Tissue Inhibitor of Metalloproteinase 1 (TIMP-1) May Be an Autocrine Growth Factor in Scleroderma Fibroblasts

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In scleroderma (systemic sclerosis, SSc), an autoimmune disorder in which excessive extracellular matrix is deposited in skin and internal organs, one of the suggested contributory factors to the development of fibrosis is a decrease in collagenase activity that may be related to levels of serum tissue inhibitors of metalloproteinases 1 (TIMP-1). We recently reported that the serum TIMP-1 levels in SSc patients were elevated compared with normal controls. To determine the biologic significance of TIMP-1 in SSc, we compared the proliferative effects of TIMP-1 between normal and SSc fibroblasts. TIMP-1 showed significant mitogenic activity for both normal and SSc fibroblasts. The mitogenic responses to TIMP-1 (33–100 ng/ml) in SSc fibroblasts, however, were significantly greater than those in normal controls and were completely neutralized in the presence of anti-TIMP-1 IgG. Moreover, anti-TIMP-1 IgG partially but significantly blocked the basal mitogenic activities of SSc fibroblasts. SSc fibroblasts produced increased amounts of TIMP-1 relative to normal fibroblasts, as confirmed by western blotting, ELISA, and RT-PCR techniques. In contrast, transforming growth factor β1 (TGF-β1) upregulated TIMP-1 production in normal fibroblasts but not in SSc fibroblasts with elevated spontaneous secretion of TIMP-1. These observations suggest that TIMP-1 may play an important role as an autocrine growth factor in the fibrotic process in SSc. ***Key words: systemic sclerosis/transforming growth factor β. J Invest Dermatol 108:281–284, 1997***

In scleroderma (systemic sclerosis, SSc), development of fibrosis is prominent in the skin and various organs. In general, tissue fibrosis reflects an imbalance between collagen production and degradation. In several studies, fibroblasts grown from SSc patients have revealed increased synthesis of collagen (LeRoy, 1974; Uitto *et al*, 1979; Fleischmajer *et al*, 1981). Although collagenase activity and collagen degradation have been reported to be normal in SSc fibroblasts (Uitto *et al*, 1979), Takeda *et al* (1994) reported that SSc fibroblasts exhibited a marked decrease in collagenase activity and production. Collagenase activity is regulated by various enzymes and cytokines (Cawston, 1986). Tissue inhibitor of metalloproteinase 1 (TIMP-1) is capable of inactivating collagenase activity. In accordance with this finding, two groups have recently reported enhanced expression of TIMP-1 in scleroderma fibroblasts (Bou-Gharios *et al*, 1995; Kirk *et al*, 1995). We have also detected elevated serum TIMP-1 concentrations in SSc patients, suggesting the overproduction of TIMP-1 *in vivo* in these patients (Kikuchi *et al*, 1995b). Interestingly, TIMP-1 has been shown to have mitogenic activities for various cell types including dermal fibroblasts (Hayakawa *et al*, 1992). Taken together, these observations suggest that TIMP-1 might be involved not only in the collagen accumulation but also in the fibroblast proliferation in SSc. We investigated the mitogenic responses to TIMP-1 in SSc fibroblasts and their TIMP-1 expression.

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Abbreviations: TIMP-1, tissue inhibitor of metalloproteinase 1; SSc, systemic sclerosis; SFM, serum-free medium.

MATERIALS AND METHODS

Cell Cultures SSc fibroblasts were obtained by skin biopsy from affected forearm skin of seven female Japanese patients with diffuse cutaneous SSc with less than a 2-y duration of skin thickening who fulfilled the American Rheumatism Association criteria for diagnosis (American Rheumatism Association, 1980). Control normal strains of fibroblasts (NS fibroblasts) were obtained from healthy donors matched with each SSc patient according to sex, age, and biopsy site and were processed in parallel. The cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Cells at the third or fourth passage were used for experiments. Incubation was performed in T-75 flasks at 37°C in 95% air and 5% CO_2.

