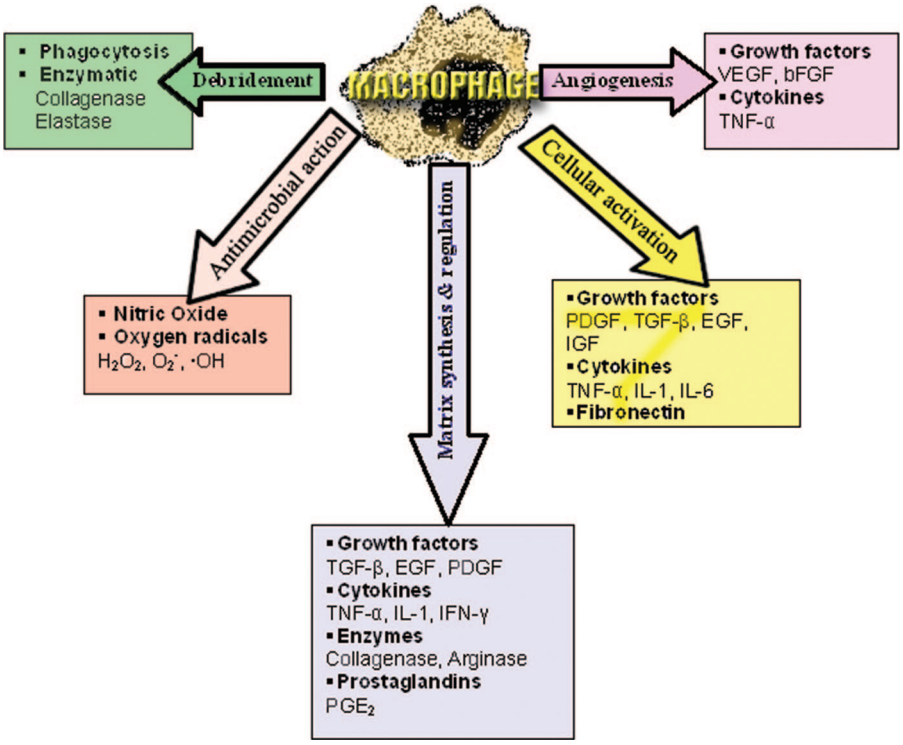


Fig. 6. The main functions of macrophages in wound healing are phagocytosis, cellular recruitment and activation, angiogenesis, regulation of matrix synthesis, and wound débridement. Effector mechanisms with examples are shown in the boxes. Reprinted with permission from Witte, M., and Barbul, A. General principles of wound healing. *Surg. Clin. North Am.* 77: 509, 1997.

amounts of NO.[10] Macrophage-synthesized NO reacts with peroxide ion-oxygen radicals to yield the more toxic peroxy nitrite and hydroxyl radicals for pathogen killing. NO helps kill *Staphylococcus aureus*, prevents the replication of DNA viruses within cells, and serves as an immune regulator.[12] Cytokines and growth factors also regulate fibroblast chemotaxis, proliferation, and collagen synthesis, as well as other cells involved in the repair process, such as endothelial cells.[111,112] Through these various functions, macrophages influence angiogenesis, fibroplasia, and extracellular matrix synthesis.

Monocytes

Upon arrival to the wound site, blood and tissue monocytes are stimulated to transform into macrophages by IL-2, TNF-α, PDGF, and IFN-γ (released by T lymphocytes).[28]

Fibroblasts

The fibroblast undergoes phenotypic changes during wound healing.[113] Fibroblasts derived from the wound are characterized by increased collagen synthesis and contraction but decreased proliferation compared with normal dermal fibroblasts; they are referred to as "wound fibroblasts."[23] Macrophage-derived cytokines trigger the phenotypic transformation of fibroblasts.[24] This has been well documented for the myofibroblastic phenotype, which is strongly induced by TGF-β1.[25] The surrounding matrix also influences the fibroblast's phenotype. Cell adhesion promoted by synthesis of the extracellular matrix molecule, fibronectin, can also result in phenotypic alteration.[114,115] Fibroblasts and endothelial cells are the primary cells in the proliferative phase. Fibroblasts migrate into the wound site from the surrounding tissue. Fibroblasts in the surrounding tissue need to become activated from their quiescent state. The growth factors and cytokines responsible for their activation and proliferation are mainly from platelets and activated macrophages. Some of them are stored in the fibrin clot and the fibroblasts themselves can be induced to release growth factors and cytokines in an autocrine manner. The most important growth factor for fibroblast proliferation is PDGF. Table 3 summarizes the effect different growth factors and cytokines have on fibroblast proliferation.

