Constitutive Thrombospondin-1 Overexpression Contributes to Autocrine Transforming Growth Factor- β Signaling in Cultured Scleroderma Fibroblasts

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The extracellular matrix (ECM) glycoprotein thrombospondin-1 (TSP-1) has been reported to activate the latent complex of transforming growth factor- β (TGF- β), the major effects of which in mesenchymal cells is stimulation of the synthesis of ECM. Previous reports suggested the involvement of an autocrine TGF- β loop in the pathogenesis of scleroderma. In this study, we examined whether TSP-1 plays a role in maintaining the autocrine TGF- β loop in scleroderma. TSP-1 expression was increased in scleroderma patients compared with in healthy controls *in vivo* and *in vitro*. TGF- β blocking antibody or TGF- β 1 antisense oligonucleotide markedly reduced the up-regulated TSP-1 expression in scleroderma fibroblasts but had little effect on normal fibroblasts. The expression of TSP-1 is up-regulated in scleroderma fibroblasts, possibly at the post-transcriptional level just like in normal fibroblasts stimulated with exogenous TGF- β 1. TSP-1 blocking peptide or antisense oligonucleotide had an inhibitory effect on the up-regulated α 2(I) collagen and phospho-Smad3 levels in scleroderma fibroblasts but had little effects on normal fibroblasts. The transient overexpression of TSP-1 up-regulated α 2(I) collagen and phospho-Smad3 levels in normal fibroblasts but had no major effect on scleroderma fibroblasts. Furthermore, these effects of transiently overexpressed TSP-1, which possibly occurred via the activation of latent TGF- β 1, were abolished by the TGF- β 1 antisense oligonucleotide. These results indicate that the constitutive overexpression of TSP-1 may play an important role in autocrine TGF- β signaling and accumulation

of ECM in scleroderma fibroblasts. (*Am J Pathol* 2005, 166:1451–1463)

Scleroderma, or systemic sclerosis (SSc), is a generalized disease which affects connective tissue in the skin and internal organs.¹ Although its pathogenesis is still unclear, it includes inflammation, autoimmune attack, and vascular damage, leading to activation of the fibroblasts.^{2,3} Fibroblasts derived from the affected skin areas of scleroderma patients are considered to be in an activated state, synthesizing increased amounts of connective tissue components, mainly collagens I, III^{4–6} and VI,⁷ fibronectin^{8,9} and proteoglycans.¹⁰ Cytokine- and cell surface receptor-mediated signaling is thought to be involved in this activation and to lead to nuclear events which increase the transcription of the genes for collagens, fibronectin and other components of the extracellular matrix in the affected tissues.¹¹ Moreover, many of the characteristics of scleroderma fibroblasts resemble those of healthy fibroblasts exposed to TGF- β ,¹² suggesting that the scleroderma phenotype may be a result of activation by an autocrine TGF- β loop.

TGF- β 1 is normally secreted as a complex composed of three proteins, which are the bioactive peptide TGF- β 1, latency-associated peptide- β 1 (LAP- β 1), and latent TGF- β binding protein-1. TGF- β 1 forms a complex with LAP- β 1 noncovalently, which is called the small latent complex, and in this configuration TGF- β 1 is unable to bind to its receptors.¹³ Thus, TGF- β must be activated before it can bind to its receptors. One mechanism of TGF- β activation, initiated by the binding of the latent

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complex of TGF- β 1 to the extracellular matrix glycoprotein TSP-1, may be particularly important *in vivo*.¹⁴

TSP-1 is a trimer of disulfide-linked 180-kD subunits. It is produced by a number of cell types. Each subunit consists of several domains that bind to matrix and cell surface proteins.¹⁵ The site responsible for activating latent TGF- β in TSP-1 is localized in a domain that consists of three type 1 repeats.¹⁶ Two amino acid sequences are implicated in TGF- β 1 activation: GGWSHW (amino acids 418 to 423) in the first type 1 repeat (or potentially DGWSPW in the second and GGWGPW in the third type 1 repeat) and KRFK (amino acids 412 to 415) between the first and the second type 1 repeats.¹⁷ It has been proposed that latent TGF- β 1 is activated as a result of binding of the KRFK sequence in TSP-1 to the LSKL sequence in the amino-terminal region of LAP.¹⁸ TSP-1 is reported to be a major activator of TGF- β 1 *in vivo* as demonstrated with TSP-1 null mice.¹⁴ Moreover, TSP-1 expression is reported to be closely related to the development of fibrosis in proliferative glomerulonephritis¹⁹ and in tubulointerstitial fibrosis.²⁰ Collectively, these reports suggest a major role for TSP-1 in pathological fibrosis by its activation of TGF- β . Although it was reported that serum levels of TSP-1 are higher in scleroderma patients than those in healthy controls,²¹ the role of TSP-1 in the pathogenesis of scleroderma has yet to be elucidated. We investigated whether TSP-1 plays a role in the accumulation of ECM observed in scleroderma fibroblasts, focusing on its contribution to the maintenance of the autocrine TGF- β loop.

Materials and Methods

Reagents

Recombinant human TGF- β 1, latent TGF- β 1 and a pan-specific neutralizing TGF- β antibody (Ab) were obtained from R&D systems Inc. (Minneapolis, MN). Actinomycin D, anti- β actin Ab (AC-15) and anti-smooth muscle actin Ab (1A4) were purchased from Sigma. Anti-Smad2/3 Ab (N-19), anti-Smad3 Ab (FL-425), anti-vimentin Ab (V9), anti-CD68 Ab (M-20) and anti-TSP-1 Ab (N-20) used for immunohistochemical staining were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphoserine Ab was purchased from Biomedica (Foster City, CA). The luciferase assay kit was purchased from Promega (Madison, WI). Anti-TSP-1 Ab (clone B5.2) for immunoblotting was purchased from Neomarkers (Fremont, CA). Anti-type I collagen Ab was from Southern Biotechnology Associates (Birmingham, AL). Anti-CD34 Ab was from Immunotech S.A. (Marseilles, France). FUGENE 6 was obtained from Roche Diagnostics (Indianapolis, IN).

