Wound healing in diabetic ulcers

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Citation for published version (APA):

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Chapter 1

General introduction and aims of the studies

Loots MAM

Diabetic Medicine 2002 (Chapter 1c: in press)
A. GENERAL PRINCIPLES OF WOUND HEALING

Wound healing in all its different aspects is an important topic in the practice of dermatology. The dermatologist is on a daily basis involved in the treatment of wounds and other cutaneous defects, performing dermatosurgery, electrocoagulation, cryosurgery, photodynamic and lasertherapy, chemical peelings and dermabrasia, phlebological and proctological surgery and sometimes cosmetic surgery. Dermatologists treat difficult-to-heal chronic ulcers of various origin, e.g. venous and arterial insufficiency, diabetes, neuropathy, decubitus, vasculitis, ulcerating skin disorders, malignancies, and many others (see chapter 2). As a consequence, the dermatologist has to possess a certain knowledge about acute wound healing, chronic wound healing, and wound treatment modalities, ranging from simple dressings to human recombinant growth factors and cultured skin equivalents. A thorough understanding of the basic principles of wound healing, down to its cellular and molecular level, is therefore essential.

The physiological process of wound repair can be subdivided in a number of consecutive but partly overlapping phases (Table 1): hemostasis, inflammation and debridement, proliferation, epithelialization, and remodeling.

Hemostasis is achieved by vasoconstriction, activation and aggregation of blood platelets, and the initiation of the coagulation cascade, with the conversion of prothrombin to thrombin and fibrinogen to fibrin as the major steps in the formation of the blood clot. Activated blood platelets release a large number of active substances, not only platelet factors that activate and attract more platelets, but also growth factors that are able to initiate wound healing, such as transforming growth factor β (TGFβ) and platelet-derived growth factor (PDGF). PDGF, along with other growth factors and glycoproteins such as fibronectin, increases cellular chemotaxis.

Inflammation represents a cellular cascade characterized by cell recruitment, first neutrophils followed by monocytes and lymphocytes. These cells are able to migrate into the wound area through the fibrin clot which serves as a provisional matrix. The neutrophils phagocytose bacteria, then kill the ingested bacteria by the production of microbiocidal substances - oxygen metabolites such as hydroxyl radicals, hydrogen peroxide and superoxide ion, later assisted by the macrophages. Circulating monocytes are attracted to the site of injury
several hours after the first neutrophils arrive and differentiate into macrophages. Neutrophils and macrophages are able to digest necrotic tissue, a process summarized as (autolytic) debridement. To accomplish this, a plethora of enzymes is being used, including lysosomal enzymes, collagenase 1 and 3, gelatinase, stromelysin, elastase, cathepsin and plasminogen activator, which converts plasminogen into enzymatically active plasmin. Apart from their role in debridement, macrophages secrete chemotactic factors, which attract additional inflammatory cells to the wound site, and produce growth factors such as PDGF, TGF-β, and fibroblast growth factor (FGF), which are necessary for the initiation and propagation of granulation tissue formation. In this way the macrophages mediate the transition from the initial inflammatory response to the early repair phase of wound healing. Helper T-cells are activated following injury. They recognize any foreign antigen on the surface of antigen-presenting cells, e.g. Langerhans cells and migrate into the wound along with the macrophages. It has been known for long that lymphocytes are present in healing wounds, but their function is not entirely clear and remains an area of interest. Both macrophages and lymphocytes disappear from mature wounds by an unknown mechanism, but in abnormal scars and chronic venous and diabetic ulcers they persist for a prolonged period of time.

Table 1. Phases in wound healing

<table>
<thead>
<tr>
<th>healing phase:</th>
<th>active cells:</th>
<th>main controlling cytokines:</th>
</tr>
</thead>
<tbody>
<tr>
<td>hemostasis</td>
<td>platelets</td>
<td>PDGF, TGF-β</td>
</tr>
<tr>
<td>inflammation and debridement</td>
<td>lymphocytes/leukocytes/macrophages</td>
<td>IL-6, IL-1, TNF-α, G-CSF, PDGF</td>
</tr>
<tr>
<td>proliferation</td>
<td>fibroblasts/endothelial cells</td>
<td>PDGF, bFGF, TGF-β, IGF, VEGF</td>
</tr>
<tr>
<td>epithelialization</td>
<td>keratinocytes</td>
<td>EGF, KGF</td>
</tr>
<tr>
<td>remodeling</td>
<td>(myo-)/fibroblasts</td>
<td>IFN-γ, TNF-α</td>
</tr>
</tbody>
</table>
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The proliferation phase (the first days to weeks after injury) involves the multiplication of fibroblasts and endothelial cells, the two major cellular components of granulation tissue. This newly formed tissue is characterized by an increased number of fibroblasts organized parallel to the epidermis, capillaries organized perpendicular to the fibroblasts and deposition of unorganized collagen type III fibrils and proteoglycans. Differentiation of fibroblasts into a contractile myofibroblast phenotype also occurs and may aid in closure of the wound by wound contraction.

