Upregulated Expression of Transforming Growth Factor-ß Receptors in Dermal Fibroblasts of Skin Sections from Patients with Systemic Sclerosis

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ABSTRACT. ***Objective.*** To investigate the expression levels of transforming growth factor-ß (TGF-ß) receptors in the skin of patients with systemic sclerosis (SSc).

Method. We examined expressions of TGF-ß type I and type II receptors (TGF-ßRI and TGF-ßRII) in skin sections of 5 patients with SSc and 5 healthy controls using *in situ* hybridization and immunohistochemical analysis.

Results. The expression levels of both TGF-ßRI and TGF-ßRII were elevated in the dermal fibroblasts of skin sections from patients with SSc in comparison to control skin sections in *in situ* hybridization and in immunohistochemical stainings. The numbers of fibroblasts expressing TGF-ßRI and TGF-ßRII in the SSc skin sections were increased in comparison to controls. The inflammatory cells around the vessels also expressed TGF-ßRI and TGF-ßRII intensively in the SSc skin sections.

Conclusion. These results suggest that the autocrine TGF-ß signaling due to the overexpression of TGF-ßRI and TGF-ßRII in dermal fibroblasts is involved in the pathogenesis of dermal fibrosis in patients with SSc. (J Rheumatol 2002;29:2558–64)

Key Indexing Terms:
FIBROSIS SIGNAL TRANSDUCTION *IN SITU* HYBRIDIZATION
IMMUNOSTAININGS TRANSFORMING GROWTH FACTOR-ß

Scleroderma or systemic sclerosis (SSc) is a connective tissue disease characterized by excessive deposition of extracelluar matrix, including collagens, in the skin and various internal organs. The regulation of production and turnover of extracellular matrix components is important for normal tissue homeostasis[1]. Overproduction of matrix components is the main pathological finding in SSc tissue fibrosis. Although the pathogenesis of SSc is poorly understood, it has been suggested that growth factors and cytokines released from inflammatory cells in the organs play important roles in the initiation of connective tissue fibrosis. Among these growth factors and cytokines, transforming growth factor-ß (TGF-ß) has recently gained attention as an important factor in the pathogenesis of SSc[2]. TGF-ß is a multifunctional cytokine that plays an important role in regulating extracelluar matrix organization, cellular growth, and differentiation in many biological systems[1,3]. TGF-ß causes the deposition of extracellular matrix by simultaneously stimulating skin fibroblasts to increase the production of extracellular matrix proteins, such as collagen, fibronectin, or proteoglycan, decreasing the production of matrix-degrading proteases, increasing the production of inhibitors of these proteases, and modulating the expression of integrins[1]. In clinical investigations, several laboratories have reported increased expression of TGF-ß in skin biopsies from patients with SSc by immunohistochemical staining or *in situ* hybridization[4-9]. In other sclerotic skin disorders including localized scleroderma, eosinophilic fasciitis, and keloids, the increased expression of TGF-ß has also been described[9-11]. Thus, the TGF-ß signaling pathway is suspected to play an important role in the pathogenesis of fibrosis.

There are 3 isotypes of receptors for TGF-ß: type I, II, and III, among which type I and II receptors are involved in the TGF-ß signaling pathway[12]. The type II TGF-ß receptor (TGF-ßRII) binds TGF-ß, and then the type I receptor (TGF-ßRI) is recruited into the forms of a heteromeric complex. TGF-ßRII transphosphorylates the glycine/serine-rich domain (GS domain) of TGF-ßRI kinase[12]. After phosphorylation of Smad2 or Smad3 by the activated TGF-ßRI, a heteromeric complex is formed with Smad4, resulting in translocation of the complex into the nucleus[13,14]. The complex can directly act as transcriptional activators and indirectly regulate gene transcription interacting with other transcriptional factors[15-18].

The overexpression of TGF-ßRI and TGF-ßRII in cultured SSc dermal fibroblasts by Northern blot analysis in an *in vitro* system has been described[19]. Further, in that study, the overexpression of these receptors was correlated with the overexpression of collagen mRNA in SSc fibro-

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blasts. However, the expression levels of these receptors have not been determined in *in vivo* analysis. To examine their expression levels *in vivo*, we carried out immunohistochemical staining and *in situ* hybridization in skin sections from patients with SSc.

