Increased Expression of TGF-β Receptors by Scleroderma Fibroblasts: Evidence for Contribution of Autocrine TGF-β Signaling to Scleroderma Phenotype

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Scleroderma fibroblasts exhibit numerous phenotypic differences when compared with healthy skin fibroblasts. Some of these differences, in particular overexpression of collagen type I and other extracellular matrix proteins, parallel the effect of transforming growth factor-β (TGF-β) on dermal fibroblasts, suggesting that the scleroderma fibroblast phenotype may result from activation of autocrine TGF-β signaling. To test this hypothesis we examined the role of TGF-β Type I and Type II receptors in regulating collagen type I transcription. We have shown that overexpression of either Type I or Type II receptors significantly (3–4-fold) increases α2 (I) collagen promoter activity in transient transfection assays in dermal fibroblasts. Addition of anti-TGF-β antibody abolished, whereas addition of plasmin enhanced, the stimulatory effect of receptor overexpression on collagen promoter activity, suggesting that this effect depends on autocrine TGF-β. Moreover, these cotransfection experiments indicated that expression levels of TGF-β receptors is a limiting factor in the autocrine regulation of collagen type I transcription by TGF-β. Comparison of the TGF-β receptor Type I and Type II mRA expression levels in scleroderma and normal fibroblasts have indicated elevated expression (2-fold) of both receptor types in scleroderma cells, which correlated with increased binding of TGF-β. Significantly, elevated TGF-β receptor levels correlated with elevated α2 (I) collagen mRNA levels. These results suggest that the elevated production of collagen type I by scleroderma fibroblasts results from overexpression of TGF-β receptors. ***Key words: collagen type I/fibrosis. J Invest Dermatol 110:47–51, 1998***

Excessive extracellular matrix (ECM) deposition in skin, lung, or other organs is a hallmark of systemic sclerosis (SSc) (LeRoy, 1992). Whereas the pathogenesis of SSc is still poorly understood, increasing evidence suggests that activation of lesional fibroblasts contributes to the fibrotic process (Kulozik *et al*, 1990; Jelaska *et al*, 1996). Numerous pleiotropic differences between cultured SSc and healthy skin fibroblasts that can contribute to excessive ECM deposition *in vivo* have been documented. These differences include: elevated expression of collagens type I, III, VI, VII, and fibronectin (LeRoy, 1974; Buckingham *et al*, 1978; Peltonen *et al*, 1990; Xu *et al*, 1991; Rudnicka *et al*, 1994); increased synthesis of glycosaminoglycans (Falanga *et al*, 1987); elevated expression of tissue inhibitor of metalloproteinase (TIMP-1) (Bou-Gharios *et al*, 1994; Kikuchi *et al*, 1995; Kirk *et al*, 1995); elevated expression of intercellular adhesion molecule (Abraham *et al*, 1991); and elevated expression of c-myc and c-myb protooncogenes (Trojanowska *et al*, 1988; Piccini *et al*, 1996). In addition, SSc fibroblasts constitutively produce pro-IL-1a (Kawaguchi, 1994) and constitutively secrete IL-6 (Feghali *et al*, 1994). SSc and healthy skin fibroblasts also differ in their responses to transforming growth factor-β (TGF-β) and basic fibroblast growth factor (bFGF). TGF-β stimulates platelet-derived growth factor-α receptor expression in SSc fibroblasts, but not in healthy skin fibroblasts (Yamakage *et al*, 1992). Conversely, bFGF stimulates expression of platelet-derived growth factor-α receptors in normal fibroblasts, but not in SSc fibroblasts (Kikuchi *et al*, 1992b; Ichiki *et al*, 1995). In addition, SSc fibroblasts are less sensitive to the stimulatory effects of cytokines that regulate collagen production such as Oncostatin M, IL-4, and TGF-β (Duncan *et al*, 1995; Lee *et al*, 1996; Kikuchi *et al*, 1992a). SSc fibroblasts also abnormally regulate collagen type I gene when placed into three-dimensional collagen gels (Eckes *et al*, 1996).

