Constitutively phosphorylated Smad3 interacts with Sp1 and p300 in scleroderma fibroblasts

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Objective. To elucidate the role of transforming growth factor-β (TGF-β)/Smad signalling in the increased expression of the collagen gene in systemic sclerosis (SSc) fibroblasts.

Methods. Dermal fibroblasts from seven patients with diffuse SSc of recent onset and from seven healthy individuals were studied. The expression levels of Smad2, Smad3 and Smad4 proteins were determined by immunoblotting. Smad3 phosphorylation and the interaction of Smad3 with Sp1 or p300 were analysed using immunoprecipitation. The effects of overexpression of Smad proteins or Sp1 on the human α2(I) collagen gene transcription were investigated with chloramphenicol acetyltransferase (CAT) assays using the −772 Colta2/CAT construct.

Results. Constitutive increased Smad3 phosphorylation was detected in SSc fibroblasts compared with normal fibroblasts. Increased interaction of Smad3 with Sp1 as well as p300 was also detected in SSc fibroblasts. The overexpression of Smad3 caused an increase of up to 5-fold in Colta2 promoter activity in normal fibroblasts, while Smad3 caused a small increase in Colta2 promoter activity in SSc fibroblasts. However, neither Smad2 nor Smad4 caused significant effects in COL1A2 promoter activity in normal fibroblasts or SSc fibroblasts. The overexpression of Sp1 caused further increase in Colta2 promoter activity stimulated by TGF-β in normal fibroblasts, but did not change Colta2 promoter activity in the presence of TGF-β in SSc fibroblasts. The combined overexpression of Smad3 and Sp1 significantly enhanced TGF-β response in normal fibroblasts, but less markedly in SSc fibroblasts.

Conclusions. These results suggested that SSc fibroblasts are less sensitive to exogenous TGF-β stimulation because they are already activated by the autocrine TGF-β loop.

Key words: Collagen diseases, Connective tissue, Cytokines.

Systemic sclerosis (SSc) is characterized by excessive deposition of extracellular matrix (ECM) in the skin, lung or other organs, prominent alterations in the microvasculature and humoral and cellular immunological abnormalities [1, 2]. Although the exact mechanisms involved in the pathogenesis of SSc are still unknown, the basic mechanism appears to involve overproduction of ECM in cutaneous and visceral tissues, which leads to the fibrosis that is responsible for most of its clinical manifestations [3, 4].

Increasing evidence suggests that activation of lesional fibroblasts contributes to the fibrotic process [5, 6]. Fibroblasts from affected SSc skin cultured in vitro produce an excessive amounts of types I, III, VI and VII collagen as well as fibronectin, glycosaminoglycans [6–11] and tissue inhibitor of metalloproteinases [12], and show elevated expression of intercellular adhesion molecules [13, 14]. Furthermore, SSc fibroblasts display transcriptional activation of collagen genes [15, 16].

The mechanism of activation of dermal fibroblasts in SSc is currently unknown, but many of the characteristics of SSc fibroblasts resemble those of healthy fibroblasts stimulated by transforming growth factor-β (TGF-β) [17, 18], suggesting that the activation of dermal fibroblasts in SSc may be a result of stimulation by autocrine TGF-β. This notion is supported by our recent findings that SSc fibroblasts express elevated levels of type I and type II TGF-β receptors, correlating with elevated levels of type I collagen gene expression, and that the blockade of endogenous TGF-β signalling with anti-TGF-β antibodies or a TGF-β1 antisense oligonucleotide abolished the increased expression of type I collagen in SSc fibroblasts [19, 20]. Type I collagen, the most abundant mammalian collagen, consists of the two α1(I) chains and one α2(I) chain, which are expressed in coordination [21]. Previous studies have characterized several responsive elements and cognate transcription factors involved in the regulation of type I collagen genes in human fibroblasts. Initial studies of the human α2(I) collagen promoter have indicated that the −376 bp to −108 bp promoter segment is sufficient to direct a high level of transcription in human fibroblasts [22]. This promoter region has been extensively studied. Stimulation by TGF-β of the human α2(I) collagen promoter is mediated by a multiprotein complex that interacts with two distinct promoter segments (−330 bp to −286 hp and −271 hp to −255 bp), termed TbRE [23]. Two of the proteins in this complex have been identified as transcription factors Sp1 and Sp3 [24–26]. Sp1 was also shown to be required for the response of the gene to TGF-β [27]. Further studies showed the important role of Smad3/Smad4 complex binding to the CAGACA motif near the Sp1 binding site in the human α2(I) collagen promoter for the full TGF-β response [28, 29]. Recent studies have shown that synergistic cooperation between Sp1 and Smad3/Smad4 is required for the TGF-β response of the collagen gene [30, 31]. Further detailed analyses showed the cooperation of p300/CBP with Smad in the TGF-β response of the collagen gene [32, 33].

