Wound healing in diabetic ulcers
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Cultured fibroblasts from chronic diabetic wounds on the lower extremity (non-insulin-dependent diabetes mellitus) show disturbed proliferation

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SUMMARY

Patients with diabetes mellitus experience impaired wound healing, often resulting in chronic foot ulcers. Hospital discharge data indicate that 6-20% of all diabetic individuals hospitalized (mostly with type 2 diabetes) have a lower extremity ulcer. Maintaining glucose levels at acceptable ranges (below 10 mmol/l) is considered to be an important part of the clinical treatment, but the exact mechanism by which diabetes delays wound repair is not yet known.

We studied this phenomenon by determining the potential of fibroblasts isolated from the ulcer sites of four patients with non-insulin-dependent diabetes mellitus to proliferate in vitro. Controls were fibroblasts isolated from normal skin of the upper leg of five healthy age-matched volunteers and of six non-insulin-dependent diabetes patients. Proliferative capacity was analysed by evaluation of plates after trypsinization and \(^{3}\)H]thymidine incorporation. Fibroblast morphology was studied by light and transmission electron microscopy.

Diabetic ulcer fibroblasts, measured by \(^{3}\)H]thymidine incorporation, proliferated significantly more slowly than the non-lesional control fibroblasts \((P < 0.00047)\) and age-matched control fibroblasts \((P < 0.00003)\). After culturing the fibroblasts for a prolonged period in high-glucose (27.5 mM) and low-glucose (5.5 mM, i.e. physiological) medium, this difference in proliferation rate between diabetic ulcer fibroblasts and nonlesional diabetic fibroblasts remained \((P < 0.0001\) for high-glucose and \(P < 0.0009\) for low-glucose on day 7). Fibroblast proliferation in all three groups was slightly lower in high-glucose than in low-glucose medium, although not significantly at any time point. 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 large dilated endoplasmic reticulum, a lack of microtubular structures and multiple lamellar and vesicular bodies. These results show a diminished proliferative capacity and abnormal morphology of fibroblasts derived from diabetic ulcers of non-insulin-dependent diabetes patients.
Fibroblast proliferation in diabetic wounds

INTRODUCTION

Several clinical and experimental studies have shown that wound healing is impaired in patients with diabetes mellitus. The majority of these patients have chronic nonhealing ulcers localized at pressure sites on the foot (Fig. 1). This entity is known as the diabetic foot. The exact mechanisms behind this phenomenon are still unclear. Our knowledge about wound healing has increased during the last decade. We are able to understand and describe the process in detail by dividing it into phases such as hemostasis, inflammation and debridement, proliferation, epithelialization and remodeling. The regulation at the molecular and cellular level, however, is still poorly understood. Based on the clinical observation that epithelialization can proceed normally in these patients as soon as the quality of the wound bed has improved, we hypothesize that the disturbances are located in the two preceding phases, in the inflammation/debridement phase or the proliferation phase.

Figure 1. Diabetic ulcer localized on the plantar surface of the left foot.
In the proliferation phase the progress of wound repair depends upon the interactions between different cell types and the extracellular matrix, which is sequentially remodeled and rebuilt during this process. The extracellular matrix is predominantly synthesized by fibroblasts. Several studies have demonstrated that the growth capacity of nonlesional fibroblasts is affected in diabetes mellitus. No studies are known, however, on the proliferation of cells actually present at the chronic diabetic wound site. In order to understand more about the role of fibroblasts in the healing of diabetic wounds, we decided to study the effects of different glucose levels on the proliferative capacity and morphology of diabetic ulcer fibroblasts compared to control fibroblast from diabetic nonlesional skin and from the skin of healthy age-matched individuals.

