Molecular Analysis of the Environments of Healing and Chronic Wounds: Cytokines, Proteases and Growth Factors

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Molecular Regulation of Wound Healing

Wound healing in the skin is a complex biological process involving inflammation, chemotaxis of cells, mitosis of epidermal, dermal and vascular cells, neovascularisation, synthesis of extracellular matrix proteins and remodelling of scar tissue. Growth factors, cytokines, proteases and hormones have been shown to regulate most aspects of these processes *in vitro*, and this has led to the hypothesis that these different classes of molecules also regulate important phases of wound healing *in vivo* [1, 2]. The im-portant implication of this hypothesis is that the impairment or imbalance of growth factors, cytokines, proteases or hormones in wounds directly promotes the establishment and maintenance of chronic wounds [3, 4]. If these two concepts are correct, then therapies which establish in chronic wounds an environment that permits these molecules to function normally should lead to the healing of chronic wounds.

The Roles of Cytokines and Growth Factors in Wound Healing

The many processes which occur during healing of skin wounds can be grouped into four general phases:

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- haemostasis;
- inflammation;
- proliferation and repair, and
- remodelling.

There is considerable temporal overlap of these phases of healing and the entire process lasts several months.

Immediately following injury, the process of blood clotting is initiated by activation of a proteolytic cascade, which ultimately converts fibrinogen into fibrin. As the fibrin molecules self-associate into a web-like net, red blood cells and platelets become entrapped. Eventually, an aggregate of fibrin, red blood cells and platelets grows large enough to form a tampon, helping to plug an injured capillary and thus stop the flow of blood. The process of blood clotting also induces platelet degranulation, which releases a burst of pre-formed cytokines and growth factors stored in the alpha granules. These cytokines and growth factors – including platelet-derived growth factor (PDGF), insulin-like growth factor-I (IGF-I), epidermal growth factor (EGF) and transforming growth factor-beta (TGF-β) – initiate two major processes:

- chemotaxis of inflammatory cells, and
- initiation of tissue repair.

The cytokines and growth factors released from platelets quickly diffuse from the wound into the surrounding tissues and chemotactically draw inflammatory cells into the injured area. Initially, neutrophils enter the wound, followed by macrophages. The neutrophils and macrophages engulf and destroy bacteria and release proteases, including neutrophil elastase and neutrophil collagenase, also known as matrix metalloproteinase eight or MMP-8. These proteases play important, beneficial roles in initiating wound healing by proteolytically removing

damaged extracellular matrix components, which must be replaced by new, intact extracellular matrix molecules if wound healing is to proceed properly.

As the concentrations of inflammatory cytokines and growth factors released from platelets decrease in an acute wound area due to diffusion, neutrophils, macrophages, activated fibroblasts and epidermal cells drawn into the wound area begin to synthesise and release new factors. Activated macrophages secrete tumour necrosis factor alpha (TNFα) and interleukin one beta (IL-1β), which have a variety of actions on different cells. TNFα and IL-1β stimulate the endothelial cells of the capillaries to express cell adhesion molecules. In response, the endothelial cells produce interleukin eight (IL-8), which induces expression of adhesion molecules on the surface of inflammatory cells. This enables the inflammatory cells to bind to vascular endothelial cells, traverse the capillary basement membrane and enter the surrounding tissues. TNFα also induces macrophages to produce IL-1β, which is mitogenic for fibroblasts and up-regulates MMP expression. Both TNFα and IL-1β directly influence the deposition of collagen in the wound by inducing synthesis of collagen by fibroblasts and by up-regulating the expression of MMPs. In addition, these cytokines down-regulate expression of the tissue inhibitors of metalloproteinases (TIMPs). Interferon gamma (IFN-γ), produced by lymphocytes attracted into the wound, inhibits fibroblast migration and down-regulates collagen synthesis.

Inflammatory cells also secrete growth factors, including TGFβ, TGF-α, heparin-binding epidermal growth factor (HB-EGF) and basic fibroblast growth factor (bFGF). The growth factors secreted by macrophages continue stimulating migration of fibroblasts, epithelial cells and vascular endothelial cells into the wound. As the fibroblasts and other cells migrate to the site of injury, they begin to proliferate and the cellularity of the wound increases. This proliferative and repair phase often lasts several weeks. If the wound is not infected, the number of inflammatory cells in it begins to decrease after a few days. Other cells in the wound – such as fibroblasts, endothelial cells and keratinocytes drawn into the wound area – begin to synthesise growth factors. Fibroblasts secrete IGF-1, bFGF, TGF-β, PDGF and keratinocyte growth factor (KGF), while the endothelial cells produce vascular endothelial cell growth factor (VEGF), bFGF and PDGF and keratinocytes synthesise TGF-β, TGF-α, and IL-1β. These continue to stimulate cell proliferation, synthesis of extracellular matrix proteins and capillary formation.