Cell Cultures

Human dermal fibroblasts were obtained by skin biopsy from the affected areas (dorsal forearm) of eight rapidly worsening patients with diffuse cutaneous scleroderma and <2 years of skin thickening.²² These areas seemed

to be the leading edge or near the leading edge when the samples were obtained. Control fibroblasts were obtained by skin biopsy from eight healthy donors. Institutional approval and informed consent from all subjects were obtained. Control donors were matched with each scleroderma patients for age, sex, and biopsy site, and control and patient samples were processed in parallel. Primary explant cultures were established in 25-cm² culture flasks in modified Eagle's medium (MEM) with 10% fetal calf serum (FCS), 2 mmol/L L-glutamine, and 50 μ g/ml amphotericin. Ascorbate was not added to the medium. Cultures of fibroblasts isolated independently from different individuals were maintained as monolayers at 37°C in 95% air, 5% CO₂, and studied between the third and sixth subpassages.

Cell Lysis and Immunoblotting

Fibroblasts were grown to subconfluence in MEM with 10% FCS. After 24 hours of serum starvation, cells were solubilized in lysis buffer (1% Triton X-100 in 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 3 mmol/L MgCl₂, 1 mmol/L CaCl₂ containing 10 μ g/ml leupeptin, pepstatin, and aprotinin, 1 mmol/L PMSF, and 1 mmol/L Na₃VO₄). For detection of TSP-1 and type I collagen, the culture medium was collected, and the amount loaded was adjusted based on the protein concentrations of the cell lysate determined with a Bio-Rad protein assay (Hercules, CA), as recommended by the manufacturer.

Proteins were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated overnight with anti-TSP-1 Ab or anti-type I collagen Ab (1:1000), washed, and incubated with horseradish peroxidase-conjugated secondary Ab for 60 minutes. After the washing, visualization was performed using enhanced chemiluminescence (Amersham Pharmacia Biotech). As a loading control, immunoblotting was also performed using antibodies against β -actin (1:2000).

Immunoprecipitation

Confluent quiescent normal and scleroderma fibroblasts were washed with cold PBS and harvested into lysis buffer (1% Nonidet P-40 in 10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L Na₃VO₄, 50 mmol/L NaF, containing 10 μ g/ml leupeptin, 10 μ g/ml pepstatin, 10 μ g/ml aprotinin, and 1 mmol/L PMSF). Each extract was precleared with 10 μ l of protein G-Sepharose for 1 hour with rotation. The beads were pelleted, the supernatant was transferred to a new tube, and 10 μ l of protein G-Sepharose beads conjugated to anti-Smad3 Ab (rabbit polyclonal IgG) was added. Immunoprecipitation was performed overnight at 4°C with rotation, after which the immunoprecipitates were washed four times with lysis buffer. After the last wash, the beads were resuspended in 30 μ l of sample buffer and boiled for 5 minutes. Proteins were subjected to immunoblotting using anti-phosphoserine Ab (mouse monoclonal IgG). After the development, the membrane was stripped and

reprobed with anti-Smad2/3 Ab (goat polyclonal IgG) to determine the total level of Smad3.

Immunohistochemical Stainings

Immunohistochemical staining was performed using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA).²³ Two-micrometer thick paraffin-embedded sections were mounted on slides, then deparaffinized by xylene and rehydrated through a graded series of ethyl alcohol and PBS. The sections were then incubated with anti-TSP-1 Ab overnight at 4°C. The immunoreactivity was visualized with Vector Red (Vector Laboratories). The sections were then counterstained with hematoxylin.

Treatment of Dermal Fibroblasts with TGF- β 1/TSP-1 Antisense Oligonucleotides, Anti-TGF- β 1 TSP-1 Neutralizing Ab or TSP-1 Blocking Peptides

We used a pan-specific neutralizing TGF- β Ab, the effectiveness and specificity of which was previously verified.²⁴ We also used a TGF- β 1 18-mer antisense oligonucleotide (GAGGGCGGCATGGGGAGG), which overlaps the promoter and transcriptional start site of the TGF- β 1 gene. This same sequence, which is specific for the TGF- β 1 isoform, has been found to be sufficient to block TGF- β 1 transcription *in vitro*²⁵ and *in vivo*.²⁶ A sense oligonucleotide was used as a control.

The effectiveness and specificity of the TSP-1 blocking antibody, a gift from J.E. Murphy-Ullrich (University of Alabama, Birmingham, AL), was well established previously.²⁷ TSP-1 antisense (GTCTGGCGATGCTG) and control (ACCGACCGACGTGT) oligonucleotides directed to TSP-1 were designed and manufactured by Biognostik GmbH (Gottingen, Germany), who owns the intellectual property rights of these sequences. TSP-1 blocking peptide GGWSHW and a negative control peptide, GGY-SHW, were manufactured by QIAGEN (Tokyo, Japan). The specificity and effectiveness of these oligonucleotides and these peptides have also been well established.^{28,29} Fibroblasts were grown to confluence and the culture medium was removed, washed with MEM to remove excess TGF- β 1, and replaced with serum-free MEM. Antisense or sense oligonucleotide, TGF- β 1 blocking antibody or a preimmune IgG was added to the media, and immunoblotting, Northern blotting or a reporter assay was performed 48 hours later. The incubation time for the TSP-1 blocking peptide or a control peptide was 96 hours.

RNA Preparation and Northern Blot Analysis

Two micrograms of total RNA extracted from confluent quiescent fibroblasts were subjected to electrophoresis on a 1% agarose/formaldehyde gel and blotted onto a nylon filter (Roche Diagnostics, Indianapolis, IN). The filter was UV cross-linked, prehybridized, and sequentially hybridized with DNA probes for TSP-1 (1.4-kb

*Bam*HI fragment of the human TSP-1 cDNA), α 2(I) collagen or GAPDH. The membranes were then washed and exposed to X-ray film.

Plasmids

The promoter region of TSP-1 (–2200/+754), which was inserted into the luciferase vector pGL3basic (Promega), was kindly provided by Dr. Hong (The Catholic University of Korea, Seoul, Korea).³⁰ The human TSP-1 cDNA, which was inserted into the expression vector pcDNA, was kindly provided by Dr. Detmar (Harvard Medical School, Charlestown, MA).³¹ The generation of a –3500 COL1A2/CAT construct consisting of the human α 2(I) collagen gene fragment (bp + 58 to –3500 relative to the transcription start site) linked to the chloramphenicol acetyltransferase (CAT) reporter gene and COL1A2/CAT constructs used for deletion analysis were described previously.³² Substitution mutations were introduced into a Smad3-binding site (located between bp –263 and –258) of the –353 COL1A2/CAT construct using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as described previously.³³ P3TP-Lux was a gift from Dr. Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY).³⁴ Plasmids used in transient transfection assays were purified twice on CsCl gradients, as described previously. At least two different plasmid preparations were used for each experiment.