MATERIALS AND METHODS

Patients. In addition to normal skin samples from 5 controls, skin biopsies were taken from 5 patients with SSc (Table 1). One patient with SSc had overlap with polymyositis. The patients fulfilled the preliminary criteria for SSc proposed by the American Rheumatism Association[20]. All patients with SSc had Raynaud's phenomenon as initial sign of the disease. According to the classification of LeRoy, *et al*[21] 3 patients had limited cutaneous SSc and 2 patients had diffuse cutaneous SSc. All these patients had clinically apparent sclerosis at the dorsum of the forearm. All biopsy specimens were obtained from the dorsum of the forearm. All samples were fixed in neutral buffered formalin, embedded in paraffin, and prepared for hematoxylin-eosin (H&E) examination. On H&E examination, all patients with SSc were found to have histological sclerosis in the dermis. One patient with diffuse cutaneous SSc showed the sclerotic stage of the disease and the other 4 patients showed clinical and histological signs of the inflammatory stage, with perivascular and periappendageal lymphocytic infiltration in the dermis. The dermal collagen bundles were packed and thickened, and the dermo-epidermal junctions were flattened. Dermal sclerosis extended into the subjacent adipose tissue.

In situ hybridization. Formalin fixed, paraffin embedded skin tissues were investigated using a slight modification of the nonradioactive *in situ* hybridization technique with digoxigenin labeled RNA probes[22]. Briefly, paraffin embedded sections were cut at a thickness of 5 μm, mounted on silane coated slides, deparaffinized, and treated with 0.2 M HCl for 15 min followed by 1.5 mg/ml proteinase K digestion for 15 min at 37°C. The sections were postfixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 min and treated with PBS containing 2 mg/ml glycine twice for 15 min each time. After rinsing with PBS, the samples were soaked twice in standard saline citrate buffer with 50% formamide and subjected to hybridization.

A 2300 bp fragment from TGF-ßRI (kindly provided by Dr. P. ten Dijke, Ludwig Institute for Cancer Research, Uppsala, Sweden)[19] and a 4000 bp fragment from TGF-ßRII cDNA (kindly provided by Dr. R. Weinberg, Whitehead Institute for Biochemical Research, Cambridge, MA, USA)[19] were subcloned into the SPT18 phagemid (Roche Molecular Biochemicals, Alameda, CA, USA) and pcDNA 3.1 (Invitrogen, San Diego, CA, USA) and used to make probes. The sense probes and antisense probes of TGF-ßRI and TGF-ßRII were labeled with digoxigenin-11-UTP using a DIG RNA-labeling kit (Roche).

The labeled RNA probe (final 1 μg/ml) in a mixture containing 50% formamide, 10% dextran sulfate, 1× Denhart's solution, 100 μg/ml tRNA, 5× saline citrate buffer, 0.25% sodium dodecyl sulfate, 1 mM EDTA, and 50 mM NaH_2PO_4 was placed on the slides and covered with a coverslip. Hybridization was performed in a humidified chamber for 18 h at 45°C, after which the specimens were washed in 2× saline citrate buffer with 50% formamide at 50°C. Unhybridized probes were digested in 2.5 μg RNase A, 500 mM NaCl, 1 mM EDTA, and 10 mM Tris HCl, pH 8.0, for 15 min at 37°C. The slides were then washed for 15 min in 2× saline citrate buffer and in 0.2× saline citrate buffer twice at 50°C. After posthybridization washing, digoxigenin labeled probes were visualized as described in the DIG nucleic acid detection kit protocol (Roche Diagnostics). We used the following grading system: + for slight staining, +++ for strong staining, and ++ for staining between + and +++, and we counted the approximate number of stained cells in a 200× magnified area.

Immunohistochemical staining. Immunohistochemical staining on paraffin embedded sections was performed using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's recommendations. Five micrometer sections were mounted on APES coated slides, then deparaffinized by xylene and rehydrated through a graded series of ethyl alcohol and PBS. The sections were then incubated with antibodies against TGF-ßRI and TGF-ßRII (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 500× in PBS overnight at 4°C. The immunoreactivity was visualized by diaminobenzidine. The sections were then counterstained with hematoxylin. We used the following grading system: + for slight staining, +++ for strong staining, and ++ for staining between + and +++.

