

Platelet-derived Growth Factor and Transforming Growth Factor- β Enhance Tissue Repair Activities by Unique Mechanisms

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Abstract. Platelet-derived growth factor (PDGF) and transforming growth factor- β (TGF- β) markedly potentiate tissue repair in vivo. In the present experiments, both in vitro and in vivo responses to PDGF and TGF- β were tested to identify mechanisms whereby these growth factors might each enhance the wound-healing response. Recombinant human PDGF B-chain homodimers (PDGF-BB) and TGF- β 1 had identical dose-response curves in chemotactic assays with monocytes and fibroblasts as the natural proteins from platelets. Single applications of PDGF-BB (2 μ g, 80 pmol) and TGF- β 1 (20 μ g, 600 pmol) were next applied to linear incisions in rats and each enhanced the strength required to disrupt the wounds at 5 d up to 212% of paired control wounds. Histological analysis of treated wounds demonstrated an in vivo chemotactic response of macrophages and fibroblasts to both PDGF-BB and to TGF- β 1 but the response to TGF- β 1 was significantly less than that observed with PDGF-BB. Marked increases of procollagen type I were observed by immunohistochemical staining in fibroblasts in treated wounds during the first week. The augmented breaking strength of TGF- β 1 was not observed

2 and 3 wk after wounding. However, the positive influence of PDGF-BB on wound breaking strength persisted through the 7 wk of testing. Furthermore, PDGF-BB-treated wounds had persistently increased numbers of fibroblasts and granulation tissue through day 21, whereas the enhanced cellular influx in TGF- β 1-treated wounds was not detectable beyond day 7. Wound macrophages and fibroblasts from PDGF-BB-treated wounds contained sharply increased levels of immunohistochemically detectable intracellular TGF- β . Furthermore, PDGF-BB in vitro induced a marked, time-dependent stimulation of TGF- β mRNA levels in cultured normal rat kidney fibroblasts. The results suggest that TGF- β transiently attracts fibroblasts into the wound and may stimulate collagen synthesis directly. In contrast, PDGF is a more potent chemoattractant for wound macrophages and fibroblasts and may stimulate these cells to express endogenous growth factors, including TGF- β , which, in turn, directly stimulate new collagen synthesis and sustained enhancement of wound healing over a more prolonged period of time.

POLYPEPTIDE growth factors such as the platelet-derived growth factor (PDGF)¹ and transforming growth factor- β (TGF- β) mediate activities considered essential for normal tissue repair processes (Deuel, 1987; Sporn et al., 1987). PDGF is a potent chemoattractant for neutrophils, monocytes, and fibroblasts (Deuel et al., 1982; Senior et al., 1983; Seppä et al., 1982) and also stimulates

these cells in activities of major importance in the wound-healing process, including the synthesis of fibronectin, collagenase, and additional growth factors (i.e., PDGF and TGF- β) (Tzeng et al., 1984, 1985; Bauer et al., 1985; Blatti et al., 1988; Paulsson et al., 1987; Pierce et al., 1989). TGF- β is also a potent chemotactic agent for monocytes and fibroblasts (Postlethwaite et al., 1987; Wahl et al., 1987) and induces the synthesis of extracellular matrix proteins (Ignatz et al., 1987; Ignatz and Massague, 1986; Chen et al., 1987; Raghov et al., 1987; Penttinen et al., 1988; Blatti et al., 1988; Roberts et al., 1988), modulates protease-induced degradation of the extracellular matrix (Lund et al., 1987; Keska-Oja et al., 1988), and stimulates the synthesis of additional growth factors (i.e., PDGF and interleukin 1) (Leof et al., 1986; Wahl et al., 1987).

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1. *Abbreviations used in this paper:* NRK, normal rat kidney; PDGF, platelet-derived growth factor; PDGF-BB, recombinant-derived human B-chain (c-sis) of platelet-derived growth factor; TGF- β , transforming growth factor β ; TGF- β 1, recombinant-derived human transforming growth factor β 1.

The direct demonstration that growth factors positively influence wound healing was obtained when both TGF- β and PDGF were found to accelerate healing of full-thickness, incisional wounds in rats (Mustoe et al., 1987; Pierce et al., 1988b). Importantly, however, PDGF and TGF- β appear to operate by different mechanisms since TGF- β but not PDGF was able to reverse the deficit in wound healing induced by systemic glucocorticoid administration (Pierce et al., 1989). In this manuscript, it is shown that recombinant derived human transforming growth factor β 1 (TGF- β 1) is more effective at lower concentrations as a chemoattractant for monocytes and fibroblasts than the recombinant derived human B-chain (*c-sis*) of PDGF (PDGF-BB) in vitro, although each is able to attract equal numbers of cells at optimal concentrations. However, PDGF-BB initiates a substantially greater influx of macrophages and fibroblasts into wounds than TGF- β 1. PDGF-BB continues to increase wound strength long after the 5–7 d in which TGF- β 1 produces maximal increases; importantly, this prolonged influence of PDGF-BB in augmenting wound strength correlates directly with the demonstration of enhanced TGF- β expression in both macrophages and fibroblasts, two cell types which are essential for normal tissue repair. The data strongly support the hypothesis that the persistent effects of PDGF on wound healing may be mediated in part through the induction of additional endogenous growth factor activities in both fibroblasts and macrophages.

Materials and Methods

Study Design and Model

A full-thickness, dorsal incisional model was used, as previously described (Mustoe et al., 1987). Four to eight rats were used for each test variable. Paired incisions were coapted with three surgical clips and injected once with individual growth factors in a collagen vehicle or with collagen vehicle alone at the time of wounding. The wounds were excised on the specific days noted and strips perpendicular to the paired experimental and control wounds (collagen alone) were obtained for tensometry and histologic analysis. From each rat, two to three pairs of strips were obtained for tensometry analysis and one to two paired samples were obtained for histologic analysis.

Preparation of Growth Factors in Collagen

TGF- β 1 (a gift from A. Ammann, Genentech, Inc., South San Francisco, CA) was purified as described (Shalaby and Ammann, 1988). Endotoxin levels were <0.1 ng/mg purified TGF- β 1. PDGF-BB (the homodimeric product of the human *c-sis* gene) was purified to homogeneity by immunoaffinity chromatography (gift of A. Thomason, Amgen, Inc., Thousand Oaks, CA). PDGF-BB was shown previously to be fully equivalent in bioactivity to platelet-purified human PDGF-AB (Pierce et al., 1988b). Growth factors were mixed into a diluted bovine collagen suspension (Zyderm II, Collagen Corp. Palo Alto, CA) and were applied once only with 1 mg collagen per incision using a tuberculin syringe. Collagen alone (control wounds) did not influence the rate of healing when compared to phosphate-buffered saline. In *in vitro* experiments, both growth factors were largely released from the collagen vehicle within 24 h (Pierce et al., 1988b). Histologically, the exogenous collagen was no longer apparent in wounds by 4 d after surgery.

Data Analysis

Analysis of variance and paired *t* tests of breaking strength scores and of differences between matched experimental and control values were performed using the SAS data system (Division of Biostatistics, Washington University, St. Louis, MO). Percent stimulation of wound breaking strength in growth factor-treated samples compared to paired controls was calculated and analyzed using a population *t* test. Breaking strength measurements

were performed blindly on a tensometer (Tensometer 10; Monsanto Co., St. Louis, MO) on precoded samples.

