Hypothesis

Pathogenesis of Systemic Sclerosis

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ABSTRACT. A hypothesis for the pathogenesis of systemic sclerosis (SSc) is proposed. Transforming growth factor-ß (TGF-ß) has received attention as an essential factor in the pathogenesis of various fibrotic disorders, including SSc, although some unknown additional factor has been sought as the second mediator of fibrotic disorders. Connective tissue growth factor (CTGF) has been shown to be closely related to the pathogenesis of SSc as follows: (1) CTGF mRNA expression was observed in the fibrotic lesions but not in the early nonfibrotic lesions or atrophic lesions. (2) Serum CTGF protein concentrations were significantly elevated, and correlated with skin sclerosis and lung fibrosis. (3) In our animal model, TGF-ß-induced subcutaneous fibrosis and subsequent CTGF application caused persistent fibrosis. Based on these data, we hypothesize that a 2-step process of fibrosis occurs in SSc: that is, TGF-ß induces fibrosis in the early stage and afterwards CTGF acts to maintain tissue fibrosis. (J Rheumatol 2003;30:755–9)

Key Indexing Terms:
SYSTEMIC SCLEROSIS FIBROSIS TRANSFORMING GROWTH FACTOR-ß
CONNECTIVE TISSUE GROWTH FACTOR

PATHOGENESIS OF SYSTEMIC SCLEROSIS

Systemic sclerosis (SSc) is a multisystem disorder of connective tissue characterized by excessive fibrosis in the skin and various internal organs such as the lungs, kidneys, esophagus, and heart. Although the pathogenesis of this disease remains unknown, 3 major abnormalities are intricately connected — autoimmunity, abnormal connective tissue metabolism, and disturbed vascular systems.

Regarding abnormal connective tissue metabolism and subsequent fibrosis, many studies suggest that several growth factors and cytokines released from inflammatory cells, endothelial cells, fibroblasts, and other cells in the involved organs play an important role in the initiation and maintenance of connective tissue fibrosis.

Among these factors, transforming growth factor-ß (TGF-ß) has gained much attention as an essential factor in the pathogenesis of SSc[1], since TGF-ß is a very potent stimulator of collagen synthesis by fibroblasts[2,3], although other growth factors have been sought as the additional pathogenetic factor in the development of fibrosis in SSc.

Transforming growth factor-ß

TGF-ß is a 25 kDa homodimer. Five distinct isoforms have been found, of which 3 are present in humans. TGF-ß1, 2, and 3 exhibit similar, but not identical, biological activities *in vitro*. TGF-ß1 acts as a growth inhibitor for most cell types[4] and also causes vascular damage, described in sera from patients with SSc[5]. In addition, TGF-ß stimulates extracellular matrix (ECM) production, including type I collagen, type III collagen, and fibronectin, in fibroblasts[2,3]. This stimulatory effect on ECM production is much stronger than those of other cytokines.

A TGF-ß response element has been well characterized in the mouse COL1A2 promoter. An initial study demonstrated that TGF-ß activation of the COL1A2 promoter is mediated via a CTF/NF-1 binding site located at –300 bp in the promoter region[6]. On the other hand, earlier studies identified TGF-ß response element containing the Sp1 binding site in human α2(I) collagen[7], and Sp1 was shown to be required for the response of the gene to TGF-ß[8]. 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-ß response[9-11]. Recent studies have shown that synergistic cooperation between Sp1 and Smad3/Smad4 are required for the TGF-ß response of the collagen gene[12-14]. Further detailed analyses showed the cooperation of p300/CBP with Smad in the TGF-ß response of the collagen gene[15,16]. Another study indicated that the interaction of Ets with Smad is also involved in the TGF-ß response of the collagen gene[17].

One other important characteristic of TGF-ß is that it is produced as a latent form. The detailed mechanisms of activation of TGF-ß *in vivo* have not been clarified. In general, the biological activities of TGF-ß are very strong, and therefore the molecule is probably produced as an inactive form in order to block excessive TGF-ß activity[3].

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Serine threonine kinase is used in TGF-ß signal transduction, which is quite different from the case of other growth factors such as platelet derived growth factor (PDGF) and epidermal growth factor (EGF), which use tyrosine kinase. TGF-ß isoforms bring together members from 2 families of receptor serine/threonine kinases, known as the type I and type II receptors. The major known function of the type II receptors is to activate the type I receptors. The type I receptors propagate the signal by phosphorylating the Smad. Each ligand may have a choice of several type I and/or type II receptors, and a given cell may express different receptor forms[18].