**MATERIALS AND METHODS**

**Clinical profile**

This study was approved by the medical ethical committee of the Academic Medical Center, Amsterdam, The Netherlands. Patients were informed about the purpose and consequences of this study, after which they were asked to provide informed consent. Chronic lower extremity ulcers, defined as existing for 8 weeks or longer, of four patients with non-insulin-dependent diabetes mellitus (NIDDM) were biopsied. Biopsies of 4 mm were taken from a central, non-granulating part of the ulcer. Two of the biopsied ulcers were located at pressure points on the plantar surface of the foot (MTP-1), one on the heel and one on the ankle region (combined diabetic and venous origin). The median ulcer duration was 5.5 months (range 2-36 months). Patients suffering from any systemic disease which might have interfered with wound healing were excluded. Fibroblasts derived from the normal upper leg skin of healthy age-matched controls and NIDDM patients were used as controls. In all patients with NIDDM adequate glycaemic control was established (with no relevant differences between the ulcer and nonlesional group) around the time of biopsy. The patient characteristics are shown in Table 1.
Fibroblast proliferation in diabetic wounds

Table 1. Clinical data of the patient and control groups

<table>
<thead>
<tr>
<th></th>
<th>NIDDM ulcer patients</th>
<th>NIDDM non-lesional controls</th>
<th>Age-matched controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number</td>
<td>4</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>4/0</td>
<td>3/3</td>
<td>4/1</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>76</td>
<td>72.7</td>
<td>84</td>
</tr>
<tr>
<td>Age range (years)</td>
<td>53-89</td>
<td>48-92</td>
<td>64-94</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>12 (11-14)</td>
<td>11 (7-24)</td>
<td></td>
</tr>
<tr>
<td>Insulin/oral hypoglycaemic agents</td>
<td>1/3</td>
<td>1/5</td>
<td></td>
</tr>
<tr>
<td>Nephropathy</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Neuropathy</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Body mass index (mean ± SD)</td>
<td>29.7 ± 8.2</td>
<td>28.7 ± 10</td>
<td></td>
</tr>
<tr>
<td>Biopsy site</td>
<td>foot, ankle</td>
<td>upper leg</td>
<td>upper leg</td>
</tr>
</tbody>
</table>

**Cell culture**

All tissue samples were minced into fine pieces and incubated in 0.25% dispase/0.25% collagenase (Boehringer Mannheim, Mannheim, Germany) for 2 hours at 37 °C. The suspension was filtered through an infusion chamber (Central Laboratory for Blood Transfusion, Amsterdam, The Netherlands), and the cells were centrifuged, washed in phosphate-buffered saline (PBS) and resuspended in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum, 100 IU/ml penicillin and 100 IU/ml streptomycin. The fibroblast cultures were maintained in a humidified atmosphere containing 5% CO₂ at 37 °C. At confluence, and all subsequent passages, the monolayer was washed three times with PBS and then trypsinized (trypsin 0.25% in PBS). During trypsinization, each flask was frequently agitated to facilitate detachment of the cells. Trypsin was inactivated by adding culture medium. Detached cells were collected and centrifuged at 1000 g for 10 minutes, washed and suspended in culture medium. The medium was changed twice a week. Cultures were periodically screened to exclude mycoplasma contamination.
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Cell proliferation studies

Cell counting
Cell proliferation was investigated in monolayer cultures on plastic. Cells were inoculated into 24-well tissue culture plates at a concentration of 5000 cells/ml culture medium per well. At specific time-points three replicate wells were counted after addition of trypsin in a Coulter counter. The culture medium was refreshed twice weekly. All experiments were performed in the presence of physiological (5.5 mM) or elevated (27.5 mM) glucose concentrations.

$[^3]H$thymidine-assays
Cells were seeded in 24-well dishes at a density of 10000 per well. Cells were grown overnight in DMEM supplemented with 2mM L-glutamine, 100 IU/ml penicillin G, 100 µg/ml streptomycin (Gibco, Glasgow, UK) and 10% (v/v) fetal bovine serum (Bio Whittaker, Wokingham, UK). The cells were cultured in medium containing 0.2% serum for an additional 24 hours. Then 10% serum was added to the cultures. After 16 hours, the cells were pulsed for 4 hours with 0.2 µCi $[^3]H$-methyl]thymidine (10 µCi/ml) per well. The labeling was terminated by washing once with PBS (Gibco). Macromolecules were precipitated with trichloroacetic acid and washed twice with PBS. Fibroblasts were then dissolved by incubation for 1 hour with 2 M NaOH and collected. After neutralization with an equal volume of 2 M HCl, the incorporated thymidine was counted in a liquid scintillation counter (Packard 1600 CA or Packard 2000 CA).

