CELLULAR AND MOLECULAR BASIS OF IMPAIRED HEALING OF DIABETIC FOOT ULCERS

HANNA GAŁKOWSKA, WALDEMAR LECH OLSZEWSKI

Department of Surgical Research and Transplantology, Medical Research Center, Polish Academy of Sciences in Warsaw
Kierownik: prof. dr hab. W. L. Olszewski

The diabetic foot syndrome represents a major problem in the health care of diabetic patients. The moment a person with diabetes suffers a foot skin abrasion, the danger of requiring amputation arises. Most commonly, diabetic patients display neuropathy and/or arterial ischemia. When coupled with impaired ability to fight infection due to defects in leukocyte function in hyperglycemia (1), these patients become largely unable to develop an adequate inflammatory response. Decreased resistance to infection is often referred to as immunopathy (2). Diabetic foot ulcers become portals for infection, a consequence rather than a cause of foot ulceration. Bacterial and/or yeast infection, tissue ischemia, and poor wound management cause diabetic foot ulcers to heal slowly and to transform into chronic wounds.

Basic aspects of normal wound healing

Small and superficial cutaneous defects can resurface mainly by epidermal migration. Partial-thickness wounds can heal both from the edges and from the present within the wound keratinocyte reservoir, like hair follicles and skin appendages. Conversely, full-thickness wounds can only heal from the edges, and contraction plays an important mechanism for wound closure (3, 4).

Wound healing occurs as a cellular response to injury and involves the activation of keratinocytes, fibroblasts, endothelial cells, macrophages, granulocytes and platelets. There are four overlapping phases of the repair process: coagulation, inflammation, migration-proliferation with matrix deposition, and remodeling (5). Soon after injury, blood platelets become activated, a fibrin plug is formed, and inflammatory cells (neutrophils and macrophages) are recruited to the wound. The fibrin plug consists of platelets embedded in fibrinogen, fibronectin, vitronectin, and thrombospondin. Platelets release a wide range of growth factors important for cell recruitment (6) and extracellular matrix (ECM) deposition (7).

Immediately after injury, the wound becomes hypoxic because of damage to the blood vessels. Hypoxia increases keratinocyte migration, angiogenesis and proliferation of fibroblasts. Within the next 2-3 days, inflammatory and dermal cells produce many cytokines, chemokines and growth factors and form granulation tissue. Many processes of normal wound healing are regulated in large part by these growth factors also produced by the epidermis. When the inflammatory phase is toned down, formation of the ECM, neo-angiogenesis, contraction and re-epithelialization occur.

In normal healing, the wound margin and bed tissue become infiltrated by macrophages, neutrophils and some lymphocytes. This inflammatory response is mediated by adhesion molecule interactions between leukocytes and vascular endothelium (8). Macrophages and granulocytes play a crucial regulatory role in transition between the inflammatory and granulation tissue formation phase of normal wound healing. Concentration of inflammatory
cells in healing normal wounds is reduced. Communication between the infiltrating leukocytes and integumentary cells (e.g. fibroblasts, keratinocytes, Langerhans cells and endothelial cells) and activation of these cells are mediated by cytokines and growth factors.

The role of endogenous growth factors in the normal healing process has been only partially elucidated (9). Proinflammatory interleukins (IL1α, IL1β, IL1-receptor antagonist, IL6, IL8) and growth factors like tumor necrosis factor (TNFα) and transforming growth factor β (TGFβ) are involved in granulocyte and macrophage infiltration. Factors like basic fibroblast growth factor (FGFβ), vascular endothelial growth factor (VEGF), granulocyte/macrophage-colony stimulating factor (GM-CSF) and IL8 are involved in angiogenesis. Platelet-derived growth factor (PDGF), TGFβ and GM-CSF produced in wounds are actively involved in fibroplasia and ECM deposition. Epidermal growth factor (EGF), FGFβ, IL6 and GM-CSF are involved in the process of wound re-epithelialization. The use of genetically modified mice for wound healing studies recently confirmed crucial roles of some these factors in the repair process. Therefore, the impaired cytokine and growth factor production in skin ulcers may bring down granulation tissue formation and maintain chronicity of the ulcer. The principal cytokines, chemokines and growth factors, and their sources and biological activity in the processes of wound healing are presented in tab. 1 and 2.