Once the initial scar forms, proliferation and neovascularisation cease and the wound enters the remodelling phase, which can last many months. During this last phase, a balance is reached between the synthesis of new components of the scar matrix and their degradation by metalloproteinases such as collagenase, gelatinase and stromelysin. Fibroblasts, the major cell type synthesising the extracellular matrix components of collagen, elastin and proteoglycans, are also a major source of the MMPs that degrade the matrix as well as the TIMPs. Further, they secrete lysyl oxidase, which cross-links components of the extracellular matrix. Angiogenesis ceases and the density of capillaries decreases in the wound site as the scar matures. Eventually, remodelling of the scar tissue reaches equilibrium, although the mature scar is never as strong as uninjured skin.

The Role of MMPs in Wound Healing

MMPs play several essential roles in wound healing, including facilitating migration of cells, removing damaged matrix and remodelling new scar matrix. Four major classes of MMPs involved in wound repair are collagenases, gelatinases, stromelysins and the membrane-type metalloproteinases [5]. Interstitial collagenases, or MMP-1, MMP-8 and MMP-13, cleave intact fibrillar type I collagen, the predominant form of collagen in skin, at a single site in the collagen molecule. Type I collagen molecules – which are ridge rods formed by the triple helix of three collagen protein chains – associate in a head-to-tail and side-by-side arrangement to form a fibril. Multiple fibrils then associate to form a collagen fibre with extremely high tensile strength (resistance to breaking). Intact collagen fibrils are very resistant to proteolytic destruction by most enzymes except col-lagenases (MMP-1). The gelatinases MMP-2 and MMP-9 are able to degrade type I collagen to small fragments only after the initial cleavage of intact collagen by MMP-1. In addition, MMP-2 and MMP-9 degrade type IV collagen, which is the major type of collagen found in basement membranes. Type IV collagen molecules are not straight, rigid rods like type I col-lagen molecules but have bends in the triple helical region. This causes type IV collagen molecules to aggregate into a sheet-like meshwork of polygonal shapes, ultimately forming a multi-layered network. Stromelysins, or MMP-3, MMP-10 and MMP-11, have broad substrate preference and can degrade several types of extracellular matrix molecules, including non-fibrillar collagens (types IV and X), matrix attachment proteins such as fibronectin and laminin, and proteoglycans. The latter are large molecules that contain a core protein linked to many

long carbohydrate chains. Proteoglycans bind large amounts of water, due to their enormous carbohydrate content, and give the extracellular matrix compressibility. The newest members of the metalloproteinase family are the membrane-type metalloproteinases or MT-MMPs. Four MT-MMPs have been identified and are designated MT1-MMP through MT4-MMP. An important biological role of the MT-MMPs appears to be the proteolytic activation of pro-MMP-2 and pro-MMP-9.

Another important process mediated by MMPs is diapedesis, or the transmigration of leukocytes through the vessel wall and into surrounding tissues in response to chemotactic growth factors and cytokines. Macrophages and neutrophils produce MMP-2, MMP-3 and MMP-9, which can degrade the basement membrane and allow cell migration out of the vessels. Once inflammatory cells migrate into the wound area, leuko-cytes secrete MMP-1, MMP-2, MMP-3 and MMP-9 – these proteases play key roles in breaking down and removing damaged extracellular matrix proteins. As the healing response continues, angiogenic growth factors such as aFGF, bFGF, TGF-α, TGF-β, TNF-α, VEGF and angiogenin stimulate new vessels to grow into the wound area. Vascular endothelial cells also produce MMPs necessary for the migration of vascular endothelial cells through tissues during the formation of new capillaries. In the resolution phase of wound repair, the new extracellular matrix of the scar is remodelled by MMPs produced by fibroblasts in the scar tissue.