In this study, we demonstrate constitutive phosphorylation of Smad3 and increased interaction of Smad3 with Sp1 and p300 in...
scleroderma fibroblasts that show increased transcription of the collagen gene. Overexpression of Smad3 increased the transcription of the human α2(I) collagen gene in normal dermal fibroblasts but not in scleroderma fibroblasts. The results correlate with the abnormality of TGF-β signalling in the up-regulated collagen gene expression in scleroderma fibroblasts.

Materials and methods

Cytokines and other material

Recombinant human TGF-β was purchased from R&D Systems Inc. (Minneapolis, MN, USA). Antibodies specific for Smad2, Smad3, Smad4, Smad2/3, Sp1 and p300 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antiphosphoserine antibody was obtained from Biomeda Corp. (Foster City, CA, USA). Protein G Sepharose was obtained from Zymed Laboratories Inc. (San Francisco, CA, USA). Monoclonal anti-β-actin was purchased from Sigma. Monoclonal antibody against human type I collagen was obtained from Southern Biotechnology Associates Inc. (Birmingham, AL, USA).

Fibroblast cultures

Human dermal fibroblasts were obtained by skin biopsy from the affected areas (the dorsal forearm) of seven patients with diffuse cutaneous SSc and <2 yr of skin thickening. Control fibroblasts were obtained by skin biopsy from seven healthy donors. Institutional approval and informed consent were obtained from all subjects. Control donors were matched with each SSc patient for age, sex and biopsy site, and control and patient samples were processed in parallel. Cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS). Fibroblast cultures independently isolated from different individuals were maintained as monolayers at 37°C in an atmosphere of 5% CO₂ and 95% air. Fibroblasts under the fifth subpassage were used for the experiments. Cell viabilities were determined by trypan blue stain.

Immunoblotting

For the preparation of cell lysates from dermal fibroblasts, cells were placed in MEM and 0.1% bovine serum albumin for 24 h. Then the conditioned medium was removed and the cells were washed with phosphate buffered saline. The cells were lysed by scraping into solubilization buffer (50 mM Tris Cl (pH 8), 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1% Nonidet P40, 1 mM sodium orthovanadate, 0.2 mM phenylmethylsulphonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin). The lysates were incubated for 30 min at 4°C and then centrifuged for 5 min at 4°C. Protein concentrations of lysates were determined using a Bio-Rad (Hercules, CA, USA) protein assay reagent. Immunoblotting was performed as described previously [26, 34]. Briefly, cell lysates (20 μg) were subjected to electrophoresis on 10% or 7.5% sodium dodecyl sulphate (SDS)-polyacrylamide gel slabs, and then electrotransferred from the gels onto nitrocellulose sheets. The nitrocellulose sheets were then incubated overnight with the indicated primary antibodies. The bound antibodies were then detected with horseradish peroxidase-conjugated secondary antibodies and immunoreactive bands were visualized with enhanced chemiluminescence (ECL) (Amersham, Arlington Heights, IL, USA) as described previously [35].