The molecular mechanism underlying these diverse phenotypic differences observed in SSc fibroblasts is presently unknown; however, many of the characteristics of SSc fibroblasts parallel those of healthy fibroblasts exposed to TGF-β (Massague, 1990), suggesting that the SSc phenotype may be a result of activation of an autocrine TGF-β loop. This study was undertaken to test this hypothesis. First we investigated the role of autocrine TGF-β in regulating collagen type I gene transcription by dermal fibroblasts. We then examined the expression of TGF-β receptors in SSc and healthy controls and correlated the level of such expression with collagen type I gene expression by the same cells. The results of our studies are consistent with the notion that elevated expression of TGF-β receptors by SSc fibroblasts is responsible for the elevated expression of collagen type I and possibly other phenotypic differences of SSc cells.

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Abbreviation: SSc, systemic sclerosis.

MATERIALS AND METHODS

Cell cultures Fibroblasts were obtained by skin biopsy from the affected areas (dorsal forearm) of patients with diffuse cutaneous SSc with less than 2 y of skin thickening. Control fibroblasts were obtained by skin biopsy of healthy donors (within several days of SSc biopsy); these were matched with each SSc patient for age, race, gender, and biopsy site and were processed in parallel.

Primary explant cultures were established as described previously (Yamakage *et al*, 1992). Fibroblasts between third and fifth subpassages were used for experiments. Dermal skin fibroblasts derived from a 2-mo-old child (GM05756 A) were obtained from Coriell Cell Repositories (Camden, NJ).

Plasmids construction Generation of a –772 COL1 A2/CAT construct consisting of the human collagen α2 (I) gene fragment (+58 to –772 bp relative to the transcription start site) linked to chloramphenicol acetyltransferase reporter gene was previously described (Tamaki *et al*, 1995). pcDNA3.1 (Invitrogen, San Diego, CA) was used to generate expression vectors for the TGF-β receptors. The TGF-β-RI cDNA (kindly provided by Dr. ten Dijke, Ludwig Institute for Cancer Research, Uppsala, Sweden) was amplified using the polymerase chain reaction technique (primer 1, TAGAAGCTTGGCGAGGC-GAGGTTTGC, primer 2, GACAAGCTTGACACAGAAGTGGCAC; both primers included the HindIII site) and was subcloned into pcDNA3.1 linearized with HindIII. The TGF-β-RII cDNA (kindly provided by Dr. Weinberg, Whitehead Institute for Biomedical Research, Cambridge, MA) was excised by EcoRI and subcloned on the EcoRI site of pcDNA3.1. The kinase deficient TGF-β-RII (TGF-RIIΔK) (kindly provided by Dr. Schneider, Molecular Cardiology Unit, Baylor College of Medicine, Houston, TX) was excised with HindIII and XbaI and subcloned on pcDNA3.1 digested with HindIII and XbaI. Plasmids used in transient transfection assays were purified by a double CsCl gradient.

Transient transfections and chloramphenicolacetyltransferase assays For transient transfections human fibroblasts (GM05756 A) were grown to 90% confluence in 100 mm dishes in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. Monolayers were washed and cells were transfected by the calcium phosphate technique with 10 μg of –772 COL1 A2/CAT and various pcDNA3.1 derivatives carrying TGF-β receptor clones as described in the text. A dose response curve using pcDNA3.1 vector had been constructed to establish the optimum amount of DNA for transfections, and therefore 1.5 μg of all the pcDNA3.1 plasmid derivatives was used. A pSV-β-galactosidase control vector (Promega, Madison, WI) (5 μg) was cotransfected to normalize for transfection efficiency. β-galactosidase activity was measured using the Galacto-Light™ chemiluminescent reporter assay system (Tropix, Bedford, MA). After incubation overnight, the medium was replaced with DMEM containing either 0.1% bovine serum albumin or 1% fetal bovine serum with or without 2 ng recombinant TGF-β1 (UBI, Lake Placid, NY) per ml. Incubation was then continued for 24 h. Cells were harvested in 0.25 M Tris-HCl, pH 8, and fractured by freeze-thawing. Extracts were normalized for protein contents as measured by Bio-Rad reagents and then incubated with butyryl-CoA and [^{14}C]chloramphenicol for 90 min at 37°C, an assay condition predetermined to be within the linear range of chloramphenicol acetyltransferase activity for these samples. Butyrated chloramphenicol was extracted using organic solvent (2:1 mixture of tetramethylpentadecane and xylene) and quantitated by scintillation counting. Each experiment was performed in duplicate.