Transmission electron microscopy
Fibroblasts to be studied by transmission electron microscopy were fixed in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer. They were postfixied in 1% OsO$_4$, dehydrated through graded ethanol and embedded in Epon LX 112 (Ladd Research Industries, Burlington, Vt., USA). Based on semi-thin sections the dermal area in the ulcer region was selected for thin sections. These were collected on copper grids, double stained with uranyl acetate and lead citrate and examined with a Philips-420 electron microscope.

Statistical analysis
The Kruskal Wallis test was used to compare the differences among the three groups of monolayer cultures. All pairwise comparisons were made with the Wilcoxon rank sum test. $[^3]H$thymidine incorporation data were evaluated using Student's $t$-test. $P$-values of 0.05, two-sided, were considered to be statistically significant.
RESULTS

Cell proliferation

Fibroblast proliferation, as measured by thymidine incorporation, is shown in Fig. 2. The bars represent the incorporation of \(^{3}\text{H}\)thymidine in populations of fibroblasts obtained from the ulcer sites of NIDDM patients, non-lesional NIDDM controls, and age-matched controls cultured in 5.5 mM glucose-containing medium (physiological). Diabetic ulcer fibroblasts showed lower cell numbers than nonlesional control fibroblasts (\(P < 0.000473\)) and age-matched control fibroblasts (\(P < 0.00003\)). Nonlesional fibroblasts also proliferated significantly more slowly than age-matched control fibroblasts (\(P < 0.0017\)).

![Figure 2](image_url)

**Figure 2.** Reduced proliferation of diabetic ulcer fibroblasts in comparison with nonlesional diabetic fibroblasts (\(P < 0.000473\)) and age-matched controls (\(P < 0.00003\)) in medium containing glucose at a physiological concentration (5.5 mM). The difference between ulcer and nonlesional fibroblast proliferation rates is even more evident in high-passage (more than ten passages) cultures (\(P < 0.000001\)). Proliferation was assessed by tritiated thymidine incorporation (DPM x 1000). The values are the means + SEM of the ulcer fibroblasts (\(n=4\) Lp and \(n=3\) Hp, in 16 independent experiments), nonlesional fibroblasts (\(n=6\) Lp and \(n=3\) Hp, in 15 independent experiments) and age-matched control fibroblasts (\(n=5\), in 8 independent experiments). Lp low (less than ten) passages, Hp high (more than ten) passages.
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After more than ten passages in culture, the difference in proliferation between diabetic ulcer fibroblasts and nonlesional diabetic fibroblasts became even more pronounced \((P < 1.14 \times 10^{-6})\). Similar results were obtained when fibroblast proliferation was measured in a collagen sponge,\(^{15}\) to mimic the extracellular matrix environment (results not shown).

In Fig. 3A and 3B all individual growth curves are shown. The curves represent the growth rate of the populations of fibroblasts cultured as monolayers in the presence of high glucose (27.5 mM, Fig. 3A) and physiological glucose (5.5 mM, Fig. 3B) concentrations. Statistical analyses were performed at comparable time-points for the three groups only during the first 13 days of cell culture to exclude the inhibitory effects on cell proliferation caused by the state of confluence. The proliferation of fibroblasts isolated from the diabetic ulcers was significantly decreased in comparison with the fibroblasts from nonlesional diabetic skin and age-matched controls \((P < 0.0001\) for high and low glucose at day 7). Nonlesional diabetic skin fibroblasts showed a significantly reduced proliferation rate compared to age-matched control fibroblasts in high-glucose \((P < 0.0135)\) and low-glucose \((P < 0.0124)\) medium.