Reagents Recombinant human TIMP-1 and monoclonal anti-human TIMP-1 IgG were purchased from Fuji Chemical (Takaoka, Japan). Recombinant transforming growth factor β1 (TGF-β1) was purchased from R & D Systems (Minneapolis, MN).

Growth Assays ([^{3}H]Thymidine Incorporation Assays) SSc and NS fibroblasts (2×10^4 cells per well) were placed in 24-well plates in MEM with 10% FBS and grown to confluency. The cells were washed with phosphate-buffered saline once followed by a 24-h incubation in MEM with 0.1% bovine serum albumin (serum-free medium; SFM). The cells were then incubated with various concentrations of TIMP-1 in the presence or absence of anti-TIMP-1 IgG for 24 h in SFM, labeled with [^{3}H]thymidine (final concentration 1 μCi/ml; New England Nuclear, Boston, MA) for 2 h, then washed three times with ice-cold phosphate-buffered saline and four times with 5% trichloroacetic acid. Five hundred microliters of 0.1 N

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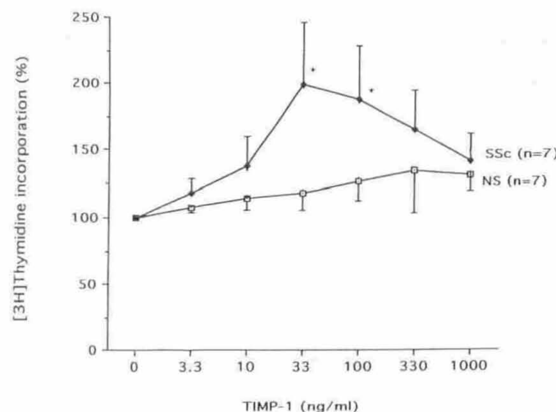

Figure 1. SSc fibroblasts show increased mitogenic response to TIMP-1 compared with NS fibroblasts. Confluent SSc or NS fibroblasts were stimulated with various concentrations of TIMP-1 for 24 h. TIMP-1 was added in 0.5 ml of serum-free medium, and cells were labeled for 2 h with [^{3}H]thymidine. Each experiment was performed in triplicate. [^{3}H]Thymidine incorporation (cpm) in the wells without TIMP-1 was set at 100% for each group. □, NS fibroblasts; ◆, SSc fibroblasts. *$p < 0.05$ compared to NS fibroblasts at the same concentration of TIMP-1 by Mann–Whitney *U* test. *Error bars*, SD (n = 7).

NaOH/0.1% sodium dodecyl sulfate were added, and the radioactivity was measured in 5 ml of ACS II (Amersham, Arlington Heights, IL) with a liquid scintillation counter. All experiments were performed in triplicate.

Measurement of TIMP-1 Levels in Serum and Conditioned Medium TIMP-1 concentration was determined with a sandwich enzyme immunoassay kit (Fuji Chemical Industries, Takaoka, Japan) that used two kinds of monoclonal antibodies against human TIMP-1 (Kikuchi *et al*, 1995a). SSc and NS fibroblasts (2×10^4 cells per well) were seeded in 24-well plates in MEM with 10% FBS and grown to confluency, and then medium was replaced with 0.5 ml of SFM followed by incubation for a further 72 h. The conditioned medium was then collected and clarified by centrifugation, and the cells remaining in the dishes were treated with trypsin and counted electronically. TIMP-1 concentration in the wells was determined as described above and collected by cell numbers per well. Serum TIMP-1 levels in the SSc patients were determined as described (Kikuchi *et al*, 1995a).

