Wound healing in diabetic ulcers

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Chapter 6

Fibroblasts derived from chronic diabetic ulcers differ in their response to stimulation with EGF, IGF-I, bFGF and PDGF-AB compared to controls

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SUMMARY

Patients with diabetes mellitus experience impaired wound healing, often resulting in chronic foot ulcers. Healing can be accelerated by application of growth factors like platelet-derived growth factor (PDGF). We investigated the mitogenic responses, measured by ^{3}[H] Thymidine incorporation, of fibroblasts cultured from diabetic ulcers, non diabetic ulcers, and non-lesional diabetic and age-matched controls, to recombinant human PDGF-AB, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and insulin-like growth factor (IGF-I). We determined the optimal concentration of these factors, and investigated which single factor, or combination of factors, added simultaneously or sequentially, induced the highest mitogenic response. For single growth factor additions, in all fibroblast populations significant differences in mitogenic response to different growth factors were observed with PDGF-AB consistently inducing the highest response and IGF-I the lowest ($p \leq 0.043$). IGF-I produced only a 1.7 fold stimulation over control in diabetic ulcer fibroblasts, versus 2.95 fold for chronic ulcer, 3.2 fold for non-lesional ($p=0.007$) and 5 fold for age-matched fibroblasts ($p = 0.007$). The highest mitogenic response induced by EGF was significantly less for chronic ulcer fibroblasts compared with age-matched and non-lesional controls ($p<0.03$), chronic ulcer fibroblasts also needed significantly more EGF to reach this optimal stimulus ($p<0.02$ versus age-matched and non-lesional controls).
The simultaneous addition of FGF-IGF, PDGF-IGF and FGF-PDGF to diabetic ulcer fibroblasts always produced a higher stimulatory response than sequential additions ($p \leq 0.05$). Also the addition of bFGF, PDGF-AB and EGF prior to IGF-I induced a higher ^{3}[H] Thymidine uptake in all fibroblasts compared to the combination of each in reverse order. Significant differences were observed when comparing the combinations of growth factors with the highest stimulatory responses (PI, FP and EP added simultaneously) to a double dose of PDGF, with the highest mean rank for the combination PI ($p = 0.018$). In conclusion, combinations such as PDGF-AB and IGF-I may be more useful than PDGF-AB alone for application in chronic diabetic wounds.

INTRODUCTION

Patients with diabetes mellitus are at risk for developing chronic wounds. The increased risk can be mainly attributed to the vascular and neurological disorders, but in addition to this, the diabetic condition itself is associated with delayed wound healing.[1-3] It is estimated that 15% of diabetic patients will develop an ulcer on the feet or ankles at some time during the disease course.[4] The socioeconomic implications are therefore considerable. Appelqvist et al. calculated the costs of treatment per ulcer episode in diabetic patients in Sweden.[5] The total average cost for patients with primary healing was $5.859 (6.245 Euro's) and for healing with amputation $39.167 (41.743 Euro's).

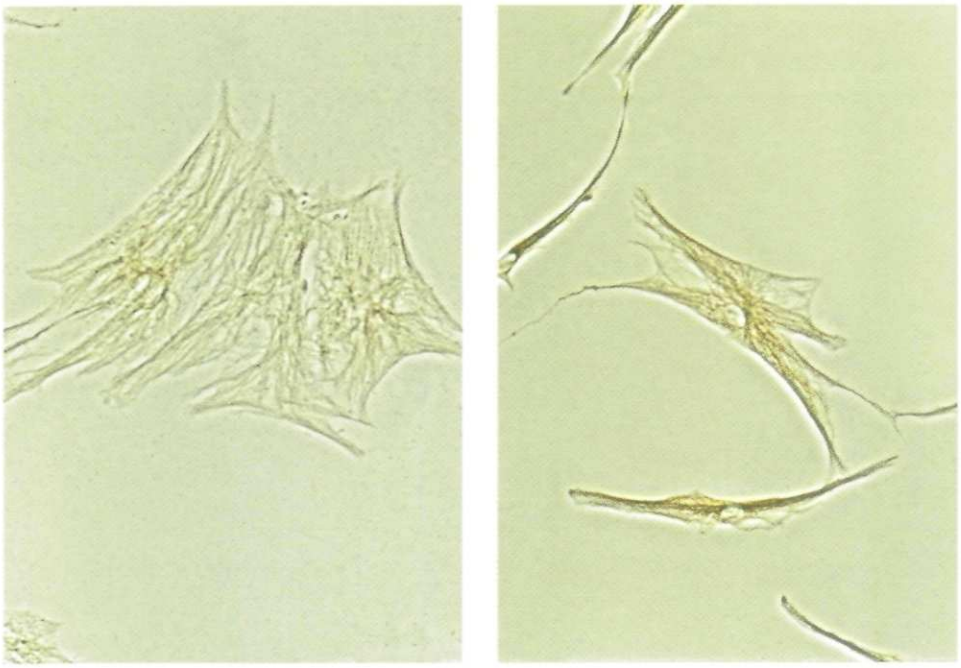
Not surprisingly, many recently developed bio-engineered medical devices and treatment modalities, such as cultured skin equivalents, extracellular matrix components, and recombinant human growth factors, focus on the diabetic foot as primary indication.

