

# Selective Upregulation of Platelet-derived Growth Factor $\alpha$ Receptors by Transforming Growth Factor $\beta$ in Scleroderma Fibroblasts

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## Summary

Transforming growth factor  $\beta$  (TGF- $\beta$ ), a multifunctional cytokine, is an indirect mitogen for human fibroblasts through platelet-derived growth factor (PDGF), particularly the A ligand- $\alpha$  receptor arm of that system. TGF- $\beta$  effects on PDGF  $\alpha$  receptor expression were studied in vitro using ligand binding techniques in three human dermal fibroblast strains: newborn foreskin, adult skin, and scleroderma (systemic sclerosis, SSc). Each cell strain responded differently to TGF- $\beta$ . In newborn foreskin fibroblasts, PDGF  $\alpha$  receptor number decreased in a dose-dependent manner after exposure to low concentrations of TGF- $\beta$  (0.1–1 ng/ml). Responses of normal skin fibroblasts were varied, and mean net receptor number was unchanged. Increases in PDGF  $\alpha$  receptor number by TGF- $\beta$  occurred consistently with SSc fibroblasts and low concentrations of TGF- $\beta$  (0.1–1 ng/ml) were particularly stimulatory. Increased surface expression of  $\alpha$  receptor subunit by TGF- $\beta$  in SSc fibroblasts correlated with increased new PDGF  $\alpha$  receptor synthesis as demonstrated by radioimmunoprecipitation analysis of metabolically labeled cells and with increased steady-state levels of corresponding mRNAs. In normal adult skin fibroblasts, TGF- $\beta$  had no effect on either synthesis or mRNA expression of  $\alpha$  receptor subunits. Proliferative responses to PDGF-AA after pretreatment with TGF- $\beta$  correlated positively with effects of TGF- $\beta$  on expression of  $\alpha$  receptor subunit. Decreased mitogenic responses to PDGF-AA were observed in foreskin fibroblasts, small changes in responses in adult fibroblasts, and significant increases in SSc fibroblasts. Thus, costimulation with PDGF-AA and TGF- $\beta$  selectively enhanced proliferation of fibroblasts with the SSc phenotype. Immunohistochemical examination of SSc and control skin biopsies revealed the presence of PDGF-AA in SSc skin. Data obtained by ligand binding, immunoprecipitation, mRNA, and mitogenic techniques are consistent with the hypothesis that activation of the PDGF-AA ligand/ $\alpha$  receptor pathway is a characteristic of the SSc fibroblast and may contribute to the expansion of fibroblasts in SSc.

Scleroderma (systemic sclerosis, SSc)<sup>1</sup> is a complex connective tissue disease of unknown etiology, in which fibrosis develops in the skin and internal organs in association with vascular insufficiency. Scleroderma skin fibroblasts express a phenotype distinct from that of normal skin fibroblasts. Characteristics of this phenotype include: enhanced mRNA expression of several extracellular matrix genes (collagens I, III, VI; fibronectin, proteoglycan), and an abnormality of cell growth that includes insensitivity to the induction of quiescence by serum deprivation and persistent *c-myc* mRNA expression (1, 2). Since the matrix characteristics are recreated

in vitro by exposure of diploid fibroblasts to TGF- $\beta$  (3), we have studied the role of TGF- $\beta$  in the cell growth regulation of SSc (4). We report herein a significant upregulation of platelet-derived growth factor (PDGF)  $\alpha$  receptors after TGF- $\beta$  exposure, a phenomenon that is limited to SSc fibroblasts.

PDGF, a major smooth muscle cell and fibroblast mitogen, exists as a dimer of two homologous polypeptide chains, A and B. Heterodimer (PDGF-AB) and homodimer (PDGF-AA, PDGF-BB) isoforms have been purified from natural sources and are biologically active (5, 6). Human platelets contain PDGF-AB (40%), PDGF-AA (30%), and PDGF-BB (30%) (7). Two distinct PDGF receptor subunits, denoted  $\alpha$  and  $\beta$ , have been identified. The  $\alpha$  receptor subunit binds all three isoforms of PDGF, whereas the  $\beta$  receptor subunit binds PDGF-BB with high affinity, PDGF-AB with lower

<sup>1</sup> Abbreviations used in this paper: PDGF, platelet-derived growth factor; SFM, serum-free medium; SSc, systemic sclerosis.

affinity, and does not bind PDGF-AA (8, 9). The  $\alpha$  and  $\beta$  receptor subunits are structurally and functionally related and, together with *c-kit* product and CSF-1 receptor, belong to a subfamily of tyrosine kinase receptors (10). Dimerization of receptor subunits is required for activation, and several substrates for activated receptor recently have been identified, including phosphoinositol (PI) kinase (11), phospholipase C- $\gamma$  (12, 13), Raf-1 kinase (14), and GAP protein (15, 16). Interestingly, there appear to be differences in the targets of the postreceptor signals transduced by  $\alpha$  and  $\beta$  receptor subunits (17, 18). Relative number and ratio of  $\alpha$  to  $\beta$  receptors vary in different cell types and correlate with mitogenic responses to specific PDGF isoforms (8). In NIH-3T3 cells, where the number of  $\alpha$  and  $\beta$  receptors are similar, both PDGF-AA and BB stimulate DNA synthesis with equal potency. In nonimmortalized, nontransformed human fibroblasts that predominantly express  $\beta$  subunits on the cell surface, higher constitutive mitogenic responses to PDGF-BB than to PDGF-AA are usually observed.