Western Blotting SSc and NS fibroblasts (10^6 cells per dish) were placed in 1-cm^2 dishes in MEM with 10% FBS and grown to confluency, and then medium was replaced with SFM and cells were incubated for 72 h. The conditioned medium was then collected and clarified by centrifugation, and the cells remaining in the dishes were treated with trypsin and counted electronically. The conditioned medium from SSc and NS fibroblasts was concentrated 25-fold in an ultrafiltration device. The concentrated samples were normalized for cell number and subjected to electrophoresis on 10–20% sodium dodecyl sulfate polyacrylamide gels. Proteins were electrotransferred from the gels onto nitrocellulose sheets, which were then incubated with monoclonal anti-TIMP-1 IgG for 3 h. Filters were then incubated with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Cappel, Durham, NC) for 1.5 h, and immunoreactive species were visualized with 5-bromo-4-chloro-3-indolyl phosphate (Sigma, St. Louis, MO) and nitroblue tetrazolium (Research Organics, Cleveland, OH).

Reverse Transcriptase-Coupled Polymerase Chain Reaction (PCR) RT-PCR was performed by using a GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, CT) according to the manufacturer's instructions. Briefly, total RNA from fibroblasts was isolated by the guanidium isothiocyanate method (Chomczynski and Sacchi, 1987). Aliquots of 1 μg of total RNA were reverse-transcribed by using random hexamer primers. For cDNA amplification, PCR was performed for 20 cycles. The cDNA concentration was first normalized by the expression of glyceraldehyde-3-phosphate dehydrogenase (GADPH). The primers used were 5′S primer, nucleotides 71–96, and 3′AS primer, nucleotides 1030–1053 (Arcari *et al*, 1984), which yielded a 983-bp product. The primer set based on the published sequences of human TIMP-1 cDNA was 5′S primer, nucleotides 94–114, and 3′AS primer, nucleotides 594–614 (Docherty *et al*, 1985). The PCR product for TIMP-1 was 520 bp. The primers were mRNA-specific in that the recognition sites of the upstream and downstream primers resided in separate exons or at intron–exon boundaries in the genomic sequence. The RT-PCR were separated by agarose gel electrophoresis and visualized by using ethidium bromide staining. For quantification, the cDNA concentration was first normalized by the expression of GADPH. Two microcuries of deoxycytidine 5′-triphosphate were added in the reaction mixture, and cDNA concentrations of different batches were compared. cDNA derived from 150 ng of total RNA was prepared and diluted 1:2, 1:4, 1:8, 1:16, and 1:32 in water. The PCR products were electrophoresed in a 2% agarose gel, the 983-bp bands were excised, and the radioactivity incorporated into the DNA was measured by Cerenkov scintillation counting. A standard curve was drawn from the radioactivities and the relative levels of GADPH gene expression were thus calculated. Similar experiments were performed by using TIMP-1 primers to determine the relative expression of the gene in comparison with the corresponding GADPH gene expression.

Table I. Anti-TIMP-1 IgG Blocks the Mitogenic Activity in Scleroderma Fibroblasts[a]

	% Inhibition	
	NS (n = 4)	SSc (n = 4)
SFM	100	100
anti-TIMP-1 IgG	94 ± 12	72 ± 11[b]
TIMP-1 (100 ng/ml)	133 ± 12	192 ± 12
TIMP-1 + anti-TIMP-1 IgG	105 ± 5[b]	106 ± 6[b]

[a] Confluent cells NS and SSc fibroblasts were incubated in SFM with TIMP-1 (100 ng/ml) and in the presence or absence of anti-TIMP-1 IgG (100 μl, 1:50 dilution) for 24 h. Cells were labeled for 2 h with [^{3}H]thymidine. Each experiment was performed in triplicate. [^{3}H]Thymidine incorporation (cpm) of the cells in SFM was arbitrarily set at 100% in each group. Each value represents the mean ± SD of four experiments.

[b] Values significantly different from corresponding values without anti-TIMP-1 IgG by the paired Wilcoxon's test; $p < 0.05$.

Statistics We used Mann–Whitney *U* test, Wilcoxon's paired test, or linear regression for statistical analyses.