Growth factors play a key role in initiating and sustaining the different phases of tissue repair.[6,7] 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.[8,9] 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. Each growth factor uses a distinct tyrosine kinase receptor but the receptors share common signal transduction pathways. Platelet-derived-growth factor *(PDGF-AB),* released from the α-granules of platelets, is a major serum mitogen and induces fibroblast proliferation, matrix production, and maturation of connective tissue. *Basic fibroblast growth factor (bFGF)* has its main stimulatory effect on the growth and differentiated function of fibroblasts and on the proliferation of vascular smooth muscle cells and endothelial cells. Therefore, it has a major function as an 'angiogenesis peptide'. *Epidermal growth factor (EGF)* is also released from α-granules of platelets and stimulates cell proliferation by binding to the EGF receptor in a variety of tissue types. *Insulin-like growth factor (IGF-I)* is transferred from blood and local sites of production to its cellular target via a sequence of binding proteins, whose affinities are modulated by the pH of the wound environment. Alterations in the levels of binding proteins, and elevations of IGF-I antagonists have been found in situations associated with defective repair, including diabetes, malnutrition, uraemia and jaundice.

A number of clinical and experimental studies with growth factors have been published with sometimes contradictory results. Up to date only PDGF-BB has proven to be effective, in a clinical controlled study, for the treatment of diabetic ulcers. This was shown by Steed and the Diabetic Ulcer Study Group, they studied recombinant human PDGF-BB (rhPDGF-BB) and reported significant differences in the percentage of patients that reached complete wound healing (48% treatment group versus 25% control group) at the end of 20 weeks.[10] In fetal mesangial cells, IGF-I stimulated protein and proteoglycan synthesis, this stimulation increased with high glucose concentration.[11] Topical application of PDGF-AB, TGF-α and EGF has been reported to be successful in accelerating healing of full-thickness wounds in normal mice and bFGF normalized the delayed wound healing response in diabetic mice.[12-14]
Although there is some evidence that these growth factors are or might be successful clinically, little work is done on the working mechanisms of single or combinations of growth factors at the cellular level in humans. Sprugel et al. studied combinations of growth factors (PDGF, bFGF, EGF and TGFβ) in a wound chamber model in rats and found no different response compared to single factors.[15] However, they used single growth factors in subthreshold doses. Lynch et al. applied PDGF-BB and IGF-I to partial thickness porcine skin wounds or to canine bone regeneration models and found that the two growth factors did act synergistically to enhance tissue repair.[16-18] Greenhalgh et al. further established the synergy of PDGF and IGF-II in full thickness skin wound in diabetic mice.[19]
In this study, we investigated which of the afore mentioned growth factors has the highest stimulatory effect on fibroblasts obtained from chronic diabetic ulcers. Although these fibroblasts are difficult to culture, it appears to be crucial to use this specific population. In previous experiments we studied proliferation rates of diabetic ulcer fibroblasts and controls by cell counting (in time) and Thymidine incorporation assays. Both methods showed that diabetic ulcer fibroblasts have an impaired proliferative capacity compared to non-diabetic ulcer fibroblasts, non-lesional diabetic and age-matched fibroblasts.[20] Another observation by light microscopy was that diabetic ulcer fibroblasts were usually large and widespread, suggestive of a hypertrophic phenotype, in contrast to the spindle shaped structure of the age-matched control fibroblasts. Transmission electron microscopic observations revealed multiple lamellar and vesicular bodies and a lack of microtubular structures. Based on previous reports suggesting that a factor in the serum of diabetic patients might inhibit microtubule formation,[21] we hypothesized that a disturbance in microtubules might contribute to the observed diminished diabetic ulcer fibroblast proliferation rate. We performed a qualitative immunohistochemical staining for

anti-α tubulin on diabetic ulcer fibroblasts and controls but could not observe any differences (Fig. 1A,B).

