IN VIVO INCISIONAL WOUND HEALING AUGMENTED BY PLATELET-DERIVED GROWTH FACTOR AND RECOMBINANT *c-sis* GENE HOMODIMERIC PROTEINS

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Platelet-derived growth factor (PDGF)[1] was initially identified as a potent mitogen in human serum for mesenchymally derived cells (1–3). PDGF is present in platelet α granules and released from internal stores in association with platelet activation at sites of injury (4, 5). PDGF is a disulfide-linked heterodimeric protein comprising A (non-*sis*) and B (*sis*) polypeptide chains (6–9). The B chain of PDGF is structurally and functionally highly homologous to the protein product of the v-*sis* oncogene, the transforming gene of simian sarcoma virus (10, 11), and is encoded by the normal cellular homologue of v-*sis*, the c-*sis* proto-oncogene (12, 13). The c-*sis* and v-*sis* genes have been cloned and expressed in Chinese hamster ovary (CHO) cells at high expression levels (Thomason, A., et al., submitted for publication), permitting for the first time the direct application of this growth factor into wounds in experimental animals to test a functional role of PDGF in tissue repair.

Since PDGF and related proteins are released from platelets, monocytes, and fibroblasts, and PDGF stimulates both chemotaxis and activation of inflammatory cells and fibroblasts in vitro (14–21), PDGF is thought to be an important vulnerary agent for tissue repair processes in vivo. Another polypeptide growth factor stored in the α granules of platelets, transforming growth factor type β (TGF-β), is also considered important in tissue repair (22). Both PDGF and TGF-β increase collagen formation, DNA content, and protein levels in wound chambers implanted in rats (23, 24), and TGF-β stimulates the reversible formation of granulation tissue when injected subcutaneously into newborn mice (25). We have successfully demonstrated acceleration of wound healing in full-thickness linear incision wounds made in rat skin (26). A single application of human TGF-β at the time of wounding advanced by 2–3 d the breaking strength

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[1] *Abbreviations used in this paper:* DMEM, Dulbecco's modified essential medium; h, human; NRK, normal rat kidney cells; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor β.

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required to rupture the incision margins after 1 wk. The present studies demonstrate marked augmentation of wound healing using human PDGF (hPDGF) and recombinant *c-sis* homodimers (rPDGF-B), and reveal unique patterns of response when compared with the results obtained with TGF-β. Furthermore, the recombinant *c-sis* homodimers on a molar basis are functionally as fully active as PDGF in in vitro mitogenic and chemotactic assays.

Materials and Methods

Preparation of Growth Factors. Human PDGF was purified to homogeneity from outdated platelets as described (6). Recombinant human *c-sis* homodimers were produced in Chinese hamster ovary cells after transfection with and amplification of the *c-sis* gene. The protein was purified to apparent homogeneity from conditioned medium using a procedure that included affinity chromatography over an anti-v-*sis* protein mAb column (Thomason, A., et al., submitted for publication). Purity was established by evaluating iodinated hPDGF and rPDGF-B in polyacrylamide gels by autoradiography. No endotoxin was detectable in rPDGF-B preparations. Antisera raised against hPDGF (anti-$PDGF_2$) showed a high degree of crossreactivity with rPDGF-B using a solid-phase ELISA as described (27), but was not crossreactive with other proteins in platelet extracts.

Mitogen Assay. Normal rat kidney cells (NRK, clone 49F; American Type Culture Collection, Rockville, MD) were grown to confluence in 10% FCS/Dulbecco's modified essential medium (DMEM), trypsinized, and plated into 96-well microtiter plates (Falcon Labware, Oxnard, CA) at a density of 2×10^4 cells/well in FCS-DMEM. Cells were confluent within 3 d. Growth factors were assayed by addition of 200 μl [^{3}H]thymidine (120 nCi/well) in serum-free DMEM/Hams' F12 medium (1:1, vol/vol), supplemented with 35 mg/liter human transferrin, 5 mg/liter bovine insulin, 1.2 μl/liter ethanolamine, and 4.3 μg/liter sodium selenite. After 24 h, cells from triplicate wells were trypsinized and harvested with an automated microharvester (Flow Laboratories, Inc., McLean, VA), fixed onto glass fiber filter paper with trichloroacetic acid, and counted. Human serum (10%) from a normal individual was added as a positive control and background values were subtracted from experimental values. In some experiments, 6 μg anti-$PDGF_2$ IgG, a rabbit polyclonal blocking antibody, was added per well just before addition of growth factors.