RESULTS

Mitogenic Response to TIMP-1 in SSc Fibroblasts Is Elevated Compared to NS Fibroblasts We first investigated the growth responses of NS and SSc fibroblasts at the fourth passage to various concentrations of TIMP-1 (3.3–1000 ng/ml). The mean ± SD of [^{3}H]thymidine incorporation (cpm) in the wells that received no reagent in the SFM only was 264 ± 32 cpm/well (mean ± SD) in NS fibroblasts and 278 ± 43 cpm/well in SSc fibroblasts, and the difference between the two was not significant. Thus, the mean

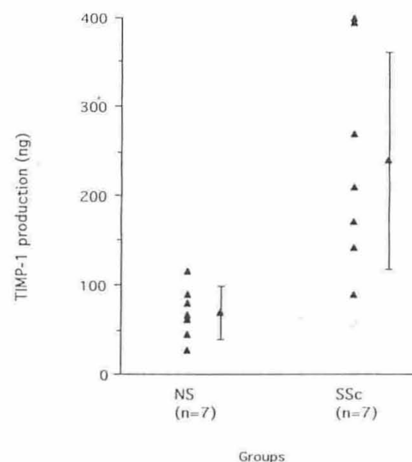

Figure 2. TIMP-1 production is elevated in SSc fibroblasts. SSc or NS fibroblasts (2×10^4 cells per well) were seeded in 24-well plates in MEM with 10% FBS and grown to confluency, and then medium was replaced with SFM for 72 h. TIMP-1 production (nanograms per 2×10^4 cells) was measured by enzyme-linked immunosorbent assay in NS and SSc fibroblasts. TIMP-1 production in SSc fibroblasts was significantly elevated relative to that in NS fibroblasts. *Error bars*, SD (n = 7).

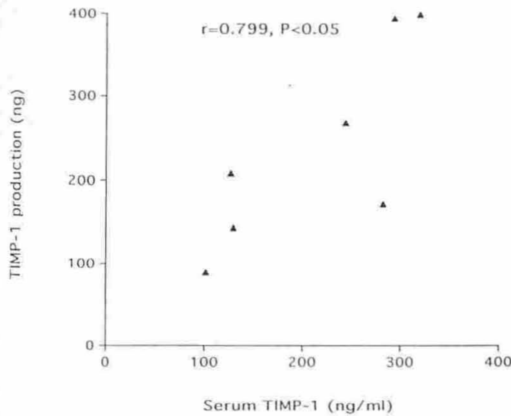

Figure 3. Serum TIMP-1 level in each SSc patient is correlated with the *in vitro* TIMP-1 production of fibroblasts derived from the same patient. The relationships between spontaneous TIMP-1 production (nanograms per 2×10^4 cells) by fibroblasts and serum TIMP-1 levels (nanograms per milliliter) in seven SSc patients are shown. r = 0.799; $p < 0.05$ by linear regression.

[^{3}H]thymidine incorporation in the SFM for each group was arbitrarily set at 100%, and the effects of TIMP-1 were estimated as a percentage ratio at each concentration. As shown in **Fig 1**, the mitogenic response to exogenous TIMP-1 became maximum when the concentration of TIMP-1 was 33 ng/ml in SSc (199 ± 47%) fibroblasts and 330 ng/ml in NS fibroblasts (135 ± 31%), respectively. SSc fibroblasts showed significantly greater mitogenic responses than NS fibroblasts when TIMP-1 was added at 33–100 ng/ml, suggesting that SSc fibroblasts are much more sensitive to the mitogenic capability of TIMP-1 than NS fibroblasts. Anti-TIMP-1 IgG (1:50 dilution) completely blocked the TIMP-1-induced mitogenic response of both NS and SSc fibroblasts **(Table I)**. Moreover, anti-TIMP-1 IgG partially but significantly decreased the basal mitogenic activity of SSc fibroblasts but not of NS fibroblasts **(Table I)**.