For each individual growth factor, the optimal concentration to reach a maximal mitogenic effect was determined. In addition, combinations of growth factors were investigated, added simultaneously or sequentially. The sequential experiments were performed to investigate the hypothesis that also for diabetic ulcer fibroblasts, growth factors may act as competence factors or progression factors.



A B

Figure 1. The network of microtubules (anti-α tubulin antibody) in chronic diabetic ulcer fibroblasts (A) and age-matched control fibroblasts (B).

MATERIALS AND METHODS

Clinical profile

5 patients with non-insulin-dependent diabetes mellitus and a chronic lower extremity ulcer, defined as existing for 8 weeks or longer were biopsied. Punch biopsies (4 mm) were taken from a non-granulating part of the chronic ulcers to make sure we were studying chronic non-healing tissue and not newly arrived fibroblasts. These ulcers were mainly neuropathic in origin with transcutaneous oxygen measurements of ≥ 30 mmHg. The mean ulcer duration was 11 months. Patients suffering from any systemic disease which might interfere with wound healing were excluded. As controls we used fibroblasts derived from chronic non-diabetic ulcers (3 neuropathic and 1 decubitus ulcer on the foot), normal upper leg skin of NIDDM patients (n=7) and healthy age-matched controls (n=7). The neuropathic, non-diabetic ulcers were located on the feet of ex-Morbus Hansen (Leprosy) patients. The patient characteristics are shown in Table 1. 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 gave their informed consent.

Table 1. Clinical data in diabetic ulcer and control groups

Patient Groups	*Diabetic ulcers*	*Chronic ulcers*	*Non-les. diab.*	*Age-matched*
Total nr.	5	4	7	7
Sex (F/M)	4/1	1/3	4/3	6/1
Age, mean	74	52	70.4	85
Age, range	53-89	33-85	49-92	77-96
Ulcer duration (months, mean)	11	27.5		
Duration diabetes (years, mean)	14		12	
Insulin / OHA	2/3		2/5	
Biopsy site	foot, ankle	foot	upper leg	upper leg

F= female, M=male, OHA=oral hypoglycaemic agents

Laboratory methods

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), the cells were centrifuged, washed in Phosphate Buffered Saline (PBS) and resuspended in culture medium (Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum (FCS), 100 IU/ml penicillin and 100 IU/ml streptomycin. PBS, FCS, DMEM, penicillin and streptomycin were purchased from Gibco BRL, Breda, The Netherlands. The fibroblast cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. At confluence, and all subsequent passages, the monolayer was washed 3 times with PBS and then trypsinized (trypsin 0.25% in PBS). During trypsinization, we frequently agitated each flask to facilitate detachment of the cells. Trypsin was inactivated by adding FCS and 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. For all experiments cell cultures were used with passages below P10.

^{3}[H] Thymidine-incorporation assays

10,000 cells were seeded per well in 24-multi well dishes. Cells were grown overnight in DMEM supplemented with 2mM L-glutamine, 100 IU/ml penicillin G, 100 µg/ml streptomycin and 10%(v/v) Fetal Calf Serum. After 24 hours the cells were cultured in medium containing 0.2% FCS for an additional 24 hours. PDGF-AB, IGF-I, bFGF and EGF were tested alone and in combinations. Each growth factor was added in increasing concentrations from 0.05-10 ng/ml (and 5-80 ng/ml for IGF-I) to determine optimum concentrations.

Since in general the response to growth factor additions graphically takes the form of an S-curve, the results are expressed as ED50 (effective dose) and ED90. The ED50 and ED90 are defined as the growth factor concentration (in ng/ml) at which 50% or 90% of the maximum value reached during the plateau phase was reached (Fig. 2). To make inter-experimental comparisons possible, *ED90 levels* were expressed as a ratio (increase of ^{3}[H] Thymidine incorporation over that of control (0.2% FCS)).

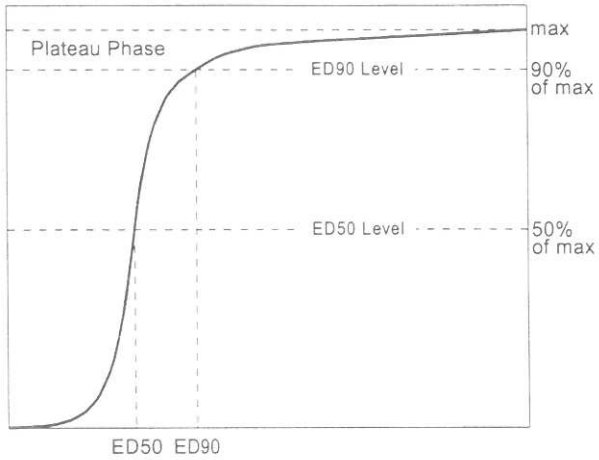


Figure 2. Schematic representation of the S-shaped dose response curve of the fibroblasts. The ED50 (effective dose) and ED90 are defined as the growth factor concentration (in ng/ml) at which 50% (ED50 level) or 90% (ED90 level) of the maximum value reached during the plateau phase was reached.