Immunoprecipitation

Immunoprecipitation was performed as described previously [15, 36, 37]. Briefly, 500 μg of total cellular protein was incubated with antibodies to the Smad3 (2 μg/ml) at 4°C overnight, followed by a 2 h incubation with Protein G Sepharose at 4°C. After three washes in lysis buffer, the immunocomplexes were resolved by SDS-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane and incubated with antibodies specific for phosphoserine, Sp1 or p300. The membrane was washed and then incubated with the secondary antibodies for 60 min. As a loading control, the reaction was also performed using antibodies against Smad2/3.

Plasmid constructions

Generation of a −772 COL1A2/CAT construct consisting of the human α2(I) collagen gene fragment (+58 to −772 bp relative to the transcription start site) linked to the chloramphenicol acetyltransferase (CAT) reporter gene was done as previously described [24, 25]. Expression vectors for human Smad2 [38], Smad3 [39] and Smad4 [40] were described previously. The expression vector of Sp1 (CMVSp1) was also described [36]. Plasmids used in transient transfection assays were twice purified on CsCl gradients. At least two different preparations of plasmids were used for each experiment.

Transfections and constructs

Transient transfections were performed as described previously [24–26, 34–37]. Fibroblasts were transfected by the lipofection technique (FuGene 6 Transfection Reagent, Boehringer Mannheim, Indianapolis, IN, USA) with various amounts of constructs. The pSV-β-galactosidase control vector (Promega, Madison, WI, USA) was transfected to normalize for transfection efficiency. The cells were stimulated with cytokines for 24 h in the absence of serum, and incubation was continued for 48 h. Cells were harvested in 0.25 M Tris-HCl (pH 8) and fractured by freeze-thawing. Extracts were incubated with butyryl-CoA [14C]chloramphenicol for 90 min at 37°C. ButyLATED chloramphenicol was extracted using an organic solvent (2:1 mixture of tetramethylpentadecane and xylene) and quantified by scintillation counting. Each experiment was performed in duplicate. In order to normalize for small variations in transfection efficiencies, the data were standardized with β-galactosidase activity using a chemiluminescent reporter assay for β-galactosidase (Galacto-Light Plus™, Tropix, Bedford, MA, USA).

Statistical analysis

Statistical analysis was carried out with the Mann–Whitney test for the comparison of means. P values less than 0.05 were considered significant.

Results

Expression of Smad2, Smad3 and Smad4 in dermal fibroblasts

Recent studies have shown the important role of Smad3/Smad4 complex binding to the CAGACA motif in the human α2(I) collagen promoter for the TGF-β response [28, 29]. First, to investigate the expression of Smad2, Smad3 and Smad4 in dermal fibroblasts we performed immunoblot analysis. As shown in Fig. 1, Smad2, Smad3 and Smad4 proteins were detected in dermal fibroblasts. The expression levels of Smad2, Smad3 and Smad4 were quite heterogeneous in normal fibroblasts as well as SSc fibroblasts. There were no significant differences in the expression levels of Smad2, Smad3 or Smad4 proteins between normal and SSc fibroblasts.
Effects of overexpression of Smad proteins on COL1A2 transcription

Next we examined the effects of overexpression of Smad proteins on COL1A2 transcription and response to TGF-β. Basal COL1A2 activity increased in SSc fibroblasts compared with normal fibroblasts. Among the Smad expression plasmids tested, co-transfection with the Smad3 plasmid increased the basal as well as the TGF-β-mediated COL1A2 transcription in normal fibroblasts (Fig. 2). Smad3 caused an increase of up to 5-fold in the basal COL1A2 promoter activity in normal fibroblasts. On the other hand, Smad3 caused only a small increase in the basal as well as the TGF-β-mediated COL1A2 promoter activity in SSc fibroblasts, and its effect was not statistically significant (Fig. 2). Neither Smad2 nor Smad4 caused significant effects in COL1A2 promoter activity and TGF-β response in normal fibroblasts or SSc fibroblasts (Fig. 2).

