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The Stimulatory Effects of PDGF and TGF-β1 on Dermal Fibroblast Attachment

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We investigated the effects of various growth factors (platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), transforming growth factor-α (TGF-α), transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α), keratinocyte growth factor (KGF)) on fibroblast attachment to plastic plates. It is thought that cell attachment to plastic plates in vitro may represent the step between cell migration and proliferation in vivo during wound healing. Among the growth factors examined, only PDGF and TGF-β1 significantly increased fibroblast attachment to both uncoated and collagen-coated plates in a concentration-dependent manner. The addition of anti-PDGF antibody abolished the enhancing effect of PDGF but not that of TGF-β1, suggesting that the effect of TGF-β1 is not through the autocrine induction of PDGF-related activities secreted by the fibroblasts themselves. These data suggest that PDGF and TGF-β1 regulate fibroblast attachment to the suitable environment in the process of dermal wound healing in vivo. ***Key words: platelet-derived growth factor; transforming growth factor-β1; wound healing; chemotaxis.***

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Dermal wound healing after injury is a complex biological process involving an acute inflammatory response followed by tissue regeneration. As the acute events of inflammation begin to subside, fibroblasts increase and participate in the repair process by forming granulation tissue with collagen deposition.

Growth factors have been reported to enhance the repair process in animal models by increasing the degree of cellularity and the amount of collagen accumulated (1–4). Platelets are shown to be a major source of various growth factors such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) and epidermal growth factor (EGF) (5, 6), and these growth factors released from platelets at the sites of injuries play an important role in wound healing. Vascular endothelial cells are also considered to be a source of growth factors such as basic fibroblast growth factor (bFGF) and other PDGF-related activities (7, 8).

The growth factors described above are growth stimulatory substances for dermal fibroblasts (9–13). Moreover, PDGF acts as an chemoattractant for fibroblasts (14, 15), and TGF-β induces the synthesis of extracellular matrix proteins such as collagen and fibronectin (16–18).

In the process of dermal wound healing, the participation of fibroblasts may involve the following four steps: 1) migration toward the site of wound; 2) attachment to the suitable environment for proliferation; 3) proliferation; 4) production and accumulation of matrix proteins.

It is thought that cell attachment to plastic plates in vitro may represent the step between cell migration and proliferation in vivo described above (20, 21). However, little is known about the regulation of cell attachment process in vitro. In the present study, we investigated the effects of various growth factors on fibroblast attachment to uncoated and collagen-coated plastic plates. In addition, the mitogenic and chemotactic activity of these growth factors was measured.

MATERIALS AND METHODS

Growth factors and anti-PDGF IgG

The growth factors, PDGF purified from human platelets, basic FGF purified from bovine brain, TGF-β1 purified from human platelets, recombinant human transforming growth factor-α (TGFα) and recombinant human tumor necrosis factor-α (TNF-α), were obtained from R&D Systems Inc. (Minneapolis, Minn., USA). EGF purified from rat submandibular glands was purchased from Biomedical Technologies Inc. (Stoughton, Mass., USA). Recombinant human KGF was obtained from Boehringer Mannheim Biochemica (Indianapolis, IN., USA). Anti-PDGF IgG was prepared from goat serum immunized with purified human PDGF, as described previously (19).

Cell culture

Human adult skin fibroblasts were grown from explants of skin biopsies from 4 healthy adult donors. Cells were cultured in T-25 flasks in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS). Fibroblasts prior to the fifth passage were used. All cultures were maintained at 37°C in an atmosphere of 5% CO_2 and 95% air.