RNA preparation and northern blot analysis Fibroblasts were grown to confluence in DMEM supplemented with 10% fetal bovine serum, and then incubated for 48 h in serum-free medium (DMEM plus 0.1% bovine serum albumin). Total RNA was extracted and analyzed by northern blot as described previously (Yamakage *et al*, 1992). Filters were sequentially hybridized with radioactive probes for TGF-β-RI, TGF-β-RII, proα2 (I) collagen, and GAPDH. The filters were scanned with a phosphorimager scanner (Molecular Dynamics, Sunnyvale, CA).

Display of TGF-β receptors by ^{125}I-labeled TGF-β Confluent SSc and healthy fibroblasts grown in 6 well plates were incubated for 3 h at 37°C in phosphate-buffered saline to remove bound TGF-β. Next, cells were incubated with ^{125}I-TGF-β (100 pM) for 3 h at 4°C in binding buffer (25 mM HEPES, 3 mM phenylmethylsulfonyl fluoride, 1 mg soybean trypsin inhibitor per ml). Unbound ^{125}I-TGF-β was then washed off. Cross-linking was performed by incubation with 30 mM disuccinimityl suberate for 30 min at 4°C in binding buffer. Cells were then washed with 250 mM sucrose, 1 mM ethylenediamine tetraacetic acid, 3 mM phenylmethylsulfonyl fluoride, and 1 mg per ml soybean trypsin inhibitor. After medium removal cells were solubilized in 1% Triton X-100, 3 mM phenylmethylsulfonyl fluoride, and 1 mg per ml soybean trypsin inhibitor for 3 h at room temperature. Cell lysates were centrifuged at 12,000 *g* for 20 min. Supernatants were collected and protein concentration was measured. Equal amounts of proteins were electrophoresed through 3–12% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by autoradiography.

RESULTS

Collagen type I transcription is under autocrine control by TGF-β in human dermal fibroblasts Expression vectors carrying cDNA for the TGF-β-RI and -RII receptors were cotransfected either individually or together with the α2 (I) collagen promoter/CAT reporter gene construct (-772 COL1 A2/CAT) into human dermal fibroblasts. Overexpression of either receptor type increased (3–4-fold, $p < 0.02$) the basal activity of the collagen promoter (**Fig 1**). Simultaneous cotransfection of both receptors did not have a significantly greater effect on the basal collagen promoter activity than cotransfection with either receptor type alone. Cells cotransfected with TGF-β receptors were responsive to TGF-β stimulation, and the more vigorous response was observed in cells cotransfected with both TGF-β receptors. To test the specificity of the effects generated by overexpression of TGF-β receptors we employed a kinase deficient mutant of the TGF-β-RII (TGF-β-RIIΔK). Previous studies from other systems indicated that such mutation exhibits a dominant-negative effect resulting in blocking of TGF-β responses (Brandt *et al*, 1993; Yamamoto *et al*, 1996). Cotransfection of the TGF-β-RIIΔK