Constitutive Smad3 phosphorylation in SSc fibroblasts

Following ligand activation, signalling from TGF-β receptors to the nucleus occurs predominantly by phosphorylation of Smad proteins [41]. Because only the overexpression of Smad3 showed a significant effect on COL1A2 promoter activity in dermal fibroblasts, we focused on Smad3. Phosphorylation of Smad3 by TGF-β occurs principally on serine residues within the C-terminal [41]. Therefore, Smad3 phosphorylation was assessed using antiphosphoserine antibody. As shown in Fig. 3, constitutive Smad3 phosphorylation was detected in SSc fibroblasts but not in normal fibroblasts. In addition, immunoblotting was performed to determine the expression levels of type I procollagen using monoclonal antibody against human type I collagen. The levels of Smad3 phosphorylation were not completely correlated with the expression levels of type I procollagen protein in SSc fibroblasts, but the SSc fibroblasts with more increased levels of Smad3 phosphorylation (S4 and S5) expressed higher levels of type I procollagen protein than those with less increased levels of Smad3 phosphorylation (S1 and S2) (Fig. 3).

Increased interaction of Smad3 with Sp1 and CBP/p300 in SSc fibroblasts

Recent studies have shown that synergistic cooperation between Sp1 and Smad3 is required for TGF-β response of the collagen gene [30, 31]. The interaction of Smad3 and Sp1 was determined using immunoprecipitation. The cell lysates were immunoprecipitated by the antibodies specific for Smad3 and the interaction of these proteins was detected with antibody specific for Sp1. As shown in Figs 4A and B, increased interaction of Smad3 with Sp1 was detected in SSc fibroblasts compared with normal fibroblasts. To rule out the possibility that SSc fibroblasts express more Sp1 protein than normal fibroblasts, immunoblot analysis was performed. Sp1 protein of ~105 kDa was detected in dermal fibroblasts and there was no significant difference in the amount of Sp1 protein between SSc and normal fibroblasts (Fig. 4C). Furthermore, cooperation of p300/CBP with Smad3 in the TGF-β response of the collagen gene has been reported [32, 33]. Therefore, the interaction of Smad3 and p300 was determined using immunoprecipitation. As shown in Figs 4A and B, an increased interaction of Smad3 with p300 was detected in SSc fibroblasts compared with normal fibroblasts. The interaction of Smad3 with Sp1 was correlated with that of Smad3 with p300. To rule out the possibility that SSc fibroblasts express more p300 protein than normal fibroblasts, immunoblot analysis was performed. A p300 protein of ~300 kDa was detected in dermal fibroblasts and there was no significant difference in the amount of p300 protein between SSc and normal fibroblasts (Fig. 5C).

Effects of overexpression of Sp1 on COL1A2 transcription

As described above, the increased interaction of Smad3 with Sp1 was detected in SSc fibroblasts compared with normal fibroblasts. Therefore, we examined the effects of overexpression of Sp1 on COL1A2 transcription and the response to TGF-β. Consistent with a previous study [35], the overexpression of Sp1 slightly changed the basal COL1A2 promoter activity in both normal and SSc fibroblasts (Fig. 6A). Next, the effect of overexpression of Sp1 on COL1A2 promoter activity induced by TGF-β was examined. The results indicated that the overexpression of Sp1 caused a further increase in stimulation of COL1A2 promoter activity in normal fibroblasts (Fig. 6A). These results are consistent with results showing that synergistic cooperation between Sp1 and Smad3 is required for the TGF-β response of the collagen gene [30, 31]. However, the overexpression of Sp1 did not change TGF-β-mediated COL1A2 promoter activity significantly in SSc fibroblasts (Fig. 6A). Furthermore, we examined the effects of combined overexpression of Smad3 and Sp1 on COL1A2 transcription and response to TGF-β. As shown in Fig. 6B, the combined overexpression of Smad3 and Sp1 significantly enhanced...
the TGF-β response in normal fibroblasts (4.5-fold), but less markedly in SSc fibroblasts (2.1-fold). These results suggest that SSc fibroblasts are already activated by autocrine TGF-β/Smad signalling, and that SSc fibroblasts are less sensitive to exogenous TGF-β stimulation even in the presence of forced combined overexpression of Smad3 and Sp1.