For correct wound healing, a balance between tissue proteases and their inhibitors is necessary (10, 11). Proteases are essential in initial wound debridement, as well as for angiogenesis, epithelialization and remodeling of scars. Inflammatory cells, fibroblasts, endothelial cells, and keratinocytes produce zinc-dependent matrix metaloproteases (MMPs) at different times during wound healing. Their activity is under the control of tissue inhibitors of MMPs (TIMPs).

Early in the remodeling phase, the provisional wound matrix that consists predominantly of fibrin and fibronectin is replaced with

Table 1. The principal cytokines, chemokines and growth factor families involved in wound healing

<table>
<thead>
<tr>
<th>Factor</th>
<th>Source</th>
<th>Biological activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1</td>
<td>macrophages, keratinocytes, endothelial cells</td>
<td>fibroblast and keratinocyte chemotaxis, collagen synthesis</td>
</tr>
<tr>
<td>IL8</td>
<td>neutrophils, macrophages, fibroblasts, keratinocytes</td>
<td>chemotaxis of neutrophils and macrophages, keratinocyte maturation</td>
</tr>
<tr>
<td>IL10</td>
<td>macrophages, keratinocytes</td>
<td>inhibition of proinflammatory IL1, IL6 and TNFα production, and fibroblast and macrophage and granulocyte activation</td>
</tr>
<tr>
<td>TGFβ</td>
<td>platelets, macrophages, fibroblasts, keratinocytes</td>
<td>fibroblast chemotaxis and proliferation, collagen, MMPs and TIMPs synthesis, angiogenesis, ECM deposition</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelets, macrophages, fibroblasts, keratinocytes</td>
<td>activation and chemotaxis of fibroblasts and macrophages, angiogenesis, synthesis of collagen, MMPs, TIMPs, ECM deposition</td>
</tr>
<tr>
<td>FGF</td>
<td>macrophages, endothelial cells, fibroblasts</td>
<td>angiogenesis, fibroblast and keratinocyte proliferation and migration, ECM deposition</td>
</tr>
<tr>
<td>EGF</td>
<td>keratinocytes, fibroblasts, endothelial cells</td>
<td>keratinocyte proliferation and migration, collagen synthesis, ECM deposition</td>
</tr>
<tr>
<td>IGF</td>
<td>fibroblasts, macroporphages, keratinocytes</td>
<td>fibroblast and keratinocyte proliferation, angiogenesis, collagen synthesis, ECM deposition</td>
</tr>
<tr>
<td>MCP1</td>
<td>keratinocytes, endothelial cells, macrophages fibroblasts</td>
<td>chemotaxis of macrophages and neutrophils, angiogenesis, ECM deposition, keratinocyte migration</td>
</tr>
</tbody>
</table>

Table 2. Effect of growth factors and cytokines on wound healing processes

<table>
<thead>
<tr>
<th>Process</th>
<th>Involved factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil and macrophage infiltration</td>
<td>TGFβ, IL8, MCP1, IL10 (-)</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>VEGF, PDGF, FGFβ, IL8, MCP1</td>
</tr>
<tr>
<td>Fibroplasia</td>
<td>PDGF, TGFβ, IGFs, CTGF</td>
</tr>
<tr>
<td>ECM deposition</td>
<td>FGFβ, IGF1, NGF, CTGF, TGFβ, MCP1</td>
</tr>
<tr>
<td>Scarring</td>
<td>TGFβ, IGF1, CTGF, IL10 (-)</td>
</tr>
<tr>
<td>Reepithelialization</td>
<td>FGFβ, EGF, HB-EGF, IGFs, NGF, MCP1, TGFβ (-)</td>
</tr>
<tr>
<td>negative regulation</td>
<td></td>
</tr>
</tbody>
</table>
collagen type III and I produced by fibroblasts. These collagen fibers greatly increase the tensile strength of the scar matrix. Furthermore, fibroblast-to-myofibroblast differentiation contributes to proper wound healing. To re-populate wound bed, fibroblasts proliferate and acquire a migratory phenotype by de novo developing contractile bundles. Such activated pro-myofibroblasts, with increasing stress in the ECM, develop into myofibroblasts neo-expressing α-smooth muscle actin (12). Incorporation of actin into stress fibers significantly augments the contractile activity of fibroblasts and hallmarks the contraction phase of wound healing. In the final stages of remodeling, the high density of myofibroblasts and new blood vessels in the scar decreases as these cells undergo programmed cell death (apoptosis).