Single-factor experiments

For **single-factor experiments**, growth factors dissolved in DMEM with 0.2% fetal calf serum, were added to the cells and incubated for 16 hours. For the **combination-of-factors experiments,** two growth factors were added simultaneously for 24 hours. For each factor, not the *median* but the *highest* concentration required to reached ED90 level in the solo experiments was used. In this way all fibroblast populations were maximally stimulated (in the plateau phase of the dose-response curve). This was done because of the fact that the optimum growth factor concentration for one patient may be irrelevant for other patients. In case of sequential addition of growth factors, cells were treated for 8 hours with one growth factor followed by 16 hours with the second growth factor. The cells were pulsed for 4 hours by adding 20 µl/ 0,2 µCi [3H-methyl] thymidine (10 µCi/ml) per well. The labelling was terminated by washing once with PBS and macromolecules were precipitated by adding 500 µl trichloroacetic acid 10% to each well for 10 minutes. Again the cells were washed this time twice with PBS and 250 µl 2N NAOH was added for 1 hour. After harvesting of the fibroblasts and neutralization with equal volume of 2M HCl, the incorporated ^{3}[H] Thymidine was counted in a liquid scintillation counter (Packard 1600 CA or Packard 2000 CA). Each concentration or combination of growth factors was repeated in three wells.

Synergism and additive effects of combinations of growth factors
For synergistic and additive effects of combinations of growth factors individual fibroblast populations were analyzed in each category of fibroblasts (n=4). An effect was defined as synergistic if the stimulation caused by the combination of growth factors was ≥30% greater than the sum of the stimulation achieved by each growth factor. An additive effect was observed if the combined effect was between 90% and 130% of the sum of the effects of the individual growth factors.

Statistical analysis

The data were analysed by the statistical program SPSS for Windows (SPSS Inc. Chicago IL USA). The **Kruskal Wallis test** was used for the comparison of data from the *single growth factor experiments* (ED50 and ED90 experiments). When significant differences were observed with the Krukal Wallis test, the **Mann Whitney U test** was used for further analysis. The **Friedman test** (paired, non-parametric test) was applied for all paired data from the *combination-of factors experiments* (sequential versus simultaneous etc.). When significant differences were observed, the **Wilcoxon signed ranks test** was applied. *P*-values of <0.05 were considered to be statistically significant.[22]

RESULTS

Mitogenic effects and optimal concentrations of single growth factor additions

The ED90 levels of individual growth factors on diabetic ulcer, chronic ulcer, non-lesional diabetic and age-matched control fibroblasts and their optimum concentration (defined as the concentration at which the ED90-level was reached) are shown in Fig. 3. **PDGF-AB** was able to stimulate diabetic ulcer fibroblasts 7.5 fold over control value, nonlesional fibroblasts were stimulated 9.3 fold, chronic ulcer fibroblasts 11.6 fold, and age-matched fibroblasts 16.3 fold. Statistical significant differences among the fibroblast groups were not observed. The concentration of PDGF-AB at which the ED90 level was reached was 1 ng/ml (median) for diabetic ulcer fibroblasts and age-matched fibroblasts, and 2 ng/ml (median) for chronic wound fibroblasts and non-lesional diabetic skin fibroblasts.

For **IGF-I**, the ED90 level was most frequently reached at 20 ng/ml (median), chronic wound fibroblasts needed more IGF-I (40 ng/ml) compared to controls to reach the optimal levels, although not significant. The diabetic ulcer fibroblasts responded poorly to IGF-I. Only a 1.7 fold stimulation over control was obtained for diabetic ulcer derived fibroblasts, versus 2.95 fold for chronic ulcer, 3.2 fold for non-lesional ($p=0.007$) and 5 fold for age-matched fibroblasts ($p=0.007$). When all fibroblast groups were exposed to the same concentration, 20 or 40 ng/ml IGF-I, significantly lower mitogenic responses were obtained for diabetic ulcer fibroblasts compared to non-lesional and age-matched controls as well ($p \leq 0.05$).