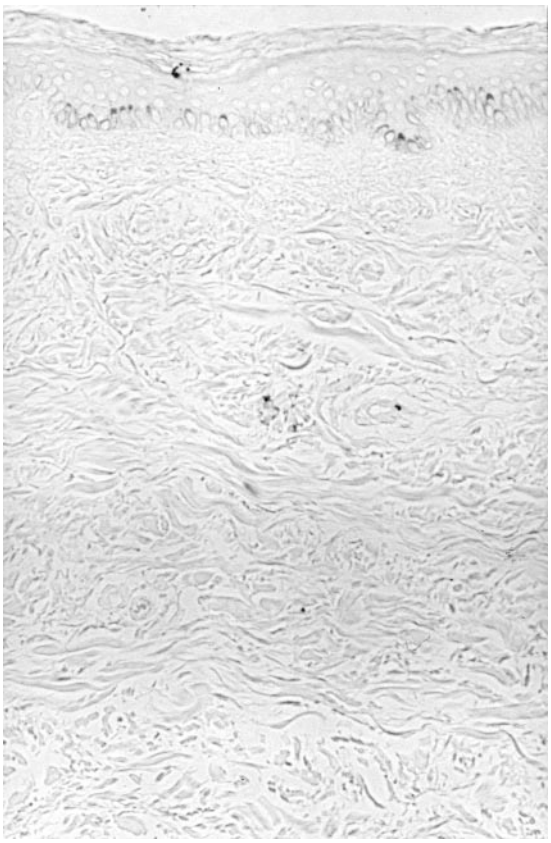
RESULTS

In situ hybridization using sense probes of TGF-ßRI and TGF-ßRII. We investigated the background of our *in situ* hybridization technique using sense probes of TGF-ßRI and TGF-ßRII in control and SSc skin sections. There was no staining or coloration by *in situ* hybridization except for the basal pigmentation in the epidermis in sections from control and SSc skin (Figure 1A).

Table 1. Results of *in situ* hybridization and immunohistochemical staining from sections of SSc and normal skin.

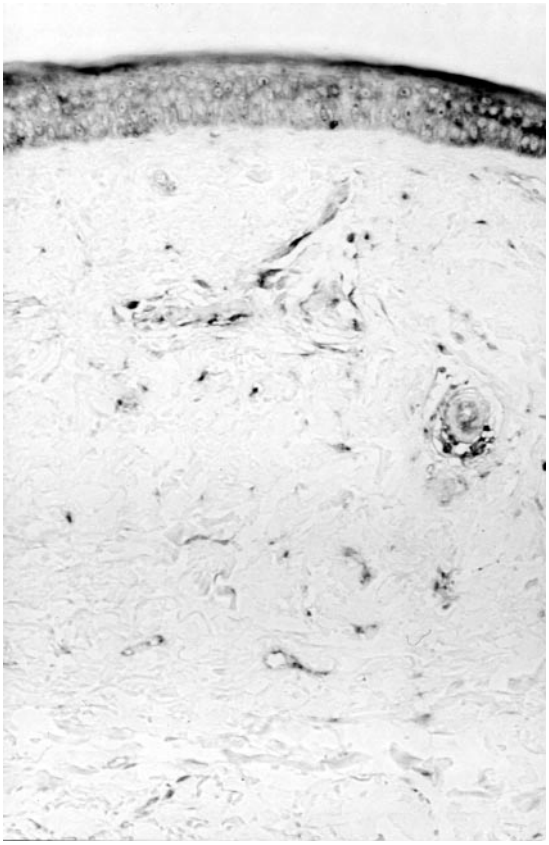
	In situ Hybridization						Immunohistochemical Staining			
	Type I Receptor			Type II Receptor			Type I Receptor		Type II Receptor	
	Epidermis	Fibroblasts	No. *	Epidermis	Fibroblasts	No.*	Epidermis	Fibroblasts	Epidermis	Fibroblasts
Case										
1	+	+	10–20	++	+	10–20	++	++	++	+++
2	++	++	10–20	++	++	> 30	++	+++	++	+++
3	++	+	5–10	++	++	> 30	+++	+	++	++
4	+	++	1–5	++	++	> 30	+++	++	+++	+++
5	+++	++	> 30	++	++	> 30	–	–	++	++
Control										
1	++	+	0–1	++	+	1–5	++	++	++	+
2	++	–	0	+	–	0	++	+	++	+
3	+	–	0	+	–	0	++	–	++	–
4	++	+	0–1	++	+	1–5	++	++	+	–
5	++	–	0	++	+	0–1	+	–	+	–