Histologic Analysis and Immunoperoxidase Staining

Matched paired samples of experimental and control wounds from each rat were fixed in acetic acid–ethanol (1:99, vol/vol). Paired hematoxylin- and eosin-stained sections were coded and then analyzed microscopically by two independent observers for the degree of cellularity (using an arbitrary scale from 0 to 4), for cell types within wounds, and for new granulation tissue within the scar. Immunoperoxidase staining of samples was performed using rabbit anti-rat procollagen type I or type III monospecific IgG fraction (the generous gift of K. Cutroneo, University of Vermont, Burlington, VT), or rabbit anti-TGF- β 1 (R & D Systems, Minneapolis, MN) at appropriate dilutions using established methods (Pierce et al., 1989). Paired sections were incubated in primary antisera (2 μ g/ml) and then incubated with appropriate dilutions of biotin-conjugated goat anti-rabbit IgG (Bethesda Research Laboratories, Gaithersburg, MD) followed by streptavidin–horseradish peroxidase (Bethesda Research Laboratories). After development in 3,3'-diaminobenzidine tetrahydrochloride (Bethesda Research Laboratories) slides were lightly counterstained with Harris hematoxylin. Negative controls consisted of parallel sections incubated with comparable dilutions of irrelevant primary antisera. Paired wound sections were coded and analyzed microscopically by two independent observers. In some experiments, another TGF- β 1 specific antibody, anti-A1/30, was used (a gift of J. McPherston, Collagen Corp., Palo Alto, CA) (Ellingsworth et al., 1986).

Chemotaxis

Chemotaxis was assayed using modified Boyden chambers (Senior et al., 1983). Peripheral blood mononuclear cells were separated on Ficoll–Hypaque gradients from peripheral blood obtained from healthy volunteers. Fetal bovine ligament fibroblasts were obtained as described (Mecham et al., 1981). Growth factors were serially diluted in DME before use, and five high-power fields (fibroblasts) or grids (monocytes) were counted from triplicate samples. Anti-TGF- β 1 (R & D Systems), a rabbit polyclonal neutralizing antibody, was premixed and serially diluted with TGF- β 1 in some experiments to assess the specificity of the chemotactic response.

RNA Isolation and Northern Blot Analysis

Normal rat kidney fibroblasts (NRK, Clone 49F, American Type Culture Collection, Rockville, MD) were maintained in DME-supplemented 10% fetal bovine serum (Pierce et al., 1988b). Cells were grown to confluency without a renewal of media over 6 d to ensure serum starvation. At various times during treatment with 50 ng/ml PDGF-BB or PDGF-BB plus 10 μ g/ml cycloheximide (Sigma Chemical Co., St. Louis, MO) in serum-free DME, total cellular RNA was isolated by a modified guanidine thiocyanate procedure (Chomczynski and Sacchi, 1987). Briefly, cells were lysed in 4 M guanidine thiocyanate, extracted with water-saturated phenol/chloroform/isoamyl alcohol, and the remaining RNA was precipitated twice in ethanol. Equal amounts (15 μ g) of RNA (estimated by absorbance at 260 nm and by ethidium bromide staining) were separated on standard denaturing formaldehyde–agarose gels, blotted onto nitrocellulose (Schleicher and Schuell, Inc., Keene, NH), and prehybridized overnight as described (Pierce et al., 1989). The blots were transferred to a fresh hybridization solution containing 0.5×10^6 cpm/ml of a nick-translated TGF- β 1 probe (1.6-kb insert of the sp65Mur β a.s. plasmid, gift of R. Derynck, Genentech, Inc.) labeled to a specific activity of 8×10^6 cpm/ μ g. After an overnight hybridization at 42°C, the blot was washed and exposed to Kodak X-OMAT film (Eastman Kodak Co., Rochester, NY) for 4 d. Bands on the autoradiogram were quantitated on a scanning laser densitometer.

Results

TGF- β 1-induced Chemotaxis In Vitro

Monocytes and fibroblasts were tested for chemotactic responsiveness towards TGF- β 1 (Fig. 1 A). Peak chemotactic activity towards both monocytes and fibroblasts was observed at 20 fM (0.5 pg/ml); anti-TGF- β 1 antiserum spe-

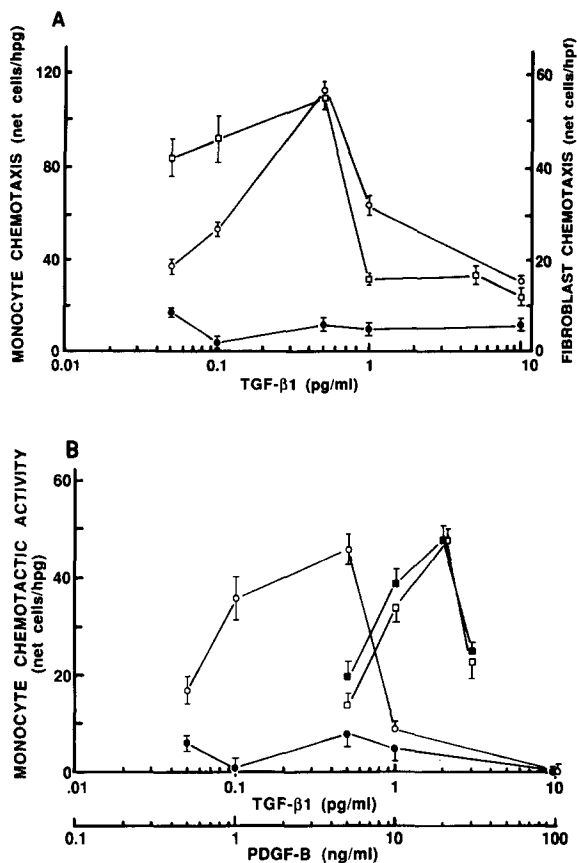


Figure 1. Chemotaxis of monocytes and fibroblasts in response to TGF-β1. TGF-β1 was serially diluted and tested for chemotactic activity in modified Boyden chambers. (A) For monocytes, the positive control, 10^{-8} M f-met-leu-phe, was 113 ± 4.7 cells per high power grid (hpg). To test for specificity, anti-TGF-β was mixed with TGF-β1 and serially diluted. Anti-TGF-β was present at 100 ng/ml when TGF-β1 was 10 pg/ml. Anti-TGF-β alone was not chemotactic and had no effect on the f-met-leu-phe response. For fibroblasts, the positive control, 30 ng/ml human platelet-purified PDGF-AB, was 57 ± 3.6 cells per high power field (hpf). (□) Fibroblasts; (○) monocytes; and (●) monocytes plus anti-TGF-β1. (B) Anti-TGF-β abrogated the monocyte response to TGF-β1, however, it had no effect on the PDGF-BB-induced chemotactic response. Anti-TGF-β also had no effect on PDGF-BB-induced fibroblast chemotaxis which peaked at 30 ng/ml (data not shown). (□) PDGF-BB; (■) PDGF-BB plus anti-TGF-β1; (○) TGF-β1; and (●) TGF-β1 plus anti-TGF-β1.

cifically and fully reversed the chemotactic potential of TGF-β1. As reported previously, PDGF-BB was highly effective as a monocyte chemoattractant, with optimal activity at 0.8 nM (20 ng/ml) (Pierce et al., 1988b). Importantly, anti-TGF-β1 had no effect on the PDGF-BB-generated chemotaxis on either monocytes (Fig. 1 B) or fibroblasts (data not shown). The recombinant growth factors were fully as active as TGF-β and PDGF purified from human platelets; the optimum migration of cells in response to TGF-β as a chemoattractant was equal to that of PDGF. The chemotactic activity of PDGF is not mediated by trace contamination with TGF-β.