For **bFGF**, the ED90 level was most often reached at 0.5 ng/ml (median) for diabetic ulcer and chronic ulcer fibroblasts, which was slightly higher than the concentration of 0.25 ng/ml (median) required for non-lesional diabetic controls and age-matched controls, although not significant. For all concentrations used, no significant differences were observed among the four fibroblast groups.

For **EGF**, significant differences in mitogenic responses and optimum concentrations were observed among the fibroblast groups with the Kruskal Wallis test. The highest mitogenic response induced by EGF was significantly less for chronic ulcer fibroblasts compared with age-matched ($p=0.025$) and non-lesional controls ($p=0.034$). Chronic ulcer fibroblasts also needed significantly more EGF to reach this optimal stimulus ($p=0.024$ versus age-matched and $p=0.008$ versus non-lesional controls).

When comparing the mitogenic effects induced by PDGF-AB, bFGF, IGF-I and EGF with the Kruskal Wallis test, PDGF-AB was assigned the highest mean rank for all fibroblast groups, and IGF-I the lowest ($p \leq 0.043$).

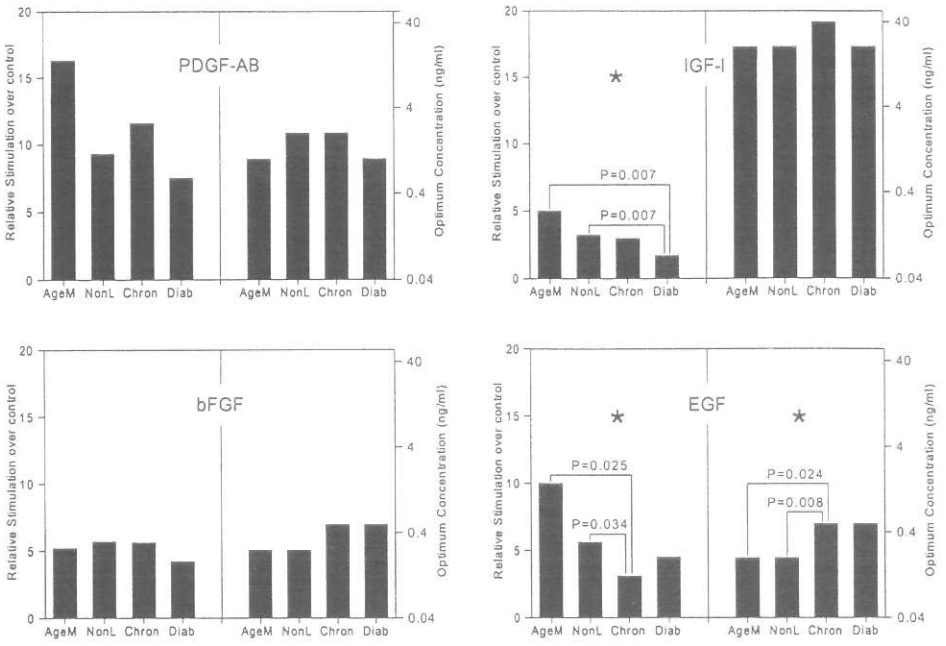


Figure 3. The ED90 levels of PDGF-AB (A), bFGF (B), IGF-I (C) and EGF (D) and their optimal concentrations on diabetic ulcer, chronic ulcer, non-lesional diabetic and age-matched control fibroblasts. The error bars represent medians. IGF-I; only 1.7 fold stimulation over control was obtained for diabetic ulcer derived fibroblasts versus 2.95 fold for chronic ulcer, 3.2 fold for non-lesional (p=0.007) and 5 fold for age-matched fibroblasts (p=0.007). EGF; a significantly lower maximum mitogenic response was induced in chronic ulcer fibroblasts versus non-lesional (p=0.034) and age-matched fibroblasts (p=0.025), chronic ulcer fibroblasts also needed significantly more EGF to reach their optimal stimulus (p=0.024 versus age-matched and p=0.008 vs. non-lesional controls). (All experiments were performed in triplo, and the number of patients/cellstrains/experiments, respectively, were as follows: Diabetic ulcer fibroblasts: PDGF, 4/9/9; IGF, 4/8/8; FGF, 5/5/5; EGF, 4/4/4. Chronic ulcer fibroblasts: PDGF, 3/3/3; IGF, 3/3/3; FGF, 3/3/3; EGF, 3/3/3. Nonlesional diabetic fibroblasts: PDGF, 5/7/7; IGF, 3/7/7; FGF, 5/5/5, EGF, 4/4/4. Age-matched fibroblasts: PDGF, 5/7/7; IGF, 6/6/6; FGF, 4/4/4; EGF, 5/5/5.)