Chemotaxis. Chemotaxis was determined in modified Boyden chambers, as previously described (15, 28). PBMCs and polymorphonuclear leukocytes (PMNs) were separated on Ficoll-Hypaque gradients from peripheral blood obtained from healthy volunteers. Fetal bovine ligament fibroblasts were obtained from explants of ligamentum nuchae as described (29). Growth factors were serially diluted in DMEM before use, and five high power fields (fibroblasts) or grids (PMNs and monocytes) were counted from triplicate samples.

Preparation of Growth Factors in Collagen. A bovine collagen suspension (Zyderm II; Collagen Corp., Palo Alto, CA) with or without the addition of specific concentrations of growth factors was applied using a tuberculin syringe containing 1 mg collagen per incision. Preliminary experiments in vitro using ^{125}I-PDGF suspended in collagen showed about half the radioactivity was released into surrounding medium during the first hour, and the remaining 50% was released from the collagen suspension during the next 23 h. Despite the apparent delayed release of ^{125}I-PDGF from collagen suspensions in vitro, it remains possible that very different kinetics of release occur in vivo. However, preliminary experiments in vivo revealed that rPDGF-B applied to wounds in a saline vehicle was not active in augmenting healing. No significant differences in wound breaking strength were found between collagen-treated wounds and saline-treated wounds in the present studies (26).

Linear Incision Model. 300–350-gm young adult male Sprague-Dawley rats (Sasco, Inc., Omaha, NB) had paired 6-cm full-thickness linear incisions placed through the skin 1.5 cm on either side of the midline as described (26). Wounds were coapted with three surgical clips and the collagen–growth factor mixture was applied to one wound and

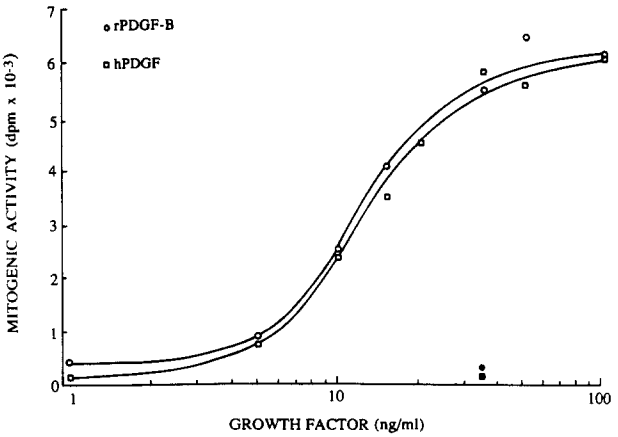

FIGURE 1. Mitogenic activity of hPDGF and rPDGF-B. Confluent NRK fibroblasts were incubated with growth factors for 24 h at specified dilutions in the presence of [^{3}H]thymidine. For antibody blocking experiments, 6 μg anti-$PDGF_2$ was added to each well just before the addition of growth factor (□, hPDGF; ○, rPDGF-B; ■, antibody + hPDGF; ●, antibody + rPDGF-B).

collagen alone to the contralateral wound. Thus, each animal served as its own control. The maximum load (breaking strength) tolerated by wounds harvested at various days after wounding was measured with a tensometer (Tensometer 10; Monsanto Co., St. Louis, MO) using two or three 8-mm wide skin strips from each wound as described (26). Measurements were not performed on wounds showing evidence of infection, excessive hemorrhage, or poor coaptation (<5% of all wounds).

Data Analysis. Eight rats were usually used for each variable to be tested in an experiment. The control and treated wounds from each rat each had two to three matched strips, 8 mm wide, perpendicular to the incision removed for tensometer analysis, and one to two matched, paired samples taken for histologic analysis. Analysis of variance and paired *t*-tests of breaking strength scores and differences between matched experimental and control values were performed using the SAS data analysis system (Division of Biostatistics, Washington University, St. Louis, MO). Breaking strength measurements were performed blindly on coded samples.

Histology. Matched paired samples of experimental and control wounds from each rat were placed immediately after harvest into acetic acid/ethanol (1:99, vol/vol) fixative and processed 24–48 h later. Paraffin-embedded paired growth factor–treated and control thin sections were stained with hematoxylin, counterstained with eosin, and analyzed by two independent observers in a blinded fashion by light microscopy. Wounds were scored on a scale from 0 to 4 for cellularity and the formation of granulation tissue as a function of time after wounding. Reticulin and trichrome staining were performed by routine methods.