Recent studies indicate that selected growth factors have the capacity to modulate proliferative responses to PDGF isoforms by affecting the expression of PDGF receptor subunits. For example, we have observed increased responses to all three PDGF isoforms implying increased receptor  $\alpha$  subunit expression after pretreatment of human skin fibroblasts with TGF- $\beta$  (19). Increased proliferative responses to PDGF-AA and specific induction of PDGF  $\alpha$  receptor subunit, but not  $\beta$  receptor subunit, were observed in osteoblast-enriched cultures in response to IL-1 (20), and in vascular smooth muscle cells in response to basic fibroblast growth factor (FGF) (21). However, in smooth muscle cells, foreskin fibroblasts, and chondrocytes, decreased synthesis of  $\alpha$  receptor subunit after exposure to high concentrations of TGF- $\beta$  correlated with decreased proliferative effects of TGF- $\beta$  (22). Similarly, TGF- $\beta$  decreased mitogenic responses to PDGF-AA in NIH-3T3 cells, an effect that correlated with decreased  $\alpha$  subunit number, while  $\beta$  subunits were slightly increased (23). In general, PDGF  $\alpha$  receptor expression is downregulated by TGF- $\beta$  in cells whose constitutive  $\alpha$  receptor expression is high and unregulated in cells whose constitutive expression is low.

Growth factors, such as PDGF isoforms, are expressed and considered to be important during embryogenesis (24) and normal tissue remodelling (25). In addition, recent evidence links them to a number of proliferative diseases. Several studies have indicated the presence of PDGF-BB and PDGF  $\beta$  receptors in atherosclerotic plaques (26–28), in pulmonary fibrosis (29, 30), in synovium from rheumatoid arthritis (31, 32), as well as in SSc (33, 34). These observations are consistent with a role for PDGF-BB ligand/ $\beta$  receptor interaction in smooth muscle cell proliferation in diseases with vascular involvement.

Because of the possible relevance to the process of fibrosis of our recent finding that TGF- $\beta$  increases responsiveness to PDGF isoforms in diploid dermal fibroblasts via upregulation of  $\alpha$  receptor subunit (19), further investigation of this arm of the PDGF system was initiated. We present evidence herein that SSc fibroblasts exhibit distinct upregulation of

$\alpha$  receptor subunits by TGF- $\beta$  and increased responsiveness to PDGF-AA, as compared with healthy adult and newborn foreskin fibroblasts. Such characteristics of SSc fibroblasts may be important for the in vivo expansion of these cells. The identification of PDGF-AA in scleroderma dermal biopsies supports this hypothesis.

## Materials and Methods

**Cells.** Fibroblasts were obtained by skin biopsy from affected areas (dorsal forearm) of patients with diffuse cutaneous SSc with <2 yr of skin thickening. Control fibroblasts were obtained by biopsy of healthy donors (within several days of SSc biopsy) matched with each SSc patient for age, race, sex, and biopsy site, and processed in parallel. Newborn foreskin fibroblasts were obtained from the delivery suites of affiliated hospitals. All biopsies were obtained with informed consent and institutional approval. Primary explant cultures were established in 25-cm<sup>2</sup> culture flasks in DMEM supplemented with 10% FCS, 2 mM-glutamine, and 50  $\mu$ g/ml gentamicin. Amphotericin (5  $\mu$ g/ml) was included for the first week only. Monolayer cultures were maintained at 37°C in 5% CO<sub>2</sub> in air. Fibroblasts between the third and fifth subpassages were used for experiments. Fetal fibroblast strains (GM-00011, GM-05386, GM-08447) and foreskin fibroblast strain AG0152 were obtained from the Mutant Cell Repository (Camden, NJ).

**<sup>125</sup>I PDGF Binding Assay.** Saturation binding assays were done according to the method of Bowen-Pope and Ross (35). Cells ( $2 \times 10^4$ /well) were plated in 24-well trays in DMEM supplemented with 10% FCS, grown to confluence, and incubated for 24 h in DMEM with 0.1% BSA. Two wells from each plate were used for cell number determination (mean  $\pm$  SD =  $5.8 \pm 0.5 \times 10^4$  for SSc cells,  $5.8 \pm 1.2 \times 10^4$  for normal cells, and  $5.6 \pm 0.6 \times 10^4$  for foreskin cells). Cells were then incubated with various concentrations of TGF- $\beta$  (R&D Systems, Inc., Minneapolis, MN) for 8 h, which has previously been shown to be the optimal time for maximal induction of PDGF  $\alpha$  receptor (see reference 19). Control cells were incubated in DMEM/0.1% BSA (serum-free medium; SFM) during this time. Medium containing TGF- $\beta$  was removed, and varying concentrations (0.125–8 ng/ml) of <sup>125</sup>I-labeled PDGF-AA (sp act, 70  $\mu$ Ci/ $\mu$ g; purchased from Upstate Biotechnology, Inc., Lake Placid, NY; custom iodinated by Biomedical Technologies, Inc., Stoughton, MA) were added in binding buffer for 2 h at 4°C. Cells were then washed with PBS/0.1% BSA, harvested with solubilization buffer, and total cell-associated <sup>125</sup>I was determined. Nonspecific binding was determined by addition of 100-fold excess of unlabeled PDGF-AA or PDGF-BB (UBI Inc.). Binding isotherms were analyzed as described by Scatchard (36), and receptor number was calculated according to the following formula: receptor number =  $(R_1) (2 \times 10^{-4}) (6.023 \times 10^{23})$ /cell number; where  $R_1 = x$  intercept from Scatchard plot M/L;  $2 \times 10^{-4}$  = reaction volume in liters;  $6.023 \times 10^{23}$  = Avogadro number.

