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Proliferation and Mitogenic Response to PDGF-BB of Fibroblasts Isolated from Chronic Venous Leg Ulcers is Ulcer-Age Dependent[1]

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Several pathophysiologic mechanisms have been proposed to explain slow-healing leg ulcers, but little is known about the growth behavior of cells in these wounds. Platelet-derived growth factor-BB applied topically to chronic wounds has shown beneficial effects, although the effects have been less pronounced than would have been expected based on studies on acute wounds. The objective of this study was to compare fibroblasts in culture obtained from chronic wounds (non-healing chronic venous leg ulcers), acute wounds and normal dermis regarding growth, mitogenic response to platelet-derived growth factor-BB and levels of platelet-derived growth factor α-receptor and β-receptor. Fibroblasts were obtained by an explant technique and expanded *in vitro* using fibroblast growth medium supplemented with 10% fetal bovine serum and used for the assays at their third passage. Growth of chronic wound fibroblasts (n = 8) was significantly (p < 0.05) decreased compared with those from acute wounds (n = 10) and normal dermis (n = 5). Fibroblasts from ulcers older than 3 y grew significantly (p < 0.01) slower than those from ulcers that had been present for less than 3 y. Morphology and size of fibroblasts from the oldest chronic wounds deviated substantially from those of acute wounds and normal dermis, and resembled *in vitro* aged or senescent fibroblasts. Mitogenic response of chronic wound fibroblasts to human recombinant platelet-derived growth factor-BB was also reduced with ulcer age. No significant differences were found in the amount of either platelet-derived growth factor α-receptor or β-receptor among the three groups. The features decreased growth related to ulcer age, altered morphology, and reduced response to platelet-derived growth factor, indicating that fibroblasts in some chronic wounds have approached or even reached the end of their lifespan (phase III). This might provide one explanation for the non-healing state and therapy resistance to topical platelet-derived growth factor-BB of some venous leg ulcers. ***Key words: ageing/growth factors/senescence/wound healing.*** ***J Invest Dermatol 112:463–469, 1999***

Several pathophysiologic mechanisms have been proposed to explain slow/non-healing leg ulcers. Overproduction of reactive oxygen species, hypoxia, imbalances in levels of cytokines and proteolytic enzymes, excessive fibrin deposition, and failure of keratinocyte migration on the chronic wound bed have been presented as possible explanations for the defective healing of leg ulcers (Coleridge Smith, 1994; Falanga *et al*, 1994). Little is known, however, about growth and other biologic activities of cells in these wounds.

Fibroblasts are one of the key cells in wound repair. Apart from producing the major extracellular components of collagen, elastin, and proteoglycans, fibroblasts also make mitogens for keratinocytes, fibroblasts, and endothelial cells (Werner *et al*, 1994). Fibroblasts probably migrate from neighboring tissues, although a bloodborne origin has also been proposed (Bouissou *et al*, 1988; Bucala *et al*, 1994). At the wound site, fibroblasts change into a less proliferative but a more contractile and collagen synthetic phenotype (Regan *et al*, 1991; Germain *et al*, 1994; Ågren *et al*, 1996). These functional alterations can be mimicked *in vitro* by adding wound fluid to cultured normal skin fibroblasts (Regan *et al*, 1991). This finding indicates the control provided by factor(s) present in the wound environment.

The main regulators of the cellular activities in wound repair are polypeptide growth factors (Steenfos, 1994). Platelet-derived growth factor (PDGF) is mitogenic and chemotactic for connective tissue cells such as fibroblasts, and appears to be an important autocrine and paracrine factor during wound repair (Grotendorst *et al*, 1985; Ågren *et al*, 1996). Controlled clinical trials with one of the three isoforms of PDGF, PDGF-BB homodimer have not produced the same beneficial effects, however, when applied topically to chronic wounds as when applied to acute, experimental wounds (Robson *et al*, 1992; Pierce *et al*, 1994; Robson, 1997).

PDGF activates cells via specific cell surface receptors: α-receptor

Manuscript received March 26, 1998; revised December 10, 1998; accepted for publication December 14, 1998.

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Abbreviations: FBM, fibroblast basal medium; FGM, fibroblast growth medium.

[1]Presented in part at the Gordon Research Conference on Wound Repair, July 2–7 1995 and at an IBC meeting on Fibrosis in Washington, DC, April 18–19 1996.

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and β-receptor. The presence of PDGF receptors appears to be upregulated during human wound healing compared with normal skin, as demonstrated by *in situ* hybridization and immunohistochemistry (Reuterdahl *et al*, 1993; Peus *et al*, 1995). Immunostaining for both types of receptors is predominantly localized to fibroblast-like cells in granulation tissue from both acute and chronic wounds, whereas endothelial cells stain exclusively for the β-receptor. Pierce *et al* (1994) observed that the number of fibroblasts increased in healing chronic wounds but not in non-healing chronic wounds treated with PDGF-BB, whereas in placebo-treated chronic wounds no difference in fibroblast number was seen between healing and non-healing wounds. This observation indicates that there are fibroblasts in some chronic wounds that do not respond to PDGF-BB, possibly due to deficient numbers of PDGF receptors or dysfunctional intracellular signal transduction.

In order to test this hypothesis, we have compared fibroblasts cultured from chronic as well as from acute wounds, and also from normal skin with respect to their growth and mitogenic response to growth factors, especially to PDGF-BB. Furthermore, the levels of PDGF α-receptor and β-receptor on the different fibroblast strains were estimated.