Cell attachment assay

Cell attachment assay was carried out with the use of a modified Kleinman's method (20, 21). Briefly, confluent cells were detached from the tissue culture flask by treatment with 0.1% trypsin/0.1% EDTA for 10–15 min at 37°C. The cells were washed twice in MEM containing 2 mg/ml bovine serum albumin (BSA) and by centrifugation at 500 × g for 5 min. The cells were resuspended at a concentration of 2×10^4 cells/ml in MEM with an appropriate concentration of FCS and seeded in 24-well plastic plates. Growth factor at a concentration of 10 ng/ml was added. Plastic plates without coating and plates coated with collagen derived from rat tail tendon (Iwasaki Glass, Japan) were used. The cells were allowed to attach to the plastic plates for 16 h. The unattached cells were removed and the plates were washed three times with MEM. Then attached cells were detached with trypsin/EDTA and counted with an electric cell counter (Coulter, Hialeah, FL). Data are expressed as the percentage of cells that attached to the plastic plate; each value represents the mean of quadruplicate assays of each fibroblast cell line.

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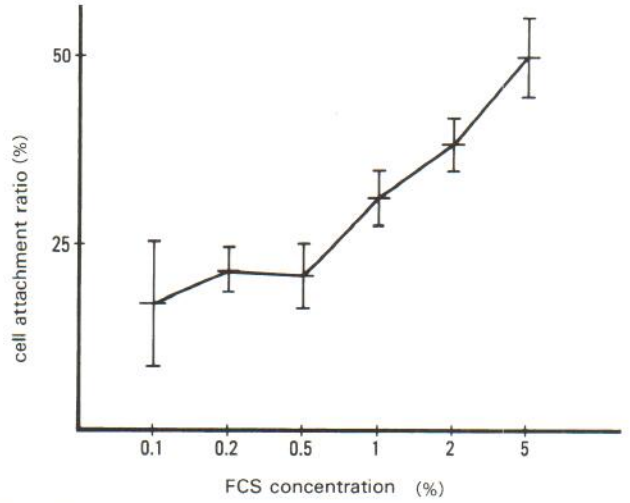

Fig. 1. Percentages of cell attachment in MEM at various concentrations of FCS. The percentage of attached cells remained steady at almost 20% in MEM with 0.1–0.5% FCS and increased in a concentration-dependent manner between 0.5 and 5% FCS. Each *error bar* represents the mean ± SD of quadruplicate assays.

Growth assay

Growth assay was performed as described previously (19, 22–24). A confluent culture of fibroblasts was trypsinized and 1×10^4 cells were seeded in 24-well plates in MEM with 10% FCS. The cells were cultured for 4–6 days until a confluent growth was obtained, and then growth factor at 10 ng/ml was added. After 24 h, the cells were labelled for 2 h with 1 μCi/ml of [^{3}H]-thymidine. The cell layer was washed three times with phosphate-buffered saline and five times with ice-cold 5% trichloroacetic acid and dissolved in 1 ml of 0.1 N NaOH and 0.1% sodium dodecyl sulfate. An aliquot of this extract was counted in a Beckman scintillation counter.

Chemotaxis assay

Chemotactic activity was measured using the modified Boyden chamber, as described previously (25, 26). Growth factors at a concentration of 10 ng/ml in MEM containing 0.2 mg/ml BSA were added to the lower well of the blind well chamber. The lower well was then covered with a collagen-coated polycarbonate filter (Nucleopore, 8 μm-diameter pores). The upper well was fixed in place and trypsinized fibroblasts (3×10^5 cells/assay) in MEM containing 2 mg/ml BSA were added to the upper well. The chamber was incubated for 4 h at 37°C in an atmosphere of 5% CO_2 and 95% air. The filter was removed and cells were fixed and stained by Diff-Quik stain (Harleco). The cells on the upper surface of the filter were removed by scraping with a rubber policeman, and the cells on the lower surface, which were migrated cells, were counted in three microscopic fields.

Statistical analysis

Statistical analysis was carried out using Student's *t*-test for comparison of means. *P* values less than 0.05 were considered significant.

RESULTS

Cell attachment ratio at various concentrations of FCS

In order to investigate the stimulatory effects of the growth factors on fibroblast attachment, we first examined the suitable concentration of FCS for use in the cell attchment assay. As shown in Fig. 1, the cell attachment ratio remained at almost 20% in MEM with 0.1–0.5% FCS and increased in a concentration-dependent manner between 0.5% and 5%. Trypsinized fibroblasts attached to plastic plates almost completely (95%) in the medium containing 10% FCS. Therefore, the condition of MEM with 1% FCS was used in all subsequent cell attachment assays.