The overall activities of MMPs in the wound environment are determined by several parameters, including their rate of synthesis, the activation of latent MMPs and the levels of specific inhibitor proteins of MMPs, the TIMPs. Two major TIMPs involved in wound healing are TIMP-1 and TIMP-2. TIMP-1 forms a strong complex with activated MMP-1, MMP-2 and MMP-3. TIMP-2 binds and inhibits both active and latent forms of MMP-2. Synthesis of MMPs and TIMPs is extensively regulated by growth factors and cytokines and there is often coordinated synthesis of the MMPs, TIMPs and extra-cellular matrix proteins by growth factors and cytokines. For example, IL-1, EGF and bFGF up-regulate the expression of MMP-1, MMP-3, TIMP-1 and collagen. TGF-β, a very powerful inducer of scar formation, up-regulates collagen synthesis while down-regulating MMP expression and up-regulating TIMP-1 production by fibroblasts. These examples demonstrate how growth factors, cytokines, MMPs and TIMPs regulate the key processes of normal healing.

The Roles of Endocrine Hormones in Regulating Wound Healing

Classical endocrine hormones are molecules synthesised by a specialised tissue then secreted into the bloodstream, which carries them to a distant target tissue. There they interact with specific cellular receptor proteins and influence the expression of genes that ultimately regulate the physiological actions of the target cell. It has been known for decades that alterations in endocrine hormones can alter wound healing. For example, diabetic patients frequently develop chronic wounds, due to multiple direct and indirect effects of the inadequate insulin action on wound healing. Patients receiving anti-inflammatory glucocorticoids for extended periods are at risk of impaired wound healing, due to direct suppression of collagen synthesis in fibroblasts and extended suppression of inflammatory cell function. Recently, Ashcroft and colleagues [6] reported that healing of skin biopsy sites in healthy, post-menopausal women was significantly slower than in healthy pre-menopausal women. Molecular analysis of the wound sites indicated that levels of TGF-β1 protein and mRNA were dramatically reduced in post- compared to pre-menopausal women. However, the healing of wounds in post-menopausal women taking oestrogen replacement therapy occurred as rapidly as in pre-menopausal women. Further, molecular analysis of wounds in post-menopausal women taking oestrogen replacement therapy revealed elevated levels of TGF-β protein and mRNA that were similar to levels in the wounds of pre-menopausal women.

These data indicate the substantial extent of the interaction that can occur between classical endocrine hormones and growth factors in regulating wound healing.

Biochemical Differences in the Molecular Environments of Healing and Chronic Wounds

Because cytokines, growth factors, proteases and endocrine hormones play key roles in regulating acute wound healing, it is possible that alterations in the actions of these molecules could contribute to the failure of wounds to heal normally. Several methods can be used to assess differences in the molecular environments of healing and chronic wounds. Homogenates of wound biopsies can be used to measure levels of mRNAs and proteins, while histological sections of biopsies can be used to immunolocalise proteins in wounds. Fluids that spontaneously collect in acute surgical wounds and chronic skin ulcers can also

be used to analyse the molecular environment of healing and chronic wounds. Several important concepts have emerged from the molecular analyses of acute and chronic wound environments.

The first major concept to emerge from analysis of wound fluids is that the molecular environments of chronic wounds have reduced mitogenic activity compared to those of acute wounds. For example, when fluids collected from acute mastectomy wounds are added to cultures of normal human skin fibroblasts, keratinocytes or vascular endothelial cells, the acute wound fluids consistently stimulate DNA synthesis of the cultured cells. In contrast, the addition of fluids collected from chronic leg ulcers typically does not stimulate DNA synthesis of the cells in culture (Figure 1). Also, combining acute and chronic wound fluids inhibits the mitotic activity of the acute wound fluids. Similar results were reported by several other groups of investigators, who found that acute wound fluids promote DNA synthesis while chronic wound fluids do not [7-9].