TGF-β1-induced Augmentation of Wound Breaking Strength

We tested increasing doses of TGF-β1 applied at the time of

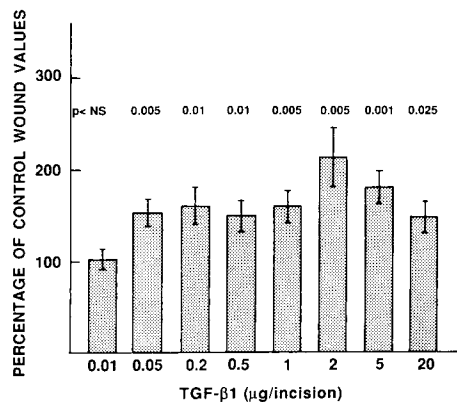


Figure 2. Dose-dependent stimulation of wound breaking strength by TGF-β1. TGF-β1 was added in a collagen suspension once at the time of surgery, and wound breaking strength was tested 5 d later. Paired experimental and control (collagen vehicle alone) incisions were placed on groups of six rats for each concentration tested and a two-tailed, paired *t*-test was used to assess significance between matched, paired wound strips. Each dose was tested on at least two occasions. 100% represents control wound breaking strength, which was 121 ± 21 g (mean \pm SD).

surgery and analyzed the breaking strength of wounds 5 d later (Fig. 2). 2 μg TGF-β1 per incision maximally increased the strength required to disrupt incisional wounds (212% of paired controls, $p < 0.005$). Peak stimulation of wound breaking strength by TGF-β1 was observed 5 and 7 d after wounding; the wound healing was accelerated by 2–3 d over the first week relative to control wounds (Fig. 3 A). The functional activity of TGF-β1 was identical to TGF-β purified from human platelets in time course, dose response, and in optimum enhancement of healing (Mustoe et al., 1987). Significantly, no evidence of delayed reepithelialization was found in TGF-β1-treated wounds.

Differences between TGF-β1- and PDGF-BB-induced Wound Repair

The enhancement of wound healing after single applications of optimal concentrations of TGF-β1 or PDGF-BB were compared for 49 d (Fig. 3 C). Striking differences were observed; compared to PDGF-BB, TGF-β1 generally enhanced wound strength to a greater degree 5 d after wounding while at 7 d both growth factors consistently increased wound breaking strength equally, averaging 150–200% of paired control values (Fig. 3 A). However, TGF-β1 no longer accelerated healing beyond the initial 2-wk period. PDGF-BB continued to significantly enhance the breaking strength of treated wounds through the 49 d under study (Fig. 3 B). The healing of PDGF-BB-treated wounds was accelerated 28 d after wounding by 5–10 d compared to paired controls; the differences between PDGF-BB-treated and control wounds amounted to ~15% of the total wound breaking strength of the treated wounds between 28 and 49 d (Fig. 3 B). No evidence of hypertrophic scar formation was observed in PDGF-BB-treated wounds (see histologic analysis below).

In other experiments, suboptimal doses of PDGF-BB and TGF-β1 were tested in combination to identify potential additive, synergistic, or antagonistic effects. An additive effect of

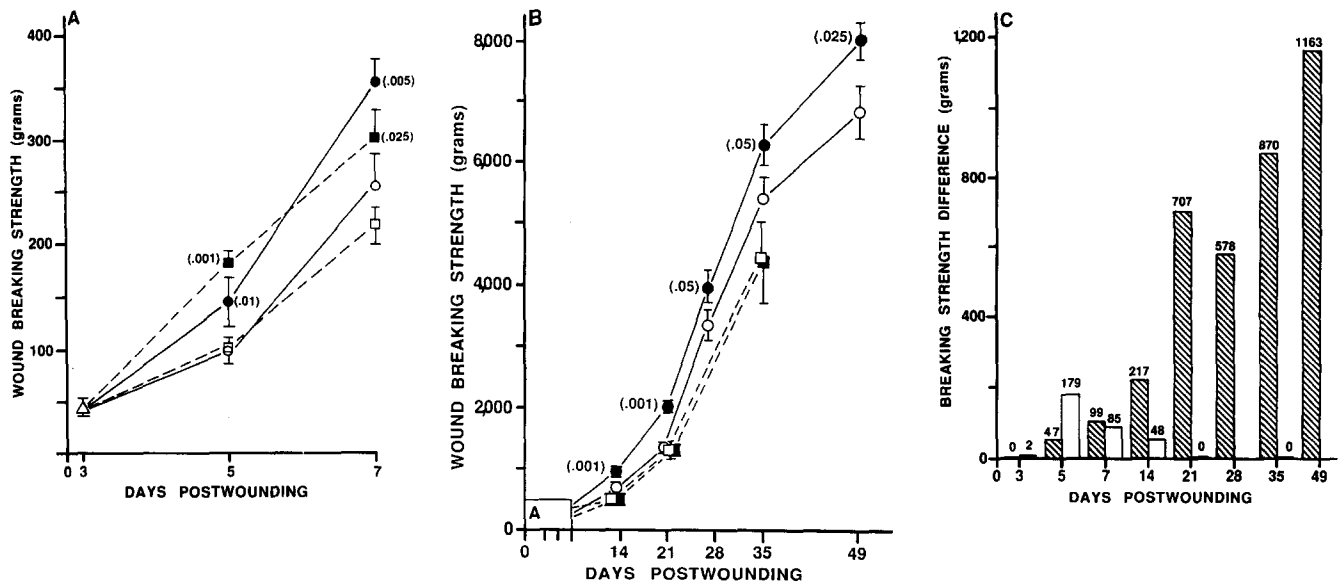


Figure 3. Pair incisional wounds received either growth factor ($2 \mu\text{g}$ TGF- β 1, $20 \mu\text{g}$ PDGF-BB) or collagen alone on the day of surgery, and 11–14 paired growth factor–treated and control strips were harvested on each day indicated for maximum breaking strength determinations (mean \pm SEM). A two-tailed, paired *t*-test was used to calculate the *P* value. (A) Acceleration of healing induced by PDGF-BB or TGF- β 1 during the first week after surgery. (B) Effects of growth factors applied at the time of surgery through 49 d (PDGF-BB) or 35 d (TGF- β 1). (●) PDGF-BB; (○) control; (■) TGF- β 1; (□) control. (C) Differences in breaking strength measurements between growth factor–treated and paired control wounds, calculated from the values presented in A and B. (■) PDGF-BB; (□) TGF- β 1.

suboptimal concentrations of both growth factors was observed on day 7 (data not shown) but, by day 21, only the PDGF-induced augmentation of wound healing was observed. When added together at optimal concentrations, the breaking strength required to disrupt the wounds was precisely that expected for PDGF-BB or TGF- β 1 alone at day 7, and for PDGF-BB alone at day 21 (data not shown).

Analysis of Cellular Influx into Wounds

The cellular influx into TGF- β 1-treated wounds was analyzed and quantitatively compared with matched, paired control wounds (Table I). Increased macrophage and fibroblast influx occurred within 3–5 d of wounding in TGF- β 1-treated

($2 \mu\text{g}$) wounds ($p < 0.01$). This enhancement of cell migration was qualitatively and quantitatively decreased relative to the enhancement found previously in PDGF-BB-treated wounds (Pierce et al., 1988b); PDGF-BB induced a large increase in the influx of neutrophils on days 1 and 2 and of macrophages and fibroblasts on days 3–5. The PDGF-BB enhancement of cell migration was substantially above the cellular influxes induced in TGF- β 1-treated wounds (Fig. 4). The influx of cells in response to PDGF-BB in vivo correlated well with chemotactic responses in vitro. Wounds treated with lower concentrations of TGF- β 1 demonstrated less of a cellular influx, in contrast to the more potent effects observed at lower concentrations of TGF- β 1 in the chemotaxis assay. TGF- β 1, thus, is nearly 40,000-fold more potent on a mole/

Table I. Quantitative Analysis of Cellular Influx in PDGF-BB- or TGF- β 1-treated Wounds*

Days after wounding	Growth factor	Difference in cellularity [†]	Difference in granulation tissue [‡]	Predominant cell type
1–2	TGF- β 1	+0.08 \pm 0.25		Neutrophil, macrophage
3–5	TGF- β 1	+0.64 \pm 0.22§		Macrophage, fibroblast
7–10	TGF- β 1	+0.12 \pm 0.26		Fibroblast
14	TGF- β 1	+0.14 \pm 0.21	+0.18 \pm 0.38	Fibroblast
21	TGF- β 1	+0.08 \pm 0.26	+0.17 \pm 0.29	Fibroblast
14–21	PDGF-BB	+0.70 \pm 0.24	+0.75 \pm 0.26	Fibroblast
28–49	PDGF-BB	-0.36 \pm 0.24	-0.36 \pm 0.37	Fibroblast

* Growth factor–treated and paired control wound sections were harvested on the days indicated and processed, embedded, and stained with hematoxylin and eosin. They were coded and blindly rated by two individuals on a scale from 0–4 for cellularity and new granulation tissue within the scar (from day 14 onward). TGF- β 1 ($2 \mu\text{g}$) or PDGF-BB ($20 \mu\text{g}$) were applied to incisions once on the day of wounding. A score of 0 represented baseline cellularity in unwounded dermis and no granulation tissue. Cellularity in PDGF-BB-treated wounds during the first 2 wk after wounding was previously reported, and was significantly increased above controls from days 1–14 (Pierce et al., 1988b).