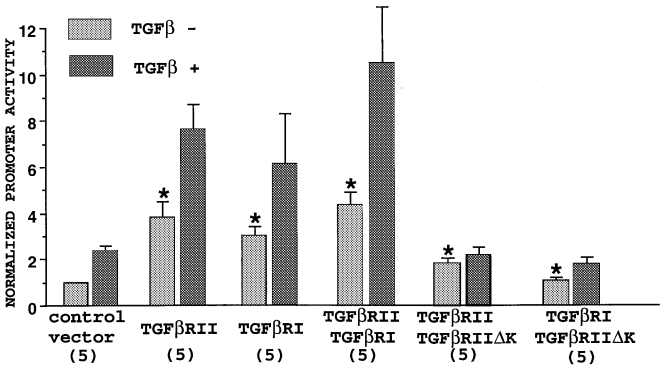

Figure 1. Overexpression of TGF-β type I and type II receptors activates collagen promoter activity in human dermal fibroblasts. Human dermal fibroblasts were transiently cotransfected with 10 μg of α2 (I) collagen promoter/CAT reporter gene construct and with either 3 μg of the pcDNA3.1 vector (control) or 1.5 μg of pcDNA3.1 carrying wild-type cDNA for the TGF-β type I (TGF-β-RI) and type II (TGF-β-RII) receptors or kinase deficient TGF-β type II receptor (TGF-β-RIIΔK). To ensure equal amounts of cotransfected expression vectors under each condition, 1.5 μg of pcDNA3.1 was added to the cotransfections with each single TGF-β receptor derivative. The day after transfections some dishes were stimulated with 2 ng per ml of TGF-β for 24 h (*solid bars*). The graph depicts means ± SEM of the collagen promoter activities relative to the activity of promoter cotransfected with pcDNA3.1 vector that was arbitrarily set at 1 (all values are normalized for transfection efficiency by cotransfection with the pSV-β-galactosidase plasmid). The number of experiments to calculate the mean is shown in parentheses. * indicates statistically significant results ($p < 0.02$, Mann–Whitney U test).

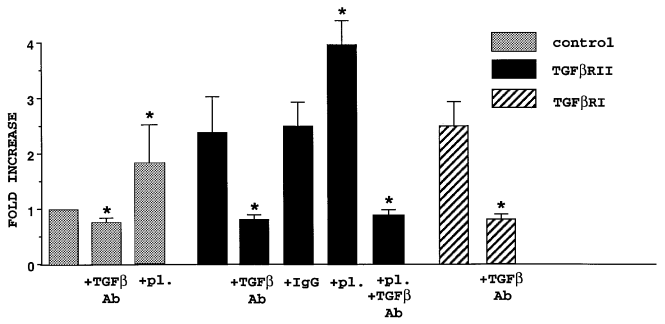

Figure 2. Autocrine TGF-β regulates α2 (I) collagen transcription in human dermal fibroblasts. Human dermal fibroblasts were transiently cotransfected with 10 μg of α2 (I) collagen promoter/CAT reporter gene construct and 1.5 μg of the pcDNA3.1 vector (control), or 1.5 μg of pcDNA3.1 carrying wild-type cDNA for the TGF-β type-1 (TGF-β-RI) and type II (TGF-β-RII) expression vectors. Some of the cotransfected cells were incubated with anti-TGF-β neutralizing antibody (20 μg per ml), nonspecific IgG (20 μg per ml), or plasmin (0.1 u per ml). The graph depicts means ± SEM (from four experiments) of the collagen promoter activities relative to the activity of promoter cotransfected with pcDNA3.1 vector that was arbitrarily set at 1. * indicates statistically significant results ($p < 0.02$, Mann–Whitney U test).

with either the wild-type TGF-β-RII or TGF-β-RI significantly decreased basal activity of the α2 (I) collagen promoter as compared with cells cotransfected with the wild-type receptor alone and completely abolished TGF-β stimulation of this promoter (**Fig 1**).