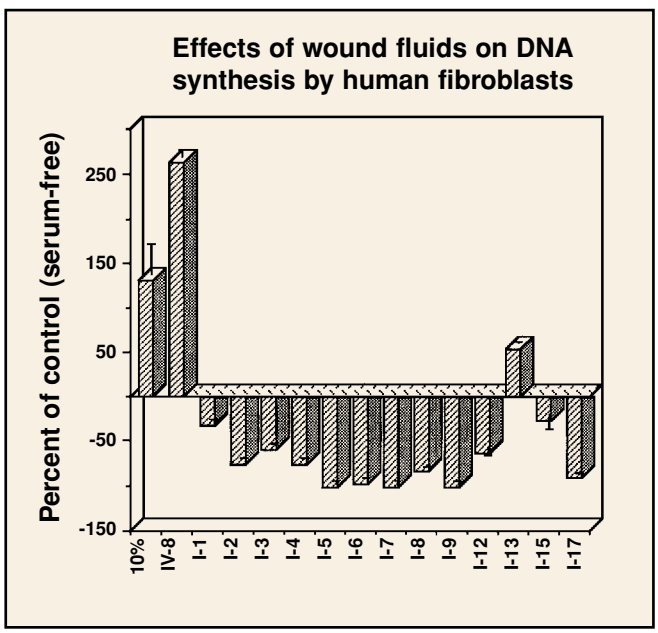

Figure 1. Effect of fluids from healing acute and chronic wounds on DNA synthesis of fibroblasts: cultures of normal human skin dermal fibroblasts were incubated in chemically defined medium containing tritiated thymidine and supplemented with 10 per cent addition of calf serum (10 per cent), acute wound fluid (IV-8) or fluids from a series of chronic skin ulcers (samples I-1 to I-17). After 24 hours, the levels of radioactivity were measured and expressed as the percentage of DNA synthesis compared to fibroblasts cultured in serum-free control medium.

The second major concept to emerge from analysis of wound fluids is that the cytokine environment of chronic wounds is substantially more pro-inflammatory than the molecular environment of acute wounds. For example, the ratios of two key inflammatory cytokines, TNFα and IL-1β, and their natural inhibitors, P55 and IL-1 receptor antagonist, are significantly higher in mastectomy than chronic wound fluids (Table 1). At the 1994 meeting of the European Tissue Repair Society, Trengove *et al* [10] also reported high levels of the inflammatory cytokines IL-1, IL-6 and TNFα in fluids collected from the venous ulcers of patients admitted to hospital. More importantly, the levels of cytokines significantly decreased in fluids collected 2 weeks after the chronic ulcers had begun to heal. Harris *et al* [9] also found that cytokine levels were generally higher in wound fluids from non-healing than healing ulcers. These data suggest that chronic wounds typically have elevated levels of pro-inflammatory cytokines and the molecular environment changes to a less pro-inflammatory cytokine environment as chronic wounds begin to heal.

Table 1. Ratios of inhibitor/cytokines in fluids from healing and chronic wounds.

	P55/TNF-"	IL-1RA/IL-1
Healing wounds	12:1	480:1
Chronic ulcers	4:1	7:1

A third important concept emerging from analysis of wound fluids is that protease activity in chronic wounds is significantly elevated compared to acute wounds. The average level of protease activity in mastectomy fluids – determined using a general substrate for MMPs, Azocoll – is low (0.75 µg collagenase equivalents/ml, *n=20*), with a range of 0.1-1.3 µg collagenase equivalents/ml (Figure 2). This suggests that protease activity is tightly controlled during the early phase of wound healing. In contrast, the average level of protease activity in chronic wound fluids (87 µg collagenase equivalents/ml, *n=32*) is approximately 116-fold higher *($p<0.05$)* than in mastectomy fluids. Al-so, the range of protease activity in chronic wound fluids is rather large (from 1 to 584 µg collagenase equivalents/ml). More importantly, the levels of protease activity tend to decrease in chronic venous ulcers 2 weeks after the ulcers begin to heal (Figure 3). Yager *et al* [11] also found 10-fold higher levels of MMP-2 protein, 25-fold higher levels of MMP-9 protein and

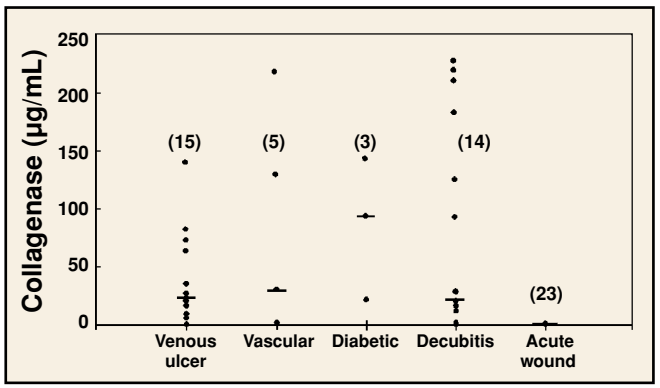

Figure 2. **Protease levels in fluids from healing and chronic wounds: fluids were collected from acute healing surgical wounds or different types of chronic wounds and levels of protease activity measured using Azocoll as the substrate. The numbers in parentheses indicate the number of wound fluids in each group. Protease levels are expressed as microgram equivalents of collagenase per millilitre of wound fluid.**