† The mean (\pm SEM) difference of scores between growth factor–treated and paired control wounds was calculated for 6–12 sections per group. Median values were nearly identical to means, indicating the data were normally distributed. Granulation tissue (scar) was assessed from day 14 on wounds.

§ $P < 0.01$, two-tailed, paired *t*-test.

|| $P < 0.02$.

mole basis in comparison to PDGF in *in vitro* chemotactic activity; *in vivo*, TGF- β 1 is <10-fold more active on a mole/mole basis. More importantly, PDGF-BB at optimal concentrations induces a greater influx of cells into wounds *in vivo* than does TGF- β 1. The chemotactic potential of PDGF-BB on wound cellularity was sustained through 21 d in contrast to the transient effects of TGF- β 1. The sustained increase in cellular influx correlated directly with increased granulation tissue at this time and with the persistent increases in breaking strength in PDGF-treated wounds (Fig. 5). Interestingly, during the second month after wounding, the cellularity and granulation tissue content of PDGF-BB-treated wounds, in contrast to the wound breaking strength, was less than that found in paired controls. This time-dependent trend in cellularity and granulation tissue was reproducible, but was not statistically significant.

Analysis of Procollagen Types I and III in Wounds

The formation of new collagen in wounds is considered the most important correlate with developing strength within the wound (Levenson et al., 1968; Madden and Peacock, 1968). Immunohistochemical analyses of procollagen type I and type III content in growth factor-treated and control wounds were performed. In each group, although the numbers of fibroblasts per high-power field were substantially greater in the growth factor-treated groups, the proportions of the fibroblasts (~40–50%) containing procollagen type I 3–7 d after wounding were similar. The results suggested that in wounds not treated with growth factors, an adequate endogenous signal is provided to induce those fibroblasts present to generate new collagen. The enhanced healing, therefore, correlated directly with the significant increase in the numbers of fibroblasts attracted into wounds treated with PDGF-BB or TGF- β 1, which in turn accounted for the substantial increase in total procollagen type I observed relative to the content of procollagen type I in matched control wounds (Fig. 6). No differences in percentage staining or staining intensity of procollagen type I or III were observed in comparisons of TGF- β 1- and PDGF-BB-treated wounds at days 3–7. Fewer than 3% of the wound fibroblasts were shown to have detectable levels of intracellular procollagen type III in either growth factor-treated or control wounds (data not shown).

Analysis of Intracellular TGF- β in Wounds

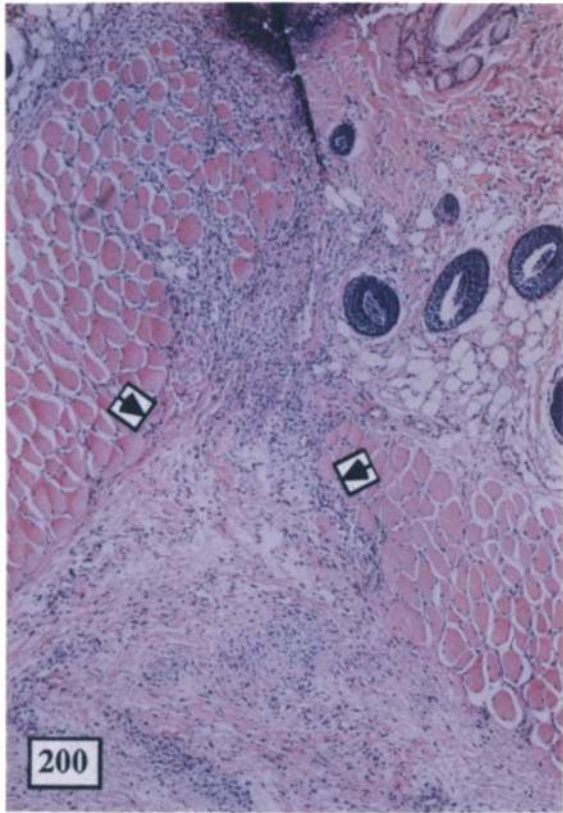
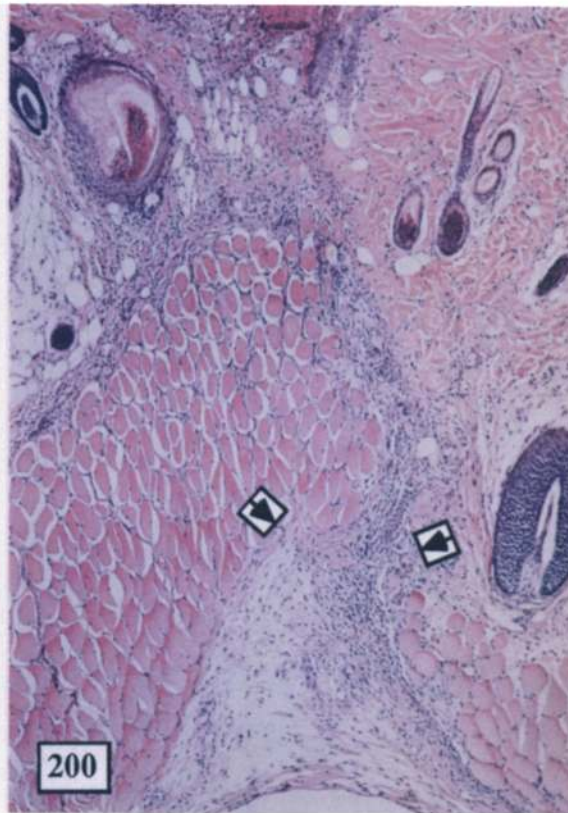
Since procollagen synthesis is perhaps the major contributor to increased wound strength, the prolonged influence of PDGF-BB is in apparent conflict with the inability of PDGF to directly promote procollagen synthesis in fibroblasts *in vitro* and in wounds from glucocorticoid-treated experimental animals (Roberts et al., 1986; Pierce et al., 1989). PDGF-BB was shown to induce expression of TGF- β in a monocyte line (Pierce et al., 1989) and thus may act through the monocyte/macrophage to provide TGF- β 1 for stimulation of procollagen synthesis in fibroblasts. However, the macrophage is not prominent in tissue sections of treated wounds after 7–10 d (Pierce et al., 1988b), suggesting that PDGF might act at the level of the fibroblast in 7–10-d wounds over and above its influence on the macrophage early in the wound-healing process. Sections from 2, 4, and 7 d PDGF-BB-treated and paired control wounds were analyzed with a monospecific anti-TGF- β antiserum. Specific intracellular

staining of TGF- β was identified in normal wounds. Remarkably, increased numbers of macrophages (days 2 and 4) and fibroblasts (days 4 and 7) containing intracellular TGF- β were found in PDGF-BB-treated wounds (Fig. 7). Anti-A1/30, a second specific anti-TGF- β 1 antiserum, was used to confirm this result. The results indicated that PDGF was capable of inducing increased intracellular TGF- β levels *in vivo*, both in the macrophage and in the fibroblast. A similar analysis of TGF- β 1-treated wounds with anti-TGF- β was not productive due to background staining. However, when quiescent NRK cells were stimulated *in vitro* by 50 ng/ml PDGF-BB, a marked time-dependent increase in TGF- β mRNA was observed (~10-fold increase, Fig. 8), confirming that the increased intracellular TGF- β observed in wound fibroblasts can arise from a direct stimulatory action by PDGF and that the increase in procollagen type I may arise from the subsequent autocrine stimulatory influence of newly synthesized TGF- β 1.