MATERIALS AND METHODS

Patients, wounds, and normal skin Fibroblasts from wounds were obtained from eight patients [aged 68 ± 5 y (mean ± SEM), four females] with chronic wounds (venous leg ulcers) and 10 patients (59 ± 5 y, six females) with acute wounds (traumatic), as well as from normal whole skin of the medial upper arm of five healthy volunteers (45 ± 4 y, three females). The chronic wounds had been present for more than 6 mo (6 mo, 1, 1.5, 3, 6, 10, 10, and 20 y), were larger than 50 cm^2 in size, and were excised for subsequent autologous skin transplantation due to the non-healing state of the ulcers. Punch biopsy specimens (4 mm) were also taken from adjacent, clinically diagnosed lipodermatosclerotic skin and normal skin in five of the eight patients (aged 64 ± 6 y) with chronic wounds (1, 1.5, 6, 10, and 20 y old ulcers). The 10 acute wounds comprised seven with 14–45 d old exuberant granulation tissue, two expanded polytetrafluoroethylene tubes with 10 d old subcutaneous granulation tissue (Jorgensen *et al*, 1996), and one 4 d old 6 mm punch biopsy wound. No sites showed any clinical signs of infection. Furthermore, none of the patients had diabetes mellitus or were given systemic antibiotics, glucocorticoids, or chemotherapeutic agents. Informed consent was obtained from all patients participating in the study, which was approved by the local ethical committee.

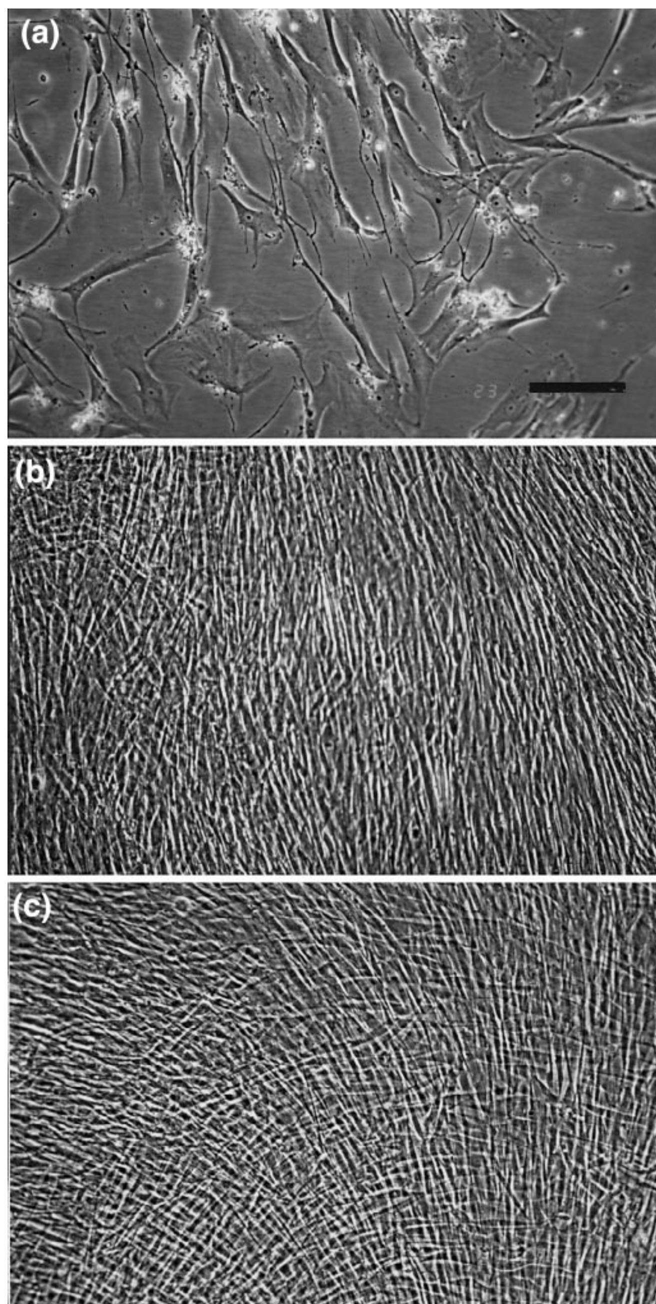
Cell cultures Granulation tissue was obtained from central non-epithelialized parts of the open wounds or from the expanded polytetrafluoroethylene tubes and used as explants. From the normal skin 6 mm whole dermis punch biopsies were used as explants after excision of the epidermis. The tissue specimens were immersed in ice-cold phosphate-buffered saline (PBS), kept at 5°C and washed three times in 50 ml ice-cold PBS before explanting, which occurred within 4 h after harvesting. Tissues were minced (1 mm^3), explanted on 60 mm Petri dishes (Greiner, Frickenhausen, Germany) and fibroblast outgrowth supported with fibroblast growth medium (FGM, Clonetics, Walkersville, MD) supplemented with 10% heat-inactivated mycoplasma-screened fetal bovine serum (FBS, Gibco BRL, Grand Island, NY). FGM is composed of fibroblast basal medium (FBM) supplemented with basic fibroblast growth factor (bFGF, 1 ng per ml, final concentration), insulin (5 μg per ml), gentamicin (50 μg per ml), and amphotericin-B (10 ng per ml). The same batch of serum was used throughout the study. The explant procedure was compared with enzymatic digestion of granulation tissue obtained from the two polytetrafluoroethylene tubes. Fibroblasts were cultured either from explanted minced tissue as described above or from minced tissue that had been digested with 200 U bacterial collagenase type IA (Sigma, St Louis, MO) per ml FBM for 18 h at 37°C in a humidified atmosphere of 5% CO_2/air essentially as described by Regan *et al* (1991). Cell growth was similar for the two procedures with a mean saturation density on post-plating day 10 (see *Growth kinetics* below) of 6.9×10^4 for the fibroblasts grown from explants compared with 7.3×10^4 for collagenase-treated tissue. Initial experiments also showed that the growth of human dermal fibroblasts, obtained from explanted skin from the posterior lower leg of a healthy 30 y old male, increased significantly when using FGM supplemented with 10% FBS compared with using Dulbecco's modified Eagle's medium (DMEM) with Glutamax, 4.5 g glucose per liter and supplemented with 10% FBS. The saturation density, achieved with DMEM with 10% FBS, was $2.1 \pm 0.1 \times 10^4$ compared with $1.2 \pm 0.1 \times 10^5$ for fibroblasts cultured in FGM supplemented with 10% FBS. Furthermore, FGM with 10% FBS supported outgrowth of fibroblasts from chronic wound tissue. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2/air. Culture medium was changed twice weekly. Subcultivation was performed using mycoplasma-tested trypsin/ethylenediaminetetraacetic acid (EDTA) (0.05%/0.02% in PBS pH 7.4; Biological Industries, Kibbutz Beit Haemek, Israel) and cells were split at a ratio of 3:1 at confluence (Hayflick and Moorhead, 1961). Cells of the third passage were used for the assays. Phase-contrast microphotography was performed using an inverted microscope (Nikon TMS, 10 × objective) on all fibroblast strains. Selective cell strains were checked immunocytochemically for vimentin (Boehringer Mannheim, Germany), cytokeratins 5, 6, 8, and 17 (M 821; DAKO, Glostrup, Denmark), and von Willebrand factor (A 082; DAKO). All fibroblast strains examined were found positive for vimentin, and negative for cytokeratins and von Willebrand factor. The cell strains checked for mycoplasma by polymerase chain reaction were found negative.