Transient Transfection, Luciferase Assays, and CAT assays

Fibroblasts were grown to 50% confluence in 100-mm dishes as described above. After 4 hours of incubation with serum-free medium, cultures were transfected with 2 μ g of various COL1A2/CAT constructs, along with 1 or 3 μ g of the TSP-1 expression vectors or corresponding empty constructs, using FuGENE6.³⁵ To control for minor variations in transfection efficiency, 1 μ g of pSV- β -galactosidase vector (Promega, Madison, WI) was included in all transfections. Cells were harvested 48 hours after transfection. To determine the promoter activity of COL1A2/CAT, each extract normalized for protein content was incubated with butyl-CoA and [¹⁴C]chloramphenicol for 90 minutes at 37°C. Butylated chloramphenicol was extracted using an organic solvent (2:1 mixture of tetramethylpentadecane and xylene) and quantitated by scintillation counting. Regarding TSP-1-Lux (–2200/+754), cells were transfected in the same manner as COL1A2/CAT constructs, and the indicated reagents were added after 24 hours incubation. To determine the promoter activity of TSP-1, each extract normalized for protein content was assayed for luciferase activity. Each experiment was performed in duplicate.

(A) Normal stained
with anti-TSP-1 Ab

(B) SSc stained with
anti-TSP-1 Ab

(C) SSc stained with
anti-CD34 Ab

(D) SSc stained with
anti-CD68 Ab

Figure 1. Comparison of TSP-1 expression levels in dermal tissue between scleroderma patients and healthy controls. Paraffin sections of normal and scleroderma dermal tissue were subjected to immunohistochemical analysis as described in Materials and Methods. The immunoreactivity of TSP-1 (**arrow**) was more strongly detected around the cells between collagen bundles in scleroderma (**B**) than in normal tissue (**A**). The cells positive for anti-TSP-1 Ab between collagen bundles were not stained with anti-CD34 Ab (**C**) or anti-CD68 Ab (**D**). Original magnification, $\times 200$.

Statistical Analysis

Data presented as bar graphs are the means $\pm$ SD of at least three independent experiments. Statistical analysis was performed by the Mann Whitney *U*-test. *P* values less than 0.05 were considered significant.

Results

Comparison of TSP-1 Expression Levels in the Dermal Tissue between Scleroderma Patients and Healthy Controls

To compare the expression levels of TSP-1 between normal and scleroderma dermal tissues, we performed immunohistochemical staining on paraffin-embedded

sections. The expression of TSP-1 was more strongly detected in scleroderma tissues, especially between collagen bundles, than in normal tissues (Figure 1, A and B). Since TSP-1 expression can be found in endothelial cells, we also examined CD34 expression in scleroderma tissues to make sure that the cells positive for TSP-1 between collagen bundles were not endothelial cells. As shown in Figure 1C, CD34 expression between collagen bundles was almost negative. In addition, CD68 expression could not be detected (Figure 1D), suggesting that the TSP-1-positive cells were not macrophages. Moreover, the cells positive for anti-TSP-1 Ab were also stained with anti-Vimentin Ab and anti-SMA Ab (data not shown). These results imply that the cells positive for TSP-1 between collagen bundles seem to be fibroblasts.

Comparison of Levels of Type I Procollagen Expression, Smad3 Phosphorylation and TSP-1 Expression between Cultured Normal and Scleroderma Dermal Fibroblasts

We compared the levels of type I procollagen protein, Smad3 phosphorylation (p-Smad3) and TSP-1 protein between normal and scleroderma fibroblasts by immunoblotting. Consistent with a previous report,²² scleroderma fibroblasts expressed increased levels of type I procollagen protein and p-Smad3 (2.1-fold and 2.7-fold, respectively. Figure 2A). The two bands which represented $\alpha 1(I)$ procollagen and $\alpha 2(I)$ procollagen in this immunoblotting showed that the ratio of the $\alpha 1$ to $\alpha 2$ chain was 0.25:1 or 1:1 rather than the expected 2:1. We previously demonstrated that the altered ratio of the $\alpha 1$ to $\alpha 2$ chain in this immunoblotting assay is attributed to the difference in the immunoreactivity of anti-type I collagen Ab to the $\alpha 1$ and $\alpha 2$ chain rather than to the lack of ascorbic acid in the conditioned medium.³⁶

The expression of TSP-1 protein was also up-regulated in scleroderma fibroblasts (2.4-fold). Next, we determined TSP-1 mRNA expression by Northern blotting. The expression of TSP-1 mRNA as well as that of $\alpha 2(I)$ collagen mRNA was up-regulated in scleroderma fibroblasts (2.3-fold and 2.1-fold, respectively. Figure 2B).

Constitutive Overexpression of TSP-1 Most Likely Depends on Autocrine TGF- β Signaling in Scleroderma Fibroblasts

To examine the possibility that the up-regulated expression of TSP-1 in scleroderma fibroblasts is due to the stimulation by autocrine TGF- β , we first investigated the effect of the anti-TGF- β blocking Ab on the expression of TSP-1 protein and mRNA. The anti-TGF- β Ab had little effect on the protein or mRNA expression in normal fibroblasts. In contrast, anti-TGF- β Ab significantly reduced the expression of TSP-1 protein and mRNA in scleroderma fibroblasts (Figure 3, A and B). The reduced levels were slightly higher than those of normal fibroblast although the difference was not statistically significant. The treatment with a preimmune rabbit IgG had no effect on the expression of TSP-1 protein and mRNA. In addition, we used TGF- $\beta 1$ antisense oligonucleotide to inhibit endogenous TGF- $\beta 1$ synthesis. The antisense oligonucleotide had little effect on TSP-1 protein or mRNA expression in normal fibroblasts (Figure 3, A and B). In contrast, treatment with the TGF- $\beta 1$ antisense oligonucleotide markedly reduced the expression of TSP-1 protein and mRNA significantly in scleroderma fibroblasts. The reduced levels were slightly higher than those of normal fibroblast although the difference was not statistically significant. The sense oligonucleotide had no such inhibitory effects. These results imply that the up-regulated expression of TSP-1 in scleroderma fibroblasts depends mainly on the stimulation by autocrine TGF- β .