Discussion

Direct evidence supports both PDGF and TGF- β as important transducers of tissue repair mechanisms (Deuel, 1987; Pierce and Deuel, 1989). PDGF was the first growth factor shown to be chemotactic for monocytes and neutrophils (Deuel et al., 1982; Senior et al., 1983); recently TGF- β purified from platelets was also found to be a potent chemotactic protein (Postlethwaite et al., 1987; Wahl et al., 1987). Both growth factors are released by degranulating platelets at the initiation of wound repair (Deuel, 1987; Assoian and Sporn, 1986), and both growth factors may be expressed by cells active in inflammation and repair, producing an augmented cascade of activities leading to increased macrophage and fibroblast influx. The increased numbers of these cells in PDGF or TGF- β -treated wounds directly support this hypothesis. Both PDGF- and TGF- β -like growth factors are present in monocytes/macrophages (Derynck et al., 1985; Shimokado et al., 1985; Martinet et al., 1986; Cheng et al., 1987; Assoian et al., 1987; Fig. 7), which are normally directed to sites of wounds within the first 24–48 h (Liebovich and Ross, 1975; Hunt et al., 1983; Pierce et al., 1988b). Activated macrophages also represent a significant source of other cytokines which may function in tissue repair, including interleukin 1 and tumor necrosis factor (Nathan, 1987; Rappolee et al., 1988).

The present investigations firmly establish that PDGF-BB and TGF- β 1 function identically to their natural counterparts purified from human platelets in chemotactic assays *in vitro* and in the enhancement of breaking strength in incisional wounds *in vivo*. Both PDGF-BB and TGF- β 1 were incapable of synergistic effects when analyzed at suboptimal or optimal concentrations, suggesting that procollagen synthesis, which best correlates with breaking strength in healing wounds, is rate limiting. Previous data obtained *in vitro* indicated that PDGF cannot directly increase procollagen synthesis in fibroblasts (Roberts et al., 1986), in contrast to TGF- β (Rossi et al., 1988). However, in the present experiments the marked increase in the numbers of fibroblasts attracted to both PDGF-BB- and TGF- β 1-treated wounds were equally intense in procollagen type I immunostaining, implying that, although PDGF-treated wounds have markedly increased in

A**B**

procollagen type I, PDGF mediates procollagen synthesis in healing wounds only indirectly. The indirect effect of PDGF on fibroblast procollagen synthesis was further tested at a time when the influence of PDGF-BB had extended well beyond the period of macrophage influx into wounds. Wounds treated with PDGF-BB continued to manifest increased strength above paired controls through day 49 and contained significantly greater numbers of fibroblasts and increased granulation tissue within the wounds through day 21. Because PDGF-BB induces and sharply enhances the expression of the TGF- β gene in quiescent macrophages and in fibroblasts in vitro (Van Oberrghen-Schilling, 1988, Pierce et al., 1989; Fig. 8), one distinction between TGF- β 1 and PDGF-BB may be the initial requirement of the macrophage and subsequently the fibroblast for the activation of endogenous growth factors such as TGF- β to transduce the external signal of PDGF-BB. Furthermore, the persistent stimulation of the fibroblasts attracted to the wounds through the potent chemotactic potential of PDGF-BB may lead to new expression of endogenous TGF- β within wound fibroblasts and subsequent expression of procollagen type I. This hypothesis is consistent with the induction of TGF- β mRNA synthesis observed in vitro in PDGF-treated fibroblasts. Other possible mechanisms which may be responsible for the observed prolonged effect of PDGF are currently under study, and may include the regulation of collagenase synthesis (Bauer et al., 1985), which is required for wound remodeling. Although

TGF- β also stimulates expression of TGF- β in fibroblasts in vitro (Van Oberrghen-Schilling, 1988), the transient cellular influx observed in TGF- β 1-treated wounds may not provide an adequate stimulus for the initiation of a cascade effect, as observed in PDGF-BB-treated wounds.

PDGF also sharply stimulates fibroblast expression of PDGF-AA chain (Paulsson et al., 1987; Pierce G. F., V. R. Masakowski, B. Tong, and T. F. Deuel, manuscript submitted for publication). PDGF, thus, may activate an autocrine loop to effect a positively regulated cascade of growth factor activities during normal tissue repair. Finding increased numbers of macrophages and fibroblasts which contain TGF- β in PDGF-BB-treated wounds offers strong support to this hypothesis. Despite the ability of epidermal growth factor to induce PDGF-AA in cultured fibroblasts (Paulsson et al., 1987), a single application of epidermal growth factor does not recruit cells into incisional wounds and does not augment wound breaking strength (unpublished observations). Thus, the unique inductive and chemotactic effects of PDGF-BB and TGF- β 1 in vivo are not generalized activities mimicked by other growth factors.

Important in vivo roles of TGF- β and PDGF may also include the regulation of procollagen type I, thrombospondin, fibronectin, collagenase, glycosaminoglycan, and fibronectin receptor synthesis by TGF- β (Ignatz and Massague, 1986; Ignatz et al., 1987; Raghov et al., 1987; Chen et al., 1987; Penttinen et al., 1988; Chua et al., 1985; Edwards et