Table I. *Effects of growth factors on fibroblast attachment*

Each value represents the mean ± SD of quadruplicate assays. N.D.: not done.

Growth factor	Percentage of cells attached	
	to uncoated plates	to collagen-coated plates
Control	43.5±10.7	52.6± 7.8
PDGF	67.5±21.9*	78.4±11.0**
EGF	42.3± 9.6	51.9± 6.5
bFGF	41.8±11.1	52.6± 9.2
TGF-α	41.5± 4.3	50.1±10.6
TGF-β	70.5±21.3***	89.1± 9.8***
TNF-α	45.8±13.4	N.D.
KGF	42.3±12.8	54.6± 8.9

*$p < 0.05$, compared with control, **$p < 0.02$, ***$p < 0.01$.

Effects of growth factors on fibroblast attachment

Both PDGF and TGF-β1 promoted fibroblast attachment to uncoated plastic plates (Table I). The proportion of attached fibroblasts in the absence of growth factors was 43.5 ± 10.7% (mean ± standard deviation), whereas in the presence of PDGF or TGF-β1 the proportions were significantly higher (67.5 ± 21.9%; $p < 0.05$, 70.5 ± 21.3%; $p < 0.01$, respectively). The increase of fibroblast attachment by PDGF or TGF-β1 was concentration-dependent between 0 and 10 ng/ml (Fig. 2). In

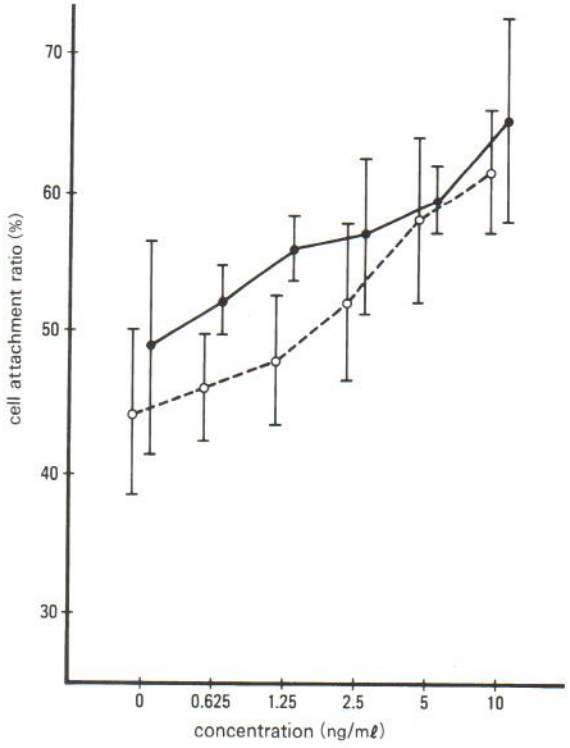

Fig. 2. The increase of fibroblast attachment by PDGF or TGF-β was concentration-dependent between 0 and 10 ng/ml. The details are the same as in Fig. 1. ○: PDGF; ●: TGF-β.