Previous studies have indicated that human fibroblasts secrete large amounts of TGF-β (White-Needleman *et al*, 1990; McWhirter *et al*, 1994), some of which may be in its active form. Thus, an increase in the basal activity of the collagen promoter in cells overexpressing TGF-β receptors may result from the action of such autocrine TGF-β. Alternatively, overexpression of TGF-β receptors may contribute to the heteromeric receptor complex formation, resulting in ligand-independent signaling, a possibility implicated in other experimental systems (Feng and Derynck, 1996). To resolve this question additional experiments were performed. First, TGF-β activity was blocked by adding anti-TGF-β neutralizing antibody (**Fig 2**). Under this condition a relatively small (15–20%) but consistent ($p < 0.02$) decrease of the collagen promoter activity was observed in cells cotransfected with the control vector; however, addition of the antibody completely abolished stimulation of collagen promoter caused by overexpression of TGF-β-RII or TGFβ-RI. We next compared the effect of plasmin (a known activator of latent TGF-β) on the collagen promoter activity in cells cotransfected with control vector or TGF-β-RII. As shown in **Fig 2**, addition of plasmin increased collagen promoter activity by about 50%, indicating the presence of latent TGF-β in dermal fibroblast cultures. These experiments suggest that α2 (I) collagen transcription is ligand dependent and that autocrine TGF-β activity contributes significantly to the constitutive collagen transcription in dermal fibroblasts.

SSc fibroblasts express increased levels of TGF-β type I and type II receptors Because of the role established for autocrine TGF-β signaling through type I and type II receptors in controlling collagen type I transcription in cultured dermal fibroblasts, we examined receptors in scleroderma fibroblasts. Because SSc fibroblasts overexpress collagen type I mRNA and protein (LeRoy, 1974; Jimenez *et al*, 1986), and exhibit elevated activity of the collagen type I promoters (Kikuchi *et al*, 1992a; Hitraya and Jimenez, 1996), we considered it important to examine expression of TGF-β receptors in SSc fibroblasts. Expression of the TGF-β Type II and Type I receptor mRNA along with α2 (I) collagen mRNA in four pairs of SSc and normal fibroblasts was measured (**Fig 3*a***). SSc fibroblasts express increased levels of both receptor mRNA (2-fold increase, $p < 0.02$), and correlating increases in collagen mRNA levels (2.5–3-fold increase, $p < 0.02$) (**Fig 3*b***).

In addition, we measured TGF-β receptor binding activity by cross-linking ^{125}I-TGF-β to TGF-β receptors in SSc and normal human dermal fibroblasts. Note that binding was specific, as determined by a cold competition assay using 200 × excess of unlabeled TGF-β (data not shown). As shown in **Fig 4**, there is increased binding to TGF-β receptors in SSc fibroblasts.

In conclusion, SSc fibroblasts exhibit elevated expression of the TGF-β-RI and -RII receptors that correlates positively with elevated expression of the collagen α2 (I) gene.