Resurfacing of a wound and regeneration of epidermis during normal wound healing takes place by migration, proliferation, differentiation, and apoptosis of keratinocytes on the wound borders. These processes are also involved in homeostatic mechanisms in healthy skin. Epidermis is continuously renewed, and this process is ensured by antigen p63+ and integrin β1+ (CD29) epidermal stem cells that reside in the basal layer (13, 14). Post-mitotic differentiating keratinocytes that express cytokeratin 10 are continuously lost for the skin surface undergoing senescence and/or apoptosis. Acute skin injury was shown to induce keratinocyte hyperproliferation associated with cytokeratin 16 and 17 expression (15). Recent advances in wound biology clarified some molecular pathways governing keratinocyte re-epithelialization at the wound site, i.e., the integration of keratinocyte integrins with dermal ECM and changes in expression of cytokeratin filaments in migrating keratinocytes at the wound margin (16, 17).

In vitro studies in a skin model including dermal fibroblasts and epidermal keratinocytes showed morphogenesis of dermal-epidermal junctions (DEJ), a complex macromolecular structure establishing the boundary between epidermis and dermis, which must be formed de novo during resurfacing of the wound (18, 19). The major components of DEJ are laminins, collagen type IV and VII, perlecan, and nidogen. Basement membrane (BM) also includes ECM proteins such as tenascin, fibrillin-1 and types III and I collagens. In vitro studies indicated that the presence of both fibroblasts and keratinocytes are crucial for optimal production and localization of BM components. Keratinocytes were absolutely required for the preferential localization at the BM of all proteins synthesized by fibroblasts, and on the other hand, the presence of fibroblasts was required for correct deposition of type VII collagen and laminin 5 at the BM. In the absence of fibroblasts, these proteins were completely or partially retained within keratinocytes.

Impaired healing of diabetic foot ulcers

Clinical evidence suggests that diabetic ulcers, like other types of chronic wounds (venous leg ulcers, pressure sores), do not follow an orderly and reliable progression of wound healing. In the case of diabetic ulcers, several etiologic factors like neuropathy, ischemia, wound infection and wound size (20) predominantly determine healing impairment.

Peripheral sensory neuropathy

Peripheral sensory neuropathy is a prominent component of diabetic neuropathy (21) and skin denervation increases with diabetic duration (22, 23). Sensory nerves communicate tactile sensation to the central nervous system and contribute to the inflammatory response with the release of neuropeptides such as substance P (SP) (24) and calcitonin gene-related peptide (CGRP) (25). Sensory nerve fibers are involved in local monitoring and modulation of host immune defense (26) and can play an integral role in wound healing. Our study (27) demonstrates severe denervation of skin both in sensory neuropathic and non-neuropathic type 2 diabetes patients with active foot ulcers. Both groups do not differ significantly in the expression of SP, CGRP and nerve growth factor (NGF).

Infections in diabetic foot ulcers

There are five stages of clinical signs of severity of wound infection: (1.) contamination (presence of non-replicating microorganisms in wound), (2.) colonization (presence of replicating organisms adherent to wound bed without causing cellular damage of tissue), (3.) critical colonization (increasing bacterial burden, no classic signs of infection, absent or abnormal granulation tissue), (4.) local infection (bacterial burden of >10^6 organisms per gram of tissue, with classic signs of infection: redness, heat, swelling, and pain), and (5.) syste-
Cellular and molecular basis of impaired healing of diabetic foot ulcers

Bacteria do have an important role to play in permitting a degree of inflammatory response in acute wound healing. Colonizing or sub-infective levels of bacteria appear to accelerate neutrophils, monocytes and macrophages to infiltrate wounds and to form granulation tissue. However, in the presence of wound infection, infiltrating leukocytes produce cytotoxic and proteolytic enzymes (MMPs) and free oxygen radicals, which together with bacterial enzymes result in tissue damage and decreased growth factor production, reduced numbers of fibroblasts and disorganized collagen production (30).