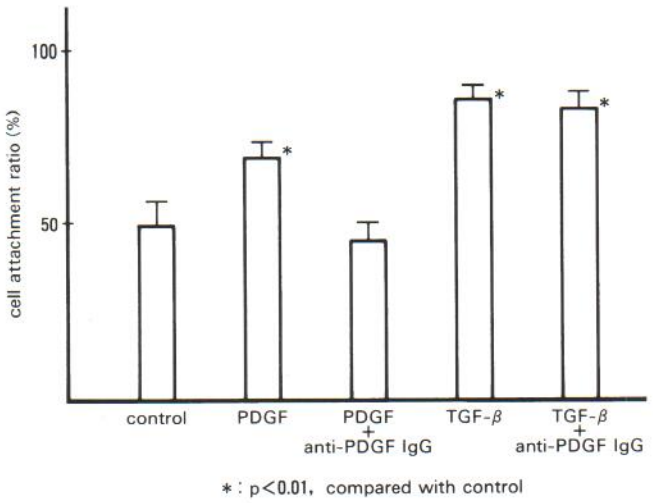

Fig. 3. Anti-PDGF IgG blocked the enhancing effect of fibroblast attachment by PDGF nearly completely. There was a statistically significant difference between the percentage of cell attachment of control and that with PDGF ($p < 0.01$), but there was no significant difference between the percentage of cell attachment of control and that with PDGF and anti-PDGF IgG. However, anti-PDGF IgG had little effect on the enhancement of fibroblast attachment by TGF-β. There was no significant difference between the percentage of cell attachment with TGF-β and that with TGF-β and anti-PDGF IgG. The details are the same as in Fig. 1.

contrast, the other growth factors showed little effects on fibroblast attachment.

When collagen-coated plastic plates were used, similar results of enhancement of fibroblast attachment by PDGF or TGF-β1 were observed, although the percentages of fibroblasts attached to collagen-coated plates were greater than those to uncoated plates in all tested conditions. According to these results, we performed subsequent cell attachment assays in uncoated plates.

Effect of anti-PDGF IgG on fibroblast attachment induced by PDGF or TGF-β1

Previous studies have shown that skin fibroblasts treated with TGF-β1 secrete factor with PDGF-related activities (25); this factor has recently been named connective tissue growth factor (CTGF) (8). Therefore, we next asked whether the increased cell attachment by TGF-β1 was through CTGF production or other mechanisms. We conducted antibody neutralization experiments using anti-PDGF IgG, which has previously been shown to block PDGF-related activities by CTGF secreted from skin fibroblasts treated with TGF-β1 (25). As shown in Fig. 3, anti-PDGF IgG had little effect on the enhancement of fibroblast attachment by TGF-β1, although this antibody blocked the effect of PDGF nearly completely.

Effects of growth factors on fibroblast proliferation and migration

The effects of growth factors on fibroblast proliferation and migration were examined. As shown in Table II, EGF, bFGF, PDGF and TGF-α stimulated DNA synthesis in confluent quiescent cultures of skin fibroblasts 24 h after the addition of growth factors, whereas only PDGF showed increased chemotactic activity. TGF-β1 had little effect on DNA synthesis 24 h after its addition (Table II) but showed delayed mitogenic activity another 24 h later (data not shown).

Table II. *DNA synthesis-inducing activity and chemotactic activity of growth factors*

Values are mean ± SD. N.D.: not done.

Growth factor	^{3}H-thymidine incorporation	Chemotactic activity
Control	2467± 373 (cpm)	47.0± 7.2 (cells)
PDGF	12048±1542*	82.3± 4.2*
EGF	8713± 305*	44.3± 4.0
bFGF	12400± 906*	N.D.
TGF-α	8290± 789*	40.7±11.0
TGF-β	3200± 459	36.3± 3.2

*$p < 0.01$, compared with control.

Effect of pretreatment of growth factors to cell attachment

A confluent cell layer in MEM with 10% FCS was incubated with 10 ng/ml growth factor for 24 h prior to the cell attachment assay. Only PDGF and TGF-β1 increased the percentage of cell attachment (Fig. 4). Under the culture conditions of this experiment, DNA-synthesis should have been stimulated by EGF, bFGF, PDGF and TGF-α but not by TGF-β1, as shown in the previous experiment on cell proliferation (Table II). The fact that PDGF and TGF-β1 enhanced cell attachment suggests that fibroblast attachment is independent of DNA-synthesis.