* Approximate numbers of positively stained cells in 200× magnified areas. – to +++: approximate intensity of stained cells.



A

B



C

D

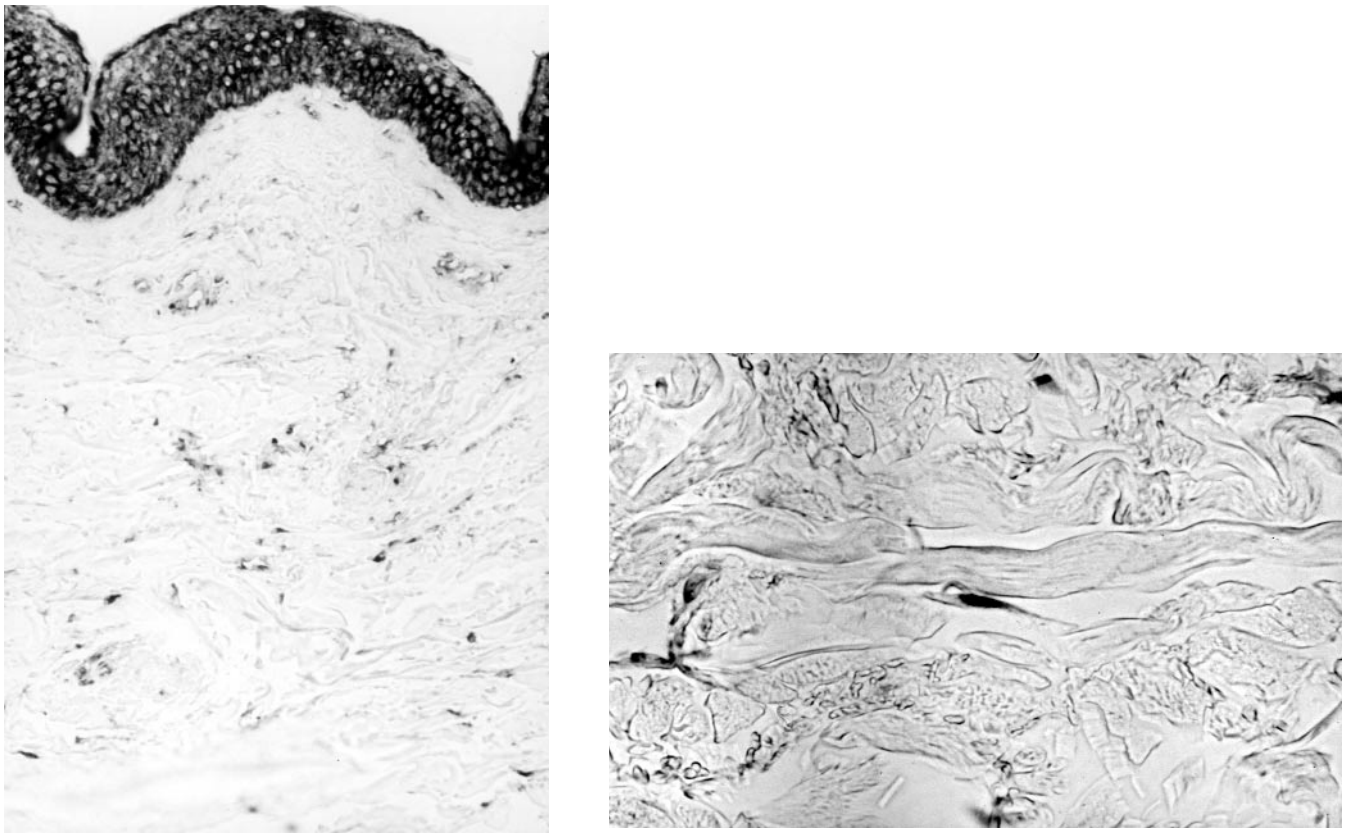


Figure 1. A. Representative *in situ* hybridization staining using TGF-ß type II receptor sense probe for skin section from a patient with SSc. There is no staining or coloration except for basal pigmentation by melanin. *In situ* hybridization was performed in normal (B) and scleroderma skin sections (C) using TGF-ß type I receptor antisense probe. Strong staining was found in the epidermis and appendageal tissues, and moderate staining in blood vessels in both normal and SSc skin sections. Strong staining of spindle shaped cells was found in the dermis of scleroderma skin sections, but only weak staining was found in normal skin sections. *In situ* hybridization was also performed in normal (D) and scleroderma skin sections (E) using TGF-ß type II receptor antisense probe. Strong staining was found in the epidermis and appendageal tissues, and moderate staining was found in blood vessels in both normal and SSc skin sections. Strong staining of spindle shaped cells was found in the dermis of scleroderma skin sections, but only weak staining was found in normal skin sections. Panel F: a higher magnification of *in situ* hybridization of SSc skin by TGF-ß type II receptor antisense probe. Staining was found in the spindle shaped cells between collagen bundles. The spindle shaped cells seemed to be dermal fibroblasts morphologically. Original magnifications, A–E ×200; F ×1200.