**Discussion**

The accumulation of ECM is one of the most prominent features of fibrosis. TGF-β signaling has been implicated in the pathogenesis of fibrosis, including SSc [42, 43]. It has been shown that SSc fibroblasts do not secrete increased levels of TGF-β [20, 44]. On the other hand, we have demonstrated overexpression of TGF-β receptors in SSc fibroblasts, indicating a possible mechanism of autocrine TGF-β activity by overexpression of TGF-β receptors [20, 45–47]. These previous findings established the notion that autocrine TGF-β signalling may play a central role in the pathogenesis of SSc. To further understand autocrine TGF-β signalling in SSc, investigation of the signalling pathway downstream of TGF-β receptors in SSc is important.
Recent studies have identified Smad proteins as major downstream targets of TGF-β receptor kinases, with important roles in intracellular TGF-β signalling in vertebrates [48]. Based on their structure and function, Smads are classified into three subgroups. The receptor-regulated Smads, such as Smad2 and Smad3, are directly phosphorylated and activated by TGF-β receptors. Smad4, representing a distinct subfamily, oligomerizes with the receptor-regulated Smads and together these complexes translocate into the nucleus where they activate the transcription of TGF-β-inducible genes [49]. Smad6 and Smad7 inhibit TGF-β signalling by receptor-regulated Smads.

This is the first study to demonstrate the increased interaction of Smad3 with p300 in SSc fibroblasts and investigate the effects of overexpression of Smad proteins on collagen gene expression in SSc fibroblasts. Among the Smad expression plasmids tested, only co-transfection with a Smad3 plasmid exhibited an effect on COL1A2 transcription in normal dermal fibroblasts. Neither Smad2 nor Smad4 caused significant effects on COL1A2 promoter activity in normal or SSc fibroblasts, which indicates that Smad3 is most important in collagen gene expression.

In this study, Smad3 was constitutively phosphorylated in SSc fibroblasts. Furthermore, Smad3 caused an increase of up to 5-fold in basal COL1A2 promoter activity in normal fibroblasts, while Smad3 caused only a small increase in COL1A2 promoter activity in SSc fibroblasts. This may be a result of up-regulated autocrine TGF-β signalling in SSc fibroblasts. This notion is supported by our recent findings that (i) SSc fibroblasts secrete amounts of TGF-β similar to those secreted by normal fibroblasts but express elevated levels of TGF-β receptors, and this correlates with elevated levels of type I collagen mRNA [20], and (ii) SSc fibroblasts express increased levels of Smad7, which may be due to constitutive autocrine TGF-β signalling in SSc fibroblasts, but the Smad7–Smurf-mediated inhibitory effect on TGF-β signalling is impaired in SSc fibroblasts [50]. Furthermore, the forced overexpression of TGF-β receptors was shown to increase COL1A2 activity in normal fibroblasts [19]. We have also recently shown that overexpression of thrombospondin-1 (TSP-1) plays a role in maintaining autocrine TGF-β signalling in SSc fibroblasts [51]. In that study, TSP-1 expression was increased in SSc fibroblasts and TSP-1 blocking antibody or antisense oligonucleotide had an inhibitory effect on Smad3 phosphorylation in SSc fibroblasts [51]. This suggests that overexpression of TGF-β receptors and TSP-1 may activate TGF-β, up-regulate TGF-β signalling and induce Smad3 phosphorylation.

Recent studies have shown that synergistic cooperation between Sp1 and Smad3 is required for the TGF-β response of the type I collagen gene [30, 31]. In this study, the increased interaction of Smad3 with Sp1 was detected in normal fibroblasts. It has also been demonstrated that Smad3 and transcriptional coactivators p300 and CREB-binding protein (CBP) are involved in TGF-β stimulation of the human α2(I) collagen gene [32, 33]. We showed that the increased interaction of Smad3 with p300 was also detected in SSc fibroblasts. These results also indicate the important role of autocrine TGF-β signalling in up-regulated collagen gene expression in SSc fibroblasts.