Kawakami, *et al* reported that type I and type II TGF-ß receptor mRNA expression levels were higher in SSc fibroblasts than in healthy fibroblasts[19]. Ihn, *et al* examined the expression of TGF-ß receptor proteins in SSc fibroblasts, which were found to express increased levels of TGF-ß type I and type II receptor proteins[20]. These results suggest that although SSc fibroblasts express TGF-ß in quantities similar to those produced by healthy fibroblasts, the levels of TGF-ß type I and type II receptors produced by SSc fibroblasts are greater than those produced by normal fibroblasts.

The forms of TGF-ß signal via serine/threonine kinase transmembrane receptors, which phosphorylate cytoplasmic mediators of the Smad family[21-23]. The ligand-specific Smad1, Smad2, Smad3, and Smad5 interact directly with, and are phosphorylated by, activated TGF-ß type I receptors. The resulting Smad heterocomplexes are then translocated into the nucleus, where they activate target genes, binding DNA either directly or in association with other transcription factors. Members of the third group of Smad, the inhibitory Smad6 and Smad7, prevent phosphorylation and/or nuclear translocation of receptor-associated Smad[21–23]. Recently, an Smad7 defect was reported in SSc fibroblasts[24].

In protein kinase C, the PKC-delta isoform has been found to be critical in the early stage of TGF-ß1 signaling related to gene transcription[25,26]. PKC-delta participates in upregulation of collagen gene transcription in SSc fibroblasts[27].

Thus, TGF-ß has attracted much attention as a key factor in various fibrotic disorders, as described[1], because it potently increases ECM production by many cells.

However, studies have suggested that TGF-ß expression at a skin fibrotic site is limited to the early stage in various skin fibrotic disorders, including SSc and localized scleroderma[28,29]. In addition, our studies revealed that a single application of any TGF-ß isoform is insufficient to induce persistent fibrosis[30].

Connective tissue growth factor

Connective tissue growth factor (CTGF) has been suggested to play an important role in the development of various fibroses. CTGF is a cysteine-rich peptide originally identified from cultured human umbilical endothelial cell (HUVEC) supernatants that exhibits PDGF-like chemotactic and mitogenic activities on mesenchymal cells, and appears to be antigenically related to PDGF A and B chain peptides[31]. Human foreskin fibroblasts produce high levels of CTGF mRNA and protein after activation with TGF-ß but not other growth factors such as PDGF, EGF, or basic fibroblast growth factor (bFGF)[32]. In the wound chamber model, coordinated expression of TGF-ß followed by CTGF in regenerating tissue has been observed, suggestive of a cascade process for control of tissue regeneration and repair[32]. Thus, CTGF is a candidate autocrine stimulator released in response to TGF-ß in skin fibroblasts, as shown in Figure 1, and also appears to participate in the pathologic process of fibrosis.

CTGF mRNA is overexpressed in a large number of fibrotic conditions, including SSc[33], localized scleroderma[34], keloid[34], atherosclerosis[35], renal fibrosis[36], inflammatory bowel disease[37], chronic pancreatitis[38], lung fibrosis[39], and liver fibrosis[40]. CTGF mRNA expression was also confirmed in other skin fibrotic disorders including cutaneous fibrohistocytic and vascular tumors[41].

Although it is likely that CTGF expression is not specific for fibrosis in SSc, several lines of evidence indicate that CTGF is closely involved in fibrosis in SSc. The CTGF receptor was identified as lipoprotein receptor-related protein/α_2-macroglobulin[42].

CTGF and SSc

Our studies strongly suggest that CTGF is involved in the pathogenesis of SSc as follows.

CTGF mRNA expression in fibroblasts in SSc sclerotic lesions. When tissues from patients with SSc were examined by *in situ* hybridization with the antisense CTGF probe, dermal fibroblasts were all positive in all 12 cases that showed histologic sclerosis[32]. Positive signals were more abundant in the sclerotic stage than in the inflammatory stage. Moreover, no CTGF mRNA expression was observed in tissue from the atrophic stage of SSc or from the prescleroticstage. Our finding of elevated CTGF mRNA expression in SSc lesions was confirmed using cultured fibroblasts derived from SSc lesions[43,44].

Serum concentrations of CTGF in SSc. By ELISA, we examined serum samples from patients with SSc[45]: serum concentrations of CTGF were elevated in these patients (Figure 2). Since elevated CTGF levels were not observed in patients with systemic lupus erythematosus and polymyositis/dermatomyositis, it is likely that elevation of CTGF levels is specific for SSc among autoimmune connective tissue diseases. Further, the findings that CTGF levels correlated with the extent of skin sclerosis and the severity of pulmonary fibrosis suggest that CTGF plays a critical role in the development of fibrosis in SSc.