Table 7. Various Collagen Types Grouped by Major Collagen Families

Type	Tissue Distribution
Fibril-forming collagens	
I	Bone, dermis, tendon, ligaments, cornea
II	Cartilage, vitreous body, nucleus pulposus
III	Skin, vessel wall, reticular fibers of most tissues (lungs, liver, spleen, and so on)
V	Lung, cornea, bone, fetal membranes; together with type I collagen
XI	Cartilage, vitreous body
Basement membrane collagen	
IV	Basement membranes
Microfibrillar collagen	
VI	Widespread: dermis, cartilage, placenta, lungs, vessel wall, intervertebral disc
Anchoring fibril	
VII	Skin, dermal-epidermal junctions; oral mucosa, cervix
Hexagonal network-forming collagens	
VIII	Endothelial cells, Descemet's membrane
X	Hypertrophic cartilage
Fibril-associated collagens with interrupted triplet helices (FACIT)	
IX	Cartilage, vitreous humor, cornea
XII	Perichondrium, ligaments, tendon
XIV	Dermis, tendon, vessel wall, placenta, lungs, liver
XIX	Human rhabdomyosarcoma
XX	Corneal epithelium, embryonic skin, sternal cartilage, tendon
XXI	Blood vessel wall
Transmembrane collagens	
XIII	Epidermis, hair follicle, endomysium, intestine, chondrocytes, lungs, liver
XVII	Dermal-epidermal junctions
Multiplexins	
XV	Fibroblasts, smooth muscle cells, kidney, pancreas,
XVI	Fibroblasts, amnion, keratinocytes
XVIII	Lungs, liver

Adapted from Gelse, K., Pöschl, E., and Aigner, T. Collagens: Structure, function, and biosynthesis. *Adv. Drug Deliv. Rev.*55: 1531, 2003.

Keratinocytes

Keratinocytes located next to the wound receive their movement orders from the fibroblasts. Cytokines IL-1 and TNF-α upregulate KGF gene expression in fibroblasts.[99] In response, fibroblasts secrete KGF-1, KGF-2, and IL-6; this causes keratinocytes to proliferate, migrate into the wound, and then differentiate into the epidermis.[20] Keratinocyte migration is sensitive to the extracellular matrix environment. Collagen types I and IV, fibronectin, and vitronectin all seem to facilitate keratinocyte migration. Collagen, in the absence of cytokines, can still drive keratinocyte migration.[28] The stimulated keratinocytes also secrete IL-6 and NO, which provides additional positive stimulation for other keratinocytes to migrate and proliferate, thereby perpetuating the process. As the keratinocytes proliferate to "fill in the hole," they will need a new capillary network. Keratinocytes initiate neovascularization by secreting VEGF, which is synthesized by keratinocytes at the wound edge.[12] Recall that VEGF expression is stimulated by IL-1, TNF-α, KGF, and TGF-β.

Endothelial Cells

Dermal endothelial cells respond to VEGF by proliferating and forming capillary tubes. Endothelial cells synthesize NO, which increases VEGF production. As the capillaries form, endothelial cells express endothelial nitric oxide synthase, which generates even more NO that protects the tissue from hypoxia and ischemia by inducing vasodilation and protecting against reperfusion injury.[10]

Collagen

There are 21 known collagens. Their synthesis occurs as it does for any other protein within the cell. The collagen molecule is characterized by the repeating sequence Gly-X-Y, with X often being proline and Y often being hydroxyproline. The molecule undergoes the following eight posttranslational steps until it is secreted as procollagen[116]: (1) cleavage of the signal peptides; (2) hydroxylation of the proline or lysine amino acids in the x-position to 4-hydroxyproline or 4-hydroxylysine; (3) hydroxylation of some proline residues to 3-hydroxyproline; (4) glycosylation of some hydroxylysine molecules with galactose or glucose; (5) addition of oligosaccharides to the propeptides; (6) association of the c-terminal propeptides; (7) formation of interchain and intrachain disulfide bonds; and (8) formation of the triple helix, which starts at the c-terminal end and goes to the n-terminal end.