**Statistics.** PDGF  $\alpha$  receptor numbers for pairs of control and SSc fibroblasts were compared using Student's *t* test for paired variables. Differences between foreskin and normal and SSc cells were analyzed using the Student's *t* test for unpaired variables.

**DNA Synthesis.** Cells were plated in 24-well trays in DMEM with 10% FCS and grown to confluence, followed by 24 h incubation in DMEM with 0.1% BSA (SFM). Cells were then incubated with 1 ng/ml of TGF- $\beta$  (R&D Systems Inc.) for 8 h. Control cells received no TGF- $\beta$ . TGF- $\beta$  was washed off, and cells were stimulated with 10 ng/ml of PDGF-AA homodimer (UBI Inc.) for 24 h in the absence of serum or other growth factors. Cells were labeled

with [<sup>3</sup>H]thymidine (sp act 3.22 Bq/mmol, final concentrations 1 μCi/ml; New England Nuclear, Boston, MA) for 2 h, washed three times with cold PBS, and four times with TCA. 500 μl of 0.1% NaOH/0.1% SDS was added and the radioactivity of 250-μl aliquots was measured in 5 ml of PCS II (Amersham Corp., Arlington Heights, IL) using a liquid scintillation system (Packard, Sterling, VA).

**RNA Preparation and Northern Blot Analysis.** Cells were grown to confluence in DME supplemented with 10% FCS in 15-cm-diameter dishes (Falcon Labware, Oxnard, CA), washed three times with SFM, followed by a 24-h preincubation in this medium. Medium was replaced with DME containing 1 ng/ml of TGF-β for 8 h. Total RNA was extracted using an acid guanidinium thiocyanate-phenol-chloroform method (37). 20 μg of total RNA was subjected to electrophoresis on 1% agarose/formaldehyde gel and blotted onto nylon filters (ICN Biochemicals, Inc., Cleveland, OH). The filters were baked, prehybridized, and hybridized as follows. After a 2-h incubation at 42°C in prehybridization buffer (50% deionized formamide, 5× Denhardt's solution, 1% SDS, 5× SSPE, and 100 μg/ml herring sperm DNA), filters were hybridized overnight with 5 × 10<sup>6</sup> cpm/ml of labeled probe at 42°C in the same buffer, followed by two washes in 2× SSPE for 15 min at room temperature, two washes in 2× SSPE/2% SDS for 45 min at 55°C, and two washes in 0.1× SSPE for 15 min at room temperature. Filters were exposed to Kodak X Omat AR film at -70°C for 5 d. The probes (1.6-kb BamI fragment from 3' non-coding region of α receptor cDNA; generous gift from S. Aaronson, National Institutes of Health and glyceraldehyde 3-phosphate dehydrogenase) were labeled with a random primed DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN).

**Metabolic Labeling.** Fibroblasts were grown to near confluence in 10-cm<sup>2</sup> dishes in DME containing 10% FCS. The cells were washed three times with DME containing 0.1% BSA (SFM) and incubated with SFM for 1 h. The cells were then incubated with or without 1 ng/ml of TGF-β (Genzyme, Boston, MA) for 5 h, washed twice with methionine-free medium, and incubated for 20 min at 37°C, then labeled with 200 μCi/ml of <sup>35</sup>S-methionine in methionine-free medium for 3 h in 10% CO<sub>2</sub>. After washing twice with ice-cold PBS, the cells were lysed with 1 ml of triple detergent lysis buffer (0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, 1 μg/ml aprotinin, 100 μg/ml PMSF, 0.02% sodium azide, 50 mM Tris-HCl, pH 8.0, 0.15 M NaCl) for 1 min at 4°C. Chilled lysate was scraped and centrifuged at 12,000 g for 2 min at 4°C. The protein content and radioactivity of each clarified extract were determined using a protein assay and scintillation counting procedure (Bio-Rad Laboratories, Richmond, CA).