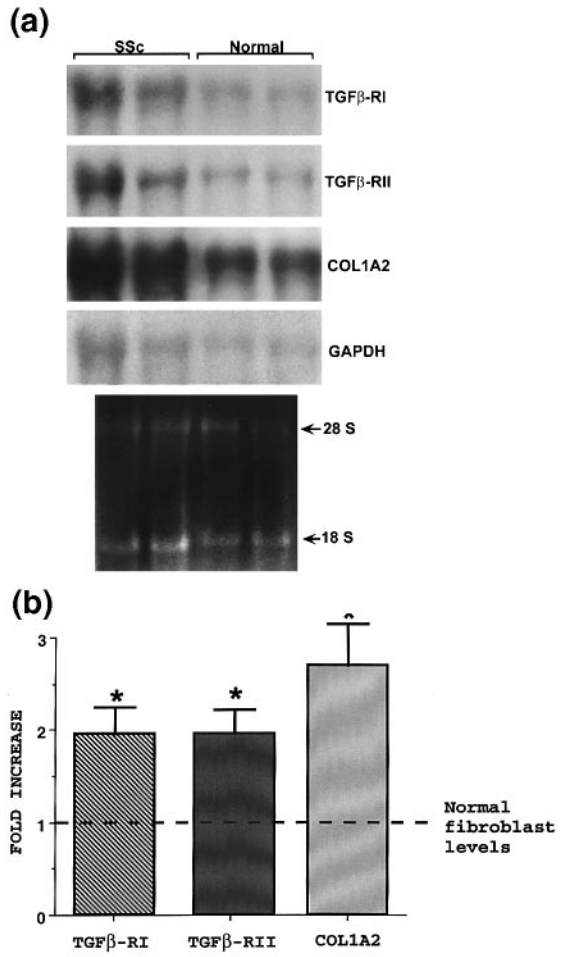

Figure 3. Increased TGF-β receptor expression correlates with the α2 (I) collagen expression in scleroderma fibroblasts. (*a*) Total RNA was isolated from SSc and control fibroblasts and analyzed simultaneously by northern blot. Blots were sequentially hybridized with probes for TGF-βRI, -RII, COL1 A2, and GAPDH and quantitated using a phosphorimager. A representative northern blot is shown in the top panel. Density of the band in normal fibroblasts for each mRNA was set at 1 (- - - - -). Values from the SSc fibroblasts indicate band density relative to control. All values were corrected for the loading differences, as determined by GAPDH mRNA intensity. Quantitative analysis (mean ± SEM) of four SSc/normal fibroblast pairs is presented (*b*). * indicates statistically significant results ($p < 0.02$).

DISCUSSION

TGF-β is currently recognized as a central mediator in various fibrotic diseases including scleroderma (LeRoy *et al*, 1989; Border and Ruoslahti, 1992). The principal effect of TGF-β on mesenchymal cells is its stimulation of ECM production leading to excessive ECM deposition. TGF-β has been shown to increase expression of collagen types I, III, VI, VII, and X, fibronectin matrix glycoproteins, and proteoglycans (Massague, 1990). Stimulation of ECM production by TGF-β is further enhanced by its inhibitory effect on matrix degradation decreasing synthesis of proteases and increasing levels of protease inhibitors (Massague, 1990). Different cell types, including immune, endothelial, and mesenchymal cells, may serve as a source of TGF-β in the fibrotic lesion. Furthermore, sustained production of TGF-β in the lesion may be maintained by autoinduction (Obberghen-Schilling *et al*, 1988). Whereas it is now recognized that constitutively activated cells in the lesions may also contribute to the disease progression, the mechanism of this activation is unknown. One possible mechanism may involve the establishment of an autocrine cytokine loop. Such a loop may be initiated *in vivo* by exposure to certain cytokines and may continue to operate in the cell culture conditions. Cytokines known to induce ECM production, such as TGF-β, IL-4, OSM, or IL-1, are candidates for such autoinduction, acting separately or in concert.

In this study we have begun to examine the role of autocrine TGF-β signaling in regulating ECM production in cultured dermal fibroblasts and a possible contribution of the autocrine TGF-β loop to the SSc phenotype. Using dermal fibroblasts overexpressing TGF-β-RI and R-II receptors, we have demonstrated a functional and direct positive correlation between TGF-β receptor expression levels and collagen α2 (I) gene transcription both in the presence and in the absence of added ligand (**Fig 1**). Significantly, increased collagen transcription was observed in the absence of serum or other growth factors suggesting that autocrine TGF-β is constitutively produced and activated by cultured fibroblasts. This was further confirmed in experiments in which the action of autocrine TGF-β was blocked by the addition of anti-TGF-β antibody or enhanced by the addition of plasmin (**Fig 2**). These experiments also indicate that levels of TGF-β receptors are the limiting factor in the autocrine (as well as paracrine) regulation of collagen type I transcription by TGF-β, because the overexpression of either type I or type II receptors increased collagen transcription to a greater extent than addition of an optimal amount of exogenous TGF-