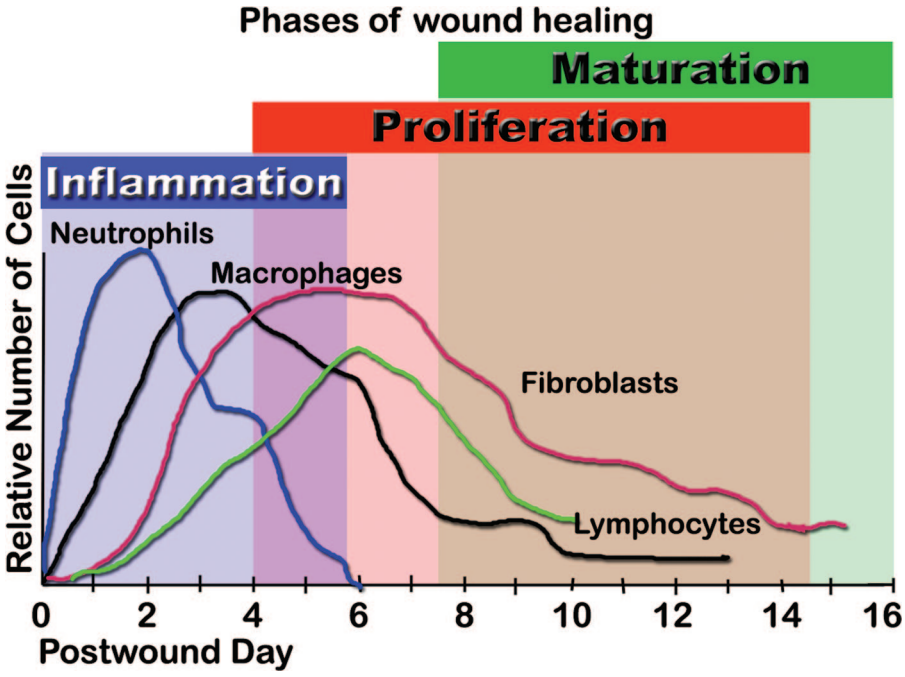


Fig. 7. The time course of the different cells appearing in the wound during the healing process. Macrophages and neutrophils are predominant during inflammation, whereas lymphocytes peak somewhat later and fibroblasts are predominant during the proliferative phase. From Witte, M., and Barbul, A. General principles of wound healing. *Surg. Clin. North Am.* 77: 509, 1997.

After posttranslational modifications are complete, the triple helix is secreted as procollagen into the extracellular environment, where the propeptide ends are specifically cleaved by procollagen C-proteinases and procollagen N-proteinases. This cleaving process is directly responsible for the decrease in the solubility of the molecule. Then the process of fibril formation begins. The cross-linking of fibrils occurs after several lysine and hydroxylysine residues have their free amino acid group transformed to aldehyde residues by the enzyme lysyl oxidase. Cross-linking occurs between these aldehyde groups and amino acid groups of the nontransformed lysine or hydroxylysine residues.[35] The collagen types and tissue distribution are shown in Table 7.

T Lymphocytes

T lymphocytes migrate into the wound after inflammatory cells and macrophages on the fifth day after injury, during the proliferative phase, and peak at day 7.[117] It was long thought that lymphocytes, although predictably present in the wound, made no significant contribution to wound healing. However, a series of experimental studies indicated a significant role for T lymphocytes in this process.[118] Adult thymectomy in rats increases wound maturation and cross-linking of collagen.[119] This effect is reversed by placement of intraperitoneal thymic grafts at the time of thymectomy. Interestingly, neonatal or intrauterine thymectomies, which prevent T-cell maturation, have no effect on wound-healing parameters. Postnatal or adult thymectomies have a more selective effect by preventing induction of suppressor T cells. The administration of thymic hormones (thymopentin and thymulin) to nude mice decreases wound-breaking strength and collagen levels.[120] This suggests that the thymus has an inhibitory effect on wound healing and that this effect may be mediated by T-suppressor lymphocytes.

Studies of $CD4^+$ immature effector T cells have the potential to differentiate into an inflammatory T cell or a helper T cell; each has distinct cytokine profiles. Both T cell types express IL-3 and granulocyte-macrophage CSF.[121,122] Inflammatory T cells also express IL-2, IFN-γ, and TNF-β, whereas helper T cells express IL-4, IL-5, IL-6, IL-10, and IL-13.[123] Histological studies of healing wounds comparing $CD4^+$ and $CD8^+$ cells have convincingly demonstrated that T lymphocytes do regulate wound healing. The inflammatory T cells are proinflammatory, helper T cells are suppressive, and there is a relationship of $CD4^+$ to $CD8^+$ ratios that shows increased $CD4^+$ is upregulatory and $CD8^+$ is downregulatory.[124,125] Therefore, it

appears that T lymphocytes may control the proliferation phase of wound healing.

Figure 7 summarizes the appearance of different cell types during wound healing.

CONCLUSIONS

Wound healing is a well orchestrated and choreographed process whose score we are just now beginning to understand. Wound healing is extremely complex, and the descriptions of the most important pathways have been abbreviated and simplified. As research continues and our comprehension grows, our current perceptions and hierarchies of importance will undoubtedly need to change.

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