As soon as a sufficient amount of granulation tissue has been formed adjacent to the wound margin, epithelialization will follow automatically. The keratinocytes finally close the wound by a combination of proliferation and migration. The process of epithelialization can be accelerated by creating a moist wound environment. In dry wound healing, the keratinocytes have to migrate underneath the dried-out fibrin crust, which requires more time.

Finally, in the remodeling phase, resolution of the scar tissue occurs. Collagen type III is replaced by collagen type I with an increasing amount of cross linkage bounds between the collagen fibrils. As scars get older, the tissue starts to consist of thick collagen bundles, organized parallel to the epidermis, few capillaries, few fibroblasts and thin immature elastin fibers. The microscopic structure of normal dermis is characterized by a more basket weave pattern of collagen type I bundles and thick mature elastin fibers. It has previously been shown that apoptosis is involved in the loss of granulation tissue cells including fibroblasts and small vessels as scar formation occurs. Compared with normal skin, tissue of a healed wound is less functional, less strong and can be cosmetically disfiguring.
B. PATHOPHYSIOLOGY OF DIABETES MELLITUS

Diabetes mellitus is defined by the WHO as a chronic elevation of the concentration of blood glucose,\textsuperscript{15,16} which characteristically poses a long-term risk to the patient of developing progressive disease of the retina and kidney, damage to peripheral nerves, and aggravated atherosclerotic disease of the heart, legs and brain. The number of people with diabetes worldwide is expected to rise from about 150 million in 2000 to 220 million in 2010 and 300 million in 2025.

Hyperglycemia can be caused by a decreased production or by an impaired action of insulin or both. There are several other hormonal and neural influences that can modulate glucose homeostasis, for instance cortisol and glucagon. Diabetes was originally considered as a state of insulin deficiency, but the last 25 years the concepts about the disease have changed. In fact, many diabetic patients do secrete insulin. Diabetes can be distinguished in the lean, ketosis prone, juvenile-onset type (insulin-dependent or type 1, IDDM) and the obese, non-ketosis-prone, maturity-onset form (non-insulin-dependent or type 2, NIDDM).

Insulin dependent diabetes mellitus

Insulin dependent diabetes mellitus (IDDM), sometimes termed type 1 diabetes, is generally characterized by abrupt onset of severe symptoms, dependence on exogenous insulin to sustain life, and proneness to ketosis even in the basal state, all of which are caused by gross insulin lack (insulinopenia). IDDM is the most prevalent type of diabetes among children and young adults in developed countries, and it was formerly termed 'juvenile diabetes'. However, classification based on age at onset showed in fact that the disease can start at any age. Onset of IDDM in adults is not uncommon. Current theories about the pathogenesis of IDDM intertwine three closely related variables: 1) autoimmune mechanisms, 2) environmental factors that could serve as triggering events (viral infections with coxsackievirus B4, rubella, mumps, Epstein-Barr virus and cytomegalovirus) and 3) genetic factors.

Non-insulin-dependent diabetes mellitus

The non-insulin-dependent diabetic state is characterized by a combination of inadequate insulin secretion and resistance of peripheral tissues to its actions. For unknown reasons, beta-cell mass in patients with NIDDM may be reduced at the time of diagnosis. Furthermore, the insulin response to a glucose challenge is diminished in many individuals. The synthesis of an abnormal, biologically less-active insulin molecule as a result of mutation of the insulin
gene (mutant insulin) has been demonstrated to be the cause of NIDDM in a few patients. In NIDDM, ketosis is rare, there is a high incidence of obesity and there is no association with viral infections, islet autoantibodies, or HLA expression.

Acute complications of diabetes mellitus

**Diabetic ketoacidosis**
This is a metabolic emergency in which hyperglycemia is associated with a metabolic acidosis due to insulin deficiency. The presenting symptoms are polyuria, polydipsia, nausea, vomiting, hyperventilation (Kussmaul’s respiration) and abdominal pain. The therapy consists of low dose intravenous insulin infusions, fluid administration and electrolyte replacement under careful monitoring.

**Non-ketotic hyperosmolar coma**
A metabolic emergency in which uncontrolled hyperglycemia induces a hyperosmolar state in the absence of significant ketosis. Patients present in middle or later life with extreme dehydration. Endogenous insulin levels are sufficient to inhibit hepatic ketogenesis. Glucose production is unrestrained resulting in osmotic diuresis causing hyperosmolality. Symptoms are extreme thirst caused by dehydration, which may eventually result in stupor or coma. Therapy consists of rehydration, intravenous low dose insulin infusions and electrolyte replacement according to the prominent clinical features.