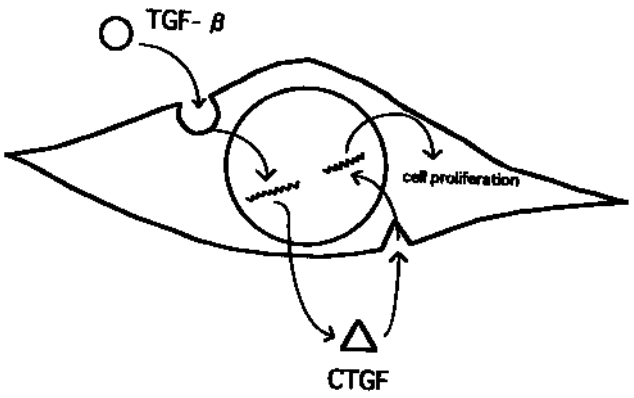

Figure 1. The relationship between TGF-ß and CTGF. CTGF acts as an autocrine growth factor when skin fibroblasts are stimulated with TGF-ß.

CTGF concentrations in patients with diffuse cutaneous SSc were elevated 1–3 years after disease onset, but not in the first year after onset. Since TGF-ß is the only inducer of CTGF identified to date[32], it is possible that TGF-ß is induced in the earliest phase of SSc and initiates fibrosis, followed by induction of CTGF that develops and/or maintains fibrosis.

An animal model of skin fibrosis by exogenous injection of TGF-ß and CTGF. In 1986, Roberts, *et al* reported that TGF-ß injection into newborn mice caused granulation tissue formation and skin fibrosis[46]. We initially tried to establish an animal model of skin fibrosis by TGF-ß injection using a similar method. We injected 800 ng of TGF-ß1, 2, or 3 into the subcutaneous tissue of newborn mice for 7 days. All types of TGF-ß caused strong granulation formation and fibrotic changes after 3 consecutive injections; however, a single injection of TGF-ß alone did not cause persistent fibrosis, and fibrosis disappeared after 7 days[30]. Therefore, we tried to establish persistent fibrosis by combination of TGF-ß and other growth factors, including CTGF, as described[30,47].

The results of these experiments are summarized in Table 1. CTGF injection caused slight edema and some cell infiltration. Similarly, bFGF injection caused slight edematous granulated tissue formation. Simultaneous injection of TGF-ß plus CTGF or TGF-ß plus bFGF resulted in fibrotic tissue