The ED50 for PDGF-AB, EGF and IGF-I did not differ significantly for the lesional, non-lesional diabetic, chronic ulcer and age-matched fibroblasts (Table 2). Diabetic ulcer fibroblasts needed significantly more bFGF to reach ED50 compared with chronic ulcer fibroblasts (p=0.024). Chronic ulcer fibroblasts required more IGF-I to reach ED50 than their controls, however this was not a significant finding.

Table 2. ED50 in single growth factor experiments (ng/ml, mean ± stdev)

	PDGF-AB	*bFGF * p=0.024*	*EGF*	*IGF-I*
Age-matched	0.65 ± 0.21	0.12 ± 0.05	0.12 ± 0.11	12.3 ± 2.9
Non-les. diab	0.55 ± 0.32	0.14 ± 0.08	0.07 ± 0.015	13.0 ± 4.9
Chronic ulcers	0.50 ± 0.13	0.08 ± 0.003	0.11 ± 0.03	20.0 ± 7.0
Diabetic ulcers	0.63 ± 0.26	0.23 ± 0.11*	0.23 ± 0.13	14.4 ± 5.6

Combinations of growth factors

Subsequently we investigated the mitogenic effects of combinations of growth factors. Remarkable was that the simultaneous addition of FGF-IGF, PDGF-IGF and FGF-PDGF to diabetic ulcer fibroblasts always led to a significantly higher stimulatory response than the sequential additions ($p \leq 0.05$) (Fig. 4). Also the addition of bFGF, PDGF-AB and EGF prior to IGF-I induced a higher ^{3}[H] Thymidine uptake in all fibroblasts compared to the combination of each in reverse order. Significant differences between different orders of addition were observed for chronic ulcer fibroblasts for the combination EI, FI and PI, with the highest stimulatory effect after the sequential addition of E-I, F-I and the simultaneous addition of PI ($p \leq 0.04$). For age-matched fibroblasts, the sequential addition of F-I elicited a significantly higher mitogenic response than the other possible combinations with FGF and IGF (p=0.04). The combination of EGF and bFGF induced generally a less than additive response than the sum of the solo addition of each and was therefore not studied any more after several experiments (not shown).

