Wound healing in diabetic ulcers
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Chapter 8

Summary and conclusions
Chapter 1: Introduction and aims of the studies

Normal versus disturbed wound healing
The physiological process of wound repair can be subdivided in several consecutive, partly overlapping phases: hemostasis, inflammation and debridement, proliferation, epithelialization and finally remodeling. In any phase complications may occur or underlying disturbances may hinder or delay the normal progress to the next phase. As a consequence, the wound healing process will be delayed. When the delay is significant the wound is called chronic.

Impairment of wound healing in diabetes mellitus
Clinical and experimental studies show that diabetic patients as a group experience impaired wound healing. There is abundant experimental evidence that neutrophil chemotaxis, phagocytosis and intracellular killing are inhibited by hyperglycemia or an yet unknown serum factor. In wounds in non-obese diabetic strains of mice (genetically diabetic mice) a reduction in collagen accumulation and tensile strength were observed when compared to non-diabetic control mice. There is clinical evidence that maintaining glucose levels at acceptable levels (below 10 mmol/l) is an important step towards improved wound healing. However, when compared with healthy subjects, the wound healing process remains perturbated for unknown reasons.

Research design
Based on the clinical observation that epithelialization can proceed normally in diabetic patients as soon as the quality of the wound bed has improved, and based on the disappointing results of clinical studies with growth factors that accelerate epithelialization, we hypothesized that the disturbances are located in the two preceding phases, the proliferation phase or the inflammation and debridement phase. The predominant cell types in these phases are granulocytes, macrophages, lymfocytes and fibroblasts. During the repair process the extracellular matrix is sequentially remodeled and rebuilt by the action of these cells and their products.

To address the question of disturbed healing in diabetic patients we therefore focused on:

- the architecture of the extracellular matrix
- the composition of the cellular infiltrate
- the proliferation rates of fibroblasts
- the effect of growth factors on fibroblast proliferation rates

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For this reasons the following groups were studied and compared with each other:

1. chronic diabetic ulcers in patients with non-insulin-dependent diabetes mellitus
2. chronic ulcers in patients without diabetes mellitus
3. normal (non-lesional) skin of the upper leg of patients with non-insulin-dependent diabetes mellitus
4. normal (non-lesional) skin of the upper leg of patients without diabetes mellitus, the so called ‘age-matched controls’

Chapter 2: Chronic wounds

The main cause of chronic leg ulcers are venous insufficiency, arterial insufficiency, diabetes, decubitus or combinations of these well known etiologic factors. The small rest group of underlying disorders consists of vasculitis, infections and rare causes like malignancies, pyoderma gangrenosum, or ulcerating skin diseases.

In Western countries, the incidence of leg ulceration is rising as a result of the ageing population and increased risk factors for atherosclerotic occlusion like smoking, obesity and diabetes. This chapter gives a complete overview of common and rare causes of leg ulceration, with a focus on differential diagnosis, and an up-to-date discussion on recent advances in treatment options.

Chapter 3: The architecture of the extracellular matrix and the composition of the cellular infiltrate in chronic diabetic ulcers

In normal wound healing, the consecutive phases such as hemostasis, inflammation and debridement, proliferation and remodeling, can be identified by characteristic patterns of cellular infiltrate and extracellular matrix (ECM) deposition. In this chapter we studied the expression of the extracellular matrix molecules fibronectin (FN), chondroitin sulfate (CS) and tenascin (TN) and the composition of the cellular infiltrate in chronic diabetic wounds, compared with chronic venous ulcers and an acute wound healing model. In the acute wound model, acute wounds were created on the upper leg at day 0 and wound healing was followed in time by collecting tissue samples at day 5, 19 and 28 days post wounding (p.w.) and 12 and 18 months p.w.