To assess the specific influence of diabetes on proliferation rates, fibroblasts were also isolated from two chronic nondiabetic wounds, a decubitus ulcer on the heel and a neuropathic ulcer on the plantar surface of the foot. These chronic wound fibroblasts had proliferated significantly more quickly than diabetic ulcer fibroblasts by day 7 in high-glucose \((P < 0.0001)\) and in low-glucose \((P < 0.0012)\) medium, did not significantly differ in proliferation rate from nonlesional diabetic fibroblasts, except on day 4 for high-glucose \((P < 0.0015,\) lower proliferation rate for ulcer fibroblasts) and showed reduced growth capacity compared to age-matched control fibroblasts (day 7, high and low glucose, \(P < 0.0015)\). Fibroblast proliferation in all groups was slightly lower in high-glucose than in low-glucose medium, although not significantly at all time points. In high-glucose medium significantly reduced numbers of chronic diabetic wound fibroblasts were noted on days 5 and 13 \((P < 0.001),\) of chronic nondiabetic wound fibroblasts on day 4 \((P < 0.0198)\) and age-matched control fibroblasts on day 12 \((P < 0.0002)\).
Fibroblast proliferation in diabetic wounds

**Figure 3A.** Time-course of proliferation of diabetic ulcer fibroblasts (n=4), nonlesional diabetic fibroblasts (n=3) and age-matched control fibroblasts (n=3) cultured as monolayers in high-glucose (27.5 mM) medium. The means (± SEM) from three independent experiments are shown. The statistical analysis for the three groups was performed on day 7. Diabetic ulcer fibroblast proliferation differed significantly from that of both of the control groups (P < 0.0001). The proliferation rate of nonlesional diabetic fibroblasts was significantly less than that of the age-matched control fibroblasts (P < 0.0135).

**Figure 3B.** Time-course of proliferation of diabetic ulcer fibroblasts (n=4), nonlesional diabetic fibroblasts (n=3) and age-matched control fibroblasts (n=3) cultured as monolayers in low-glucose (5.5 mM) medium. The means (± SEM) from three independent experiments are shown. The statistical analysis for the three groups was performed on day 7. Diabetic ulcer fibroblast proliferation differed significantly from that of both of the control groups (P < 0.0001). The proliferation rate of nonlesional diabetic fibroblasts was significantly less than that of the age-matched control fibroblasts (P < 0.0124).
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Microscopic observations

Morphological differences between diabetic ulcer fibroblasts (Fig. 4A) and age-matched controls (Fig. 4B) were observed. Diabetic ulcer fibroblasts were usually large and widely spread in the culture flask compared to the spindle-shaped morphology of the fibroblasts of age-matched controls.

Figure 4A. Fibroblasts from a diabetic chronic wound of a patient with NIDDM cultured in a nonpyrogenic cell culture flask (original magnification x 66, scale bar 60 μm).

Figure 4B. Fibroblasts from an age-matched control patient cultured in a nonpyrogenic cell culture flask (original magnification x 66, scale bar 60 μm).
We further examined the cells with transmission electron microscopy (Fig. 5). A large dilated endoplasmic reticulum was seen in the diabetic ulcer fibroblasts, indicating high protein production, and multiple lamellar and vesicular bodies, indicating a high turnover and breakdown of intracellular structures (Fig. 5A). The fibroblasts from non-lesional diabetic skin showed a slight increase in active endoplasmic reticulum and some vesicular bodies were also seen (Fig. 5B). A lack of microtubular structures seen in both ulcer and nonlesional diabetic fibroblasts suggests a disturbed cytoskeletal function. The age-matched control fibroblasts showed a normal morphology for *in vitro* conditions (Fig. 5C).

![Figure 5A](image)

**Figure 5A.** Fibroblasts cultured from a chronic wound (ankle region) of a patient with NIDDM. Note extensive dilated rough endoplasmic reticulum (*small arrow*), lamellar and vesicular bodies (*large arrow*) and the lack of cytoskeletal filaments (*original magnification x 24500, scale bar 80 nm*).
Figure 5B. Fibroblasts cultured from nonlesional skin (upper leg) of the same patient with NIDDM. Note extensive dilated rough endoplasmic reticulum (small arrow), lamellar and vesicular bodies (large arrow) and lack of cytoskeletal filaments (original magnification x 24500, scale bar 80 nm).