In situ hybridization using antisense probes of TGF-βRI and TGF-βRII in control skin sections. We investigated the expression of TGF-ßRI and TGF-ßRII mRNA in control skin sections by *in situ* hybridization (Table 1). TGF-ßRI mRNA (Figure 1B) and TGF-ßRII mRNA (Figure 1D) was expressed strongly in the follicles and the sebaceous glands, moderately in the epidermis and blood vessels, and slightly in the spindle shaped cells in the dermis.

In situ hybridization using antisense probes of TGF-βRI and TGF-βRII in patients' skin sections. We investigated the expression of TGF-ßRI and TGF-ßRII mRNA in skin sections from patients with SSc by *in situ* hybridization (Table 1). TGF-ßRI mRNA (Figure 1C) and TGF-ßRII mRNA (Figure 1E) was expressed strongly in follicles and sebaceous glands. We found spindle shaped cells with strong expression of TGF-ßRI mRNA and TGF-ßRII mRNA between collagen bundles, and moderate expression in the epidermis and blood vessels. The spindle shaped cells expressing TGF-ßRI mRNA and TGF-ßRII mRNA were increased in the tissue sections from patients with SSc. Morphologically, the spindle shaped cells were seen to be fibroblasts at higher magnification (Figure 1F); these skin samples (Figures 1C, 1E, 1F) were from a patient with diffuse cutaneous SSc.

Immunohistochemical staining using antibodies against TGF-βRI and TGF-βRII in control skin sections. We investigated the expression of TGF-ßRI and TGF-ßRII proteins in control skin sections by immunohistochemical staining (Table 1). TGF-ßRI (Figure 2A) and TGF-ßRII proteins (Figure 2C) were expressed strongly in the follicles and the sebaceous glands, moderately in the epidermis and blood vessels, and slightly in the spindle shaped cells between collagen bundles. The results from