Results

Mitogenic and Chemotactic Properties of rPDGF-B. In vitro functional activities of rPDGF-B were determined and compared with hPDGF. rPDGF-B was first shown to stimulate mitogenesis in NRK fibroblasts at picomolar levels; its activity on a mole/mole basis was fully equal to hPDGF (Fig. 1). Anti-$PDGF_2$, when added to cells just before growth factors, abrogated the response (Fig. 1). rPDGF-B was shown to be immunologically equivalent to hPDGF using this polyclonal anti-PDGF antiserum (data not shown), and thus the mitogenic response of rPDGF-B is highly specific and potent.

As a second test of the biological functionality of rPDGF-B, chemotactic activity was assayed in modified Boyden chambers. Peak chemotaxis for fibroblasts was observed at 30 ng/ml, while peak activity for monocytes was obtained at 10 ng/ml (Fig. 2*A*). These values are essentially identical to those reported previ-

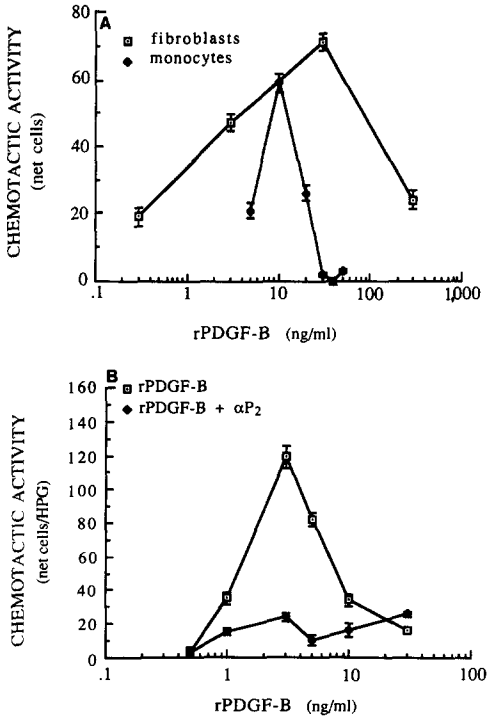

FIGURE 2. Chemotactic activity of rPDGF-B. Dilutions of growth factors were tested in modified Boyden chambers as described in the Materials and Methods. (*A*) Bovine ligamentum nuchae fibroblasts and human peripheral blood monocytes. (*B*) Human polymorphonuclear leukocytes tested in the absence or presence of anti-$PDGF_2$ (αP_2), which was mixed with rPDGF-B and then serially diluted. Anti-$PDGF_2$ was present at 140 μg/ml when rPDGF-B was 30 ng/ml. Anti-$PDGF_2$ had no effect on the chemotactic activity of FMLP.

ously for hPDGF (fibroblasts 30 ng/ml, monocytes 20 ng/ml) (14, 15). Polymorphonuclear leukocytes were maximally active at 3 ng/ml rPDGF-B (Fig. 2*B*). Previous experiments with hPDGF showed maximal reactivity of PMNs at 1–5 ng/ml (14). The magnitude of the chemotactic responses was comparable to the response obtained using optimal concentrations of the FMLP peptide. The apparent inhibition of the chemotactic response at higher concentrations of rPDGF-B reflects cell aggregation, which occurs when concentrations of agonist exceed the optimum concentration for chemotaxis, and was observed previously with hPDGF (14, 15). Addition of anti-$PDGF_2$ to samples before assay sharply reduced the chemotactic response of PMNs to 3 ng/ml rPDGF-B by 80% (Fig. 2*B*), without affecting the chemotactic activity of FMLP (data not shown), thereby establishing the specificity of chemotactic response to rPDGF-B, and not to undetectable levels of contaminating proteins.

In Vivo Activities of rPDGF-B. Since rPDGF-B appeared identical to hPDGF in stimulating inflammatory cells in vitro, a previously developed rat linear skin incision model (26) was used to test the potential of rPDGF-B to augment wound healing in vivo. When tested at 20 μg per incision, both rPDGF-B and hPDGF induced significant increases in the breaking strength of incisional wounds at day 7 (Table I). Further experiments were undertaken with rPDGF-B to determine optimal doses (Fig. 3*A*). Small but significant increases in breaking strength after 5 d were observed when 2 μg rPDGF-B were applied per incision on the day of wounding. Statistically significant increases in breaking strength to 143% and 170% of control wounds were observed at 5 and 20 μg rPDGF-B per incision, respectively.