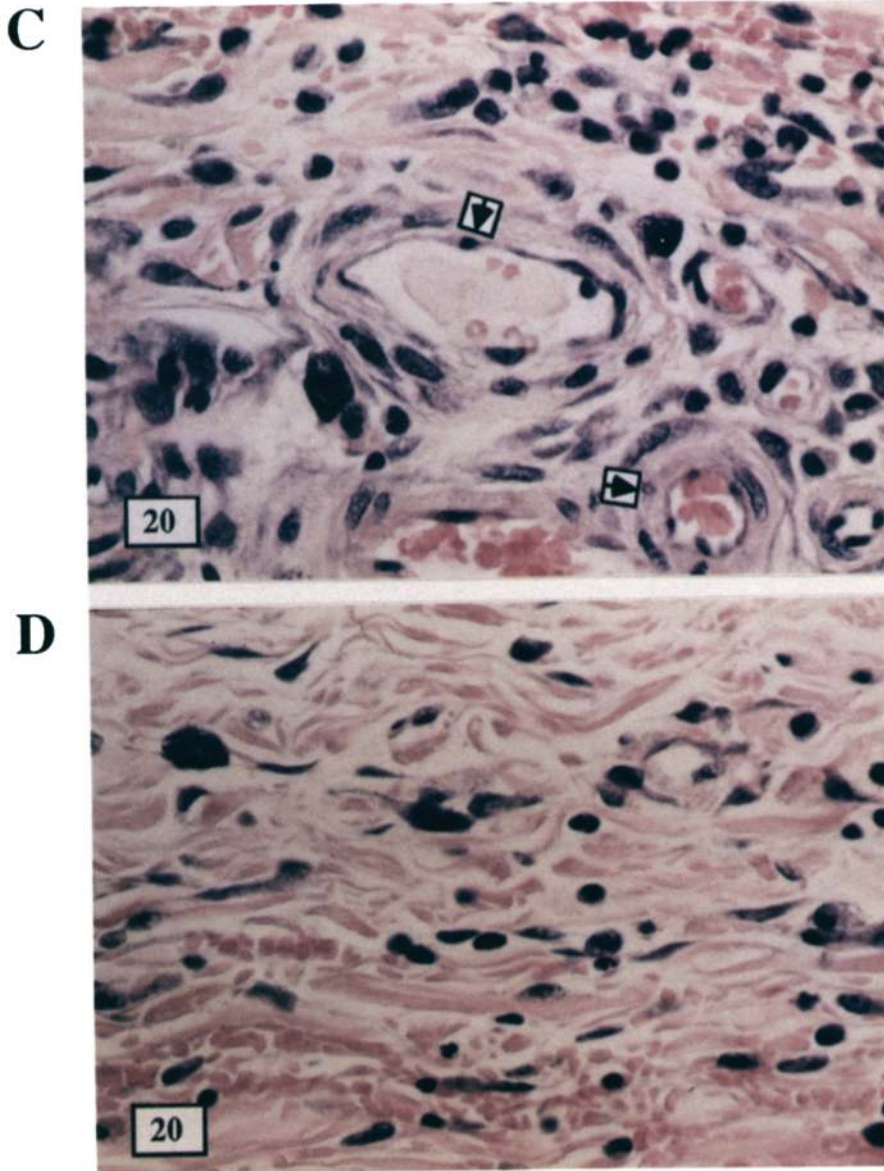


Figure 4. Cellular influx into 2 μg TGF- β 1-treated (A and C), or control (B and D) incisions 3 d after wounding. Note the increased influx of mononuclear cells (primarily macrophages and fibroblasts) throughout the TGF- β 1-treated wounds in these hematoxylin- and eosin-stained sections. New granulation tissue (arrowheads, A and B) and vessel formation (arrowheads, C and D) are occurring at the base of TGF- β 1-treated wounds, compared to the paired control. PDGF-BB-treated wounds consistently demonstrated a greater influx of macrophages and fibroblasts at this time (Pierce et al., 1988b), in comparison with TGF- β 1-treated wounds. Bars: (A and B) 200 μm ; (C and D) 20 μm .

al., 1987; Roberts et al., 1988; Bassols and Massague, 1988), and fibronectin and collagenase production by PDGF (Bauer et al., 1985; Chua et al., 1985; Blatti et al., 1988); all are essential time-dependent activities for normal tissue repair (Pierce and Deuel, 1989). The unique mechanisms of action and time course of activities we have identified for PDGF and TGF- β suggest each of these growth factors may find optimal therapeutic application in specific types of wounds in man.

The linear incision model represents an important tool for the evaluation of tissue repair activities regulated by polypeptide growth factors. Wound healing models using dead space chambers or polyvinyl sponges may induce a foreign body inflammatory response replete with foreign body giant cells that is significantly delayed in onset compared to the normal wound inflammatory response (Sporn et al., 1983;

Sprugel et al., 1987; Grotendorst et al., 1985; Davidson et al., 1985). Diffusion of factors and the failure of cells to interact with normal substrates further limit interpretation of results from these models. However, contents of wound chambers are readily accessible to extensive biochemical analyses, an advantage for analyzing extracellular matrix composition. Full-thickness biopsy models which test the acceleration of wound closure due to contraction are also not directly applicable to human wounds since, in man, wound contraction is of lesser importance. In contrast, the linear incision model corresponds precisely to the physiologically relevant time-dependent cellular and extracellular matrix activities required for normal tissue repair (Levenson et al., 1968; Madden and Peacock, 1968; Ross, 1968).

Importantly, the elucidation of the roles of PDGF and TGF- β in normal tissue repair provides valuable insights into

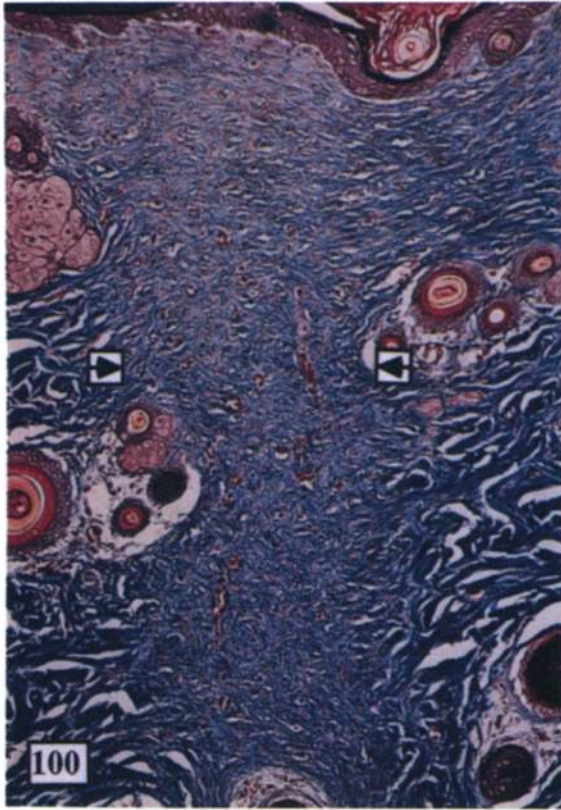
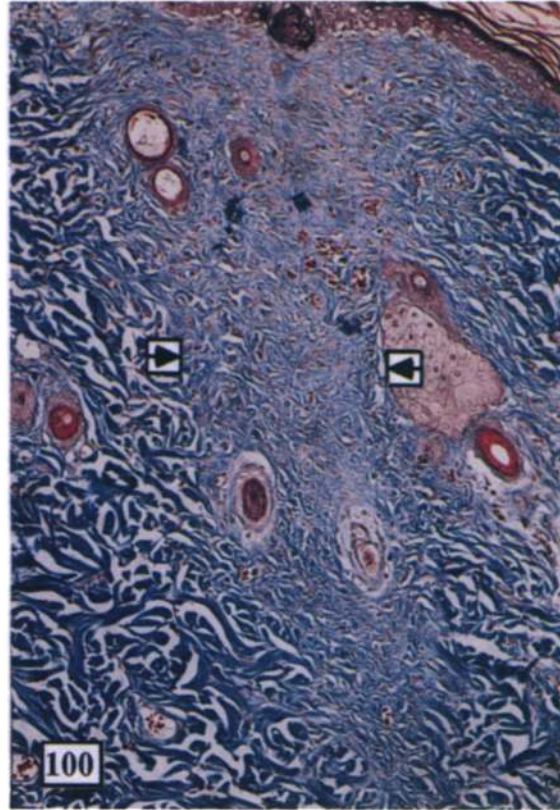
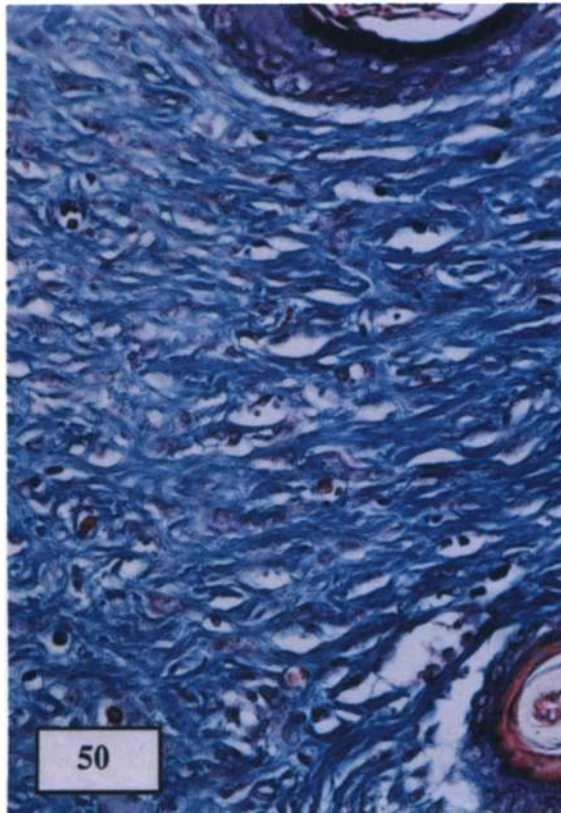
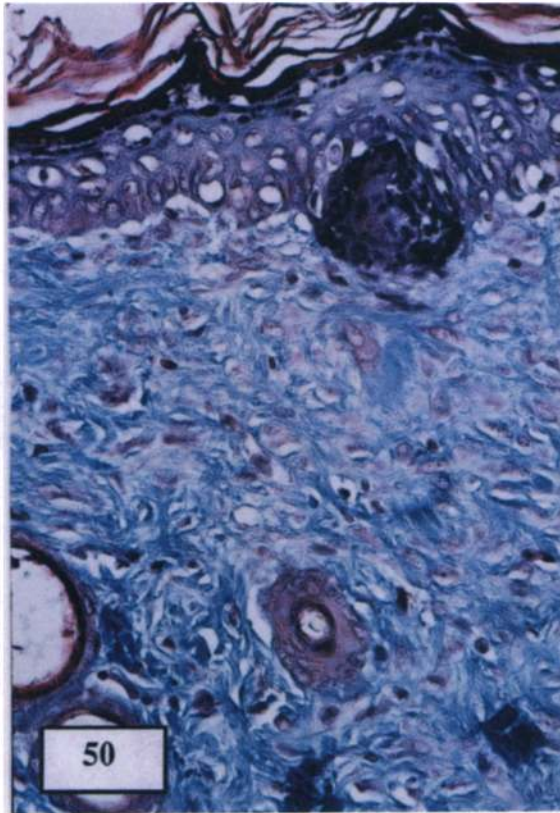
A**B****C****D**