Figure 5C. Fibroblasts cultured from a healthy age-matched individual (upper leg). Note the presence of microtubules (arrow heads) (original magnification x 24500, scale bar 80 nm).
DISCUSSION

Many of the chronic complications in diabetes are associated with defects in connective tissue metabolism. We found decreased proliferation rates for the diabetic ulcer fibroblasts compared with nonlesional diabetic skin fibroblasts from NIDDM patients and fibroblasts from age-matched controls after incorporation with \( ^{3}H \)thymidine and after culturing these cells as monolayers in high- and low-glucose medium. A smaller number of ulcer fibroblasts caused by proliferative impairment is likely to contribute to a decreased production of extracellular matrix proteins, which may be related to impaired wound healing.

The observed morphological abnormalities in the diabetic ulcer fibroblasts (large cell volumes suggestive of a hypertrophic phenotype) and a reduced proliferative capacity, even more prominent in high passages, suggest that fibroblasts from the wound environment are further along the senescent pathway than fibroblasts from non-wound areas. A detrimental effect of the chronic wound environment is illustrated by the recently reported inhibitory effect of wound fluid from nonhealing wounds on cell division. Another possible explanation could be that the function of these fibroblasts is temporarily or permanently modified by cytokines or proteolytic enzymes present in the chronic wound environment. Diabetes mellitus also brings on prematurely and with greater severity certain changes that are normally seen as degenerative changes associated with aging. Our results clearly demonstrate a decrease in proliferative capacity in the following order: age-matched fibroblasts > non-lesional diabetic fibroblasts > diabetic ulcer fibroblasts. Our results confirm reduced growth rates for diabetic skin fibroblasts (NIDDM) versus age-matched controls as observed by Rowe et al. Furthermore, chronic non-diabetic wound fibroblasts had a similar proliferation rate as diabetic non-lesional fibroblasts but a significantly higher proliferation rate than the chronic diabetic wound fibroblasts. It seems likely that diabetic wound fibroblasts are impaired as a result of cell aging in combination with, or induced by, diabetes (intrinsic aging) and the wound environment (aging).

Our results suggest that high glucose (27.5 mM) may be inhibitory to fibroblast proliferation. Turner and Biermann and Hehenberger and Hansson have shown that increasing glucose levels to a certain maximum (18 and 15.5 mM, respectively) increases fibroblast proliferation, whereas further increases lead to inhibition of proliferation. Other recent studies have shown effects of high glucose concentrations in inhibiting the proliferation and increasing the size of proximal tubule cells and in reducing the proliferation of fetal mesangial cells and umbilical endothelial cells. The auto-oxidation of glucose is
thought to generate free radicals which are responsible for delaying cell replication. Furthermore, a lengthening of the cell proliferation time and slight increases in cell death have been observed.

Transmission electron microscopy showed a lack of microtubules in the fibroblasts from the diabetic ulcers and in those from nonlesional diabetic skin. Cason et al. have reported the presence of a factor in the serum of diabetics which can impair microtubule formation in vitro, and Rousset et al. have found antitubulin antibodies in a high proportion of patients with diabetes of recent onset. Glucose has also been reported to inhibit microtubule formation in high concentrations. Microtubules can regulate cell shape and the plane of cell division, and a disturbance in these structures might contribute to the intrinsic mechanism involved in the observed diminished cell proliferation rate for nonlesional diabetic and diabetic ulcer fibroblasts and a possible deficit in specific growth factor production or release into the wound may further impair fibroblast function.

Further studies will be focused on in vitro regulation of the synthesis and deposition of new extracellular matrix by the fibroblasts, using growth factors which are known to influence fibroblast proliferative functions. The presence and function of cytoskeletal elements as e.g. tubulin, will be investigated to elucidate the underlying mechanism of the observed disturbed fibroblast proliferation.

In summary, chronic diabetic wound fibroblasts from NIDDM patients showed a significantly lower proliferation rate and an altered morphology in vitro compared to diabetic nonlesional and age-matched control fibroblasts. These results indicate an impairment of skin ulcer fibroblasts from NIDDM patients probably caused by aging due to the diabetic state and the ulcer environment.

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