**Hypoglycemia**
This is the most common complication of insulin therapy and a major cause of anxiety for patients with diabetes mellitus. Symptoms typically develop over a few minutes and most patients experience adrenergic features like sweating, tremor, a pounding heart beat and pallor and drowsiness. Any form of rapidly assimilated carbohydrate will relieve the symptoms. Unconscious patients should be given intravenous glucose or intramuscular glucagon.
Late complications of diabetes mellitus

Vascular disease
The chronic complications of diabetes can be divided into macrovascular disease (cardiovascular disease, stroke, transient ischemic attacks and peripheral vascular disease) and microvascular disease (nephropathy and retinopathy). Diabetic macroangiopathy is used for the description of diseases involving the large arteries, while microangiopathy refers to diseases involving capillaries, arterioles and venules. One of the reasons for distinction between macro- and micro vascular disease is that different cell types are involved: in macrovascular pathology these are mainly endothelial cells and smooth muscle cells, in microvascular conditions, endothelial cells, pericytes, renal glomeruli-endothelial cells, mesangial cells and epithelial cells are involved. Macrovascular disease is primarily associated with age, whereas microvascular disease is related to duration of diabetes, as described by the WHO Multinational Study of Vascular disease in Diabetes.19

Diabetic macrovascular disease mainly affects the coronary arteries, cerebral vessels and tibioperoneal arteries of the lower limbs,20 with sparing of the pedal vessels. Despite the close similarities, macrovascular disease differs from non-diabetic atherosclerotic disease in several ways; diabetic vascular disease progresses more rapidly and the lesions are more diffuse and more widespread than the classical atherosclerotic lesions which are restricted to specific sites in the vasculature. Accumulation of periodic acid Schiff (PAS)-positive material and diffuse calcification of the media of large arteries is more frequently observed in diabetic vascular disease.21,22

Microvascular complications include retinopathy and nephropathy and are associated with thickening of the capillary basement membrane. For years it was thought that ‘diabetic microangiopathy’ was also an important contributing factor in the development of diabetic ulcers. But although capillary involvement is evident in the eye and the kidneys, and may be the cause of diabetic dermopathy, the contribution of occlusive microvascular disease in the etiology of diabetic foot ulcers has not been confirmed in recent studies.23
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**Retinopathy**

Proliferative retinopathy is characterized by neovascularization of the retina. This neovascularization is triggered by occlusion of vessels as a result of intravascular coagulation or vessel wall thickening. The new vessels contain few pericytes and do not form a proper blood barrier, which leads to increased risk of bleeding and may finally result in retinal detachment and visual loss.\(^\text{24}\)

**Nephropathy**

In renal glomeruli, thickening of capillary basement membrane is a central mechanism for loss of kidney function in diabetic patients.\(^\text{23}\) Expansion of the mesangial matrix in renal glomeruli is accompanied by a gradual loss of endothelial cells and epithelial cells.\(^\text{25}\) Increased capillary permeability is observed in diabetic patients, which leads to abnormal leakage of proteins across the endothelium. Extravasated proteins may be deposited in basement membranes and contribute to their thickening. Furthermore, increased endothelial permeability causes urinary excretion of albumin, a characteristic feature of diabetic nephropathy.

**Neuropathy**

Neurological complications of diabetes occur in the peripheral nervous system, mainly affecting distal parts of the lower extremities (distal symmetric sensorimotor polyneuropathy), as well as in the brain.\(^\text{26}\) Although the underlying mechanism for the peripheral nerve lesions in diabetes is not fully understood, it is known to be a condition that is slowly progressive. There is increasing support for the ‘vascular hypothesis’ in the pathogenesis of neuropathy on the basis of either decreased circulation to the nerve or alterations in endoneurial flow. In contrast, there are studies indicating no specific changes in the endoneural vessels of diabetic versus non-diabetic patients.\(^\text{27}\)
Cutaneous diseases associated with diabetes mellitus

Necrobiosis lipoidica diabeticorum, diabetic dermopathy and diabetic bullae are, although they are not specific, skin diseases with a strong association with diabetes mellitus.\textsuperscript{29,30} Necrobiosis lipoidica, the best known cutaneous marker of diabetes mellitus, has a reported incidence of 0.3 to 1.6\% in all patients with diabetes mellitus. Necrobiosis lipoidica is not exclusive to diabetics, with almost one third of patients with this cutaneous disorder being nondiabetic. Conversely, approximately 90\% of patients that present with necrobiosis lipoidica have or eventually develop diabetes mellitus, have abnormal glucose tolerance, or report a history of diabetes in at least one parent. The condition occurs most commonly in the third or fourth decade, with the majority of patients being women. The initial lesions are well-circumscribed erythematous papules and plaques.\textsuperscript{31,32} These evolve radially into sharply defined erythematous plaques with a depressed, waxy, yellow-brown, atrophic, telangiectatic center (Fig. 1), that sometimes cover the entire pretibial areas. After minor trauma, ulceration occurs in about one third of lesions on the legs.