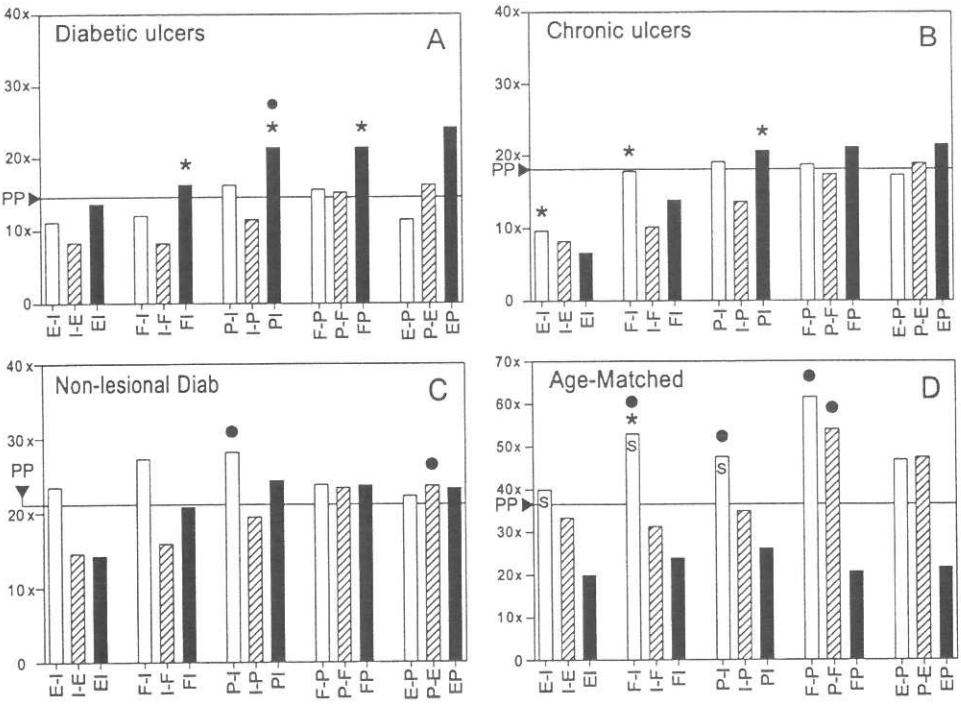


Figure 4. Mitogenic effects of combinations of growth factors on diabetic ulcers (n=4) (A), chronic ulcers (n=4) (B), non-lesional diabetic skin (n=5) (C) and age-matched controls (n=5) (D). For diabetic ulcer fibroblasts the simultaneous addition of FGF-IGF, PDGF-IGF and FGF-PDGF evoked a significant higher stimulatory response than sequential additions ($p \leq 0.05$). Significant differences were observed for chronic ulcer fibroblasts between different orders of addition of the combination EI, FI and PI (asterix on highest stimulatory response, $p \leq 0.04$) and for age-matched fibroblasts for the sequential addition of F-I ($p \leq 0.04$ compared with the sequential addition of I-F and the simultaneous addition of FI). Significant differences were also observed when comparing the combinations of growth factors with the highest stimulatory responses to a double dose of PDGF (horizontal lines marked with PP); 1) agematched fibroblasts showed a significantly higher mitogenic response after the sequential addition of F-I, P-I, F-P and P-F, 2) non-lesional fibroblasts after the sequential addition of P-I and 3) for chronic ulcer fibroblasts there was no significantly better combination of growth factors than the simultaneous addition of PP. For diabetic ulcer fibroblasts, significant differences were observed when comparing the combinations of growth factors with the highest stimulatory responses (PI, FP and EP added simultaneously) to a double dose of PDGF, with the highest mean rank for the combination PI ($p = 0.018$). Synergistic effects are indicated with a S.

Additive effects/synergism

After **simultaneous addition** of growth factors synergistic effects (stimulation by combination of growth factors ≥ 30% greater than the sum of the stimulation levels achieved by each growth factor individually) were observed for the age-matched fibroblasts with the combinations E-I, F-I (1 out of 4 cell populations, 3 showed additive effects) and P-I (1 out of 4, no additive effects were accomplished). One diabetic ulcer fibroblast population showed synergistic effects on P-I (the other 3 cell populations showed additive effects) and E-I (two other fibroblast populations showed additive effects, one cell population showed no additive effects). One non-lesional diabetic fibroblast population showed synergistic effects on the combination F-I (three other fibroblast populations showed additive effects) as well as on E-I (two other fibroblast populations showed additive effects). Chronic ulcer fibroblasts did not show any synergism after simultaneous addition. Synergistic effects were marked in Fig. 4 only when *50% or more* of the cell populations showed a synergistic effect.

In case of **sequential addition**, no synergistic effects were observed for diabetic ulcer fibroblasts at all. One non-lesional diabetic control cell population showed synergism on the combination E-I and F-I (3 others showed additive effects), 3 of the 4 cell populations of age-matched fibroblasts on F-I and two on E-I and P-I, with the remaining cell population resp. 1,2 and 2 cell populations showing additive effects (Fig. 4; synergism (S) for E-I, F-I and P-I). One out of four chronic ulcer fibroblast population showed synergism on the combination F-I, two showed additive effects.

When comparing the growth factor combinations with the highest stimulatory response with the simultaneous addition of PDGF-AB, horizontal line marked in Fig. 4, agematched fibroblasts showed a significantly higher mitogenic response after the sequential addition of F-I, P-I, F-P and P-F and non-lesional fibroblasts after the sequential addition of P-I (Fig. 4, dots). For chronic ulcer fibroblasts there was no better combination of growth factors than the simultaneous addition of PP. Diabetic ulcer fibroblasts responded with the highest stimulatory response after the simultaneous addition of PI, FP and EP (Fig. 4). When comparing these combinations with the reference line PP a significant difference was observed with the highest mean rank for the combination PI ($p = 0.018$).

DISCUSSION

The most important finding in this study is that fibroblasts cultured from diabetic ulcers and, although less pronounced, chronic non-diabetic ulcers in general reach a lower maximum mitogenic response to growth factors compared with controls. This finding is consistent with the clinical observation that chronic ulcers and especially diabetic ulcers show retarded healing and are often 'frozen' in an inert state, that requires some kind of a trigger like aggressive debridement or the external application of growth factors.

For each growth factor and for each concentration, the level of stimulation achieved in the diabetic ulcer fibroblast population was lower than in the control groups. The following order of declining levels of stimulation by growth factors was observed: age-matched > non-lesional/chronic wound > diabetic ulcer fibroblasts. This indicates that the chronic wound environment and even more pronounced the diabetic environment reduces the fibroblast's capacity to respond to growth factors, through an yet unknown mechanism that remains present during the cell passages *in vitro*. Although in this study fibroblast samples as high as P10 were used, the observed diminished responses to growth factors could not be attributed to increasing cell passages. Similar results were obtained in low and high cell passages (data not shown).