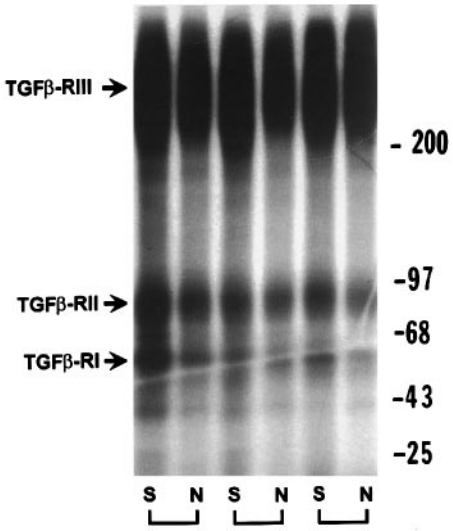

Figure 4. Display of TGF-β receptors by ^{125}I-labeled TGF-β. Cross-linking of ^{125}I-TGF-β was performed as described in *Materials and Methods*. TGF-β receptors in three pairs of SSc (S) and normal (N) fibroblast are shown.

β to cells with physiologic receptor levels (**Fig 1**). Addition of the anti-TGF-β antibody, however, only partially decreased (15–20%) constitutive transcription of collagen type I, suggesting that cell mechanisms other than TGF-β are also involved in collagen gene regulation. These cotransfection experiments presented here establish the importance of TGF-β receptor expression levels in the regulation of collagen type I transcription by autocrine TGF-β.

Both type I and II TGF-β receptor mRNA expression levels are higher in SSc than in healthy fibroblasts (**Fig 3**), which correlates with increased binding of TGF-β (**Fig 4**). This result differs from previous studies in which TGF-β receptor expression was compared in SSc and healthy fibroblast using Scatchard analysis (White-Needleman *et al*, 1990) in which receptor expression level is inferred from the level of ligand binding. Because of the multiple receptor system for TGF-β, however, levels of ligand binding do not correlate with signal transduction due to the high levels of the high affinity but nonsignaling Type III receptor (reviewed in Attisano *et al*, 1994). Therefore, Scatchard analyses are unsuitable for the quantitative analysis of the signal transducing TGF-β receptor types. We have found that TGF-β receptors are expressed at higher levels in SSc cells, and, more importantly, this elevated receptor expression correlates with the elevated collagen α2 (I) mRNA levels. Taken together with the results of cotransfection assays (**Figs 1, 2**), these findings suggest that elevated expression of TGF-β receptors by SSc fibroblasts in culture may be responsible for the elevated expression both of collagen type I and of other ECM proteins also overproduced by cultured SSc fibroblasts. It is, however, presently unclear why SSc fibroblasts exhibit diminished responses to exogenous TGF-β (Kikuchi *et al*, 1992a), despite overexpression of TGF-β receptors. It is possible that the elevated levels of the TGF-β receptors render SSc fibroblasts more sensitive to ligand. In such a case the amount of autocrine TGF-β produced by SSc cells would be sufficient to maximally activate the signaling pathway, whereas in normal fibroblasts, with their lower receptor number, higher amounts of TGF-β are necessary for receptor activation. It has been previously shown in other experimental systems (Lillien, 1995) that a 2-fold increase in receptor expression led to at least a 10-fold decrease in ligand concentration required to induce biologic response. Other mechanisms such as differences in the post-receptor signaling pathways between SSc and normal fibroblasts may also be involved. This important issue will require further studies. It will be of interest to perform cotransfection experiments directly in SSc and normal fibroblasts to test the effect of blockage of the TGF-β signaling (e.g., by overexpressing dominant-negative TGF-β-RII) on the α2 (I) collagen promoter activity in both cell types. In our experimental system, however, we were not able to perform these experiments due to the significantly low efficiency of transfection in adult dermal fibroblasts. We envision these experiments in the future studies utilizing more efficient systems of transfections (e.g., adenoviral vectors, see Yamamoto *et al*, 1996). It will also be very important to analyze *in situ* the expression of the TGF-β receptors in the SSc lesions. With the specific reagents now available, such studies are feasible.

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