\textbf{Figure 1.} Necrobiosis lipoidica diabeticorum.
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Diabetic dermopathy, also called shin spots or pretibial pigmented patches because of their usual location, is the most common finding in diabetes mellitus.\textsuperscript{32} It is not a specific marker for diabetes because 20\% of the persons without diabetes in comparable age groups show similar lesions as compared with 30\% to 60\% of patients with diabetes. Shin spots are usually small, atrophic, brownish hyperpigmented, oval or round areas distributed bilaterally but not symmetrically on the shins.

Diabetic bullae or bullosis diabeticorum is an uncommon entity occurring in approximately 0.5\% of diabetic patients. Diabetic bullae are seen in adults only, more commonly in men, usually with long-standing diabetes. The lesions appear spontaneous on the hand and feet (Fig. 2) and contain sterile, sometimes hemorrhagic fluid.\textsuperscript{34,35} The pathogenesis of this dermatosis is still unclear.

![Figure 2. Bullosis diabeticorum.](image)

Thickening or hardening of the skin can also be observed in diabetes. There are three forms of diabetic thick skin: 1) scleroderma-like syndrome (SLS) and limited joint mobility (LJM); 2) clinically unapparent but measurable increased skin thickness as compared with controls; and 3) scleredema diabeticorum.\textsuperscript{36-40} Waxy skin and stiff joints or SLS appear to be common in preadolescent and adolescent insulin-dependent diabetics.\textsuperscript{41-42} Scleredema diabeticorum is characterized by a marked increase in dermal thickness on the back and
posterior upper neck in middle-aged, overweight, poorly controlled patients with NIDDM. The pathogenesis of diabetic thick skin has not been fully elucidated. Potential explanations include the nonenzymatic glycosylation of collagen resulting in increased cross-linkage and dermal thickening.

*Acanthosis nigricans* is a skin disorder characterized by a velvety brown-black thickening of the keratin layer most commonly noticed in the neck. It is a marker for hyperinsulinemia and insulin resistance.43–45

*Granuloma annulare* may be associated with diabetes. The pathogenesis remains unknown.46 Localized lesions occur in 9.7% of the cases in patients with diabetes and generalized lesions in 21%. They can be induced by trauma and are seen typically on the dorsum of the hands, feet or elbows.

*Rubeosis faciei* has been reported in 3 to 59% of patients with diabetes mellitus. It consists of a reddish complexion secondary to venous engorgement of the superficial vessels of the face.

*Perforating dermatoses* are seen almost exclusively in patients with end-stage diabetic nephropathy. These unusual extremely pruritic lesions are caused by consist of a transepidermal expression of degenerative material, typically collagen or elastin. Multiple umbilicated hyperkeratotic, pruritic papules may be present, sometimes in a linear arrangement on the trunk or the extensor surface of the extremities, especially the legs.

*Xanthoma eruptivum* is the most common cutaneous manifestation of hyperlipidemia. Uncontrolled diabetes mellitus is a frequent cause of increased triglyceride levels in patients with familial hypertriglyceridemia. Eruptive xanthomas characteristically appear as discrete dome-shaped papules or nodules with yellow-waxy centers and an erythematous base on the buttocks, elbows and knees. They may resolve quickly with correction of the hypertriglyceridemia.

The incidence of *cutaneous infections* in diabetes mellitus shows a close correlation to the mean blood glucose levels. Both bacterial and fungal nail infections (especially Candida paronychia) and Candida infections of the female genitalia are common findings in diabetics.29,47 Balanitis is frequently seen in elderly uncircumcised diabetic men. Angular cheilitis, a possibly combined fungal and bacterial infection of the oral commissures, is not uncommon. It is a classic lesion in diabetic children and occasionally in diabetic adults although it is also frequently seen in nondiabetic adults. In some patients it may be related
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to badly fitting dentures, hypersalivation and riboflavin deficiencies. Infections of the interdigital areas, oral candidiasis and intertriginous candidiasis do occur but are less common than paronychia. Some authors report that the incidence of other dermatophytosis than paronychia is not increased at all in patients with diabetes. 48

No studies have demonstrated that furunculosis (boils) or carbuncles are more prevalent in patients with well controlled diabetes. 49 Some staphylococcal and streptococcal infections (group A and B) are over-represented in diabetes mellitus. 50,54 There is especially an increased rate of staphylococcal infections in patients continuously using subcutaneous insulin infusion. 55,56

Generalized pruritus was thought to be associated with diabetes, but recent controlled studies have failed to confirm this. Localized pruritus may be present, in conditions where Candida overgrowth is involved, such as pruritus ani and especially pruritus vulvae. 57

Insulin-induced lipoatrophy is the loss of fat at the sites of injections; this appears 6 to 24 months after injections have been started in approximately 16% of the patients using conventional insulin and less (0-2.5%) when highly purified insulin is used. 58,59

Vitiligo and lichen planus are auto-immune related disorders and occur more often in patients with IDDM.