The important role of Sp1 in fibrosis has been reported [15, 35, 36, 52]. Inhibition of Sp1 binding abolished up-regulated expression of the collagen gene in SSc fibroblasts [15]. Sp1 is also implicated in the TGF-β response of the collagen gene [23, 27].
In cultured hepatic stellate cells, overexpression of Sp1 enhanced COL1A2 transcription stimulated by TGF-β [53]. In this study, overexpression of Sp1 caused further increase in TGF-β-mediated COL1A2 promoter activity in normal fibroblasts. These results are consistent with the results that synergistic cooperation between Sp1 and Smad3 was required for the TGF-β response of the collagen gene [30, 31]. However, overexpression of Sp1 did not change TGF-β-mediated COL1A2 promoter activity significantly in SSc fibroblasts. The reason for this phenomenon is still unknown, but there is a possibility that SSc fibroblasts are constitutively stimulated by the signalling via Sp1. This notion is supported by our recent finding that increased Sp1 phosphorylation was detected in SSc fibroblasts and that the inhibition of Sp1 binding abolished the increased type I collagen gene expression in SSc fibroblasts [15].

The combined overexpression of Smad3 and Sp1 significantly enhanced the TGF-β response in normal fibroblasts, but less markedly in SSc fibroblasts. These results suggest that SSc fibroblasts are already activated by autocrine TGF-β/Smad signalling, and that SSc fibroblasts are less sensitive to exogenous TGF-β stimulation.

Several studies have shown that mice lacking Smad3 have a significantly reduced cutaneous and pulmonary fibrotic
response [54, 55], indicating an important role for Smad3 in fibrosis. Furthermore, Smad2, Smad3 and Smad4 have been reported to contribute to liver fibrosis in vitro and in vivo [56–58]. The expression levels of Smad proteins have been investigated in SSc fibroblasts [59]. Some SSc fibroblasts expressed increased levels of Smad3, but others did not [59]. However, another study reported that SSc fibroblasts showed increased phosphorylation of Smad2 and Smad3 compared with normal fibroblasts after TGF-β treatment, but the amounts of Smad2 and Smad3 in SSc fibroblasts were similar to those in normal fibroblasts [60]. Furthermore, a recent study showed that levels of phosphorylated Smad2/3 were elevated and nuclear localization of phosphorylated Smad2/3 was increased in SSc fibroblasts [61]. In this study, constitutively phosphorylated Smad3 interacted with Sp1 and p300 in SSc fibroblasts. Further studies are needed to clarify the role of Smad proteins in SSc fibroblasts.

In conclusion, we have shown that constitutively phosphorylated Smad3 interacts with Sp1 and p300/CBP in SSc fibroblasts and these transcription factors are involved in the up-regulated type I collagen expression in SSc.

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References


Clinical Vignette

Subcutaneous lesions in dermatomyositis

A 38-yr-old woman with a longstanding dermatomyositis since 1992, in clinical remission for the last 5 yr, presented to our outpatient rheumatology clinic because of back pain and low-grade fever. The current treatment consisted of methotrexate (15 mg/week) and prednisolone 5 mg every other day. Physical examination revealed a temperature of 38°C, with normal vital signs and normal muscle strength. Skin examination revealed extensive subcutaneous lesions with tenderness and swelling involving the lateral areas of the back. A plain radiograph of the pelvis revealed the presence of subcutaneous calcifications, which were confirmed by a computed tomography scan. Chest radiograph and purified protein derivative skin test were negative. Routine laboratory tests, liver and muscle enzymes, and kidney function tests were within normal limits. The urine analysis was normal. Repeated blood and urine cultures were negative, while the C-reactive protein was 12 mg/l (normal value <6 mg/l). The dose of prednisolone was increased to 30 mg/day and after 10 days of treatment the subcutaneous lesions gradually improved and became softer without swelling and tenderness. One month later the patient was asymptomatic, the subcutaneous lesions were palpable and foci of calcinosis were present without signs of inflammation. The dose of prednisolone was tapered, and colchicine was added [1].

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