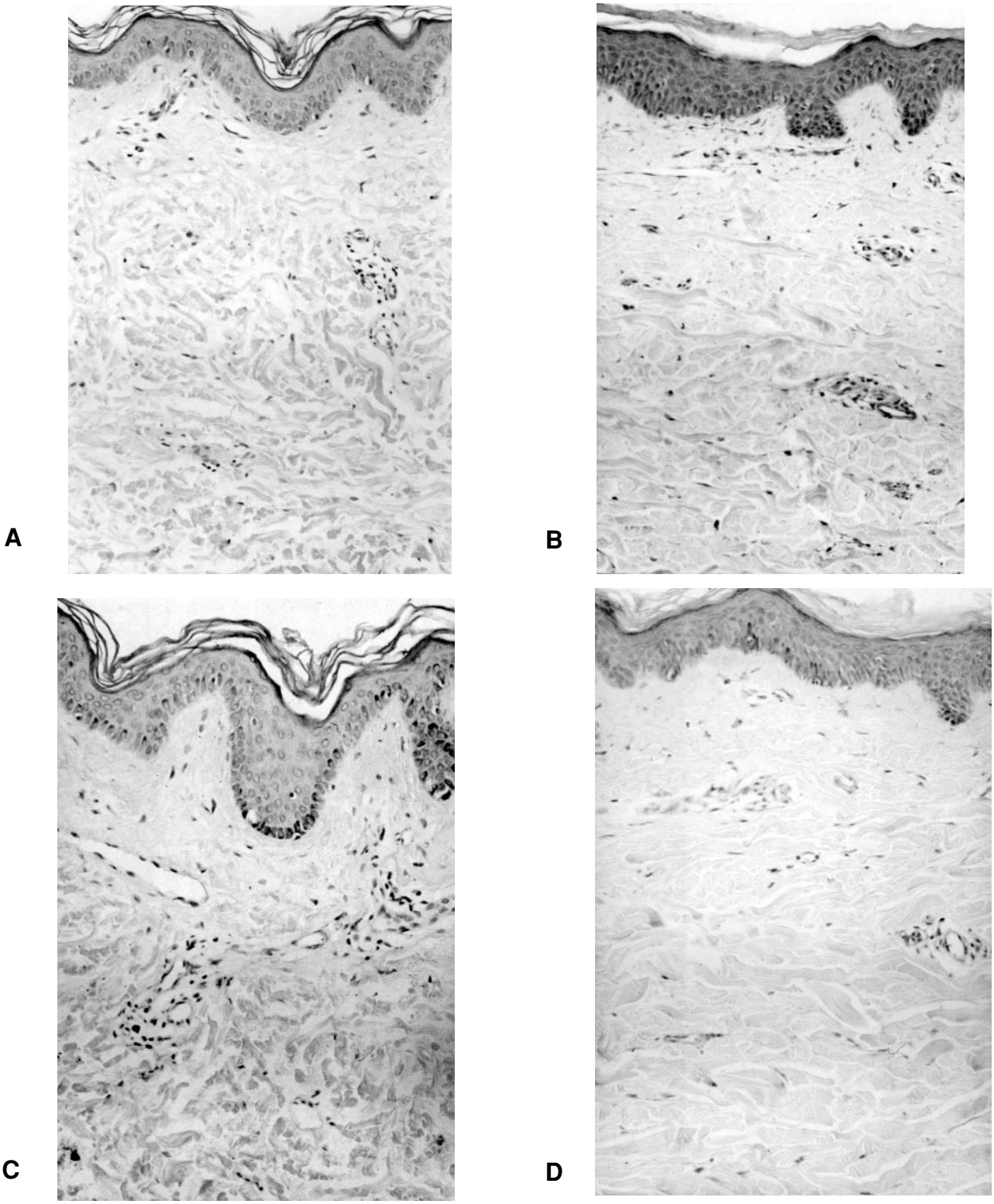


Figure 2. Immunohistochemical staining was performed in normal and SSc skin sections using polyclonal antibodies against TGF-ß type I and type II receptor. Using antibodies against type I receptor, staining was found in the epidermis, and slight staining of spindle shaped cells was found in the dermis in normal skin sections (A). Staining was found in the epidermis, in the spindle shaped cells between collagen bundles, and in the round cells around blood vessels in SSc skin sections (B). Using antibodies against TGF-ß type II receptor, staining was found in the epidermis, and slight staining of spindle shaped cells was found in the dermis in normal skin sections (C). Staining was detected in the epidermis, in spindle shaped cells between collagen bundles, and in the round cells around blood vessels in SSc skin sections (D), and the staining patterns were quite similar to those of TGF-ß type I receptors. Counterstained with hematoxylin. Original magnifications ×200.

immunohistochemical staining were similar to those of *in situ* hybridization.