DISCUSSION

Although all the growth factors tested in the present study are known to have stimulatory effects on fibroblast proliferation (9–13, 28, 29), only PDGF and TGF-β1 were shown in this study to promote fibroblast attachment to plastic plates in a concentration-dependent manner. The results of pretreatment experiments indicate that fibroblast attachment is independent

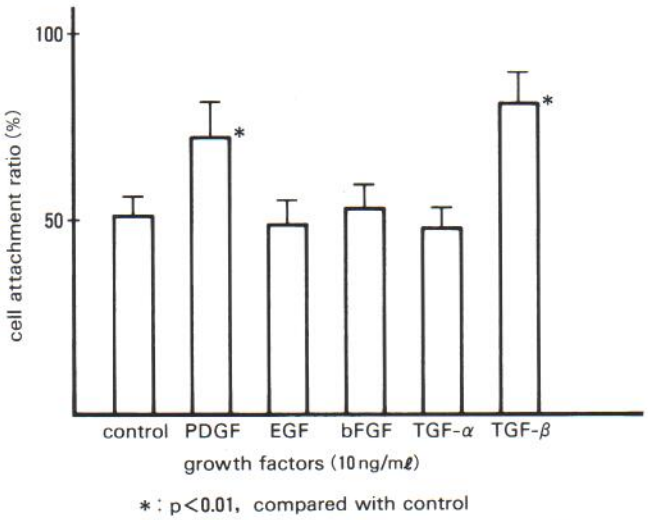

Fig. 4. The fibroblasts stimulated by PDGF, EGF, bFGF, TGF-α, or TGF-β for 24 h were used in the cell attachment assay. Even in this condition, only PDGF and TGF-β increased the ratio of cell attachment. The details are the same as in Fig. 1.

of DNA-synthesis. Previous reports have similarly shown that fibroblast chemotaxis induced by PDGF does not require DNA-synthesis (14, 21, 30).

The present study suggests that PDGF regulates the subsequent steps of dermal fibroblast action in wound healing, which are migration, cell attachment and proliferation, although EGF, bFGF and several other growth factors only stimulate DNA-synthesis. In spite of the contradictory result regarding chemoattractive activity, TGF-β also regulates at least the following three steps: cell attachment, proliferation (12) and extracellular matrix protein synthesis (16–18). Furthermore, TGF-β is known to stimulate the chemotactic migration of human fibroblasts (27). These observations indicate that PDGF and TGF-β are essential factors in dermal wound healing among various growth factors, as previously shown in in vivo studies (1–4).

The exact mechanisms of the promotion of cell attachment induced by PDGF and TGF-β1 have not been elucidated. One of the possible mechanisms of induction by TGF-β1 is through PDGF-related activities secreted by dermal fibroblasts, since TGF-β specifically induces the production of PDGF-related activities, recently named CTGF (8, 25). However, the addition of anti-PDGF IgG, which is known to block PDGF and PDGF-related activities (8, 25), did not neutralize the enhancing effect of cell attachment by TGF-β1, suggesting that TGF-β1 action is not through PDGF-related activities. Thus, the mechanisms of promotion of cell attachment by PDGF and TGF-β1 observed in the present study may be different. Chemotaxis may be related and have some relevance in fibroblast attachment.

TGF-β is a potent stimulator of the expression of extracellular matrix proteins, including fibronectin (31), various types of collagen (16, 31), matrix proteoglycans (32) and tenascin (34–37), as well as inhibitors of matrix-degrading proteases (18). Furthermore, TGF-β is known to regulate the expression of the integrin gene superfamily. TGF-β elevates concomitantly the expression of α1, α2, α3, α5, and β1 integrin subunits at protein and mRNA levels (33, 38) and increases the level of mRNA and the synthesis of vitronectin receptor subunits, αvβ3 integrin (39). The integrin gene superfamily of cell adhesion receptors plays a major role in the mediation of adhesive interactions between cells and the environment. For these reasons, TGF-β1 may promote fibroblast attachment in vivo by stimulating the expression of various extracellular matrix proteins and integrins.

In this study, PDGF and TGF-β1 were found to promote fibroblast attachment to plastic plates. It is possible that the increase of fibroblast attachment by TGF-β1 is partially due to the expression of extracellular matrix proteins and integrins. However, this mechanism remains unknown, and further investigation is now being conducted in our laboratory.

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