Figure 5. Appearance of wounds 3 wk after treatment with 20 μg PDGF-BB (*A* and *C*), or vehicle alone (*B* and *D*). Representative Masson–trichrome stained sections show the persistent, increased granulation tissue and neovascularization and fibroblast content within PDGF-BB-treated wounds, compared to control wounds at this time (*arrowheads*). The cellularity and granulation tissue content of TGF- β 1-treated wounds was identical to control wounds at this time. Quantitative analysis was performed using paired, growth factor-treated and control wounds. Bars: (*A* and *B*) 100 μm ; (*C* and *D*) 50 μm .

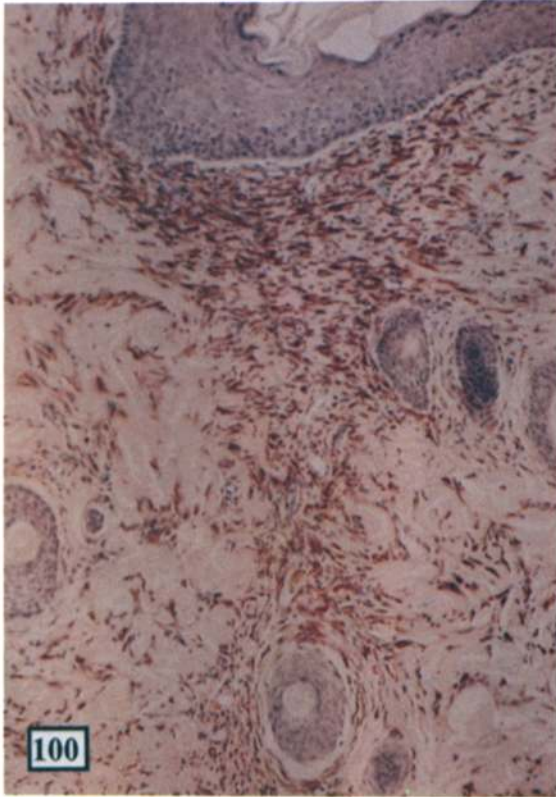
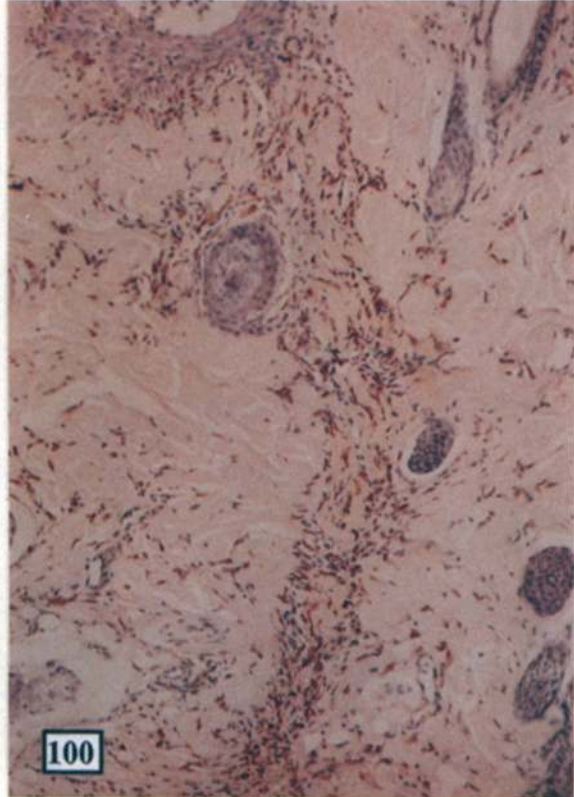
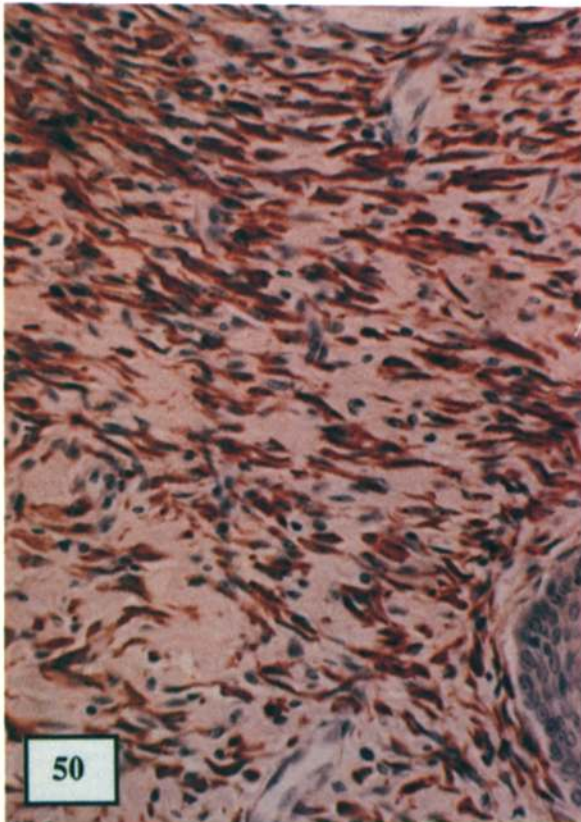
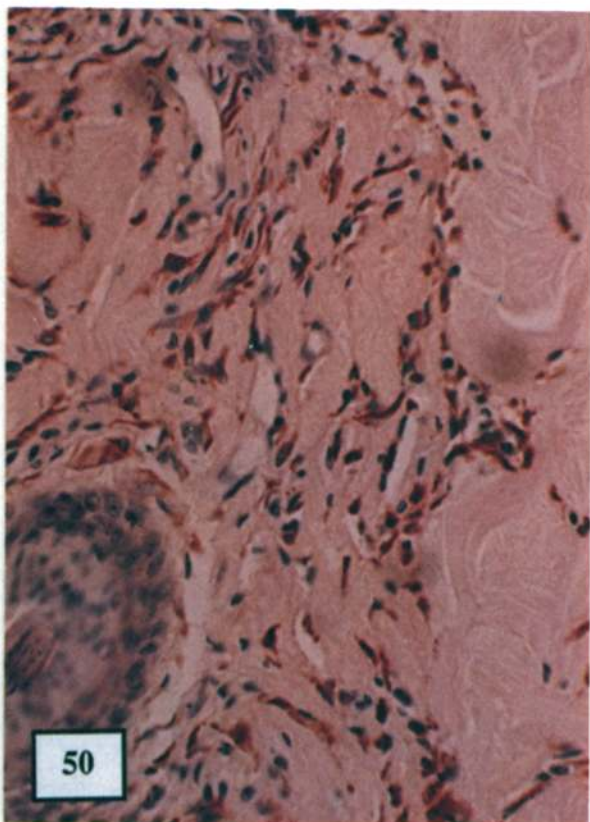
A**B****C****D**

Figure 6. Procollagen type I immunochemical staining of fibroblasts from PDGF-BB (20 μg ; *A* and *C*) or matched control wounds (*B* and *D*) 7 d after wounding. Similar proportions of fibroblasts contained intracellular procollagen type I, from all wounds. However, the increased fibroblastic influx in PDGF-BB-treated wounds resulted in an overall significant increase in procollagen type I content in the growth factor-treated wounds and this increase correlated with the observed increase in wound breaking strength. Results using TGF- β 1-treated wounds were similar to those observed using PDGF-BB. Bars: (*A* and *B*) 100 μm ; (*C* and *D*) 50 μm .