Chronic diabetic ulcers

Diabetic foot ulcers are a common complication. Fifteen percent of the patients with diabetes mellitus will eventually develop a foot ulcer, 61 and 14 to 24% of the patients with foot ulcers will undergo amputation. Only 31% of neuropathic ulcers will heal within 20 weeks, 62 and the average time until total wound healing of diabetic ulcers may be 89 days. 63 Obviously, the aforementioned factors like macroangiopathy, neuropathy, and increased susceptibility to infection can be held responsible for the development of these ulcers, and partly for their retarded healing. But there are other underlying mechanisms involved, superimposed on these gross pathological factors, mechanisms that are somehow related to glucose levels, and somehow affect wound healing on the cellular and molecular level. These mechanisms, the main subject of this thesis, are further discussed in the next chapter.
C. IMPAIRED WOUND HEALING IN DIABETES MELLITUS

Introduction

Wound healing in healthy individuals normally proceeds through general stages such as hemostasis, inflammation and debridement, proliferation, epithelialization and tissue remodeling. Many chronic wounds, and particularly chronic diabetic ulcers, fail to complete all these stages of healing. The pathological mechanisms responsible for impaired wound healing in diabetes mellitus are not yet fully understood. As in other medium- and long-term complications of diabetes mellitus, clinical and experimental observations indicate that periods of poor glycaemic control and resulting hyperglycemia are at least partially responsible.

Diabetic foot ulceration is a major problem that significantly affects the quality of life of the patient and leads to prolonged hospitalization. At least 15% of individuals with diabetes will develop foot ulcers during their lifetime. Several risk factors for ulceration have been identified. These include peripheral neuropathy (sensory, motor, and autonomic), peripheral vascular disease, structural foot deformities, limited joint mobility, increased dynamic foot pressures, previous foot ulcers and poor metabolic control. Neuropathy leads to insensitive and subsequently deformed feet, and even a minor trauma, such as pressure caused by ill-fitting shoes can proceed into a chronic ulcer (Fig. 3). Peripheral vascular disease contributes to this process by reduced tissue perfusion. Continued walking on insensitive feet leads to further tissue breakdown.

Diabetic neuropathic ulcers are, among other chronic ulcers like venous ulcers, arterial ulcers, pressure ulcers, especially noted for their slow healing rates and resistance to traditional methods of treatment. If not treated properly, these ulcers might easily become secondarily infected and even progress to gangrene or osteomyelitis, requiring amputation (Fig. 3). In 1992, 47% of all lower extremity amputations in the Netherlands (1.575) were performed in patients with diabetes mellitus.
Figure 3. Sequential amputations in a patient with neuropathy caused by NIDDM. Left: Diabetic pressure ulcer on the plantar surface of the left foot. The phalanges of rays 2, 3 and 4 had been removed previously because of osteomyelitis. Right: The same foot, three years later. A new pressure ulcer developed on the lateral surface of the first metacarpophalangeal joint, and from this ulcer the underlying joint and bones became infected and had to be removed.

Within the entire health care budget spend on the treatment of diabetes, a large proportion is consumed by diabetic foot care. The costs of treatment of diabetic foot ulcers accounts for at least £13 million in the UK and $150 million in the US per annum.67 Not surprisingly, the diabetic foot is selected by several manufacturers as a target indication for sophisticated bioengineered medical products, such as recombinant human growth factors, cultured skin equivalents, and extracellular matrix components. These products are usually quite costly, but if they are able to reduce healing time, hospitalization time, or the number of amputations, cost-benefit calculations will support their usefulness. And the potential long term benefits associated with rapid and complete healing of diabetic ulcers may be substantial. A better understanding of the effect of diabetes on wound healing is required for the development of successful treatment techniques.
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Studies on impaired wound healing in diabetes

The phenomenon of impaired wound healing in diabetes mellitus has been studied in humans as well as in animal models. The ob/ob mice and alloxan- or streptozotocin treated mice (suitable animal model for type 1 diabetes) models provide useful means for studying healing impairment. It should be kept in mind however that animal models of diabetes provide useful means of studying healing impairment caused by early changes in diabetes, but clearly do not fully reproduce the long term pathological changes in vascular and nervous function seen in human diabetic subjects. Disturbed wound healing may occur during any phase of the wound-healing process. In the following section we will summarize the reported abnormalities in the literature for each phase in wound healing. It should be noted that the different phases overlap in time (Figure 4) and that the beginning and end of a phase is dependent on multiple interacting factors.

![Diagram of wound healing phases](image)

Figure 4. Overlapping phases in wound healing.