Growth kinetics Day 0, 3.5×10^3 cells per cm^2 were seeded in 24 well plates (Nunc, Roskilde, Denmark) in 1 ml complete medium per well, and enumerated 3, 4, 5, 6, 7, 8, 9, and 10 d post-plating in triplicates using a Bürker hemocytometer. Cells were re-fed with new medium days 4 and 7 during the 10 d growth period. Growth curves were generated for each individual fibroblast strain, and the population doubling time for the individual fibroblast strains was determined graphically during their logarithmic growth and the fibroblast density determined at saturation (Freshney, 1991). All wells were inspected using inverted phase-contrast microscopy for aberrant cell behavior and for detached cells throughout the 10 d growth period.

The effect of conditioned medium from the three groups of fibroblast strains on cell proliferation was studied in a complementary experiment. Fifteen milliliters of conditioned complete medium (FGM with 10% FBS) was obtained from confluent fibroblasts, in 75 cm^2 plastic flasks grown for 72 h, from chronic and acute wounds, and dermis, respectively, from the same patients. The target cells (dermal fibroblasts) were seeded into 96 well plates (Nunc) at 3.5×10^3 cells per cm^2 and 3.5×10^4 cells per cm^2 and grown for 24 h, and then exposed to the conditioned media for 24 h. Fibroblasts were also treated with unconditioned complete media. All media were sterile-filtered (0.22 μm) before being added to the cells. Cell proliferation was measured as incorporation of the thymidine analogue, 5-bromo-2′-deoxyuridine (BrdU, 10 μM, final concentration) into the dermal fibroblasts during the last hour of incubation. The incorporation of BrdU was determined using an ELISA kit (Boehringer Mannheim) and ΔOD ($OD_{370\ nm} - OD_{492\ nm}$) read by an ELISA reader (Labsystems, Helsinki, Finland).

***In situ* incorporation of BrdU** To study further the proliferative ability, one representative fibroblast strain from each group was pulse-labelled with BrdU. Cells were seeded to about 50% confluence (10^4 cells per cm^2) on Chamber Glass Slides (Lab-Tek, Nunc) in complete culture medium and incubated for 24 h. BrdU (10 μM, final concentration) was added during the last hour of incubation. Cells were then washed three times with PBS, fixed with glycine buffer (50 mM glycine in 70% ethanol, pH 2.0). Cells synthesizing DNA were detected immunocytochemically using a kit from Boehringer Mannheim.

Mitogenic response Cells were seeded into 96 well plates (Nunc) at 1×10^4 cells per well in complete medium and incubated for 72 h. The medium was then replaced with 100 μl FBM containing 2.5% FBS per well and the cells were incubated for another 24 h. Growth factors in 25 μl FBM containing 1% (wt/vol) bovine serum albumin (BSA, Sigma) were added to a final volume of 125 μl and 25 μl FBM containing 1% BSA alone to the six control wells. Human recombinant PDGF-BB, PDGF-AA, and transforming growth factor (TGF)-β1 were obtained from Gibco BRL. Human recombinant bFGF and epidermal growth factor (EGF) were purchased from Sigma. Two-fold dilutions of each growth factor were used at six final concentrations in duplicate (PDGF, 0.6–20 ng per ml; bFGF, 0.15–5 ng per ml; EGF, 1.5–50 ng per ml; TGF-β1, 0.3–10 ng per ml). The cells were then incubated with or without growth factors for 18 h. BrdU in FBM medium (10 μl per well) was added to all wells to a final concentration of 10 μM except for the background control (unspecific binding of the anti-BrdU conjugate to the cells in the absence of BrdU), which received 10 μl of FBM medium alone. The cells were incubated for an additional 2 h and then washed three times with PBS. The effect of the growth factors on fibroblast proliferation was expressed as the percentage of BrdU incorporated, measured with the ELISA described above under *Growth kinetics*, into control-treated cells after subtraction of blank values for respective growth factor.