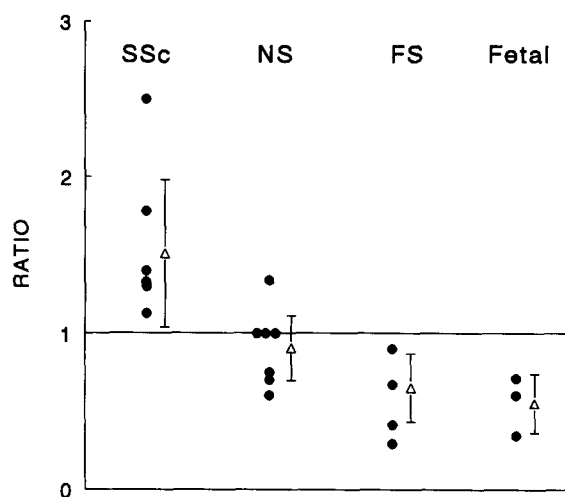
**Immunoprecipitation.** Labeled extracts were precleared by incubation with normal mouse serum for 1 h at 4°C, followed by precipitation with killed, fixed *Staphylococcus aureus* cells (Cowan strain). After preclearance, the extracts were incubated with anti-PDGF α receptor antiserum or preimmune serum at 4°C for 1 h. The immunocomplexes were absorbed by protein A-Sepharose. The gels were then washed twice with NET-gel buffer (0.1% NP-40, 1 mM EDTA, 0.25% gelatin, 0.02% sodium azide, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl) and once with 10 mM Tris-HCl, pH 7.5, 0.1% NP-40. The final immunocomplexes were dissociated from the gels using 1% SDS and analyzed by 4–15% gradient SDS-PAGE and autoradiography.

**Immunohistochemistry.** Frozen sections of skin biopsies from five SSc patients and five healthy controls were investigated for the presence of PDGF-AA ligand using polyclonal rabbit anti-human PDGF-AA antiserum (Genzyme). Punch biopsies were frozen in OCT medium (Miles Laboratories, Naperville, IL) and stored at

-70°C. Frozen sections (6-μm thick) were fixed by air drying and incubated with anti-PDGF-AA or preimmune rabbit IgG (1:40 dilution in PBS) at 37°C for 1 h. All sections were washed three times for 10 min with PBS, followed by incubation with biotinylated goat anti-rabbit IgG (R&D Systems) for 30 min at 37°C. Sections were then incubated with avidin-peroxidase complex (Boehringer Mannheim Biochemicals) for 45 min and with substrate 3,3'-diaminobenzidine (Aldrich Chemical Co., Milwaukee, WI) and H<sub>2</sub>O for 15 min. Sections were counterstained with hematoxylin.

## Results

**Effect of TGF-β on α Subunit Expression Is Cell Type Dependent.** To investigate the expression of PDGF α subunits, we measured specific binding of <sup>125</sup>I-labeled PDGF-AA (PDGF-AA binds with high affinity to PDGF α receptor subunit and does not bind to β subunit). The effect of TGF-β on the expression of PDGF α receptors was measured under experimental conditions previously established (19). In a typical experiment, cells were grown to confluence in DME supplemented with 10% FCS then transferred for 24 h to SFM. Cells were then pretreated with TGF-β (1 ng/ml) for 8 h (previously determined optimal time) followed by binding of several concentrations of <sup>125</sup>I-labeled PDGF-AA (0.125–8 ng/ml). Binding data were used for Scatchard analyses and calculation of binding site affinity and prevalence. Human fibroblasts analyzed were of different origin: normal adult skin, SSc skin, fetal skin, and newborn foreskin. Cell strains used originated from biopsies in our laboratory (except for fetal skin cell strains, obtained from Mutant Cell Repository, Camden, NJ) and were grown under identical conditions and examined only in early passage. A newborn foreskin cell line, AG01523, used for measurements of PDGF receptors (38),



**Figure 1.** Effect of TGF-β on PDGF α receptor expression in SSc, normal adult skin (NS), foreskin (FS), and fetal fibroblasts. Confluent, quiescent cells were preincubated with TGF-β (1.0 ng/ml) followed by binding of several concentrations (0.128–8 ng/ml) of <sup>125</sup>I PDGF-AA (see Materials and Methods). Data are presented as the ratio of receptor numbers per cell with TGF-β treatment to receptor numbers per cell without TGF-β treatment.

**Table 1.** Concentration-dependent Modulation of PDGF  $\alpha$  Receptors by TGF- $\beta$ 

TGF $\beta$	AG01523		FS		NS1		NS2		NS3		SSc1		SSc2		SSc3	
	Rec no.	$K_d$	Rec no.	$K_d$	Rec no.	$K_d$	Rec no.	$K_d$	Rec no.	$K_d$	Rec no.	$K_d$	Rec no.	$K_d$	Rec no.	$K_d$
ng/ml		pM		pM		pM		pM		pM		pM		pM		pM
0	36,000	90	8,600	45	3,000	60	2,500	32	10,000	30	3,500	50	5,600	60	4,200	25
0.1	14,000	50	4,500	9	2,000	20	1,200	31	9,000	50	4,000	60	8,700	80	-	-
0.5	14,000	50	-	-	2,800	40	-	-	-	-	5,400	80	-	-	4,300	25
1	10,000	40	1,400	27	3,000	40	330	12	10,000	80	4,500	50	7,600	100	6,000	27

These data (1.0 ng) represent a portion of the points of Fig. 1. Experimental conditions for all cells are the same as described for Fig. 1. FS, foreskin fibroblasts; NS, normal adult skin fibroblasts; SSc, scleroderma fibroblasts; Rec no., values represent receptor number per cell.