Phase of hemostasis
Disruption of the capillaries causes hemorrhage, platelet accumulation and activation, and coagulation of blood. Up to date no abnormalities have been reported in this wound healing phase in humans or animals with diabetes mellitus.
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Inflammation and debridement phase

Inflammatory responses
The hyperemic response observed after wounding is reported to be impaired in patients with type 1 diabetes. Blood flow increases after wounding as a result of the inflammation induced by skin injury which releases vasoactive mediators such as histamine and prostaglandines.68 Fahey et al. showed that while the initial inflammatory response in diabetic animals is similar to that seen in normal controls, the total number of leukocytes and wound fluid IL-6 levels are decreased in diabetic mice during the late inflammatory phase of healing.69 In this phase, wound repair is in transition from an inflammatory phase to a reparative phase. They conclude that impaired leukocyte migration into wound sites and an altered pattern of cytokine appearance in the wound milieu may contribute to delayed wound healing. Recently, advanced glycation end products (AGEs) have been held responsible for the delayed inflammatory cell influx.70 AGEs have been established to interfere with both extracellular matrix proteins, and with cellular function via cell surface receptor-mediated interactions. Administration of a soluble receptor fragment for advanced glycation end products (sRAGE) led to restoration of the normal wound response in a db/db mice model.71

Others have suggested that impaired leukocyte function may contribute to impaired healing in diabetics based on abundant evidence that neutrophil chemotaxis,72-75 phagocytosis,76-83 and intracellular killing84 are inhibited by hyperglycemia or a yet unknown serum factor.85-88 Others report impaired intracellular killing and decreased membrane fluidity of polymorphonuclear leukocytes.89,90

Proteolytic enzymes
It has been shown that chronic wounds often have an imbalance between proteases and protease inhibitors in the wound fluid. Main sources of proteolytic enzymes are leukocytes and macrophages. Wound infection may attribute to the increased proteolytic activity, either indirectly or directly through excess production of proteases by the bacteria. An increased proteolytic environment may lead to more rapid and/or more extensive degradation of the extracellular matrix in these wounds, which blocks or complicates the migration and attachment of regenerating cells.91-94 In addition, interruption of cell-matrix adhesion, as may occur in the case of excessive matrix degradation, may lead to apoptosis by inhibition of integrin binding to matrix proteins.95,96 Considering the frequent findings of increased protease expression in chronic wounds and reduced matrix stability, this may also be a factor leading to reduced cell survival and increased apoptotic cell death as has been suggested by Gailit and Clark.97 Another negative influence of an increased proteolytic activity in
chronic wounds is that growth factors will be deactivated more rapidly. As a consequence of this theory, some groups are considering the concept of treating chronic ulcers, and especially diabetic ulcers, with a combination of growth factors and compounds that bind or inhibit proteases.

Proliferation phase

*Fibroblasts and endothelial cells*

Darby et al.\(^9\) studied a non-obese diabetic (NOD) strain of mice (genetically diabetic mice) and reported reduced cell proliferation rates (endothelial cells and fibroblasts) in the wound tissue, a retarded onset of myofibroblast differentiation, lower procollagen I mRNA expression and increased apoptosis when compared to non-diabetic control mice. Differentiation of fibroblasts into a contractile myofibroblast phenotype (visualized by \(\alpha\)-smooth muscle actin) contributes to wound closure by wound contraction. The wounds not only showed reduced \(\alpha\)-smooth muscle actin staining but also showed a lack of orientation of fibroblasts compared to control wounds at 7 days. This alteration could affect the efficacy of wound contraction, perhaps leading to delayed and inefficient contraction. Furthermore they postulated that the observed reduced number of fibroblasts and endothelial cells in diabetic wounds versus controls could also be explained by delayed invasion of these cells into the wound from the surrounding dermis. This could result from increased matrix rigidity in the unwounded skin and the consequent diminished vulnerability to protease degradation. Dyer et al.\(^99\) and Vlassara et al.\(^100\) showed that diabetic skin collagens show increased levels of glycation compared with non-diabetic controls. Glycosylation is a non-enzymatic reaction between glucose and proteins leading to reactive advanced glycation end-products, and these in turn can result in inappropriate cross linking and increased rigidity of the matrix. Advanced glycosylation end products are postulated to accumulate in diabetes and may adversely affect extracellular matrix production, cell function, and cytokine production.\(^61,70\)

*Extracellular matrix production*

Decreases in collagen deposition have been reported in wounds of the stomach, duodenum,\(^101\) and in the musculofacial layer of the abdominal wall in diabetic patients compared to those without the disease.\(^102\) Goodson and Hunt found less collagen deposition in subcutaneously implanted stainless-steel wire-mesh cylinders in diabetic rats by measuring the hydroxyproline content of the formed granulation tissue in the cylinders.\(^103\) A reduction in subcutaneous collagen accumulation and tensile strength in wounds in streptozotocin treated and ob/ob mice has been reported by many others as well.\(^104-114\) It has been
shown that fibroblasts from chronic wounds may produce less collagen than normal dermal fibroblasts at least in vitro. Additional studies on diabetic skin fibroblast cultures also report an abnormal collagen synthesis and inhibition of linking and cross linking of collagen fibrils. A linear dose response between inhibition of collagen fibril formation and glucose concentration has been reported, leading to a decrease in bursting and tensile strength. In contrast, several in vitro studies on the effects of high glucose levels on collagen production showed no decrease or even an increase in collagen synthesis by non-insulin dependent diabetic fibroblasts versus an increase in collagen synthesis by non-diabetic fibroblasts.