TABLE I

Comparison of Breaking Strengths of Day 7 Incisional Wounds Treated with 20 μg of Either hPDGF or rPDGF-B at the Time of Wounding

Growth factor	hPDGF	Control	rPDGF-B	Control
Breaking strength (g)	233 ± 24*	162 ± 23	221 ± 16	169 ± 16
Percent stimulation of control	144		131	
n‡	8		17	
p value§	<0.02		<0.001	

* Mean ± SEM.

‡ n, number of strips tested, two to three matched strips obtained from each growth factor treated and control wound.

§ Compared with matched, paired control wounds using a two-tailed, paired t-test.

TABLE II

Histological Evaluation of rPDGF-B–treated and Untreated Wounds

Days after wounding*	Cellular influx into wounds						Granulation tissue	
	PMN‡		Macrophage		Fibroblast		rP	C
	rP	C	rP	C	rP	C		
1	2.5	1.5	1	0.5	0.5	0.25	0	0
2–3	1	0.5	3.5	2	1.7	1	1	0.5
4–7	0	0	2	1	4	2	4	2
10–21	0	0	0	0	2.3	1.7	2.7	1.3

* Three to six paired incisional wounds were analyzed per time point. Individual days analyzed include 0.5, 1, 2, 3, 4, 5, 7, 10, 14, and 21.

‡ Paraffin-embedded paired growth factor–treated and control wound thin sections were stained with hematoxylin and eosin and each parameter was graded on a scale from 0–4 as a function of days after wounding by two blinded individuals. Average scores are presented. rP, rPDGF-B treated; C, paired control wounds.

To determine whether rPDGF-B continued to enhance wound healing beyond the initial week after wounding, the time course of the rPDGF-B–induced augmentation of breaking strength was tested. Increases in breaking strength of rPDGF-B–treated wounds beginning at day 3 and extending through day 21 after wounding were observed (Fig. 3*B*). Although statistically significant differences between growth factor–treated and control wounds were observed through day 7 after wounding, even more striking differences were observed at days 10, 14, and 21 after wounding when the breaking strength of rPDGF-B–treated wounds diverged markedly from controls, with a maximum difference in breaking strength of 824 g between treated and control wounds at day 21 (Fig. 3*B*).

Analysis of Directed Cellular Migration. Semiquantitative histologic examination of treated and control wounds revealed time-dependent changes in cellular composition which correlated well with the observed differences in breaking strength (Table II). Sections obtained from both hPDGF- and rPDGF-B–treated wounds within 2 d of wounding showed an increased influx of inflammatory cells, primarily neutrophils and monocytes, into growth factor–treated wounds when compared with control wounds. Multiple sections were used to confirm these results. Increased fibroblast migration into hPDGF- and rPDGF-B–treated wounds was demonstrated at this point, although the fibroblast migration into