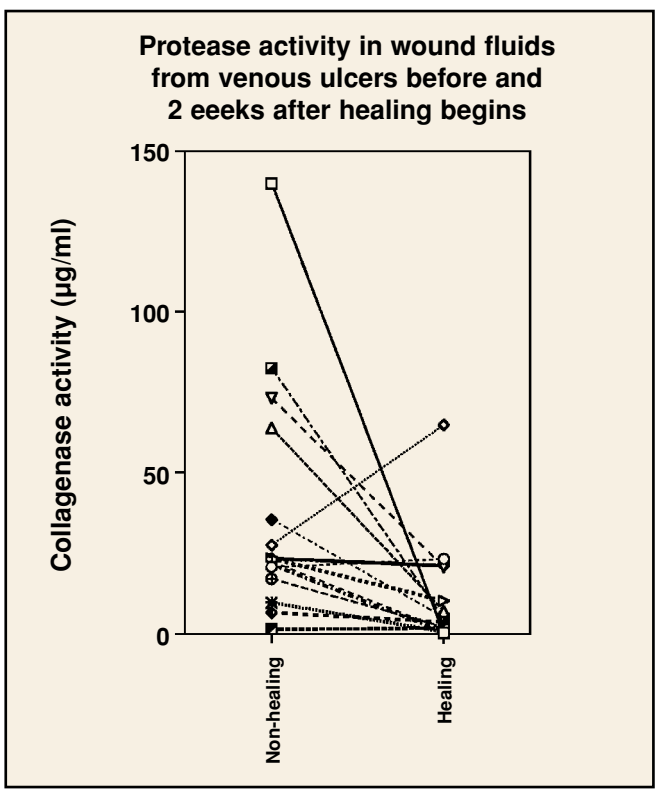

Figure 3. **Protease levels in fluids collected from non-healing and healing chronic venous leg ulcers: fluids were collected from the chronic venous leg ulcers of 15 patients at the start of hospitalisation (non-healing) and 2 weeks later, once the ulcers showed clinical evidence of healing (healing). Protease activities were measured us-ing Azocoll as the substrate. Lines connect the protease levels measured in the two samples from each patient (non-healing and healing).**

10-fold higher collagenase activity in fluids from pressure ulcers compared to surgical wound fluids using gelatin zymography and cleavage of a radioactive collagen substrate. Using immunohistochemical localisation, Rogers *et al* [12] observed elevated levels of MMPs in granulation tissue of pressure ulcers, along with elevated levels of neutrophil elastase and cathepsin G. Bullen *et al* [13] reported that levels of TIMP-1 decreased and levels of MMP-2 and MMP-9 increased in fluids from chronic venous ulcers compared to mastectomy wound fluids.

Other classes of proteases also appear elevated in chronic wound fluids. Rao *et al* [14] reported that fluids from skin graft donor sites or breast surgery patients contained intact α1-antitrypsin, a potent inhibitor of serine proteases, very low levels of neutrophil elastase activity, and intact fibronectin. In contrast, fluids from the chronic venous ulcers contained degraded α1-antitrypsin, 10- to 40-fold more neutrophil elastase activity and degraded fibronectin. Wysocki and colleagues [15] also found that chronic leg ulcers contained elevated MMP-2 and MMP-9, while Grinnell and Zhu [16] reported that fibronectin degradation in chronic wounds depended on the relative levels of elastase, α1-proteinase inhibitor and α2-macroglobulin. Besides being implicated in degrading essential extracellular matrix factors like fibronectin, proteases in chronic wound fluids have also been reported as degrading exogenous growth factors such as EGF, TGF-β1 [17] or PDGF *in vitro* [17, 18]. In contrast, exogenous growth factors were stable in acute surgical wound fluids *in vitro*. Supporting this general concept of increased degradation of en-dogenous growth factors by proteases in chronic wounds, the average immunoreactive levels of some growth factors – such as EGF and TGF-β1 – were found to be lower in chronic than in acute wound fluids, while PDGF-AB, TGF-α and IGF-1 were not [2, 17, 19]. In general, these results suggest that many chronic wounds contain elevated levels of MMP and neutrophil elastase activity. The physiological implications of these data are that elevated protease activity in some chronic wounds may directly contribute to the latter's failure to heal by degrading proteins necessary for wound healing, such as extra-cellular matrix proteins, growth factors, their receptors and protease inhibitors. Interestingly, Steed *et al* [20] reported that extensive debridement of diabetic foot ulcers improved healing in patients treated with placebo or recombinant human PDGF. It is possible that frequent sharp debridement of diabetic ulcers helps convert the detrimental molecular environment of a chronic wound into a pseudo-acute wound molecular environment.