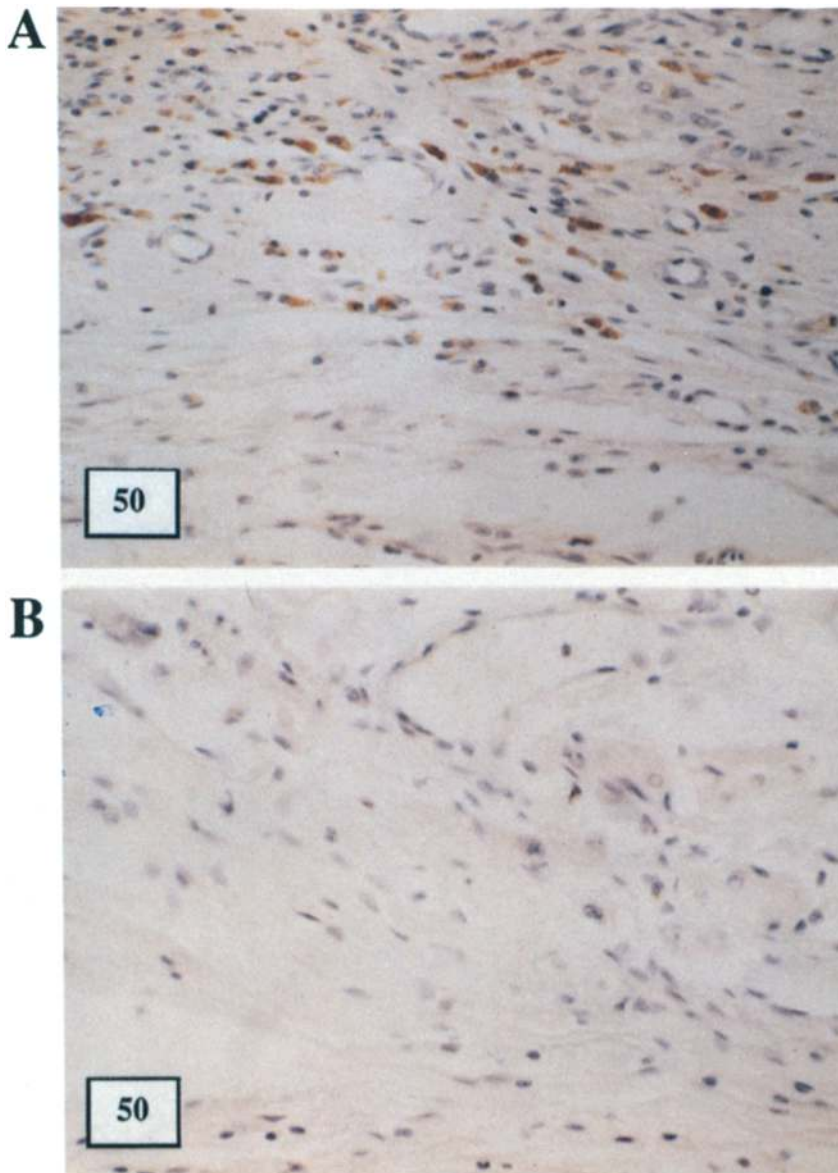


Figure 7. In situ localization of TGF- β in incisional wounds treated with 20 μ g PDGF-BB (A) or controls (B). Paired wounds were harvested on days 2, 4, and 7, and processed for immunostaining using anti-TGF- β 1 antiserum. Note the increased proportion of cells containing intracellular TGF- β in PDGF-BB-treated wounds compared to matched control wounds on day 7. These wounds were photographed immediately adjacent to the panniculus carnosus muscle layer, in the dermis. Similar results were obtained on days 2 and 4. Bars, 50 μ m.

their activities in pathological states. PDGF-like proteins have been observed in pulmonary fibrosis and are thought to be synthesized by activated alveolar macrophages (Martinet et al., 1987; Deuel and Senior, 1987). PDGF has also been found associated with atherosclerotic plaques, is made and secreted by aortic smooth muscle cells and vascular endothelial cells, and has been found in the normal arterial wall (Jaye et al., 1985; Collins et al., 1987; Barrett and Benditt, 1988; Majesky et al., 1988). Although a causal role for PDGF in pulmonary fibrosis or in the atherosclerotic process has not been established, the present results obtained with PDGF and TGF- β suggest these growth factors may be active in these microenvironments. Further elucidation of the mechanisms of action of PDGF and TGF- β should provide important insights into their roles in normal and compromised tissue repair activities, as well as their roles in fibrotic processes.

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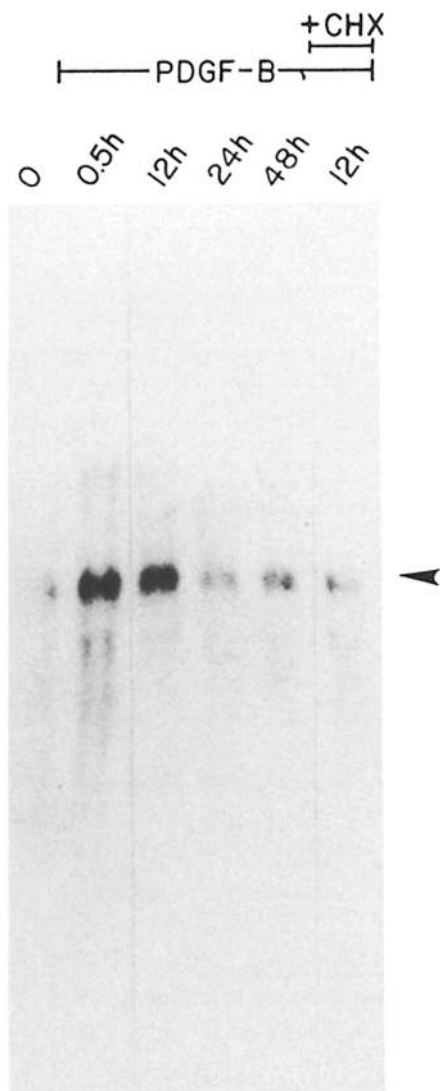


Figure 8. Northern analysis of PDGF-BB-induced stimulation of TGF- β mRNA in NRK fibroblasts. Cells were grown to confluency, serum starved, and treated with an optimal concentration of PDGF-BB (50 ng/ml) or PDGF-BB plus 10 μ g cycloheximide (CHX) for \leq 48 h. Total cellular RNA was isolated from cell samples at the times shown and hybridized to the TGF- β 1 probe. An increase in the TGF- β 2.6-kb message (arrowhead) is observed at 0.5 and 12 h of PDGF-BB treatment (\sim 10-fold stimulation above background). Treatment of NRK cells with PDGF-BB in the presence of cycloheximide showed a block in TGF- β -specific augmentation. Efficacy of cycloheximide treatment was verified by probing an identical blot with nerve growth factor I-A, a gene whose message levels are augmented in the presence of cycloheximide (Milbrandt, 1987).

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