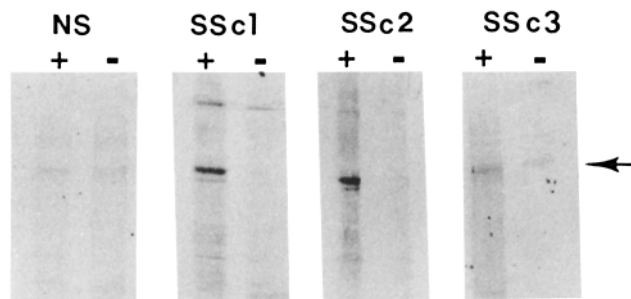
was included as an internal control. Fig. 1 summarizes results obtained from five foreskin, three fetal cell strains, and seven pairs of SSc and matched healthy control skin fibroblasts. In all foreskin and fetal fibroblasts cell strains studied, TGF- $\beta$  pretreatment decreased PDGF receptor  $\alpha$  subunit number. SSc fibroblast strains increased PDGF  $\alpha$  receptor number  $1.6 \pm 0.47$  (mean  $\pm$  SD) -fold after exposure to 1 ng/ml TGF- $\beta$ , while normal adult fibroblasts showed no increase ( $0.92 \pm 0.27$ ) ( $p = 0.03$ ). The SSc fibroblast response was very different from that of foreskin cells ( $0.61 \pm 0.25$ ) ( $p = 0.002$ ). There was no statistical difference between the foreskin and normal adult cell responses.

**Effect of TGF- $\beta$  on Expression of  $\alpha$  Subunit Is Dose Dependent.** We previously established that a dose of TGF- $\beta$  of 1 ng/ml stimulates responses to PDGF maximally in skin fibroblasts (19). However, recent studies by Battagay et al. (22) indicated that TGF- $\beta$  may be active in lower concentrations. We therefore examined the effect of different doses of TGF- $\beta$  (0.1–1 ng/ml) on the expression of  $\alpha$  subunits in three SSc and three adult skin cell strains and two foreskin fibroblasts cell lines (AG 01523 and a foreskin cell line obtained in our laboratory). As shown in Table 1, the commercially available cell line AG 01523 exhibits significantly higher constitutive receptor number compared with cell lines obtained in our laboratory. TGF- $\beta$  inhibited expression of  $\alpha$  subunit in both foreskin fibroblasts and in one adult skin fibroblast cell line in a dose-dependent manner. Two adult skin fibroblast strains were relatively insensitive to the inhibitory effects of TGF- $\beta$ . All three scleroderma cell lines showed induction of PDGF  $\alpha$  receptor expression between 0.1 and 1 ng/ml of TGF- $\beta$ .

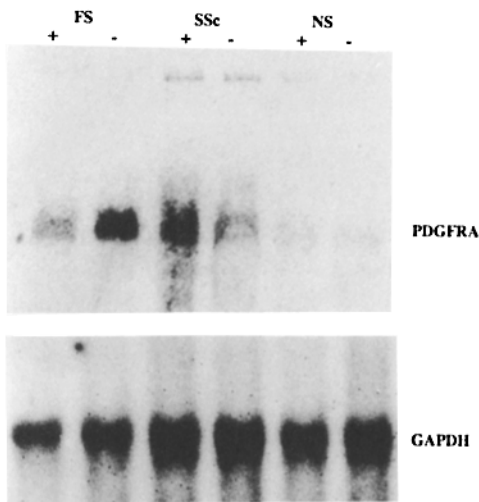
**TGF- $\beta$  Stimulates Synthesis of PDGF  $\alpha$  Receptor Protein in SSc Fibroblasts.** To investigate the mechanism of TGF- $\beta$  induction of  $\alpha$  receptor surface expression, we examined whether TGF- $\beta$  affects synthesis of new receptor subunits. SSc and adult skin fibroblasts were metabolically labeled with  $^{35}$ S-methionine in the presence or absence of TGF- $\beta$  followed by immunoprecipitation with anti-PDGF  $\alpha$  receptor mAb. As shown in Fig. 2, TGF- $\beta$  increases synthesis of  $\alpha$  subunit in SSc, while no effect is observed in normal adult skin fibroblasts.

**TGF- $\beta$  Modulates PDGF  $\alpha$  Receptor mRNA Levels.** To further investigate the mechanism of TGF- $\beta$  modulation of PDGF  $\alpha$  receptor expression, we examined the effect of TGF- $\beta$  on  $\alpha$  receptor message levels. RNA was extracted from confluent cells incubated in serum-free medium for 24 h followed by 8 h of incubation with or without 1 ng/ml of TGF- $\beta$ . As shown in Fig. 3, TGF- $\beta$  directly influences mRNA levels of  $\alpha$  receptor subunit with different effects noted in the three cell strains. In SSc fibroblasts, TGF- $\beta$  induces  $\alpha$  subunit mRNA expression levels but has no effect on message levels in adult skin fibroblasts. In agreement with studies of Battagay et al. (22), TGF- $\beta$  decreases  $\alpha$  subunit mRNA levels in foreskin fibroblasts.