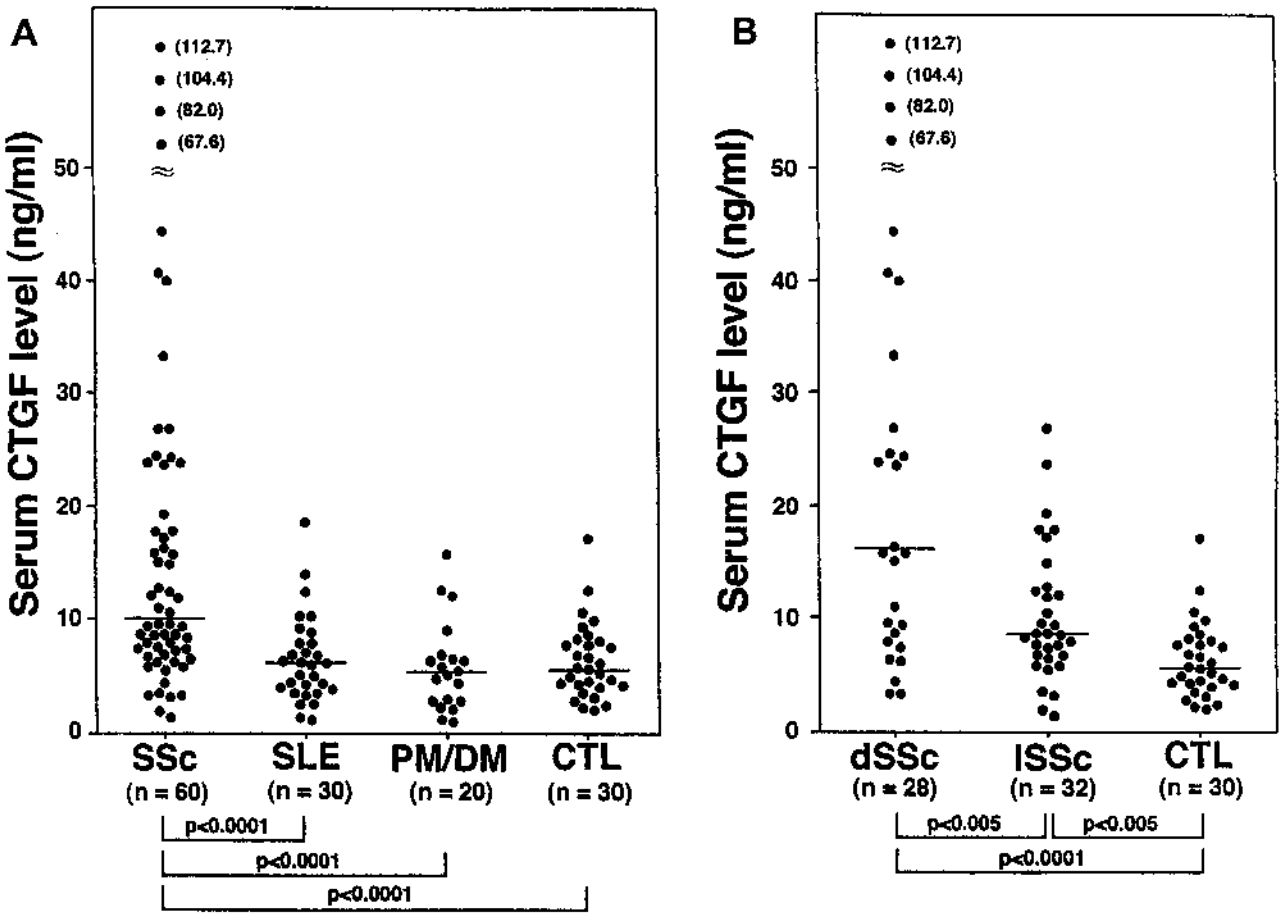

Figure 2. A. Concentrations of CTGF in sera from patients with SSc, systemic lupus erythematosus (SLE), polymyositis/dermatomyositis (PM/DM), and healthy controls (CTL). B. Concentrations of CTGF in sera from patients with diffuse cutaneous SSc (dSSc), limited cutaneous SSc (lSSc), and healthy controls. Serum concentration of CTGF was determined by ELISA. Horizontal lines show median values.

formation, consisting of fibroblast aggregation and ECM deposition, that persisted for up to 14 days, even though the injections were discontinued on Day 7. To examine the tissue response further, 2 different growth factors were injected serially: TGF-ß on Days 1–3 followed by CTGF on Days 4–7. Serial injections of CTGF or bFGF after TGF-ß caused fibrotic tissue formation. Injection of CTGF or bFGF before TGF-ß did not cause any significant change compared with TGF-ß alone (Table 1).

These results clearly demonstrate that single application of any growth factor is not sufficient to induce persistent fibrosis, despite continuous injections. Instead, interaction of multiple growth factors seems to be necessary for the induction of persistent fibrosis in this animal model. Our findings on serial application of different growth factors suggest that TGF-ß plays an important role in inducing granulation and fibrotic tissue formation. The other growth factors, CTGF and bFGF, are important in maintaining fibrosis. We conclude that induction of persistent fibrosis most likely requires 2 factors: an induction factor such as TGF-ß and a maintenance factor such as CTGF. bFGF may act synergistically by inducing CTGF, because our results suggest that either CTGF mRNA expression or the CTGF protein itself is required to develop persistent fibrotic changes[47].

THE 2-STEP FIBROSIS HYPOTHESIS IN SSC

Based on the results with TGF-ß and CTGF described above, we hypothesized that a 2-step process of fibrosis occurs in SSc. We think that TGF-ß induces fibrosis in the early stage of SSc, and then CTGF acts to maintain tissue fibrosis. TGF-ß induces CTGF mRNA, but some additional factor is required for continuous CTGF mRNA expression, because CTGF induced in an autocrine manner disappeared after 3 days' injection of TGF-ß. The mechanism of continuous CTGF expression is a key to this disorder, and efforts to reveal it are now under investigation in our laboratory.

Table 1. Histological responses to single, simultaneous, and serial injections of different growth factors into newborn mice.

	Day 4	Day 8	Day 14
TGF-ß alone	++	–	–
CTGF alone	±	–	–
bFGF alone	+	–	–
TGF-ß + CTGF	+++	+++*	+++*
TGF-ß + bFGF	+++	+++*	+++*
TGF-ß → CTGF	++	++*	++
TGF-ß → bFGF	++	++*	++
CTGF → TGF-ß	±	+	–
b-FGF → TGF-ß	+	+	–

–: no change; ±: slight edema and some cell infiltration; +: edematous granulation tissue; ++: granulation tissue consisting of lymphocytes, histiocytes, and fibroblasts; +++: fibrotic tissue consisting of fibroblast aggregation and extracellular matrix deposition, and asterisks denotes marked fibrosis. →: In the first 3 days and next 4 days, different growth factors were injected.

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