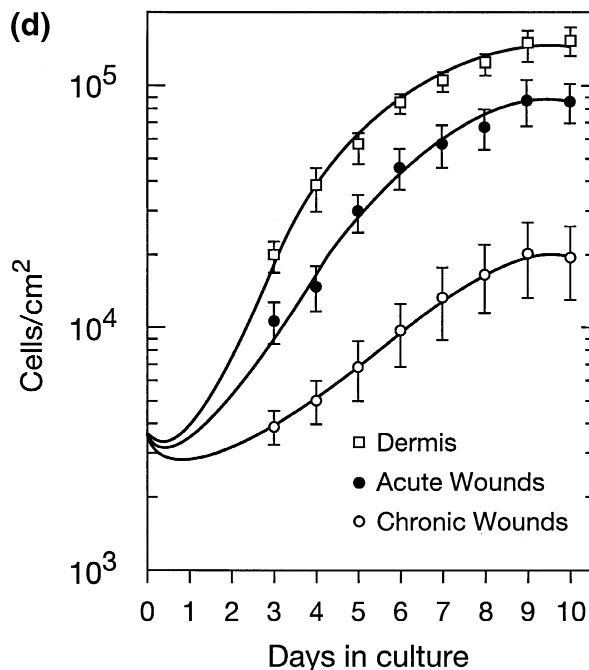


Figure 1. Different morphology and growth kinetics for fibroblasts cultured from chronic wounds, acute wounds, and normal dermis. Representative phase-contrast microphotographs of fibroblasts from chronic wounds (*a*), acute wounds (*b*), and dermis (*c*) 10 d post-plating. Note the irregular shape, large size, and sparsely growth of the chronic wound fibroblasts (*a*) compared with spindle-shaped and tightly grown acute wound (*b*) and normal dermal fibroblasts (*c*). *Scale bar*: 200 μm. Each growth curve depicted on a logarithmic y-axis in (*d*) represents the mean of eight different fibroblast strains from chronic wounds (○), 10 different fibroblast strains from acute wounds (●), and five different fibroblast strains from normal dermis (□). *Error bars*: SEM.

PDGF α-receptor and β-receptor quantification The number of α-receptors and β-receptors were quantified in detergent-solubilized cell membranes using a membrane solid phase immunoassay (slot-blot technique) with monoclonal antibodies. The immunoreactions in the slot-blots were then quantified with densitometry, which is an established quantitative method (Towbin and Gordon, 1984). To improve the precision of the assay, cell membrane proteins at three 2-fold dilutions (each dilution assayed in duplicate) from the three groups were included in the same blot. Probing with the same concentration of the two primary antibodies against the α-receptors and β-receptors and color development were carried out simultaneously to decrease variability between the immunoreactions of α-receptor and β-receptor.

Membrane extract was obtained after lysing confluent cells grown in FGM with 10% FBS with 1% (vol/vol) Triton X-100 with 3 mM phenylmethylsulfonyl fluoride for 30 min at 4°C (Ågren *et al*, 1996). Extracts were diluted to lie within the linear range of the assay in Tris(hydroxymethyl)-aminomethane (Tris)–HCl-buffered-saline (pH 7.4), added to a 48 well slot-blot apparatus (Bio-Rad, Hercules, CA) and filtered through nitrocellulose membrane (Trans-Blot, 0.45 μm, Bio-Rad). Bound PDGF receptors were immunoreacted for 18 h at ambient temperature with mouse monoclonal antibodies (10 ng per ml, Genzyme, Cambridge, MA) against the human PDGF α-receptor and the human PDGF β-receptor (10 ng per ml, Genzyme) (Hart *et al*, 1987). The primary antibodies were immunoreacted for 90 min with biotinylated swine anti-mouse IgG diluted 1:2500 (E 0453; DAKO) followed by treatment for 30 min with streptavidin complexed with biotinylated alkaline phosphatase (K 0391; DAKO) according to the manufacturer's instructions. Nitro blue tetrazolium/ 5-bromo-4-chloro-3-indolyl phosphate was applied to visualize the receptor-bound complexes. The blots were scanned (Hewlett Packard ScanJet 4). The intensities of the bands were quantified using a software from Jandel (SigmaGel), normalized to the protein content (DC Protein Assay Kit with bovine serum albumin as standard, Bio-Rad) of the cell extracts and expressed as arbitrary units per μg cell protein.

Statistics Kruskal–Wallis one-way analysis of variance was applied to the population doubling time, cell density, and PDGF-receptor data. Wilcoxon matched-pairs signed-ranks test was applied when comparing levels of α- and β-receptor within the three fibroblast groups. In case of statistical significance ($p < 0.05$), Bonferroni's correction for multiple hypotheses was used to ensure that type I errors were not multiplied. The effect of ulcer age on fibroblast growth was tested using the Student's t-test for unpaired observations. Numerical data are given as mean ± SEM.

Table I. Population doubling time was increased and saturation density decreased for chronic wound fibroblasts

	Chronic wounds	p	Acute wounds	p	Normal dermis
Number of strains	8		10		5
Population doubling time (h)	118.7 ± 39.7[a]	<0.05	32.6 ± 5.5	NS[b]	27.9 ± 3.3
Saturation density (cells per cm^2 × 10^{-4})	2.0 ± 0.7	<0.05	8.8 ± 1.7	<0.05	15.5 ± 1.9

[a]Mean ± SEM.
[b]A statistically non-significant (p > 0.05) difference.

RESULTS

Morphology and growth of the fibroblasts Fibroblasts from acute wounds did not show any morphologic difference at a light microscopic level compared with those from normal dermis. They all displayed the characteristic spindle-shaped appearance and swirl growth pattern. Chronic wound fibroblasts commonly exhibited an altered morphology with irregular cell shape, multinucleation, larger and more variable cell size, and accumulation of debris (**Fig 1*a–c***).