Figure 2. Comparison of levels of type I procollagen expression, p-Smad3 and TSP-1 expression between cultured normal and scleroderma dermal fibroblasts. **A:** For type I procollagen and TSP-1, culture media were electrophoresed through a 10% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with anti-type I collagen and anti-TSP-1 Ab. Levels of β -actin are shown as a loading control. For p-Smad3, whole cell lysates were subjected to immunoprecipitation using anti-Smad3 Ab, and p-Smad3 was detected by immunoblotting using anti-phosphoserine Ab. The same membrane was stripped and total levels of Smad3 are determined using Smad2/3 Ab. Total Smad3 levels can be appropriately regarded as a loading control. Four separate lanes for normal and scleroderma fibroblast cultures represent 4 separate primary isolates from different individuals. The levels quantitated by scanning densitometry are shown relative to the levels in normal fibroblasts (1.0). Data are expressed as the mean $\pm$ SD of five independent experiments (**bottom**). * $P < 0.05$, as compared with the value in normal fibroblasts. **B:** Northern blot analysis was performed to evaluate mRNA expression in normal and scleroderma fibroblasts. Four separate lanes for normal and scleroderma fibroblast cultures represent 4 separate primary isolates from different individuals. One result representative of five independent experiments is shown at the **top**. $\alpha 2(I)$ collagen and TSP-1 mRNA levels quantitated by scanning densitometry and corrected for the levels of GAPDH in the same samples are shown relative to the level in normal fibroblasts (1.0). Data are expressed as the mean $\pm$ SD of five independent experiments (**bottom**). * $P < 0.05$, as compared with the value in normal fibroblasts.

Figure 3. Effects of anti-TGF- β Ab and TGF- β 1 antisense oligonucleotide on TSP-1 protein and mRNA expression in normal and scleroderma fibroblasts. **A:** The effects of anti-TGF- β Ab or TGF- β 1 antisense oligonucleotide on TSP-1 protein expression were investigated by immunoblotting (**top**). Normal fibroblasts and scleroderma fibroblasts were treated with anti-TGF- β antibody or TGF- β 1 antisense oligonucleotide for 48 hours. One result representative of five independent experiments is shown. The levels quantitated by scanning densitometry are shown relative to those in normal fibroblasts treated with preimmune serum or sense oligonucleotide (1.0). Data are expressed as the mean $\pm$ SD of five independent experiments (**bottom**). * P < 0.05 versus control cells treated with TGF- β 1 sense oligonucleotide or preimmune IgG. **B:** The effects of anti-TGF- β blocking Ab and TGF- β 1 antisense oligonucleotide on TSP-1 mRNA levels were investigated by Northern blotting (**top**). Normal and scleroderma fibroblasts were treated with anti-TGF- β blocking Ab or TGF- β 1 antisense oligonucleotide for 48 hours. One result representative of five independent experiments is shown. The mRNA levels quantitated by scanning densitometry are shown relative to those in normal fibroblasts treated with preimmune serum or sense oligonucleotide (1.0). Data are expressed as the mean $\pm$ SD of five independent experiments (**bottom**). * P < 0.05 versus control cells treated with TGF- β 1 sense oligonucleotide or preimmune IgG.

Exogenous Active TGF- β 1 Increases Both TSP-1 Protein and mRNA Levels in Normal Fibroblasts

If autocrine TGF- β stimulation is one of the main causes of the constitutive up-regulation of TSP-1 in scleroderma fibroblasts, exogenous active TGF- β 1 may increase TSP-1 expression in normal fibroblasts. To test this, we investigated the effect of exogenous active TGF- β 1 on the expression of TSP-1 protein and mRNA. Normal fibroblasts were cultured until they were confluent, and then incubated for an additional 24 hours under condition of serum starvation. Cells were subsequently incubated for

Figure 4. Exogenous active TGF- β 1 increases both TSP-1 protein and mRNA in normal fibroblasts. Human dermal fibroblasts were serum-starved for 24 hours and incubated in the absence or presence of the indicated doses of TGF- β , or for the indicated time in the presence of 2 ng/ml TGF- β . **A (I)** and **B (I)**: Medium (adjusted based on the protein concentration of the cell lysate) was subjected to immunoblotting with anti-TSP-1 Ab (**top**). Levels of β -actin are shown as a loading control. TSP-1 protein levels quantitated by scanning densitometry and corrected for the levels of β -actin in the same samples are shown relative to the levels in untreated cells (1.0). Data are expressed as the mean $\pm$ SD of five independent experiments (**bottom**). * P < 0.05, as compared with the value in untreated cells. **A (II)** and **B (II)**: Northern blot analysis was performed to evaluate TSP-1 mRNA expression (**top**). Levels of GAPDH mRNA are shown as a loading control. TSP-1 mRNA levels quantitated by scanning densitometry and corrected for the levels of GAPDH in the same samples are shown relative to the levels in untreated cells (1.0). Data are expressed as the mean $\pm$ SD of five independent experiments (**bottom**). * P < 0.05, as compared with the value in untreated cells.

24 hours in the presence or absence of the indicated doses of active TGF- β 1. The TSP-1 protein expression was elevated maximally (2.2-fold) by the treatment with 2 ng/ml TGF- β 1, but was less elevated in response to treatment with higher concentrations of TGF- β (Figure 4A, left). In addition, the level of TSP-1 mRNA expression was also elevated maximally (2.5-fold) by the treatment with 2 ng/ml TGF- β (Figure 4A, right). Next, to examine the time-dependency of the effect of TGF- β on the expression of TSP-1, cells were incubated for the indicated period in the presence or absence of 2 ng/ml TGF- β . The TSP-1 protein expression was slightly elevated after the stimulation with TGF- β for 6 hours and maximally increased (2.7-fold) after 48 hours in comparison with the level in control cells (Figure 4B, left). To determine whether the TGF- β -mediated induction of TSP-1 protein expression was correlated with an increase of TSP-1 mRNA, human dermal fibroblasts were incubated in the