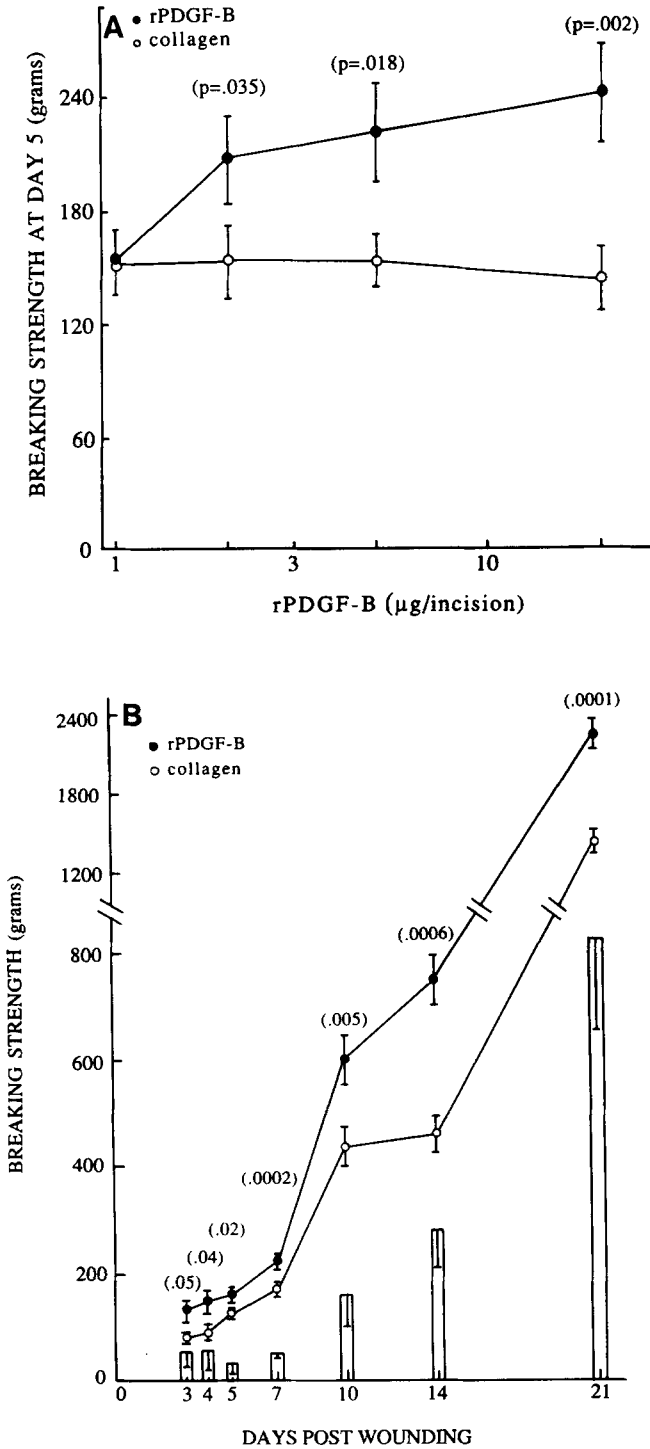

FIGURE 3. Breaking strength of linear incision wounds treated with rPDGF-B. Growth factor was applied in a collagen vehicle at the time of wounding. Matched paired experimental and control wounds were harvested at specified days, coded, and tested blindly on the tensometer for the maximum load (breaking strength) tolerated before breaking. The breaking strength scores (mean ± SEM) for 16–20 strips from eight rats (one experimental and one matched control incisional wound per rat) are represented by each data point. p values were calculated from a paired t test. (*A*) Effect of rPDGF-B on 5-d wounds, as a function of dose, compared with paired collagen control wounds. (*B*) effect of 20 μg rPDGF-B per incision as a function of time after wounding. rPDGF-B was applied on the day of surgery then wounds were harvested at the times shown and breaking strength was compared with matched control wounds containing only the collagen vehicle. Note the break on the y axis. The differences in breaking strength between paired experimental and control wounds are depicted in the histograms for each day (mean ± SEM). p values are in parentheses.