Biological Response of Chronic Wound Cells

Biochemical analyses of fluids and biopsies from healing and chronic wounds suggest there are some important molecular differences in the wound environments. However, these data only describe half the picture. The other essential component of the chronic wound environment is the capacity of the cells in a chronic wound to respond to these molecular regulators. Emerging now is interesting new data which suggest that fibroblasts in skin ulcers which have failed to heal for many years may be not be capable of responding to growth factors and exten-sively dividing as do the fibroblasts in healing wounds. Data presented by Dr Magnus Ågren at the 1998 meeting of the European Tissue Repair Society indicated that fibroblast cultures established from chronic venous leg ulcers grew to lower densities than those established from acute wounds or uninjured dermis [21]. Further, fibroblasts from venous leg ulcers that had been present more than 3 years grew more slowly and responded less well to PDGF than fibroblasts from venous ulcers present less than 3 years. These results suggest that fibroblasts in ulcers of long duration may have a decreased ability to respond to exogenous growth factors and a reduced capacity to divide (premature senescence).

Future Concepts for the Treatment of Chronic Wounds

Based on these biochemical analyses of the molecular environments of acute and chronic human wounds, it is possible to propose a general model of differences between healing and chronic wounds. As shown in Figure 4, the molecular environment of healing wounds promotes cell mitosis and is characterised by low levels of inflammatory cytokines and proteases and high levels of growth factors and cells capable of rapid div-ision. In contrast, the molecular environments of chronic wounds generally have the opposite characteristics; that is, they do not promote cell mitosis, have elevated levels of inflammatory cytokines, high levels of proteases, low levels of growth factors and cells approaching senescence. If these general concepts are correct, it may be possible to develop new treatment strategies to re-establishing in chronic wounds the balance of cytokines, growth factors, proteases, their natural inhibitors and competent cells found in healing wounds.

New treatment strategies could be designed to reduce the elevated protease levels. Fortunately, new, potent, synthetic

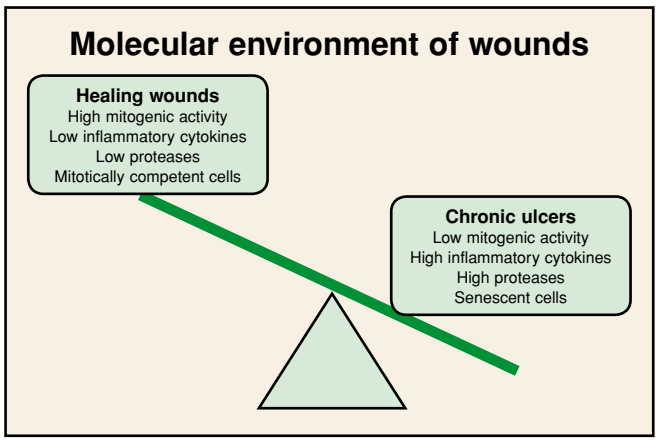

Figure 4. Diagram of the relationships of factors in healing wounds and chronic ulcers: analyses indicate that healing wounds generally have high levels of mitogenic activity, low levels of inflammatory cytokines and proteases, high levels of growth factors, and mitotically competent fibroblasts. In contrast, chronic wounds tend to have low levels of mitotic activity, high levels of inflammatory cytokines and proteases, low levels of growth factors and nearly senescent fibroblasts.

inhibitors of MMPs, as well as naturally occurring protease in-hibitors such as TIMP-1 and α1-antitrypsin, are available by way of recombinant DNA technology. The use of a recombinant growth factor, PDGF-BB, was recently approved by the United States Food and Drug Authority for diabetic foot ulcers. Treatment of chronic wounds with engineered tissue replacements such as Dermagraph® and Apligrapf® has proven effective in selected types of ulcers. The cells which populate the synthetic skin substitutes probably do not survive long-term in the wound but may secrete important cytokines, growth factors, matrix proteins and protease inhibitors that eventually recruit healthy cells around the chronic wound to migrate into it. In the future, treatment of chronic wounds with combinations of selective inhibitors of proteases, growth factors and tissue re-placements may synergistically promote healing and provide an adjuvant to the traditional treatment of chronic skin ulcers.

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