Figure 5. The TSP-1 mRNA stability and promoter activity in cultured normal and scleroderma fibroblasts. **A:** Normal fibroblasts or scleroderma fibroblasts were serum-starved for 24 hours and incubated in the absence or presence of 2 ng/ml TGF- β for 6 hours, as well as pretreated with 10 μ g/ml anti-TGF- β Ab, before the addition of 400 ng/ml actinomycin D. Northern blot analysis was performed to evaluate TSP-1 mRNA expression in untreated normal fibroblasts, normal fibroblasts treated with 2 ng/ml TGF- β 1, untreated scleroderma fibroblasts and scleroderma fibroblasts treated with 10 μ g/ml anti-TGF- β Ab. Levels of GAPDH mRNA are shown as a loading control. One result representative of five independent experiments is shown. **B:** Time-dependent degradation of TSP-1 mRNA in untreated normal fibroblasts, normal fibroblasts treated with 2 ng/ml TGF- β 1, untreated scleroderma fibroblasts and scleroderma fibroblasts treated with 10 μ g/ml anti-TGF- β Ab. The filled circles with a solid line indicate untreated normal fibroblasts. The filled squares with a dashed line indicate normal fibroblasts treated with exogenous 2 ng/ml TGF- β 1. The open circles with an irregularly dashed line indicate untreated scleroderma fibroblasts. The open squares with a finely dashed line indicate scleroderma fibroblasts treated with 10 μ g/ml anti-TGF- β Ab. * P < 0.05, as compared with the value in untreated normal fibroblasts. TGF1 antisense, TGF- β 1 antisense oligonucleotide. **C:** Normal and scleroderma fibroblasts were transiently transfected with the TSP-1 promoter-lux (-2200/+754). After incubation for 24 hours, the cells were stimulated with 2 ng/ml TGF- β 1 for an additional 18 hours. The luciferase activity was determined as the fold increase relative to that in untreated normal fibroblasts (1.0). Data are expressed as the mean $\pm$ SD of five independent experiments.

presence or absence of 2 ng/ml TGF- β under the same conditions, and mRNA expression was analyzed by Northern blotting. The expression of TSP-1 mRNA was elevated after the stimulation with TGF- β for 6 hours, and maximally increased (2.6-fold) after 24 hours in comparison with the control level (Figure 4B, right). Thus, the effect of TGF- β on the expression of TSP-1 protein paralleled that on TSP-1 mRNA.

Mechanism of Constitutive TSP-1 Overexpression in Scleroderma Fibroblasts Is Similar to That of TSP-1 Induction Observed in Normal Fibroblasts Stimulated with Exogenous TGF- β 1

To determine whether the induction of TSP-1 mRNA expression by exogenous TGF- β 1 in normal fibroblasts

and/or the up-regulation of TSP-1 mRNA in scleroderma fibroblasts was due to increased synthesis or stability of the mRNA, we examined the stability and promoter activity in both normal and scleroderma fibroblasts. Cells were incubated in the presence or absence of 2 ng/ml TGF- β 1 for 6 hours and added with actinomycin D for 4, 8, or 12 hours before RNA extraction, and the level of remaining TSP-1 mRNA was determined by Northern blotting. As shown in Figure 5, A and B, the stability of TSP-1 mRNA was significantly enhanced both in normal fibroblasts treated with exogenous TGF- β 1 and in untreated scleroderma fibroblasts, compared to untreated normal fibroblasts. Anti-TGF- β blocking antibody markedly reduced this improved TSP-1 mRNA stability in scleroderma fibroblasts. No significant increase in the stability of TSP-1 was observed in scleroderma fibroblasts treated with exogenous TGF- β 1 and no significant decrease in stability was observed in normal fibroblasts

treated with anti-TGF- β blocking Ab (data not shown). We investigated whether TGF- β 1-mediated TSP-1 induction in normal fibroblasts and/or the constitutive overexpression of TSP-1 in scleroderma fibroblasts is also regulated at the level of transcription. We used TSP-1 promoter-Lux (–2200/+754) to determine whether exogenous TGF- β 1 induces TSP-1 gene expression at the transcriptional level. This TSP-1 promoter contains regions within the 5'-flanking sequence and intron 1 that have been shown previously to be necessary for maximal expression of the TSP-1 gene in COS-1 and NIH3T3 cells.³⁷ The TSP-1 promoter activity in normal fibroblasts treated with TGF- β (2 ng/ml) was not significantly different from that observed in untreated normal fibroblasts (Figure 5C). In addition, the basal TSP-1 promoter activity in scleroderma fibroblasts was not significantly different from that in normal fibroblasts. These results indicate that the expression of TSP-1 is up-regulated in scleroderma fibroblasts at least partially at the post-transcriptional level just like in normal fibroblasts stimulated with exogenous TGF- β 1.

Constitutive TSP-1 Overexpression Might Contribute to the Up-Regulated $\alpha 2(I)$ Collagen Expression and p-Smad3 Levels in Scleroderma Fibroblasts

To examine the possibility that the up-regulated $\alpha 2(I)$ collagen gene expression in scleroderma fibroblasts is mediated by the constitutively overexpressed TSP-1, we investigated the effect of TSP-1 blocking peptide on the expression of type I procollagen protein and $\alpha 2(I)$ collagen mRNA. The treatment with TSP-1 blocking peptide

Figure 6. Effects of TSP-1 blocking peptide and TSP-1 antisense oligonucleotide on type I procollagen protein, $\alpha 2(I)$ collagen mRNA expression and Smad3 phosphorylation in normal and scleroderma fibroblasts. **A/B:** The effects of TSP-1 blocking peptide, TSP-1 antisense oligonucleotide and anti-TSP-1 blocking Ab on type I procollagen protein expression were investigated by immunoblotting (top). Normal fibroblasts and scleroderma fibroblasts were treated with TSP-1 blocking peptide for 96 hours or TSP-1 antisense oligonucleotide/anti-TSP-1 blocking Ab for 48 hours. TSP-1 protein levels in the media are also presented in **A** to show the efficacy of the TSP-1 antisense oligonucleotide. One result representative of five independent experiments is shown. The levels quantitated by scanning densitometry are shown relative to those in normal fibroblasts treated with a control peptide or sense oligonucleotide (1.0). Data are expressed as the mean $\pm$ SD of five independent experiments (bottom). * $P < 0.05$ versus control cells treated with control peptide or TSP-1 sense oligonucleotide. **C:** The effect of TSP-1 blocking peptide and TSP-1 antisense oligonucleotide on the $\alpha 2(I)$ collagen mRNA levels was investigated by Northern blotting (top). Normal and scleroderma fibroblasts were treated with TSP-1 blocking peptide for 96 hours or TSP-1 antisense oligonucleotide for 48 hours. TSP-1 mRNA levels are also shown. One result representative of five independent experiments is shown. The mRNA levels quantitated by scanning densitometry are shown relative to those in normal fibroblasts treated with a control peptide or sense oligonucleotide (1.0). Data are expressed as the mean $\pm$ SD of five independent experiments (bottom). * $P < 0.05$ versus control cells treated with a control peptide or TSP-1 sense oligonucleotide. **D:** The effect of TSP-1 blocking peptide or TSP-1 antisense oligonucleotide on Smad3 phosphorylation level (top). Normal and scleroderma fibroblasts were treated with TSP-1 blocking peptide for 96 hours or TSP-1 antisense oligonucleotide for 48 hours. One result representative of five independent experiments is shown. The levels quantitated by scanning densitometry are shown relative to those in normal fibroblasts treated with a control peptide or sense oligonucleotide (1.0). Data are expressed as the mean $\pm$ SD of five independent experiments (bottom). * $P < 0.05$ versus cells treated with a control peptide or TSP-1 sense oligonucleotide.