Although plating efficiency was not determined *per se*, a negligible number of detached fibroblasts was observed in any well prior to enumeration on the third post-plating day. Overall, chronic wound fibroblasts grew at a slower rate than those obtained from acute wounds and normal dermis (**Fig 1*d***). The mean population doubling time, as determined from the slope of the individual growth curves, was almost four times longer for chronic wound fibroblasts than for acute wound and dermal fibroblasts (**Table I**). Chronic wound fibroblasts reached a density 10 d post-plating of about a fourth of the cell density of fibroblasts from acute wounds and dermis (**Table I**). Five of the eight chronic wound fibroblast strains approached or reached a replicative senescent growth behavior. Those fibroblast strains were derived from venous leg ulcers that had been present for more than 3 y. Statistical analyses revealed that those fibroblasts (n = 5) grew to a statistically significantly ($p < 0.01$) lower saturation cell density ($6.6 \pm 2.0 \times 10^3$ cells per cm^2) than those from leg ulcers younger (n = 3) than 3 y ($4.3 \pm 0.4 \times 10^4$ cells per cm^2). The mean population doubling time was also extended by the fibroblasts from the old ulcers (166.8 ± 53.5 h) compared with fibroblasts from ulcers younger than 3 y (38.6 ± 6.6 h), although this difference did not reach statistical significance (p = 0.074). Acute wound fibroblasts reached a significantly ($p < 0.05$) lower saturation cell density than dermal fibroblasts 10 d post-plating (**Table I**).

In five of the chronic wounds, fibroblasts of peri-wound skin were also expanded *in vitro* to see if they differed from those in the chronic wounds in terms of growth. The growth of fibroblasts from the chronic venous leg ulcers was severely diminished compared with those from adjacent uninjured tissues. One biopsy from adjacent normal skin in one patient and one biopsy from adjacent lipodermatosclerotic skin in another patient were excluded due to interfering epithelial outgrowth from the biopsies. In the remaining three patients, the growth of fibroblasts from peri-ulcer lipodermatosclerotic skin appeared to be similar to fibroblasts from normal skin of the same lower limb, which again was lower in the chronic wounds (**Table II**). Thus, it appears that the fibroblasts in the chronic wounds are different regarding cellular growth from those of adjacent tissue.

The possibility of the chronic wound fibroblasts producing growth inhibitory molecules that might decrease cell growth was investigated. BrdU incorporation, expressed as ΔOD, into dermal fibroblasts from normal skin (3.5×10^3 cells per cm^2) exposed to conditioned media for 24 h from fibroblasts derived from one chronic wound and from adjacent normal skin was 0.449 ± 0.020 (n = 3) and 0.406 ± 0.020 (n = 2), respectively. The corresponding results for the acute wound and adjacent skin were 0.413 ± 0.026 (n = 5) and 0.492 ± 0.031 (n = 3), respectively. Unconditioned complete media resulted in a ΔOD of 0.780 ± 0.032 (n = 6). Similar results were obtained when the target fibroblasts were seeded at an initial higher density (3.5×10^4 cells per cm^2). These results thus indicate that the observed decreased growth of chronic wound fibroblasts was not due to an autocrine suppressive mechanism.

Table II. Population doubling time was increased and saturation density decreased for chronic wound fibroblasts compared with adjacent lipodermatosclerotic and normal skin in three patients

	Chronic wounds	Lipodermatosclerotic dermis	Normal dermis
Population doubling time (h)	111.0 ± 67.7[a]	45.9 ± 6.7	40.4 ± 4.9
Saturation density (cells per cm^2 × 10^{-4})	2.7 ± 1.2	9.1 ± 1.9	9.6 ± 0.4

[a]Mean ± SEM.

Further proof of a diminished proliferation of chronic wound fibroblasts was an abolished or decreased DNA synthesis as evidenced by no or reduced incorporation of the thymidine analog BrdU (**Fig 2**).

Mitogenic response to growth factors including PDGF-BB and levels PDGF α-receptor and β-receptor Human recombinant mitogens added to growth-arrested cells elicited DNA synthesis, indicating the presence of functional receptors on the various cell strains. BrdU-incorporation in the fibroblast strains, however, from the four chronic wounds of the longest duration (6, 10, 10, and 20 y) was below the limit of detection. PDGF-BB was the most mitogenic growth factor inducing about a 15-fold stimulation of DNA synthesis in skin fibroblasts compared with a 5–10-fold stimulation with bFGF, EGF, and PDGF-AA. TGF-β1 stimulated DNA synthesis only slightly (about 50% above control) (**Fig 3*a*** and **Table III**). The maximal response to PDGF-BB occurred at concentrations between 10 and 20 ng per ml and no further stimulation was found at concentrations above 20 ng per ml (data not shown). The maximal mitogenic response to PDGF-BB and also to bFGF and EGF, however, was diminished for the chronic wound fibroblasts compared with the acute wound and the skin fibroblast strains (**Fig 3*b*** and **Table III**).

Because PDGF activates cells via specific surface receptors (types α and β) one possible reason for the reduced response to PDGF-BB could be a lower number of PDGF α-receptors and β-receptors in the chronic wound fibroblasts. No statistically significant difference, however, was found among the three groups (**Fig 4**). Interestingly, the amount of α-receptors was about 30% lower ($p < 0.05$) than the amount of β-receptors on dermal fibroblasts, whereas there was no significant difference between α-receptors and β-receptors on either chronic or acute wound fibroblasts.