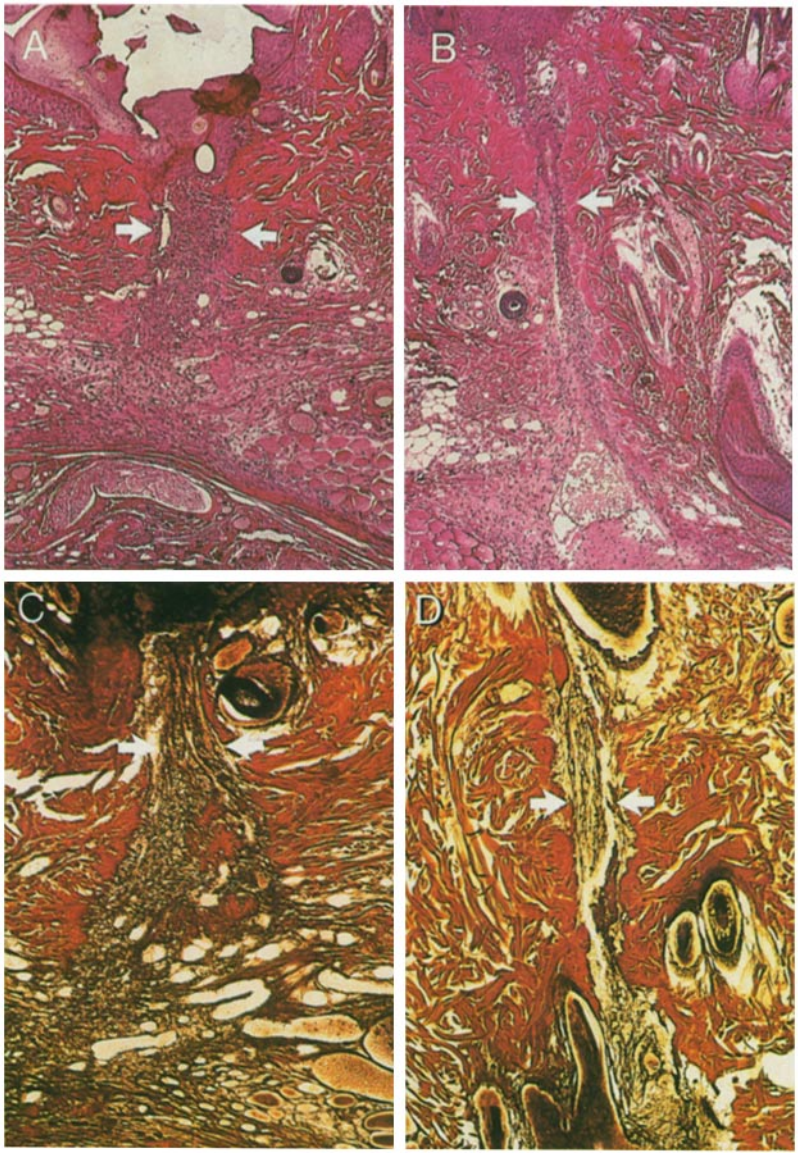

FIG 4-PART 1 COLOR

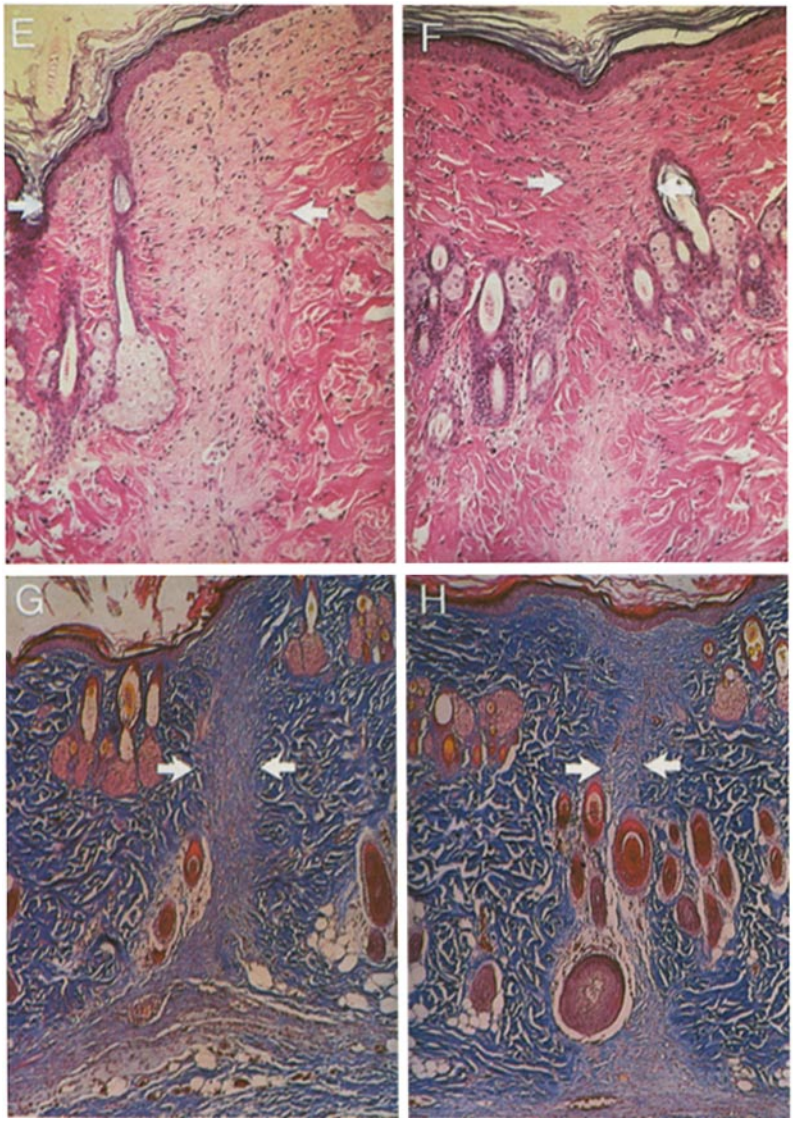

FIGURE 4. Histological evaluation of paired wounds treated with rPDGF-B or the collagen vehicle alone. All wounds were full thickness and extended through the panniculus carnosus. Sections adjacent to matched strips used for tensometry were processed, embedded, sectioned, and stained with hematoxylin and eosin (H and E), reticulin, or trichrome using routine methods. (*A–D*) day 4 wounds. (*A*) rPDGF-B, 10 μg, H and E, ×57; (*B*) matched collagen control, H and E, ×57; (*C*) rPDGF-B, 10 μg, reticulin, ×100; (*D*) matched collagen control, reticulin, ×100. (*E–H*) day 14 wounds. (*E*) rPDGF-B, 20 μg, H and E, ×100; (*F*) matched collagen control, H and E, ×100; (*G*) rPDGF-B, 20 μg, trichrome, ×57; (*H*) matched collagen control, trichrome, ×57. Arrows demarcate the wound boundaries. See text for descriptions.

wounds was substantially greater with time. By day 4 after wounding, rPDGF-B–treated wounds were markedly infiltrated with fibroblasts and new granulation tissue when compared with control sections (Table II, Fig. 4, *A* and *B*). Sections from multiple rPDGF-B wounds at each time point were analyzed through day 21. Growth factor–treated wounds consistently showed large differences in granulation tissue relative to controls, associated with the observed differences in breaking strength.

Whereas the in vivo sequence of directed cellular migration correlated well with that predicted by the sensitivity of the cells to the chemotactic signal in vitro (Fig. 2), and correlated well with increased wound breaking strength (Fig. 3), collagen deposition is the single best correlate with enhanced wound strength. Evidence of increased new collagen synthesis in rPDGF-B–treated and matched control wounds was therefore sought using the reticulin stain on day 4 wounds (Fig. 4, *c* and *d*) and by trichrome staining of day 14 rPDGF-B–treated and paired control wounds (Fig. 4, *g* and *h*). Both the increased reticulin fibers observed at day 4 and the pale trichrome staining of the collagen relative to adjacent dermal collagen at day 14 in rPDGF-B–treated wounds likely reflect primarily newly synthesized