had little effect on the expression of type I procollagen protein or $\alpha 2(I)$ collagen mRNA in normal fibroblasts. In contrast, the peptide significantly reduced the expression levels of type I procollagen protein and $\alpha 2(I)$ collagen mRNA in scleroderma fibroblasts, although the reduced levels were still higher than the basal levels in normal fibroblasts (Figure 6, A and C). The treatment with a control peptide had no such effect. Next, we used TSP-1 antisense oligonucleotide to inhibit endogenous TSP-1 synthesis. As shown in Figure 6, A and C, TSP-1 antisense oligonucleotide had little effect on type I procollagen protein and $\alpha 2(I)$ collagen mRNA expression in normal fibroblasts. In contrast, this antisense oligonucleotide caused a remarkable decrease in type I procollagen protein and $\alpha 2(I)$ collagen mRNA expression in scleroderma fibroblasts, although the decreased levels were still higher than the basal levels in normal fibroblasts. The TSP-1 sense oligonucleotide had no such inhibitory effect. In addition, the treatment with anti-TSP-1 blocking Ab also had an inhibitory effect on the expression levels of type I procollagen protein in scleroderma fibroblasts (Figure 6B). The treatment markedly reduced $\alpha 2(I)$ collagen mRNA and p-Smad3 levels in scleroderma fibroblasts (data not shown).

Likewise, the treatment with TSP-1 blocking peptide and TSP-1 antisense oligonucleotide significantly reduced the constitutively up-regulated p-Smad3 level in scleroderma fibroblasts but had little effect on basal levels in normal fibroblasts (Figure 6D). These results suggest that the up-regulated type I collagen and p-Smad3 levels in scleroderma fibroblasts at least partially depend on the overexpression of TSP-1.

Transient Overexpression of TSP-1 Increased the Levels of Type I Procollagen Protein, $\alpha 2(I)$ Collagen Promoter Activity and p-Smad3 in Normal Fibroblasts

If the constitutive overexpression of TSP-1 is one of the main causes of the constitutive up-regulation of type I collagen and p-Smad3 expression in scleroderma fibroblasts, the transient overexpression of TSP-1 should increase type I collagen and p-Smad3 levels in normal fibroblasts. To confirm this, we investigated whether transient overexpression of TSP-1 affects the levels of type I procollagen protein in normal fibroblasts. As shown in Figure 7A, the transient overexpression of TSP-1 increased the levels of type I procollagen protein in a dose-dependent manner (maximally 1.9-fold). This increase was almost completely inhibited by pretreatment with the TGF- $\beta 1$ antisense oligonucleotide for 48 hours before the transfection. This inhibitory effect of the antisense oligonucleotide was also dose-dependent. In contrast, the overexpression of TSP-1 had little effect on the expression of type I procollagen protein in scleroderma fibroblasts. Next, we examined whether the transient overexpression of TSP-1 can also increase the $\alpha 2(I)$ collagen promoter activity. As shown in Figure 7B, the transient overexpression of TSP-1 significantly increased

the promoter activity of the $\alpha 2(I)$ collagen promoter gene (2.8-fold), although the increased activity was not as high as that observed in TGF- $\beta 1$ (2 ng/ml)-treated normal fibroblasts or untreated scleroderma fibroblasts. To identify the potential regulatory elements in the COL1A2, a series of 5' deletions of the COL1A2/CAT construct were used. As shown in Figure 7B, the -773 and the -353 COL1A2/CAT constructs responded at almost the same levels as the -3500 construct to the TSP-1 overexpression. However, the deletion up to bp -186 led to a significant reduction in the level of inducibility brought to the transient overexpression of TSP-1, although a minimal up-regulation of the promoter activity was still observed. This result suggests that Smad3 might be involved in the up-regulation of the $\alpha 2(I)$ collagen gene expression in the cells transiently overexpressing TSP-1 since the potential Smad3-binding site is located between bp -263 and -258. Moreover, we introduced substitution mutations into a Smad3-binding site (CAGACA was changed to TACATA, Smad-M), using the -353 COL1A2/CAT construct and performed CAT assays using these constructs. As shown in Figure 7B, the levels of inducibility brought by the transient TSP-1 overexpression were significantly decreased in the Smad-M construct compared with the -353 construct, although they were not completely abolished. Scleroderma fibroblasts exhibited almost the same pattern of promoter activity induction as observed in the normal fibroblasts transiently overexpressing TSP-1. These results suggest that Smad3 is at least partially involved in the up-regulation of $\alpha 2(I)$ collagen gene expression in the transient TSP-1 overexpressing cells as well as in scleroderma fibroblasts. Consistent with these results, p-Smad3 levels were also increased by the transient overexpression of TSP-1 in normal fibroblasts, an effect which was abrogated by the pretreatment with TGF- $\beta 1$ antisense oligonucleotide (Figure 7C). In contrast, the overexpression of TSP-1 had very little effect on p-Smad3 levels in scleroderma fibroblasts (data not shown). These results indicate that the effect of the overexpression on $\alpha 2(I)$ collagen expression in normal fibroblasts is likely to be through the TGF- β signaling. However, considering that the stimulatory effects of transiently overexpressed TSP-1 on the promoter activity of the $\alpha 2(I)$ collagen gene were still observed when the Smad3-binding site was missing or mutated although they were significantly reduced, there seemed to be some possibility that other signal pathways, transcription factors or regulatory elements were involved in the up-regulation of $\alpha 2(I)$ collagen expression in the cells transiently overexpressing TSP-1 and scleroderma fibroblasts.