In normal skin, FN can only be detected in blood vessels, CS and TN can be found in the basement membrane. Expression of FN, CS and TN was detected
in dermal tissue early in normal wound healing (5-19 days p.w.). Abundant staining was seen 3 months p.w., returning to prewounding levels after 12-18 months p.w. In the dermis of chronic diabetic and venous ulcers with a duration of 12 months or more, a prolonged presence of these ECM molecules was noted. Compared with normal wound healing less T helpers cells were present in chronic wounds but a significantly higher number of macrophages, B-cells and plasma cells were present in the edge of both type of chronic ulcers compared with the acute wound healing model.

Chapter 4: Macrophages in chronic diabetic wounds express the activation marker MRP8/14

Macrophages play a crucial regulatory role in the transition between wound inflammation and the next phase of wound repair, granulation tissue formation. Their presence is essential for the initiation and maintenance of wound fibroblast activity. The ulcer edges of diabetic and venous ulcers are mainly populated by macrophages, however they seem to be unable to direct the repair process towards the proliferative phase. An explanation might be that the macrophages that are present are not (or partially) activated. In Chapter 4 we investigated the expression of the activation marker MRP8/14 on macrophages present at the margin of chronic non-healing diabetic wounds versus acute, healing wounds.

Activated macrophages were detected early in normal wound healing (5-19 days p.w.) where they remain present towards 4 weeks p.w. and are mainly localized in the perivascular areas. Expression of MRP8/14 on macrophages was detected through all layers of the dermis in chronic wounds, with particular dense aggregates in perivascular areas. Significantly more activated macrophages were present in diabetic ulcers compared with venous ulcers; in acute wounds, about the same amounts of activated macrophages were counted. In venous ulcers less activated macrophages were present compared with any day in the acute wound healing model, although not significant. There was a significant correlation between the age of the patients and the number of activated macrophages, in older patients less activated wound macrophages were detected. In conclusion, macrophages within chronic diabetic ulcers appear to maintain an activated phenotype as they migrate into the tissue.
Chapter 5: Diabetic ulcer fibroblasts show disturbed proliferation compared with controls

In this chapter, we investigated the most important cell in the proliferation phase of wound healing, the fibroblast. Light microscopy showed diabetic ulcer fibroblasts to be large and widely spread. Transmission electron microscopy of cultured diabetic ulcer and nonlesional diabetic skin fibroblasts revealed a large dilated endoplasmic reticulum, a lack of cytoskeleton elements (microtubuli) and multiple lamellar and vesicular bodies suggestive for accumulation of membrane structures. Diabetic ulcer fibroblasts, as measured by $^3$H Thymidine incorporation, proliferated significantly more slowly than their controls in the following declining order: age-matched fibroblasts; non-lesional diabetic fibroblasts; chronic wound fibroblasts; diabetic ulcer fibroblasts.

Chapter 6: Diabetic ulcer fibroblasts show a diminished response to growth factors compared with controls

Finally, we investigated if we could stimulate the diminished proliferation rate of diabetic ulcer fibroblasts with (combinations of) several growth factors that are reported to be important in wound healing. Growth factors are specific proteins that can act as mitogens for several target cells. For example, some are able to induce fibroblast proliferation, to promote new vessel formation, or to enhance extracellular matrix production; they also attract inflammatory cells and fibroblasts to the wound site. A number of clinical and experimental studies with growth factors have been published with sometimes contradictory results. Up to date only platelet derived growth factor (PDGF-BB) has proven to be more effective than placebo in a clinical controlled study for the treatment of diabetic ulcers. However, little work is done on the working mechanisms of single or combinations of growth factors on fibroblasts derived from diabetic ulcers. Most growth factors have been characterized by their capacity to induce mitosis in different cell types. 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 progression factors. We studied the effect of these growth factors on the proliferation rates of diabetic ulcer fibroblasts and their controls. Fibroblasts from diabetic ulcers always proliferated at a lower rate than their controls after the same growth factor addition(s), this was most pronounced after the addition of IGF-I. When comparing the mitogenic effects induced by
PDGF-AB, bFGF, IGF-1, and EGF, PDGF-AB had the highest stimulatory effect in all fibroblast groups, and IGF-1 the lowest. The simultaneous addition of FGF-IGF, PDGF-IGF and FGF-PDGF to diabetic ulcer fibroblasts always produced a higher stimulatory response than sequential additions, this effect was not observed in the control fibroblasts. Significant differences were observed when comparing the combinations of growth factors with the highest stimulatory responses (PDGF-IGF, FGF-PDGF and EGF-PDGF added simultaneously) to a double dose of PDGF: the highest stimulatory effect was obtained with the combination PDGF-IGF. In conclusion, the simultaneous application of PDGF-AB and IGF-1 seems to be more promising for diabetic ulcers than the application of PDGF alone.