collagen. The increased basophilic demarcation of the scar in the growth factor–treated wounds was also observed on hematoxylin and eosin–stained sections through day 14 (Fig. 4, *e* and *f*). Increased granulation tissue was still present at the base of the rPDGF-B–treated wound (Fig. 4*g*) at day 14, compared with the matched control (Fig. 4*h*, and Table II).

Discussion

In the present study, recombinant c-*sis* homodimers have been shown for the first time to function identically to human PDGF immunologically and in stimulating the functional activities of mitogenesis and chemotaxis in vitro at identical concentrations. Because these and previous results have demonstrated not only the positive chemotactic potential of PDGF but also its ability to activate inflammatory cells (14–18) and to stimulate the secretion of collagenase by fibroblasts (30), we wished to demonstrate the in vivo activity of PDGF in tissue repair.

Using a rat linear incision model and a single application of growth factor dissolved in a collagen suspension at the time of wounding, we observed consistent and significant stimulation of wound healing, beginning at 2 μg rPDGF-B per incision, and increasing in a dose-dependent fashion to 170% of control wounds with 20 μg rPDGF-B per incision. Stimulation by rPDGF-B was observed as early as day 3, when collagen synthesis is initially observed in this model (31, 32), and increased through day 7, producing a 2-d acceleration of healing in the first week. A single application of purified hPDGF on the day of wounding also augmented the breaking strength of incisional wounds harvested at day 7, establishing in yet another parameter that the recombinant protein was fully as active as PDGF itself. During the second and third weeks after wounding, a 4–6-d acceleration of healing was observed in rPDGF-B–treated wounds compared with control wounds treated with collagen alone. Examination of wounds at later times after surgery will be necessary to determine if the rPDGF-B–induced augmentation of healing persists, although preliminary results indicate increasing

differences in breaking strength are present through at least day 49 in rPDGF-B–treated wounds (our unpublished results).