DISCUSSION

The pathogenesis of chronic venous leg ulcers is multifactorial (Coleridge Smith, 1994; Falanga *et al*, 1994). There are only a few studies examining cells isolated from such wounds (Roberts and Harding, 1994; Herrick *et al*, 1996; Hasan *et al*, 1997; Stanley *et al*, 1997). Our results indicate that fibroblasts, crucial cells in wound repair, isolated from chronic wounds grown under optimal *in vitro* conditions have a diminished capacity to replicate. Furthermore,

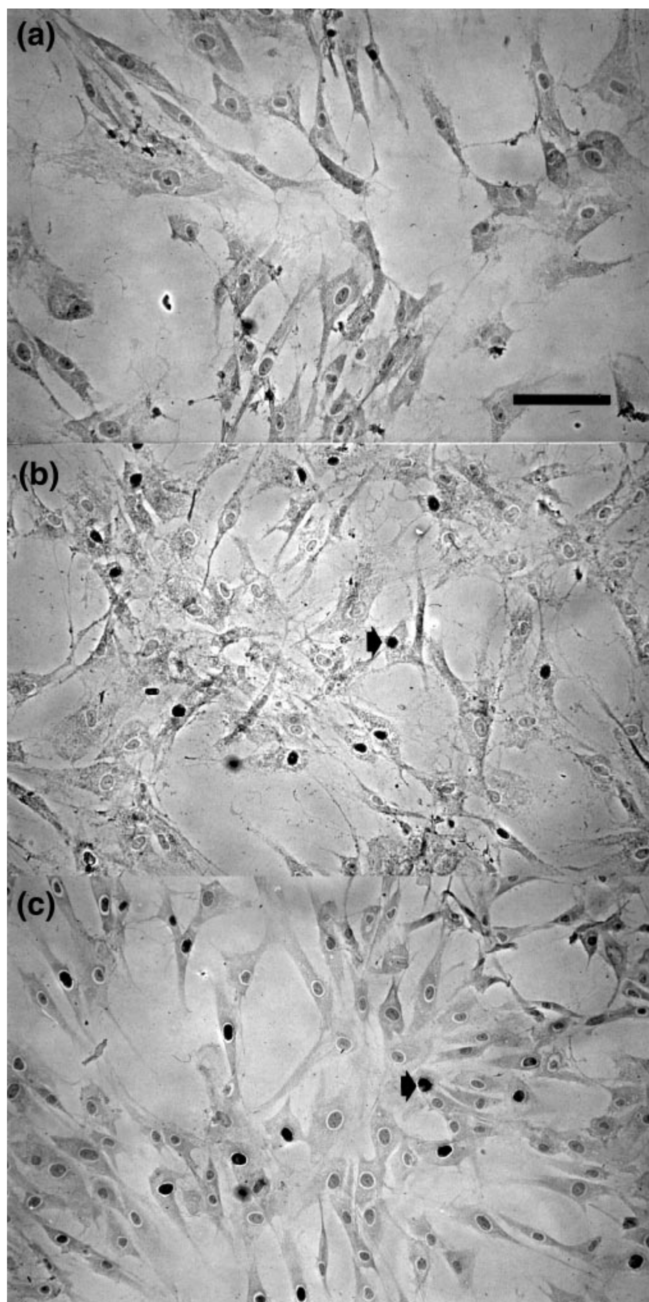


Figure 2. *In situ* incorporation of BrdU was severely diminished in chronic wound fibroblasts compared with fibroblasts from acute wounds, and normal dermis. No BrdU-positive fibroblast was found in the chronic wound fibroblasts (*a*) whereas several BrdU-positive cells (one of them arrowed in micrographs *b* and *c*) were observed amongst the fibroblasts obtained from acute wounds (*b*) and normal dermis (*c*), respectively. *Scale bar*: 100 μm.

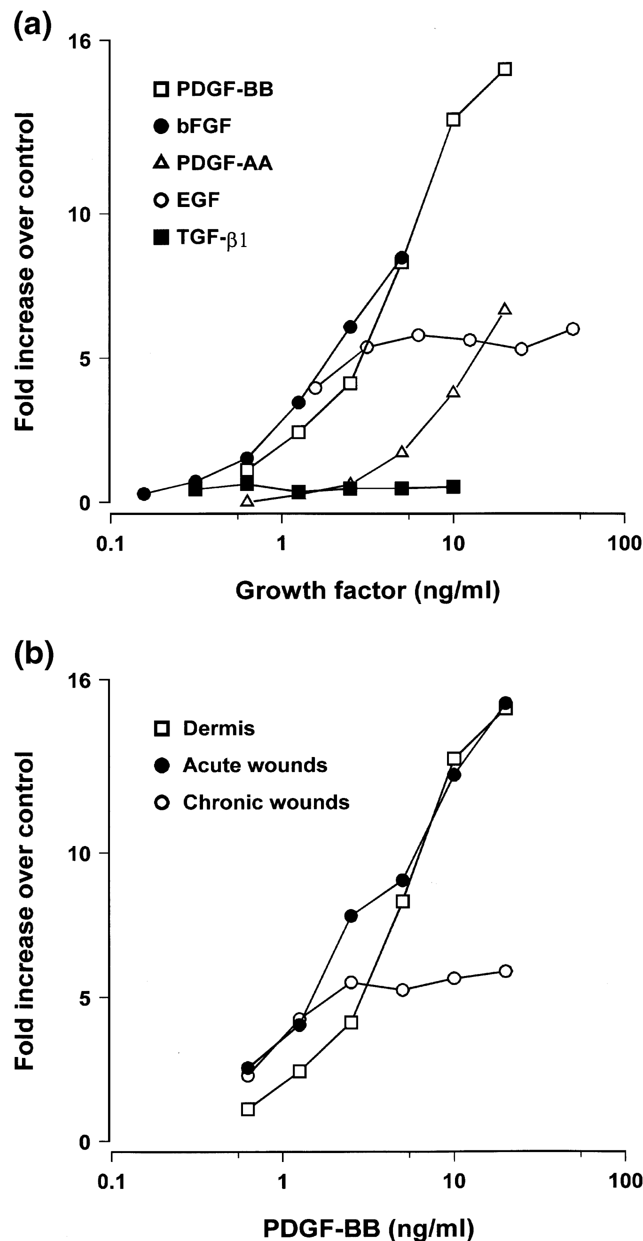


Figure 3. Dose–response effect of growth factors on DNA synthesis in normal dermal fibroblasts (*a*) and of PDGF-BB in chronic wound, acute wound, and normal dermal fibroblasts (*b*). Mitogenesis was determined in growth-arrested cells as BrdU incorporation (see *Materials and Methods*). The mitogenic response to the growth factors of the different fibroblast strains was expressed as fold increase over medium used for the growth factors alone. Error bars are omitted for reasons of clarity.

the ability to replicate appeared to decline with the duration of the ulcers from which the fibroblasts were isolated. Because decreased fibroblast proliferation appears to correlate to a correspondingly slower wound healing, at least experimentally in hamsters (Bruce and Deamond, 1991), this is a possible explanation for the reduced healing ability of chronic venous leg ulcers.