Immunohistochemical staining using antibodies against TGF-βRI and TGF-βRII in SSc skin sections. We investigated the expression of TGF-ßRI and TGF-ßRII proteins in SSc skin sections by immunohistochemical staining (Table 1). TGF-ßRI (Figure 2B) and TGF-ßRII proteins (Figure 2D) were expressed strongly in the follicles and sebaceous glands, and moderately in the epidermis, blood vessels, and the spindle shaped cells between collagen bundles. These skin samples were from the same patient with diffuse cutaneous SSc described above for *in situ* hybridization. The results from immunohistochemical staining were similar to those of *in situ* hybridization. Further, the numbers of fibroblasts that express TGF-ßRI protein and TGF-ßRII protein were increased in SSc skin sections. However, the differences of immunohistochemical staining of fibroblasts between SSc skin and normal skin were not apparent compared to the differences of *in situ* hybridization.

DISCUSSION

TGF-ß is recognized as a central mediator in various fibrotic diseases including SSc[2]. TGF-ß acts on human skin fibroblasts principally as a stimulator of extracelluar matrix production, leading to excessive extracelluar matrix deposition. TGF-ß can increase expression of collagen types I, III, VI, VII and X, fibronectin, and proteoglycans[3]. Stimulation of extracelluar matrix production by TGF-ß is further enhanced by its inhibitory effect on matrix degradation by decreasing synthesis of proteases and increasing concentrations of protease inhibitors[3]. An important mechanism for regulating the cellular response to cytokines and hormones resides at the level of receptor expression. Modulation of the levels of TGF-ßRI and TGF-ßRII expression may play an important role in the mechanism of wound healing and in the pathogenesis of various fibrogenetic disorders. Several kinds of cells including immune, endothelial, and mesenchymal cells are thought to be sources of TGF-ß in fibrotic lesions, and sustained production of TGF-ß in the lesion may be maintained by autoinduction[23,24]. Although constitutive activation of the TGF-ß signaling pathway in lesions is thought to contribute to disease progression, the mechanism of the activation is unknown. One possible mechanism may involve the establishment of an autocrine cytokine loop. Such a loop may be initiated *in vivo* by exposure to certain cytokines and may continue to operate in the cell culture conditions. Cytokines known to induce extracellular matrix production such as TGF-ß, interleukin 4 (IL-4), oncostatin M, or IL-1 are candidates for such autoinduction, acting separately or in concert.

We observed that expression of both TGF-ßRI and TGF-ßRII mRNA in skin sections of patients with SSc were elevated in comparison to control skin sections. In contrast, the staining of TGF-ßRI and TGF-ßRII mRNA in the epidermis in *in situ* hybridization showed no differences between SSc skin and normal skin. In *in vitro* analysis, the elevation of mRNA expression of these receptors was correlated with elevated α2(1) collagen mRNA levels. These results may suggest the overtransduction of TGF-ß signaling pathway, and overexpression of these receptors may induce the overexpression of extracelluar matrix including collagen[19].

These results suggest elevated concentrations of both TGF-ßRI and TGF-ßRII in skin sections of SSc; elevated concentrations of TGF-ß were reported previously[4-9]. Thus, in the tissue of patients with SSc, both TGF-ß and its type I and type II receptors are upregulated. In addition, patients with diffuse cutaneous SSc tended to show more upregulated TGF-ß receptor I and II expression than those with limited cutaneous SSc. It has been shown in other experimental systems that a 2-fold increase in receptor expression led to at least a 10-fold decrease in the ligand concentration required to induce a biologic response[25]. Thus, it may be suggested that the upregulation of TGF-ß signaling pathway is mainly due to the overexpression of TGF-ßRI and TGF-ßRII. There are several reports describing success in blocking fibrosis or reduction in production of collagen by blocking antibodies or antisense oligonucleotides against TGF-ß in animal models and in *in vitro* systems[26-29]. However, the results of this study suggest that it would also be effective to block TGF-ßRI or TGF-ßRII in the prevention of sclerosis by the TGF-ß signaling pathway. Future technical improvements would make it possible that blocking TGF-ß or TGF-ß receptors might be utilized for the treatment of SSc or other fibrotic diseases.

In summary, TGF-ßRI and TGF-ßRII are upregulated in dermal fibroblasts in the affected skin of patients with SSc, and dermal fibrosis may be induced by autocrine TGF-ß signaling due to the overexpression of both TGF-ßRI and TGF-ßRII in the skin of patients with SSc.

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