Chapter 7: The diabetic foot: advances in diabetic wound care

Foot problems are an important cause for morbidity in diabetic patients. Among persons with diabetes, approximately 15% will develop a foot ulcer during their lifetime. In the Netherlands approximately 12,000 patients had a foot ulcer in the year 2000. These ulcers are responsible for 6-20% of all hospital admissions for these patients. The duration of the hospital admission will increase with 59% if there is an ulcer. Ulceration in the diabetic foot usually results from the combination of neuropathic damage, tissue ischemia and excessive pressure loading. These ulcers might easily become infected and osteomyelitis and gangrene may develop, finally resulting in amputation.

The healing of a diabetic ulcer is slow, the mean treatment duration varies from 1.5 to 3 months, but longer healing times are no exception. Several factors are probably responsible for the impaired wound healing, such as diminished fibroblast proliferation and diminished response to growth factors, changes in the architecture of the ECM and the cellular infiltrate, decreased antimicrobial activity of leukocytes and tissue ischemia.

The main principles of treatment are relief of any pressure at the wound site, aggressive surgical debridement, adequate control of infection, arterial reconstruction if necessary, and strict control of glucose levels. The majority of diabetic ulcers respond to this treatment regimen. However, approximately 15% of all patients with ulcers under good control do not respond to conventional therapy and require the use of advanced technologies to stimulate wound repair. This chapter discusses diagnosis and established treatment options and focuses on recent advances in treatment such as special wound dressings, growth factors, extracellular matrix products, skin substitutes and hyperbaric oxygen therapy.
Conclusions

While in acute wounds the inflammatory phase is of short duration and directed at removal of bacteria and dead tissue, in chronic wounds there is a prolonged inflammatory phase. The presence of high numbers of inflammatory cells like macrophages without any evidence of autodigestion, and a large amount of extracellular matrix molecules with no signs of degradation or remodeling, suggest that these wounds are frozen in a chronic low-grade inflammatory state. The observed morphological abnormalities of the diabetic ulcer fibroblasts (large cell volumes suggestive of a hypertrophic phenotype) together with the reduced proliferative capacity and the diminished response to growth factors are suggestive for ageing. A possible explanation might be that in patients with diabetes there is an increased cell turnover due to high glucose levels which brings along prematurely changes of aging. The additional detrimental effect of the proteolytic wound environment, as observed in the diminished proliferation rates of chronic wound fibroblasts, further impairs fibroblast proliferation rates and might be responsible for the fact that diabetic ulcer fibroblasts are further along the senescent pathway than the control fibroblasts.

Our main observations in the search for disturbances in wound healing in patients with diabetic ulcers are:

- there is prolonged expression of the extracellular matrix molecules fibronectin, chondroitin sulfate and tenascin in the dermis of chronic diabetic ulcers compared with acute wounds
- a higher number of macrophages, B-cells and plasmacells and reduced numbers of CD4+ T-cells are present in the dermis of chronic wounds compared with acute wounds
- macrophages in chronic diabetic ulcers express the activation marker MRP8/14
- diabetic ulcer fibroblasts show a diminished proliferative capacity and abnormal morphology compared with control fibroblasts.
- PDGF-AB has the highest stimulatory effect on diabetic ulcer fibroblasts. The simultaneous addition of PDGF-AB and IGF-I might be even more promising for diabetic ulcers than the commercially available PDGF.

These abnormalities in the cellular infiltrate and ECM expression patterns of acute, healing versus chronic wounds indicate a stagnation in the wound healing process in the phase of inflammation and debridement and in the phase of proliferation.