It has been reported that fibroblasts derived from patients with chronic venous ulcers and patients with diabetes mellitus (ulcer and non-lesional) show premature signs of aging (senescence), resulting in abnormal morphology and reduced replicative capacity.[23-25] The influence of donor age and anatomic location on fibroblast proliferation rates is controversial. Cristofalo et al. studied 124 skin fibroblast cell lines established from donors of different ages and, when controlled for health status, no correlation for donor age and replicative life span was found.[26] Falanga et al. found no fibroblast proliferation rate differences when fibroblasts were cultured from different body sites (proximal towards distal arm).[27] Ågren et al. showed that the slowest growing wound fibroblasts were isolated from younger patients and concluded that the age of wounds is the more important determinant.[28] In this study and a previous study,[20] diabetic ulcer fibroblasts were isolated from older patients and 'younger' wounds than the chronic wound controls but proliferated at a lower rate. Diabetic ulcer fibroblasts seem to be more impaired as a result of cell aging induced by diabetes (intrinsic aging) and the wound environment (aging). A hostile chronic wound environment and diabetes causes repetitive fibroblast replication which is leading the cells further along the path of senescence. This can be confirmed by the clinical observation of a high incidence of malignancies occurring in chronic ulcers. It has also been shown that with advancing age, cells become less responsive to growth factors so that adequate stimulation for cell division is not

achieved.[29] The observed decreased mitogenic response to growth factors by diabetic ulcer fibroblasts and chronic nondiabetic ulcer fibroblasts, also observed by others,[28,30] may very well be part of this general intrinsic abnormality in chronic wounds. Furthermore, senescent fibroblasts have been shown to over-express proteolytic activity by means of an increase in the production of enzymes such as matrix metalloproteinases and to under-express protease inhibitors.[25] This might explain the modest effect on healing seen after the clinical application of growth factors on chronic wounds.

Remarkable was that the simultaneous addition of FGF-IGF, PDGF-IGF and FGF-PDGF to diabetic ulcer fibroblasts always led to a higher stimulatory response than the sequential addition. The used growth factors do not seem to act as competence or progression factors. This effect was observed occasionally for chronic ulcer fibroblasts and not at all for non-lesional and age-matched controls. The presence of more than one growth factor at the same time seems to be a requirement for diabetic ulcer fibroblasts to achieve the highest stimulatory effects on DNA synthesis. This might be due to a lower number of receptors, lower binding affinities of growth factor receptors, dysfunctional intracellular signal transduction[31] and increased expression of blocking substrates e.g. IGF-I BP-3.[32] In chronic venous ulcers no significant differences in the amount of either platelet-derived growth factor α-receptors or β-receptors on fibroblasts were found compared to acute wounds and normal dermis.[28]

The addition of bFGF, PDGF-AB and EGF prior to IGF-I always induced a higher ^{3}[H] thymidine uptake in all fibroblasts compared to the combination of each reverse order.

These observations indicate that PDGF-AB, bFGF and EGF may serve as competence factors to promote cellular responsiveness to IGF-I. This confirms the observation of Flyvberg et al. who addressed bFGF and PDGF-AB as competence factors and IGF-I as a progression factor.[8] EGF has been reported before to act as a competence factor on intestinal epithelial cells.[33]

The most promising combination of growth factors (highest stimulatory response) for diabetic ulcer fibroblasts were PDGF-AB and IGF-I, PDGF-AB and bFGF and EGF and PDGF-AB (all after simultaneous addition) with the highest mitogenic response observed after addition of PDGF and IGF-I. It has been reported that PDGF-AB has the ability to stimulate IGF-binding protein-2 synthesis which has mitogenic effects on IGF-I.[34]

Although it is difficult to isolate and culture fibroblasts from chronic leg and foot ulcers in diabetic patients, these are the proper populations of fibroblasts that should be used for *in vitro* studies of the mechanism of disturbed proliferation and determination of the effect and optimal concentrations of growth factors. Slowed cell growth may be partially responsible for the deficit in healing of diabetic ulcers. We believe that ulcer healing may be improved by exogenous

application of specific growth factors. We were able to confirm *in vitro* that PDGF, which is being used clinically, is a suitable candidate for single growth factor application. Application of combinations such as PDGF-AB and IGF-I seem to be even more promising for diabetic ulcers than the application of single growth factors.

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