TIMP-1 Expression Is Elevated in Scleroderma Fibroblasts
Because anti-TIMP-1 IgG significantly downregulated the spontaneous proliferation of SSc fibroblasts, we assumed that these cells may synthesize and secrete higher amounts of TIMP-1 than NS fibroblasts. This was the case; SSc fibroblasts showed significantly greater TIMP-1 production than NS fibroblasts as determined by enzyme-linked immunosorbent assay (59 ± 30 *vs.* 17 ± 8 ng per 2×10^4 cells, $p < 0.005$; **Fig. 2**). The mean TIMP-1 production was 3.5-fold higher in SSc fibroblasts than in NS fibroblasts. Serum TIMP-1 level in each SSc patient was significantly correlated with the *in vitro* TIMP-1 production of fibroblasts derived from the same patient ($r = 0.799$, $p < 0.05$ by linear regression, **Fig. 3**). The elevated production of TIMP-1 in SSc fibroblasts was confirmed by western blotting **(Fig 4)**. Three strains of SSc fibroblasts exhibited 4.3-fold more TIMP-1 production than NS fibroblasts as determined by densitometrical analysis.

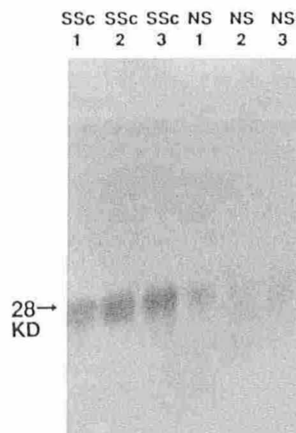

Figure 4. TIMP-1 overproduction in SSc fibroblasts is revealed by western blotting. The conditioned medium of SSc or NS fibroblasts cultures was subjected to electrophoresis on 10–20% gradient polyacrylamide gels and analyzed by western blotting.

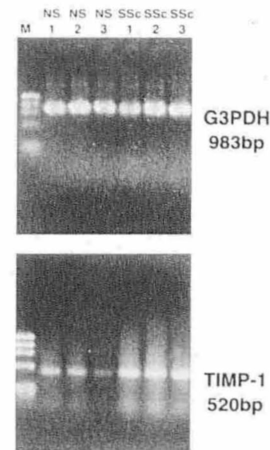

Figure 5: TIMP-1 mRNA expression is elevated in SSc fibroblasts. Expression of TIMP-1 mRNA in NS and SSc fibroblasts was examined. mRNA was amplified by reverse transcriptase-coupled polymerase chain reaction using specific primers for GADPH (G3PDH) and TIMP-1. The predicted sizes for the amplification products were as follows: GADPH, 983 bp; TIMP-1, 520 bp. M, size marker (ϕX174/*Hae*III digest) was included in the ethidium bromide agarose gel.

Furthermore, RT-PCR revealed the augmented gene expression of TIMP-1 in SSc fibroblasts **(Fig 5)**. The TIMP-1 mRNA expression level in SSc fibroblasts was 5.6-fold higher than that of NS fibroblasts as detected semiquantitatively. Since TGF-β1 is known to upregulate the expression of TIMP-1, we finally investigated the effects of TGF-β1 in TIMP-1 production of SSc and NS fibroblasts. In the presence of TGF-β1 at 1 ng/ml, TIMP-1 production was significantly increased in NS fibroblasts, whereas no significant increase was observed in SSc fibroblasts **(Table II)**.

DISCUSSION

TIMP-1 is a sialoglycoprotein with a molecular mass of 28.5 kDa that inhibits matrix metalloproteinases such as interstitial collagenase, gelatinase, and stromelysin, all of which can degrade components of the extracellular matrix (Cawston, 1986). TIMP-1 is produced by many connective tissue cells, which also produce matrix metalloproteinases. Imbalance in the activities of metalloproteinases and TIMP-1 might be important in many pathologic

Table II. Effects of TGF-β1 on TIMP-1 Production in Fibroblast Cultures[a]