**Mitogenic Responses to PDGF-AA Correlate with PDGF  $\alpha$  Receptor Expression.** Parallel to the receptor expression studies, we examined the effect of TGF- $\beta$  on the mitogenic responsiveness of fibroblasts to PDGF-AA. Briefly, cells were grown to confluence, deprived of serum in SFM for 24 h, and then divided into four groups. One group of cells was pretreated with TGF- $\beta$  (1 ng/ml) for 8 h, then stimulated with PDGF-

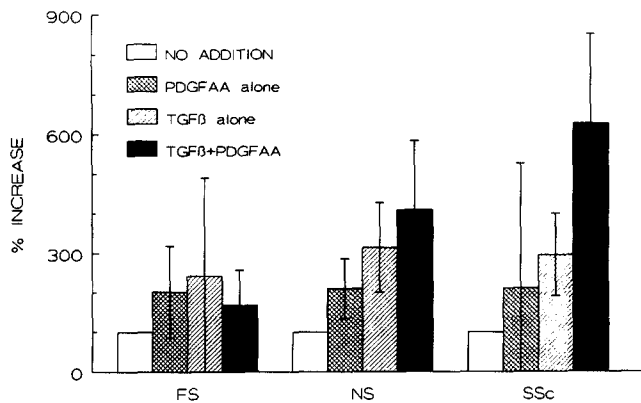


**Figure 2.** Radioimmunoprecipitation of PDGF $\alpha$  receptor subunits in SSc and adult skin fibroblasts. Cells were grown, stimulated with TGF- $\beta$ , and metabolically labeled with  $^{35}$ SO $_4$  as described (see Materials and Methods). Equal concentrations of protein from each labeled extract were immunoprecipitated with anti-PDGF  $\alpha$  receptor antibody and analyzed by 4–15% gradient SDS-PAGE and autoradiography. Three SSc cell lines and one representative normal adult skin (NS) cell line are depicted. (+) Stimulated with TGF- $\beta$ ; (–) unstimulated (1 ng/ml).



**Figure 3.** Northern blot analysis of PDGF  $\alpha$  receptor mRNA in foreskin (FS), SSc, and normal skin (NS) fibroblasts. RNA was extracted from confluent cells incubated for 8 h with TGF- $\beta$  (1 ng/ml) (+) or DME/0.1% BSA (-). (Top) 6.4-kb PDGF  $\alpha$  receptor mRNA; (bottom) GAPDH mRNA.

AA for 24 h. The second group was treated with TGF- $\beta$  only for 32 h. The third group did not receive TGF- $\beta$ ; instead, SFM was added for 8 h, then PDGF-AA was added for 24 h. A fourth or control group received no growth factors; cells were maintained in SFM. After stimulation with growth factors, [ $^3$ H]thymidine was added to all cells for 2 h and incorporation of [ $^3$ H]thymidine into DNA was determined. As shown in Fig. 4, TGF- $\beta$  alone and PDGF-AA alone are weak mitogens with similar levels of responses in all three cell types. However, after pretreatment with TGF- $\beta$ , the mitogenic responses to PDGF-AA differ significantly between all



**Figure 4.** Effects of TGF- $\beta$  preincubation on mitogenic responses to PDGF-AA in newborn foreskin (FS), adult skin (NS), and SSc fibroblasts. Confluent, quiescent cells in serum-free conditions were stimulated with TGF- $\beta$  (1 ng/ml) alone, PDGF-AA (10 ng/ml) alone, or with pretreatment with TGF- $\beta$  as described in the text. Values represent mean  $\pm$  SD from nine FS, five NS and six SSc cell lines, with each experiment performed in triplicate wells. [ $^3$ H]Thymidine incorporation (cpm) in control wells, which did not receive any growth factors, was set at 100%; values from growth factor stimulated cells represent percent increases over control.