Stanley *et al* (1997) recently reported decreased growth of fibroblasts cultured from venous leg ulcers compared with fibroblasts cultured from the normal thigh skin of the same six patients. We also found a severely decreased growth of fibroblasts from the venous ulcers compared with those from adjacent peri-ulcer skin in the same patients. Because fibroblasts in wounds exhibit a different phenotype, e.g., decreased proliferative ability, compared with skin fibroblasts, we also included fibroblasts from acute wounds to control for the influence of wound environment on fibroblasts (Regan *et al*, 1991; Germain *et al*, 1994; Ågren *et al*, 1996).

Our patients with venous leg ulcers were older than the patients with acute wounds and the healthy volunteers. Furthermore, the location of the source of the fibroblasts differed among the three groups. There appears, however, to be a disagreement on the influence of donor age and anatomic location on fibroblast functions (Cristofalo, 1996). In fact some investigators found no effect of donor age and anatomic location on fibroblast growth (Falanga *et al*, 1991; Cristofalo, 1996; Vande Berg *et al*, 1998). In our study, the age of the chronic wounds rather than the age of the donor patients was a more important determinant of cell replication rates, because the patients with the slowest growing wound fibroblasts

Table III. Chronic wound fibroblasts displayed a decreased maximal mitogenic response to growth factors[a]

Growth factor	Chronic wounds (n = 4)	Acute wounds (n = 5)	Normal dermis (n = 5)
Control (no growth factor)	0	0	0
PDGF-BB	5.9 ± 2.4[b]	15.2 ± 7.0	15.0 ± 1.6
PDGF-AA	1.5 ± 0.6	4.6 ± 2.6	6.6 ± 1.0
bFGF	2.5 ± 0.9	9.3[c]	8.5 ± 0.9
EGF	2.5 ± 1.2	8.9[c]	6.0 ± 0.6
TGF-β1	0.8 ± 0.8	0.2[c]	0.6 ± 0.2

[a]Expressed as fold increase over control (no growth factor).
[b]Mean ± 1 SEM.
[c]Only one strain from the acute wounds was tested for mitogenic response to bFGF, EGF, and TGF-β1.

were younger (aged 64 ± 8 y, n = 5) than the patients (74 ± 2 y, n = 3) with more rapidly growing cells.

The chronic wound fibroblasts showed a generally diminished mitogenic response to growth factors (**Table III**). Stanley *et al* (1997) reported similar results to ours with bFGF and EGF. The mitogenic response to PDGF-BB of chronic wound fibroblasts was also decreased. This may provide one explanation for the poor response to topical PDGF-BB seen in some cases (Pierce *et al*, 1994).

The decreased mitogenic effect of PDGF-BB on chronic wound fibroblasts did not appear to be due to fewer PDGF α-receptors or β-receptors. Preliminary studies indicated that the levels of EGF receptors, measured with an ELISA assay (Christensen *et al*, 1995), did not differ among the three groups either (E. Nexø, personal communication, 1996). The decreased mitogenic response to growth factors may be due to a reduced affinity of the receptors and/or a defective signal transduction in the chronic wound fibroblasts.

PDGF receptor levels of the α-subunits and β-subunits were similar for the dermal and wound fibroblast strains, although there were slightly more β-receptors than α-receptors on the normal dermal fibroblasts. This finding is in sharp contrast to that of other studies where 5–10-fold higher levels of β-receptors on dermal fibroblasts were found (Ichiki *et al*, 1995; Petri *et al*, 1997). These investigators used western blotting or flow cytometric techniques. Because our culture medium was optimized by supplementation with bFGF it is possible that the α-receptor was selectively upregulated under these culture conditions (Ichiki *et al*, 1995). On the other hand, Karlsson and Paulsson (1994) found similar levels of α-receptors and β-receptors in cultured human skin fibroblasts using a binding assay with radio-iodinated PDGF-AA and PDGF-BB.

Decreased growth and mitogenic response to growth factors of chronic wound fibroblasts, and possibly of other effector cells, such as keratinocytes and endothelial cells, may be a general intrinsic abnormality in some chronic wounds. The phenotype of some of our cultured fibroblasts resembles those of *in vitro* aged fibroblasts classified as phase III cells by Hayflick and Moorhead (1961), i.e., a reduced or a total loss of ability to pass from the G_1 phase into S phase of the cell cycle. It is interesting to note that patients with Werner's syndrome characterized by accelerated aging, commonly develop non-healing ulcers on the lower leg, although the etiology appears to be excessive pressure rather than a primary vascular defect (Hürlimann and Schnyder, 1991). Skin fibroblasts cultured from these patients grow slowly and the responsiveness to PDGF and FGF is diminished without a concomitant change in the number of receptors for respective growth factor (Bauer *et al*, 1986).