It is now known that the bacteria present within acute and chronic wounds live within communities encased in a self-secreted matrix of extracellular polysaccharide, termed biofilms. Within these biofilms, bacterial matrix may provide an effective defense against host immune response, since leukocytes poorly penetrate biofilms (31). Much remains to be established about the significance of the biofilm in chronic diabetic wounds since antibiotics penetrate poorly into necrotic tissues and bacterial biofilms. Chronic wound debridement may play a beneficial role not only by reducing necrotic tissue, but also by reducing the bacterial load and disruption of the bacterial biofilm. Debridement of the granulation bed can have a beneficial effect by stimulation of secretion of growth factors, cytokines and chemokines, but in infected wounds it is used to avoid the development of spreading infection.

Molecular basis of ulcer healing in diabetes

Very little is known from the clinical perspective about the infiltrate composition in chronic diabetic ulcers and only a few studies have been published. Loots et al. (32) have observed a prolonged expression of fibronectin and tenascin in dermal tissue from chronic diabetic ulcers. There was also a prolonged presence of T and B cells as well as macrophages compared with normally healing acute wounds. The level of extravasation of immune cells in diabetes could be attributed to the vascular basement membrane thickening and to intracellular production of advanced glycation end products (AGE). AGEs can alter protein function, modify extracellular matrix and activate macrophages and endothelial cells to produce factors increasing matrix production (33, 34).

Neutrophils producing elastase dominate among the infiltrating cells in the ulcer bed and the adjacent dermis of chronic venous leg ulcer (35) and in chronic pressure ulcer (36). The persistence of neutrophils and their protease enzymes is suggested to be responsible for the chronicity of these ulcers. In both diabetic and normal mice with induced neutropenia, the wound closure was accelerated, however, there was no difference in the collagen content in the wound beds compared with normal wounds (37). Our study (38) demonstrates that diabetic foot ulcers show only a few infiltrating leukocytes compared to non-diabetic inflamed skin, despite strong expression of E-selectin by vascular endothelial cells responsible for leukocyte extravasation. There was also no significant difference in recruitment of inflammatory cells into ulcers between neuropathic and non-neuropathic patients (27). We suggest that the healing process of diabetic foot ulcers may be hampered by other molecular mechanisms decreasing accumulation of leukocytes.
sma levels of MCP1 and soluble E-selectin were observed in type 2 diabetes (45). MCP1 and IL8 not only mediate leukocyte infiltration, but may participate in wound re-epithelialization and angiogenesis (46-49).

Multiple studies have demonstrated a beneficial effect of many cytokines and growth factors in the healing process, both in animal models and patients suffering from chronic venous leg ulcer (50). However, functions of most growth factors in chronic diabetic foot ulcers remain rather unconfirmed (51-54).

Chemotactic for monocytes and fibroblasts and regulating angiogenesis PDGF accelerates granulation tissue formation and extracellular matrix deposition (55). The PDGF dimers AA and BB exert their effect by interaction with two types of receptors, α and β. Our study (44) showed that immunostaining of PDGF AA and BB was rather unchanged in the epidermis and dermal endothelium in the margins of diabetic foot ulcers compared with normal foot skin. Since the expression of α and β PDGF receptors was reduced in diabetic foot ulcers, the disturbed autocrine and paracrine pathways of PDGF effect on angiogenesis and re-epithelialization might be suggested.

There is a large body of evidence for the central role of angiogenesis in wound healing (56, 57) and impaired angiogenesis can result in retarded wound healing. Growth factors such as FGFb, TGFβ1, insulin-like growth factor (IGF1) and interleukin IL15 have also been identified as potential positive regulators of angiogenesis (58, 59). Our study (44) demonstrated a lack of upregulation of FGFb and IGF1 expression and significantly reduced expression of TGFβ1 and IL15 in vascular endothelium at the edge of diabetic foot ulcers. These data suggest that there is no upregulation of these angiogenic factors in diabetic foot ulcer endothelium and this fact may be responsible for the delayed formation of granulation tissue and retarded healing of diabetic foot ulcers.

IGF1, FGFb, EGF and IL15 also stimulate keratinocyte proliferation and re-epithelialization (60, 61). Our data (44) showed only slightly enhanced expression of IGF1 in diabetic foot ulcer epidermis in the majority of specimens compared with control skin. This confirms observations of Blakytny et al. (60) carried out in diabetic foot ulcer epidermis. We also observed significantly enhanced expression of EGF and unchanged expression of IL15 in diabetic foot ulcer epidermis compared with normal foot skin. As observed by us, overexpression of EGF and TGFβ1 in diabetic foot ulcer epidermis can be responsible for the observed undisturbed keratinocyte proliferation, lack of their apoptosis and migration (62-65).