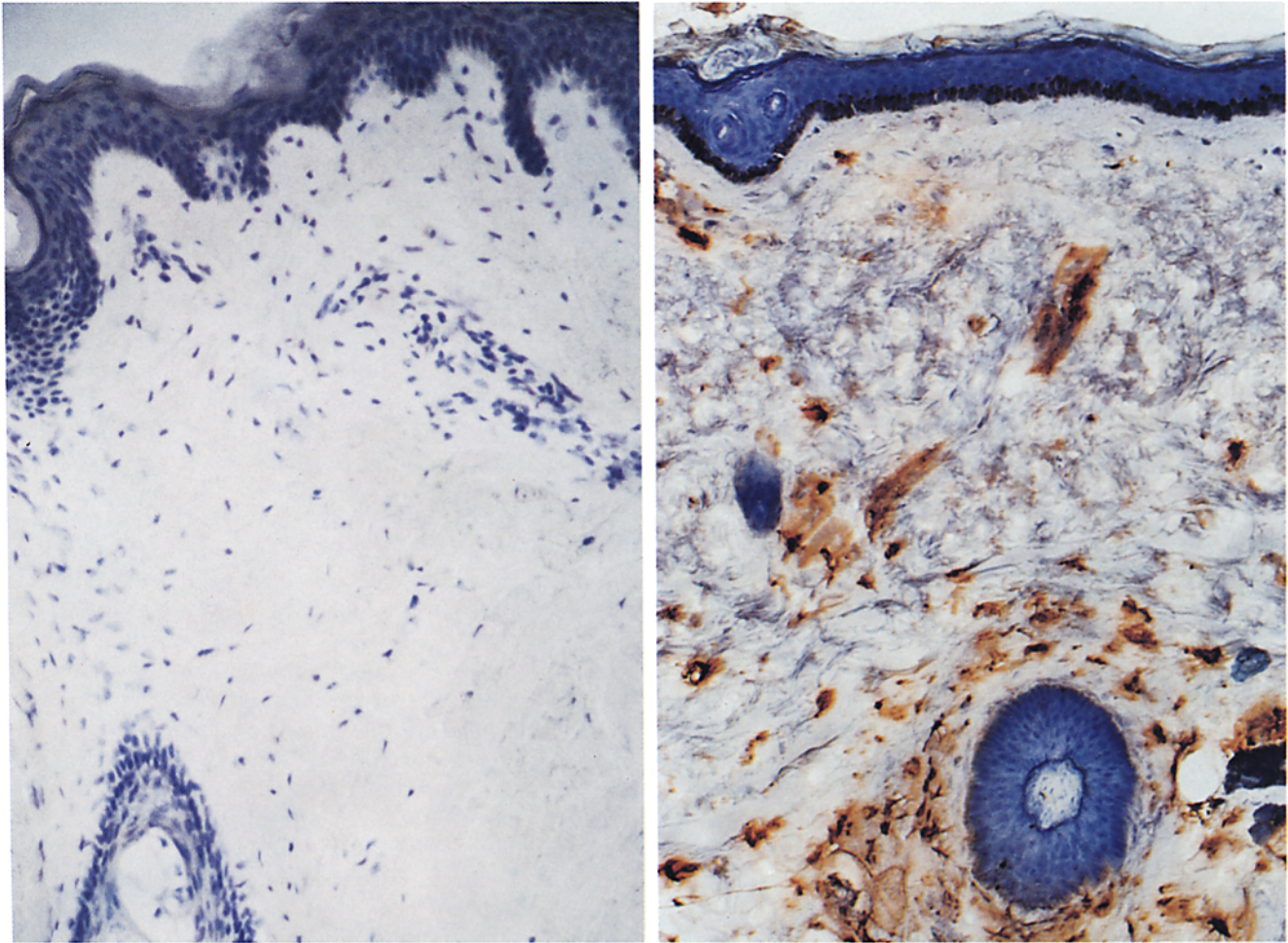
cell types and correlate with the effects of TGF- $\beta$  on expression of  $\alpha$  receptors in each cell type. In foreskin fibroblasts, the mitogenic response to PDGF-AA was abrogated by TGF- $\beta$  pretreatment in all nine cell lines tested. In adult fibroblasts, responses were variable: one cell line showed enhancement of responses to PDGF-AA, in two cell lines TGF- $\beta$  pretreatment did not have any significant effects on response to PDGF-AA, and in two cell lines inhibition of responses was observed. In six SSc fibroblast cell lines, TGF- $\beta$  pretreatment consistently enhanced responses to PDGF-AA. The relatively large variation in the magnitude of responses observed here is most likely due to heterogeneity of the fibroblast population (39), a frequent finding in fibroblast mass cultures derived from different donors. The mitogenic assay data are consistent with the data obtained by ligand binding, immunoprecipitation, and mRNA analysis.

**Expression of PDGF-AA in SSc Skin.** The present in vitro evidence, that SSc fibroblasts from involved skin selectively respond to TGF- $\beta$  by upregulating PDGF- $\alpha$  receptors, thereby enhancing fibroblast proliferative capacity if PDGF-AA is available, remains to be fully confirmed in vivo. Whereas TGF- $\beta$  has been shown to be present in involved SSc skin in close proximity to fibroblasts expressing type I collagen message (40, 41), we investigated whether PDGF-AA is also present in SSc lesions. We performed immunostaining of SSc and control skin biopsies with antiPDGF-AA-specific antibody. Control skin biopsies showed little to no specific staining. In contrast, all SSc biopsies stained positively, including hair follicles (suggesting synthesis of PDGF-AA by keratinocytes), small capillaries (endothelial cell/pericyte), as well as selective stromal cells. Fig. 5 is representative of the staining observed in involved SSc skin biopsies compared with control. Foreskin showed staining intermediate between that of adult control and SSc skin (data not shown).