Diabetic complications are often marked by increases in matrix deposition, as seen both in vitro with retinal capillary cells and in in vivo models of diabetic nephropathy. After injury, increased amounts of fibronectin accumulate, due to increased synthesis and impaired degradation, in patients with diabetes in contrast to non-diabetic individuals. Therefore the relative importance of the observed diminished granulation tissue mass, reduced collagen deposition and defects in collagen maturation remain to be defined.

**Epithelialization**

Koivukangas et al. reported a delayed restoration of epidermal barrier function after suction blister injury in patients with type I diabetes mellitus. They measured water evaporation from such wounds which was more abundant in diabetic patients versus controls. This finding and the observed higher mean increase in blood flow 4 days postwounding was suggestive of a deficit in the ability to restore the epidermal barrier.

Impaired re-epithelialization characterizes chronic diabetic ulcers, resurfacing of these wounds may fail to occur for months. On light microscopy, the prominent acanthosis regularly present at the edge of diabetic ulcers suggest that keratinocyte migration might be disturbed rather than proliferation. However, Blakytny et al. reported a lack of insulin-like growth factor I (IGF I) in the basal keratinocyte layer of diabetic skin and foot ulcers which could result in diminished proliferative activity of the keratinocytes and hence a decreased rate of re-epithelialization. Brown et al. found a reduced expression of IGF I and II in cutaneous wounds of diabetic mice, others showed that the induction of keratinocyte growth expression was reduced and delayed in genetically diabetic mice versus normal mice. The authors suggested that earlier induction of KGF might be essential for rapid re-epithelialization. Finally, a recent study showed that keratinocyte proliferation as well as differentiation were impaired in the diabetes milieu of the insulin receptor knock out mice.
Remodeling phase
Finally, in the remodeling phase, resolution of the wound tissue occurs which can last up to one year. Collagen type III is replaced by collagen type I with an increasing amount of cross linkage bounds between the collagen fibrils and thicker collagen bundles, organized parallel to the epidermis. No studies have been published so far on this phase of wound healing in diabetic patients.

In summary, these experimental and clinical wound healing studies reveal that the diabetic state is accompanied by a delayed or decreased repair capacity, most pronounced in the phase of inflammation and debridement and in the phase of proliferation. To a lesser extent the phase of epithelialization seems to be affected. This is stressed by the disappointing clinical reports on EGF in the treatment of chronic wounds. It has been demonstrated that insulin deficiency is associated with the earlier mentioned abnormalities but although insulin administration improves all these processes, certain groups of patients seem to remain prone for non-healing wounds. Other researchers found that insulin treatment neither prevents nor corrects the multiple tissue defects associated with diabetes. In conclusion, the impairment of wound healing occurring in chronic diabetic ulcers appears to be multifactorial and multilevel and needs to be further elucidated in order to come to promising new treatment strategies.
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D. AIMS OF THE STUDIES

The primary aim of this thesis was to investigate mechanisms that may be responsible for retarded wound healing in diabetic ulcers.

As apparent from the introduction, the gross pathological factors leading to diabetic ulcers are wellknown, and our knowledge of general principles of wound healing has certainly increased during the last decade. We are able to describe the healing process in detail by subdividing it in different stages such as hemostasis, inflammation and debridement, proliferation, epithelialization, and remodeling. Despite this, regulation of wound healing at the molecular and cellular level, and how the hyperglycaemic state or other phenomena associated with diabetes affect it, is still poorly understood, and hence, the main subject of this thesis.

Clinical perspective
Chronic leg ulceration affects 1% of the adult population and 3.6% of the people older than 65 years. Leg ulcers are debilitating and painful and greatly reduce the quality of life for the patient. About 400 years BC, Hippocrates wrote, “In case of an ulcer, it is not expedient to stand, especially if the ulcer be situated on the leg”. The best treatment of leg ulcers depends upon their diagnosis and underlying pathology. The majority of leg ulcers are caused by venous insufficiency, arterial disease or neuropathy, or a combination of these factors. Venous ulcers most commonly occur above the medial malleoli. Arterial ulcers often affect the toes or shin, diabetic neuropathic ulcers are usually localized on pressure points, such as the metatarso-phalangeal joints, ankles or heel region. Less frequent causes include infection, haematological disorders, vasculitis and pyoderma gangrenosum and tumors. In chapter 2, frequent and infrequent causes of leg ulceration are listed and their underlying pathology will be discussed.