Exogenous Latent TGF- $\beta 1$ Induced the COL1A2 Promoter Activity More Potently in the TSP-1-Overexpressing Cells Than in the Control Cells

To confirm that the up-regulation of $\alpha 2(I)$ collagen promoter activity by the overexpression of TSP-1 is due to the activation of latent TGF- $\beta 1$ by TSP-1, we examined the effect of exogenous latent TGF- $\beta 1$ on the -774 COL1A2/CAT promoter activity in the cells overexpress-

ing TSP-1. To suppress the endogenous TGF- β 1 synthesis, cells were pretreated with 10 μ M TGF- β 1 antisense oligonucleotide for 48 hours before the transfection. As shown in Figure 8A, the antisense oligonucleotide almost completely abolished the increase in α 2(I)

collagen promoter activity brought about by the transiently overexpressed TSP-1, while it had little effect in the controls. The addition of exogenous active TGF- β 1 increased the promoter activity significantly with or without the transient overexpression of TSP-1 (4.5- and 4.6-fold, respectively). The addition of exogenous latent TGF- β 1 increased the promoter activity significantly in the TSP-1-overexpressing cells in a dose-dependent manner (maximally 3.6-fold), while the same treatment resulted in only a small increase in the controls. In the presence of TSP-1 blocking peptide, the effect of latent TGF- β 1 in the cells overexpressing TSP-1 was diminished almost completely. These results indicate that the up-regulated α 2(I) collagen promoter activity in the cells transiently overexpressing TSP-1 is mediated by the activation of latent TGF- β 1 by TSP-1. Next, we confirmed the stimulatory effect of the overexpression of TSP-1 on TGF- β signaling using p3TP-Lux which is a TGF- β -responsive reporter gene. As shown in Figure 8B, the addition of exogenous latent TGF- β 1 increased the luciferase activity of p3TP-Lux significantly in the TSP-1-overexpressing cells in a dose-dependent manner (maximally 5.9-fold), while the same treatment caused little increase in the controls. Taken together, these results suggest that transient TSP-1 overexpression stimulates TGF- β signaling by the activation of latent TGF- β 1, which leads to the induction of α 2(I) collagen promoter activity.

Discussion

Studies show that TGF- β is a profibrotic cytokine, and that the autocrine TGF- β loop may play an important part in the pathogenesis of scleroderma. However, TGF- β is secreted as a latent complex, which must be activated before it can bind to its receptors.³⁸ The activation of TGF- β in the extracellular microenvironment is likely to be

Figure 7. Effects of transient TSP-1 overexpression on the levels of type I procollagen expression, and Smad3 phosphorylation in normal fibroblasts. **A:** Type I procollagen protein expression in the culture medium of normal or scleroderma fibroblasts transfected with the indicated doses of empty vector or TSP-1 expression plasmid, as well as pre-treated with TGF- β 1 sense or antisense oligonucleotide 24 hours before the transfection. The medium (adjusted based on the protein concentration of cell lysates) was subjected to immunoblotting with anti-type I collagen Ab. TSP-1 protein levels in the medium are also shown. One representative result of five independent experiments is shown at the **top**. The levels quantitated by scanning densitometry are shown relative to those in untreated normal fibroblasts (1.0). Data are expressed as the mean $\pm$ SD of five independent experiments (**bottom**). * P < 0.05, as compared with the value in empty vector-transfected fibroblasts pre-treated with TGF- β 1 sense oligonucleotide. **B:** The effects of TSP-1 overexpression on the α 2(I) collagen promoter activity. Normal or scleroderma fibroblasts were co-transfected with 3 μ g of TSP-1 expression plasmid or empty vector, and 2 μ g of various deleted or mutated COL1A2/CAT constructs. The mean $\pm$ SD from five separate experiments are shown. * P < 0.05, as compared with the fold-increase relative to the control (vector-transfected normal fibroblasts) on transfection with -3500 COL1A2/CAT constructs. **C:** Smad3 phosphorylation levels in normal fibroblasts transfected with indicated doses of empty vector or TSP-1 expression vector, as well as pretreated with TGF- β 1 sense or antisense oligonucleotide (**top**). The transfected cells were also stimulated with exogenous 2 μ g/ml of TGF- β for 1 hour before protein extraction (**lanes 4 and 5**). One result representative of five independent experiments is shown. The levels quantitated by scanning densitometry are shown relative to those in empty vector-transfected fibroblasts pre-treated with TGF- β 1 sense oligonucleotide (1.0). Data are expressed as the mean $\pm$ SD of five independent experiments (**bottom**). * P < 0.05 as compared with the value in empty vector-transfected fibroblasts pre-treated with TGF- β 1 sense oligonucleotide.

Figure 8. Exogenous latent TGF- β 1 induced the COL1A2 promoter activity more potently in the TSP-1-overexpressing fibroblasts than control fibroblasts. **A:** Normal fibroblasts were cultured in MEM containing 10 μ mol/L TGF- β 1 antisense oligonucleotide (antisense oligo) for 48 hours. Then, the cells were co-transfected with 3 μ g of empty vector or TSP-1 expression vector and 2 μ g of -773 COL1A2-Lux construct. The fibroblasts were stimulated with the indicated concentrations of TGF- β 1 or latent TGF- β 1 for the last 24 hours, and the luciferase activity was determined. In some experiments, latent TGF- β 1 stimulation was performed in the presence of TSP-1 blocking peptide (1 μ mol/L). The mean $\pm$ SD from five separate experiments are shown. * P < 0.05 versus control cells treated with 10 μ mol/L TGF- β 1 sense oligonucleotide. L-TGF- β 1 represents latent TGF- β 1. **B:** Normal fibroblasts were cultured in MEM containing 10 μ mol/L TGF- β 1 antisense oligonucleotide (antisense oligo) for 48 hours. Then, the cells were co-transfected with 3 μ g of empty vector or TSP-1 expression vector and 2 μ g of p3TP-Lux construct. The fibroblasts were stimulated with the indicated concentrations of TGF- β 1 or latent TGF- β 1 for the last 24 hours, and the luciferase activity was determined. In some experiments, latent TGF- β 1 stimulation was performed in the presence of TSP-1 blocking peptide (1 μ mol/L). The mean $\pm$ SD from 5 separate experiments are shown. * P < 0.05 versus control cells treated with 10 μ mol/L TGF- β 1 sense oligonucleotide. L-TGF- β 1 represents latent TGF- β 1.

involved in the pathogenesis of scleroderma, since previous reports showed that scleroderma fibroblasts of the affected area do not secrete increased levels of TGF- β 1, although autocrine TGF- β signaling may contribute to the accumulation of ECM observed in scleroderma.^{22,39} We hypothesized that TSP-1 plays a substantial role in the activation of TGF- β in scleroderma fibroblasts for the following reasons: 1) the significance of TSP-1 has been demonstrated in other fibrotic disorders;²⁰ 2) serum levels of TSP-1 were reported to be increased in scleroderma patients; and 3) scleroderma patients often develop refractory skin ulcers, which were also observed in TSP-1 transgenic mice.³¹