## Discussion

In this study we demonstrate that fibroblasts from involved SSc skin, in contrast to healthy controls or newborn foreskin fibroblasts, exhibit upregulation of expression of PDGF  $\alpha$  receptors in response to TGF- $\beta$ . The present data confirm the work of others (22), showing that, for foreskin strains, low TGF- $\beta$  concentrations (0.1 ng/ml) lead to 50% decreases of PDGF  $\alpha$  receptor number. Expression of PDGF  $\alpha$  receptors in normal adult skin fibroblasts is variable but essentially unchanged after TGF- $\beta$ . Differences between SSc and normal adult skin fibroblasts exposed to TGF- $\beta$  with regard to surface expression of  $\alpha$  receptors subunits calculated by Scatchard analysis are confirmed by radioimmunoprecipitation analysis with anti PDGF  $\alpha$  receptor mAb. In addition, these differences in TGF- $\beta$  modulation of  $\alpha$  receptor membrane expression in these three cell types correlate with the effect of TGF- $\beta$  on  $\alpha$  receptor subunit mRNA levels. In foreskin fibroblasts, there is inhibition of  $\alpha$  receptor mRNA expression by TGF- $\beta$ ; small and variable changes in expression are observed in healthy adult fibroblasts; and in SSc fibroblasts,  $\alpha$  receptor mRNA is substantially increased after TGF- $\beta$ . Levels of  $\alpha$  receptor expression correlated positively with mitogenic responses to





**Figure 5.** Immunohistochemical evidence for PDGF-AA in scleroderma skin. Frozen sections of skin biopsy of scleroderma patient (*right*) and healthy control (*left*) were stained for the presence of PDGF-AA ligand using polyclonal rabbit anti-human PDGF-AA antiserum as described in Materials and Methods. Sections of scleroderma and normal skin incubated with nonimmune rabbit serum as a control did not show detectable staining (data not shown).

exogenous PDGF-AA. After pretreatment with TGF- $\beta$ , foreskin fibroblasts showed decreased response to PDGF-AA, while SSc fibroblasts showed substantial increases.

Constitutively increased synthesis of extracellular matrix associated with increased expression of extracellular matrix genes is well documented in SSc fibroblasts (1). Our previous studies have indicated proliferation abnormalities of those cells manifested as persistent expression of *c-myc* protooncogene and increased proliferation in low serum concentration (2). Results from the present study (upregulation of PDGF  $\alpha$  receptor and increased proliferative responses to PDGF-AA) add new characteristics to the SSc fibroblast phenotype, extend the range of growth abnormalities of SSc cells, and suggest mechanisms for selective proliferative advantages in vivo. For example, if present in vivo in the vicinity of fibroblasts, PDGF-AA and TGF- $\beta$  may provide a selective stimulus for expansion of fibroblasts with the SSc-like phenotype. Unlike PDGF-BB, which binds equally to  $\alpha$  and  $\beta$  receptors, PDGF-AA uses only  $\alpha$  receptor for postreceptor signalling (8). We therefore examined whether PDGF-AA is present in SSc skin;

detection of TGF- $\beta$  and PDGF-BB ligand has been previously described in SSc (33, 40, 41). We found that while PDGF-AA is essentially absent in healthy skin, it is consistently found in SSc dermis localized around capillaries in the interstitium and around the hair follicles.

The sources of these cytokines in lesional SSc dermis are unknown. The vascular lesion in systemic sclerosis is manifested clinically as Raynaud phenomenon and hypertensive renal crisis accompanied by platelet activation (42). Platelets contain both PDGF-AA and TGF- $\beta$  in granules and their release into lesions as a result of the vascular damage is likely. Also, mononuclear cell infiltrates present in SSc dermis contain macrophages and T cells (43). Macrophages are capable of secreting both TGF- $\beta$  and PDGF-AA (29, 44) and activated T cells are known to release TGF- $\beta$  (45). Fibroblasts themselves are capable of releasing TGF- $\beta$  and PDGF-AA (46, 47). Thus, ample sources of these cytokines exist in SSc dermis, and their combined effect on proliferation of fibroblasts with the SSc phenotype (high matrix production) may help to explain the characteristic fibrotic lesions.

At present, mechanisms of fibrosis in SSc and other fibroproliferative diseases are poorly understood. Factors such as PDGF and TGF- $\beta$ , which are consistently found in fibrotic lesions, may directly stimulate fibroblasts to proliferate and synthesize extracellular matrix. Data presented in this study strengthen the hypothesis that fibroblasts are not simply passive components of fibrotic responses. Characteristics of scleroderma fibroblasts such as increased matrix synthesis and abnormal proliferation probably play a role in the development of fibrosis; presently, however, the origin of these abnormal fibroblasts in the lesion is unknown. One possibility is that

normal fibroblasts, due to exposure to TGF- $\beta$  and/or other cytokines, become permanently activated. Alternatively, expansion of a subpopulation of fibroblasts constitutively expressing increased matrix genes may occur due to their enhanced proliferative responsiveness to growth factors present in the lesion (PDGF A/ $\alpha$  system). The heterogeneous responses of healthy adult fibroblasts observed in our study favor the second alternative. A complete hypothesis concerning mechanisms of fibrosis in SSc must include vascular/microvascular injury and helper T cell activation, discussed elsewhere (1).

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