Reagent	TIMP-1 Production	
	NS (n = 4)	SSc (n = 4)
No addition	17 ± 8	59 ± 30
TGF-β1 (1 ng/ml)	42 ± 16[b]	63 ± 31

[a] SSc and NS fibroblasts (2×10^4 cells per well) were seeded in 24-well plates in MEM with 10% FBS and grown to confluency, and then medium was replaced with SFM followed by incubation for 72 h in the presence or absence of TGF-β1 (1 ng/ml). TIMP-1 production (nanograms per 2×10^4 cells) was measured by enzyme-linked immunosorbent assay in NS and SSc fibroblasts. Each value represents the mean ± SD.
[b] Values significantly greater than NS fibroblasts in the absence of TGF-β1 by the paired Wilcoxon's test; $p < 0.05$.

conditions associated with excess deposition of extracellular matrix such as liver cirrhosis, rheumatoid arthritis, and SSc. A recent study demonstrated that human TIMP-1 has potent growth-promoting activity in a wide range of human cells including fibroblasts (Hayakawa *et al*, 1992). There have been several reports showing elevated mitogenic activity in the sera of SSc patients for dermal fibroblasts (LeRoy *et al*, 1983; Potter *et al*, 1984). In our previous study, the mitogenic effects of sera derived from SSc patients were partially but significantly blocked by anti-TIMP-1 IgG (Kikuchi *et al*, 1995b). In this study, we showed the significantly increased mitogenic response to recombinant TIMP-1 in SSc fibroblasts relative to that in NS fibroblasts. The observation that anti-TIMP-1 IgG blocked the basal mitogenic activity in SSc fibroblasts suggests an autocrine growth mechanism by TIMP-1. In keeping with this finding, we also confirmed that the TIMP-1 synthesis in SSc fibroblasts was significantly elevated at both mRNA and protein levels. We previously reported that serum TIMP-1 level in SSc patients reflected their disease severity (Kikuchi *et al* 1995b). It is also interesting that serum TIMP-1 level in SSc patient was correlated with the *in vivo* TIMP-1 production of fibroblasts derived from the patients. Unemori *et al* (1990) reported that TIMP was secreted in both apical and basal directions by endothelial cells, whereas collagen, fibronectin, and several matrix-degrading metalloproteinases were secreted selectively in the basal direction. The apically directed TIMP could account for the relatively high concentration of serum TIMP, especially when overproduction of TIMP occurs by fibroblasts.

The molecular basis underlying our finding of increased TIMP-1 level in SSc fibroblasts is not yet known. The cytokines TGF-β (Overall *et al*, 1991), tumor necrosis factor α (Chua *et al*,1990), interleukin 1 (Murphy *et al*, 1985), and interleukin 6 (Lotz and Guerne, 1991) are potent inducers of TIMP-1. All these factors, especially TGF-β, are considered to be important in the pathogenesis of fibrotic diseases such as SSc. For example, there have been some reports that TGF-β expression is increased in SSc lesions, especially in the earlier stages (Gruschwitz *et al*, 1990; Gabrielli *et al*, 1993; Higley *et al*, 1994). We have previously reported that the increased mitogenic response to TGF-β1 in SSc fibroblasts is mediated by another growth factor, connective tissue growth factor (Kikuchi *et al*, 1995a). The signal transduction mechanism responsible for the growth promoting activity of TIMP-1 is still unknown, but induction of another cytokine in fibroblasts may mediate this growth activity. It should be mentioned that TGF-β upregulated the TIMP-1 production only in NS but not in the SSc fibroblasts. The hyporesponsiveness of SSc fibroblasts to TGF-β may reflect the early exposure to TGF-β *in vivo*. Thus, the current results emphasize the importance of TIMP-1 expression in the pathogenesis of SSc, suggesting that the elevated production of TIMP-1 may contribute to the autocrine proliferation of fibroblasts and excess accumulation of collagen that ultimately leads to tissue fibrosis. Our results suggest that an autocrine mechanism involving TIMP might be important in fibrotic diseases.

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