First, we showed that TSP-1 expression was increased in cultured scleroderma fibroblasts as well as in scleroderma tissue sections. We concluded that the constitutive overexpression of TSP-1 in scleroderma fibroblasts possibly depends on stimulation by autocrine TGF- β based on two results obtained. First, eliminating the autocrine TGF- β loop led to a down-regulation of the TSP-1 expression in scleroderma fibroblasts. Second, exogenous TGF- β 1 increased TSP-1 expression in normal fibroblasts. We previously reported that the overexpression of α 2(I)collagen, which is another component of the ECM overproduced by scleroderma fibroblasts, also depends on an autocrine TGF- β loop.²² An outstanding characteristic of TSP-1 that α 2(I)collagen does not have is the ability to activate latent TGF- β . Based on findings, we hypothesized that constitutively overexpressed TSP-1 is involved in the formation of an autocrine TGF- β loop and that this autocrine TSP-1-TGF- β positive feedback loop causes scleroderma fibroblasts to overproduce other components of ECM such as type I collagen. To establish this hypothesis, we investigated whether the constitutive overexpression of TSP-1 makes a contribution to the maintenance of the autocrine TGF- β loop in scleroderma fibroblasts. Both the suppression of endogenous TSP-1 production and the blockade of secreted TSP-1 to activate latent TGF- β could down-regulate p-Smad3 as well as α 2(I)collagen expression in scleroderma fibroblasts. These treatments had little effect on normal fibroblasts. Conversely transient TSP-1 overexpression led to an increase in p-Smad3 as well as type I collagen protein in normal fibroblasts, but not in scleroderma fibroblasts which are in an activated state due to autocrine TGF- β signaling and constitutively overexpress TSP-1. Transiently overexpressed TSP-1 also had stimulatory effects on the promoter activity of the α 2(I)collagen gene in normal fibroblasts, although the effects were significantly reduced when the Smad3-binding site was missing or mutated. In addition, the effects of transiently overexpressed TSP-1 were abolished by the suppression of endogenous TGF- β 1 synthesis, which indicated that endogenous TGF- β production was required for the action of TSP-1. These results combined support the hypothesis described above.

Mori et al⁴⁰ reported that anti-TGF- β Ab could not alter the activated state of Smad 3 observed in scleroderma fibroblasts, which seems to be inconsistent with our finding that the TSP-1 antisense oligonucleotide and blocking peptide decreased the elevated levels of p-Smad3 in

scleroderma fibroblasts. This inconsistency may be partly due to the difference of the methods. We examined the effect of anti-TGF- β Ab by determining p-Smad3 levels in whole cell lysates, whereas they focused on the distribution of p-Smad2/3. Holmes et al^{41,42} showed that elevated levels of CTGF in scleroderma fibroblasts, which seem to be associated with the deposition of matrix, are independent of Smads or TGF- β signaling. Their result does not coincide with our result that the promoter activity of the $\alpha 2(I)$ collagen gene in scleroderma fibroblasts was significantly reduced when the Smad3-binding site was missing or mutated. A possible explanation for this discrepancy may be that the phenotype of the scleroderma fibroblasts we obtained could be regarded as that of the leading edge fibroblasts since we obtained them from the forearms of rapidly worsening SSc patients, while they obtained samples from lesional areas of SSc patients.

That the autocrine TSP-1-TGF- β positive feedback loop causes scleroderma fibroblasts to overproduce components of the ECM is a novel notion which helps to explain the mechanism of abnormal phenotypes of scleroderma fibroblasts. However, this notion based on our present findings cannot explain all abnormal features of scleroderma fibroblasts. The result that the blockade of endogenous TSP-1 synthesis did not completely normalize the up-regulated $\alpha 2(I)$ collagen gene expression or increased Smad3 phosphorylation in scleroderma fibroblasts, and that the transient overexpression of TSP-1 in normal fibroblasts could not make $\alpha 2(I)$ collagen expression or phosphorylated Smad3 levels as high as those in scleroderma fibroblasts or exogenously TGF- β 1-stimulated cells indicated that TSP-1 is just partly responsible for these abnormal up-regulations. Therefore, several other molecules may be required for the full activation of TGF- β .

Some reports show that TSP-1 increased active TGF- β levels in the culture media of fibroblasts or other cells, which were measured by ELISA or some other method.^{43–45} However, we previously reported that active TGF- β levels in the culture media of scleroderma fibroblasts, which were also measured by ELISA, were as high as those of normal fibroblasts.²² Considering that scleroderma fibroblasts have been reported to overexpress TGF- β receptors^{22,23} and that inhibition of the function of TSP-1 leads to down-regulation of TGF- β signaling in scleroderma fibroblasts, it might be reasonable to assume that up-regulated autocrine TGF- β signaling in scleroderma fibroblasts are maintained partly by elevated sensitivity to TGF- β via the overexpressed TGF- β receptors and partly by overexpressed TSP-1 activating latent TGF- β . The reason why active TGF- β levels in the culture media of scleroderma fibroblasts are about the same as those of normal fibroblasts can be explained by the speculation that scleroderma fibroblasts bind and consume more active TGF- β than normal fibroblasts, resulting in less amounts of active TGF- β left in the media. The change of total TGF- β levels will not be detected even if more active TGF- β is consumed, because only a small portion of total TGF- β is likely to be consumed by the cells.²² In contrast to SSc fibroblasts, the consumption of TGF- β by the cells transiently overexpressing

TSP-1 is likely to be about the same as that of normal cells. Therefore, the concentration of active TGF- β in the media of the cells transiently overexpressing TSP-1 can be higher than that of normal cells. It seems to be very difficult to determine which of the two factors, elevated sensitivity to active TGF- β and overexpressed TSP-1, is more important for the maintenance of up-regulated autocrine TGF- β signaling in scleroderma fibroblasts because the two factors work cooperatively in a complex and continuous way. Further study will be necessary for the complete understanding of the mechanism for the maintenance of up-regulated autocrine TGF- β signaling in scleroderma fibroblasts.

In conclusion, we demonstrated that the constitutive overexpression of TSP-1 in scleroderma fibroblasts might play an important role in the maintenance of autocrine TGF- β signaling.

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