Since most of the rPDGF-B is released from the collagen vehicle during the first 24 h in vitro (see Materials and Methods), we suspect that the growth factor induced a cascade effect initiated by cells migrating into wounds in response to the initial stimulus. As tissue macrophages and fibroblasts approach the wound and thus are exposed to higher concentrations of rPDGF-B, these cells in turn become activated, releasing additional growth factors and other chemotactically active peptides (18, 19, 21). The observed differences in rPDGF-B–treated wounds at later weeks after wounding thus appear to result from a multiplicity of cellular events triggered and to some extent sustained by rPDGF-B. These results are in contrast to findings using human TGF-β in the same experimental model (26), in which a more marked stimulatory effect was observed in the first week after wounding. However, differences between TGF-β-treated and control wounds remained constant or decreased during the second week. TGF-β stimulates the synthesis of extracellular matrix proteins, such as procollagen type I and fibronectin, directly (33–35), while hPDGF does not appear to directly stimulate procollagen synthesis but does activate collagenase gene expression (30), which is required for wound remodeling. Remodeling begins after the initial deposition of collagen and is needed for the structural integrity of the scar (36, 37). Thus TGF-β may directly stimulate collagen synthesis by wound fibroblasts while hPDGF may be acting later in healing or indirectly to induce the secretion of other growth factors such as TGF-β (Masakowski, V. R., and G. F. Pierce, unpublished observations) and additional PDGF-like polypeptides by wound macrophages, endothelial cells, etc., which can then directly stimulate collagen synthesis by fibroblasts (38). Thus rPDGF-B and human TGF-β appear to augment wound healing by different mechanisms, as perhaps reflected in differences in collagen degradation and synthesis. However, it cannot be excluded that in vivo, the two growth factors may be released from the collagen vehicle at very different kinetic rates. Experiments to analyze these hypotheses are in progress.

Wounds analyzed histologically within the first week showed a marked increase in neutrophils, monocytes, and fibroblasts in hPDGF- and rPDGF-B–treated wounds and a large increase in granulation tissue by day 4 after wounding, in comparison to matched, paired control incisions analyzed on the same days after wounding. These results thus correlate well with earlier in vitro observations and an in vivo study using wound chambers (14–18, 23). Importantly, although the analysis is only semiquantitative, the observations that increased numbers of inflammatory cells and fibroblasts migrate into incisional wounds during healing correlates with the predicted migration of these cells based upon the optimal doses required for chemotaxis in vitro. Taken together, these findings support a highly important role of PDGF as a chemoattractant in facilitating wound healing, over and above its role in mitogenesis.

The findings of similar results in vivo using hPDGF, an A-B chain heterodimer, and rPDGF-B, a B-B chain homodimer, are interesting with respect to the proposed function of the A chain (non-*sis*) of PDGF (PDGF-A), which is 60% homologous to the B chain (10). PDGF-A was recently shown to be synthesized

and released by fibroblasts in culture which had been activated by PDGF (20), suggesting important extracellular autocrine and paracrine functions of PDGF-A. The recent cDNA cloning of the A chain (39, 40) should permit direct analysis of functional differences between A-A and B-B homodimeric proteins (41) and the PDGF A-B heterodimer. Moreover, the availability of purified recombinant human PDGF in much larger quantities than the quantities of PDGF that can be readily isolated from platelets should permit evaluation of PDGF in the treatment of impaired wound healing.

Summary

Human platelet-derived growth factor (hPDGF) is likely to be important in stimulating tissue repair, based upon its in vivo chemotactic and stimulatory activities for inflammatory cells and fibroblasts and upon the presence of PDGF and related proteins in platelets, macrophages, and activated fibroblasts, cell types that make up the milieu of the healing wound. Recombinant human c-*sis* (rPDGF-B), homodimers of the B chain of PDGF, were compared with hPDGF in vitro. rPDGF-B was immunologically similar to hPDGF and, at identical concentrations, similar to hPDGF in stimulating fibroblast mitogenesis and chemotaxis of polymorphonuclear leukocytes, monocytes, and fibroblasts. Purified hPDGF and rPDGF-B were also tested in vivo for potency in a model of tissue repair using a linear incision wound through rat dermis. A single application of hPDGF or rPDGF-B (2–20 μg/wound) in a slow release vehicle at the time of wounding resulted in a dose-dependent, statistically highly significant increase of breaking strength of treated wounds. Wound healing in animals treated with rPDGF-B was 170% stronger and accelerated by 2 d during the first week over control wounds and by 4–6 d over the next 2 wk. Histologic evaluation of growth factor–treated wounds correlated the in vitro chemotactic activity and the accelerated healing of wounds with a striking inflammatory cell infiltrate early after wounding, markedly increased formation of granulation tissue by 4-d, and increased fibrosis by 14 d in comparison to control wounds. The results thus demonstrate that rPDGF-B is fully active in in vitro tests of mitogenesis and chemotaxis and, for the first time, demonstrate directly that PDGF significantly advances wound healing in incisional wounds of experimental animals.

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