Our study also demonstrates that the main deficiency in dermal diabetic foot tissue is related to low expression of angiogenic factors (44). In contrast, there is strong expression of factors responsible for mobilization and extravasation of leukocytes, despite the limited accumulation of immune cells we observed previously (38). It may account for poor angiogenesis and disturbed granulation tissue formation. On the other hand, we find enhanced expression of factors responsible for keratinocyte proliferation and migration suggests an undisturbed capacity of these cells present in the margins of diabetic foot ulcers (65). At the edge of diabetic foot ulcers, keratinocytes were p63+, CD29+, PCNA+, p53-. This may suggest that the impaired epithelialization of diabetic foot ulcers is not caused by the lack of epidermal stem cells, distorted epidermal cell proliferation, differentiation or apoptosis. Rather, retarded healing may reflect the distorted organization of granulation tissue, caused by impaired nutrition supply, infection and high levels of MMPs, and prevent keratinocytes from migration at the ulcer bed.

Several studies have found elevated levels of proteases and reduced levels of their inhibitors in chronic wounds. Higher concentrations of MMP-2, -8 and -9 and reduced concentrations of tissue inhibitors of MMP (TIMPs) were observed in diabetic wounds (66, 67). In clinical studies, therapy with dressings that contain gelatin (Promogran), the substrate for elastase and MMPs were used (68-70). Topical use of a doxycyline gel, an antibiotic of the tetracycline family with anti- MMPs activity, also showed improved healing of chronic diabetic foot ulcers (71). It seems that modeling of the granulation tissue creating proper conditions for formation of dermal-epidermal junctions between fibroblasts and keratinocytes might be a future therapeutic modality.

Molecular surgery: an integrative approach to diabetic ulcer healing

Proper debridement, defined as the removal of the hyperkeratotic, infected and necro-
tic tissue from a wound, is essential as it accelerates diabetic foot ulcer healing (72). Debridement should be done in a sequential fashion until no callus or hyperkeratotic tissue is seen in the periphery of the wound and no scar or infection is present in the bed of the wound.

Among the decisions that the clinician must make is to select the most appropriate antibiotic regimen, usually in the face of inadequate microbiological information. Initial treatment is empirical in about two-thirds of cases, but some basic principles can improve antibiotic therapy for diabetic foot infections (29, 73).

In the past 10 years, several growth factors, including recombinant human EGF, FGFb and PDGF have been produced and used in the clinic. Some results are found to be encouraging while others were not (74). At present, only topical application of recombinant human PDGF has been approved by the U.S. Food and Drug Administration and by the European authorities for the treatment of diabetic neuropathic ulcers (54, 75, 76). Recombinant PDGF-BB (becaplermin) was examined by Margolis et al. (77) in 2,394 patients with neuropathic ulceration and 802 patients (33.5%) healed. This compares with 5, 806 (25.8%) of those not receiving becaplermin (P<0.0001). Amputation rates were 4.9% in drug users and 6.4% in non-drug users (P<0.0001), with a 35% reduction in the risk for amputation. NGF was used only in three diabetic patients with foot ulcers and healing was achieved after five to 14 weeks of treatment (78). The authors attributed the improved effect to stimulation of angiogenesis by NGF.

Another approach was diabetic ulcer repopulation with autologous fibroblasts. Studies were conducted on the use of such a skin substitute with a good healing rate (79) and a multicenter, randomized, controlled clinical trial has documented a total of 79 diabetic ulcers at a 65% healing rate at 11 weeks (80).

Other technologies for molecular analyses, such as genomics and proteomics, could potentially be applied to people with diabetic wounds in the future. One of the major steps is the integration of these resources into synergistic therapies, starting at the cellular and molecular level.

REFERENCES

18. Marionnet C, Pierrard C, Vieux-Chagnpleau C et al.: Interactions between fibroblasts and keratinocytes in morphogenesis of dermal epidermal junc-

Received: 4.09.2007 r.
Adress correspondence: 02-106 Warszawa, ul. Pawińskiego 5