Study hypothesis and outline
Diabetic neuropathic ulcers are, among the above mentioned other types of leg ulcers, noted for their slow healing rates and resistance to traditional methods of treatment. If not treated properly, these ulcers might easily become secondarily infected and osteomyelitis or gangrene may develop, finally resulting in amputation. But even with adequate treatment, approximately 15% of all diabetic patients with chronic ulcers will not respond. To achieve better wound healing rates in more patients, it is necessary to unravel which phases, cells, cytokines, matrix proteins, or other molecules might be effected by diabetes. Based on the clinical observations that epithelialization can proceed normally in diabetic ulcers as soon as the quality of the wound bed has improved, and that
clinical studies with growth factors that accelerate epithelialization are not very successful, we hypothesized that the disturbances had to be located in the two preceding phases, the inflammation and debridement phase, or the phase of proliferation followed by matrix deposition, which is the most time-consuming phase. Consequently, our in vitro studies focused on these phases and their predominant cell types.

The predominant cell types in the inflammation and debridement phase are lymphocytes, granulocytes and macrophages. The major function of granulocytes in wounds is to eliminate contaminating bacteria, the function of lymphocytes in (non)healing wounds remains a topic of interest. Macrophages play a crucial regulatory role in the transition between wound inflammation and the next phase of wound repair, granulation tissue formation.

The proliferation phase is characterized by proliferation of endothelial cells and fibroblasts and the deposition of extracellular matrix (ECM) molecules. During the repair process the ECM is sequentially remodeled and rebuilt by the action of different cell types and their products. Fibronectin (FN) acts as a scaffold for new matrix deposition, chondroitin sulphate (CS) is a disaccharide forming glycosaminoglycan (GAG) providing high hydrative capacity in the temporary matrix, and tenascin (TN) is probably important for maintaining tissue homeostasis by interfering with cell migration and proliferation and by inhibiting cell adhesion to FN.

Very little is known about the expression of these above mentioned extracellular matrix molecules and the composition of the cellular infiltrate in chronic diabetic wounds. Therefore the expression of different ECM molecules and the composition of the infiltrate, which is assumed to be a ‘frozen picture’ of a deficient healing stage, in chronic diabetic wounds was characterized. The results of these studies are summarized in chapter 3. The cellular infiltrate and the expression of different ECM molecules in skin biopsies taken from the edge of chronic ulcers (defined as existing for > 8 weeks), diabetic ulcers and of acute wounds in time was characterized quantitatively. To assess the influence of the diabetic state on the infiltrate, biopsies were also taken from patients with chronic non-diabetic wounds, upper leg skin from patients with NIDDM (nonlesional) and upper leg skin from healthy age-matched volunteers.

In chapter 4, the role of macrophages in delayed wound healing in chronic diabetic and venous ulcers is evaluated. Macrophages are considered to be important in wound healing. They are reported to be abundantly present in chronic ulcers, but it was not yet studied whether they are activated, functionally active in chronic diabetic ulcers. This was investigated by analyzing their state of activation with the marker MRP-8/14 in skin biopsies of chronic diabetic ulcers.
Chapter 1

The fibroblast, as pivotal cell in the proliferation phase, was the main target of the following studies. Although it is more difficult to isolate and culture fibroblasts from chronic diabetic ulcers than from intact skin in diabetic and non-diabetic patients, these are the proper populations of fibroblasts that should be used for in vitro studies. Chapter 5 describes the proliferation rates of fibroblasts isolated from diabetic ulcers, which seemed rather impaired, compared to controls.

Chapter 6 consists of the search for the proper concentrations of growth factors or combination of growth factors to enhance fibroblast proliferation. It is unlikely that single growth factors are released from platelets and macrophages under physiologic conditions. A common view is that a cocktail of several growth factors with different activities might be required for optimally enhanced healing. Several well-characterized growth factors are available for clinical use. Most growth factors have been characterized by their capacity to induce mitosis in different cell types in vitro. These mitogens can be grouped according to their sequence of action in the cell cycle. Competence factors, exemplified by fibroblast growth factor (bFGF) and platelet derived growth factor (PDGF-AB) act by making quiescent cells (in G0 Phase of the cell cycle) competent to respond to a second group of growth factors, the progression factors. Insulin-like growth factor (IGF-I) and epidermal growth factor (EGF) appear to drive cells through G1 of the cell cycle into DNA synthesis and are examples of the progression factors. In this chapter, the optimal concentrations of individual and combined growth factors, and their maximum mitogenic effects were determined with diabetic ulcer fibroblasts and controls.

In chapter 7 the clinical entity ‘the diabetic foot’ is discussed. In this chapter, the main principles of treating diabetic ulcers are discussed, including issues as diagnosis, preventive measures, antibiotic regimens, and vascular surgery. Experimental findings are extrapolated to the clinical setting, and recent advances in diagnosis and wound care are discussed.

Chapter 8 and 9 contain a general discussion and summary of the conclusions drawn in our studies.

The experimental studies that formed the basis of this thesis were supported by a research grant from the Dutch Organization for Scientific Research (NWO-AGIKO programma chronisch zieken, grant nr. 940-37-003, ‘Disturbed wound healing in diabetes’) and by the Dutch Diabetes Foundation (Diabetes fonds Nederland, grant nr. 94.124, ‘Improved healing of chronic diabetic wounds by stimulation of extracellular matrix formation’).
REFERENCES

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