It is unclear what causes the phenotypic changes of fibroblasts in chronic venous leg ulcers. It has been shown, however, that the production of free oxygen radicals is elevated in venous disease (Whiston *et al*, 1994). Oxidative stress induces irreversible changes, such as diminished cell growth and growth factor response, and an altered morphology, in cultured human diploid fibroblasts (Chen and Ames, 1994). Encouraging results with treatment of venous ulcerations with pentoxifylline and with topical sulfydryl-containing agents that bind oxygen-derived free radicals have also been reported (Salim, 1992; Coleridge Smith, 1994). It is also possible that the fibroblasts have exhausted their proliferative capacity

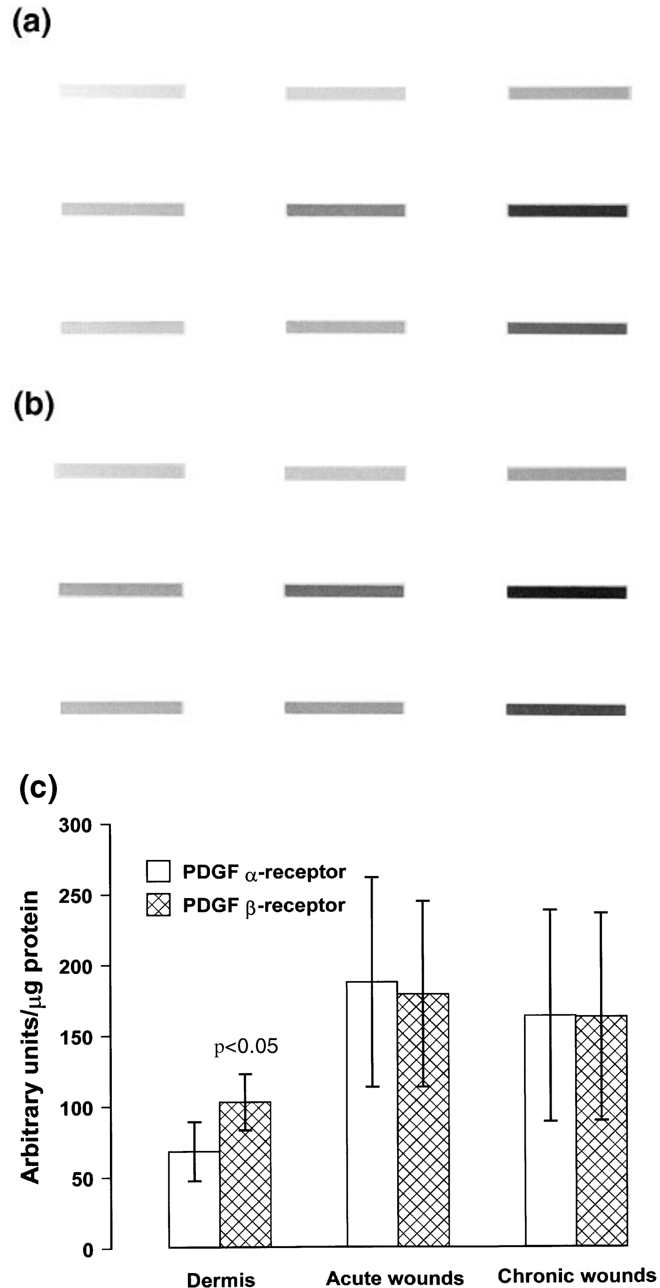


Figure 4. PDGF α-receptor and β-receptor levels in dermal, chronic wound and acute wound fibroblasts. Fibroblast membrane extracts (200 μl diluted extract was added to each slot) from normal dermis (top three horizontal bands in each blot), from chronic wounds (middle three horizontal bands), and from acute wounds (bottom three horizontal bands) were filtered through a nitrocellulose membrane, which was probed with monoclonal antibodies against the PDGF α-receptor (*a*) and the PDGF β-receptor (*b*), respectively. Left three vertical bands in each blot represent a 1:2000 dilution of extract, middle three vertical bands in each blot a 1:1000 dilution, and the right three vertical bands in each blot a 1:500 dilution. PDGF α-receptor and β-receptor levels were determined by densitometry of the slot-blots (see *Materials and Methods*). There was no statistically significant difference among dermal (n = 5), chronic wound (n = 8), and acute wound (n = 10) fibroblasts regarding the levels of α-receptors or β-receptors (α-receptors, p = 0.683; β-receptors, p = 0.842) (*c*). *Error bars*: SEM.

because of repetitive replication due to the chronic inflammation leading to the production of mitogenic cytokines (Vande Berg *et al*, 1998).

Although we studied only cell proliferation it is tempting to speculate that chronic wound fibroblasts suffer from other functional deficiencies, as loss of cell proliferation is commonly associated with other cellular changes (Cristofalo, 1996). For example, fibroblasts from venous leg ulcers appear to make less collagen than fibroblasts from normal skin, possibly due to a decreased response to TGF-β1 (Herrick *et al*, 1996; Hasan *et al*, 1997).

It should be emphasized that the chronic wounds studied represent severe cases not responding to conservative treatment, the only remaining alternative being transplantation with a split skin graft.

Because our findings are based on *in vitro* studies there is a question of phenotype stability of the fibroblasts when taken from their original environment to culture conditions. Cells, however, were analyzed at an early passage and *in vitro* cultured cells generally reflect the basic processes taking place *in vivo*, although the dynamics may differ (Cristofalo, 1996; Vande Berg *et al*, 1998). Owing to heterogeneity among fibroblasts in wounds, an error may also have been introduced by using an explant procedure possibly favoring the most rapidly growing fibroblasts (Vande Berg *et al*, 1998). We could not see a major difference, however, in the replication of fibroblasts obtained from explants compared with those obtained after enzymatic digestion of granulation tissue.

We hypothesize that old chronic wounds are populated by fibroblasts or subpopulation of fibroblasts that are exhausted/dysfunctional with a limited ability to replicate, and possibly to produce proper extracellular matrix elements and keratinocyte-stimulating factors (Werner *et al*, 1994).

In conclusion, our cellular findings provide one explanation for the failure of some chronic venous leg ulcers to heal, which opens up areas of investigation for new treatment strategies.

The excellent technical assistance of Mette Villingshøj is acknowledged. Coloplast A/S, Denmark funded the study and Sally Dabelsteen was supported by a grant (2411271 SSVF 12–2298, 9600821).

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