Involvement of αvβ5 Integrin in the Establishment of Autocrine TGF-β Signaling in Dermal Fibroblasts Derived from Localized Scleroderma

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Localized scleroderma (LSc) is a connective tissue disorder limited to skin and subcutaneous tissue, which may share pathogenic processes with systemic sclerosis (SSc). We previously demonstrated that upregulated expression of integrin αvβ5 might contribute to autocrine TGF-β signaling in SSc fibroblasts. Based on these data, we presently focused on αvβ5 and assessed its involvement in pathogenesis of LSc. We initially demonstrated that LSc fibroblasts might be activated by the stimulation of autocrine TGF-β. Consistent with SSc fibroblasts, expression levels of αvβ5 were elevated in LSc fibroblasts *in vitro* and *in vivo*. Anti-αvβ5 antibody partially reversed expression levels of type I procollagen and MMP-1 and constitutive DNA–Smad3 binding in LSc fibroblasts. In LSc fibroblasts pretreated with antisense TGF-β1, exogenous latent TGF-β1 stimulation increased expression of type I procollagen in an αvβ5-dependent manner. The luciferase activities of TMLC cells, Mv1Lu cells stably expressing a portion of the plasminogen activator inhibitor 1 promoter, co-cultured with LSc fibroblasts were significantly elevated compared with those co-cultured with normal fibroblasts and were significantly reduced in the presence of anti-αvβ5 antibody. Anti-αvβ5 antibody reversed the myofibroblastic features of LSc fibroblasts. These results indicate that upregulated expression of αvβ5 contributes to autocrine TGF-β signaling in LSc fibroblasts.

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INTRODUCTION

Localized scleroderma (LSc) is a connective tissue disorder limited to the skin and subcutaneous tissue, often involving the muscular tissues beneath the cutaneous lesions. The absence of Raynaud's phenomenon, of acrosclerosis, and of involvement of internal organs differentiates it from systemic sclerosis (SSc) (Jablonska and Rodnan, 1979). However, the two diseases may share similar pathogenetic processes, since abnormal collagen metabolism (Higley *et al.*, 1994; Kubo *et al.*, 2001; Asano *et al.*, 2004a, b, c; Asano *et al.*, 2005a) and autoimmunity (Kahaleh, 1993; Takehara and Sato, 2005) are considered to be fundamental characteristics of both. Elevated collagen synthesis by skin fibroblasts derived from involved lesions is one of the common characteristics of the two conditions and may be closely related to their pathogenesis.

TGF-β1 is a multifunctional cytokine that regulates the growth, differentiation, and function of various cell types (Wahl, 1994). The principal effect of TGF-β1 on mesenchymal cells is its stimulation of extracellular matrix deposition. TGF-β1 has been shown to increase the expression of collagen types I, III, VI, VII, and X, fibronectin, and proteoglycans (LeRoy, 1974; Buckingham *et al.*, 1978; Falanga *et al.*, 1987; Raghow *et al.*, 1987; Peltonen *et al.*, 1990; Xu *et al.*, 1991; Rudnicka *et al.*, 1994; Jelaska *et al.*, 1996). Stimulation of extracellular matrix production by TGF-β1 is further enhanced by its inhibitory effect on matrix degradation, decreasing the synthesis of proteases and increasing levels of protease inhibitors (Massague, 1990). The involvement of TGF-β in the pathogenesis of dermal fibrosis in SSc has been well-studied. Since many of the characteristics of SSc fibroblasts resemble those of healthy fibroblasts stimulated by TGF-β1 (LeRoy *et al.*, 1989; Massague, 1990), the dermal fibroblast activation in SSc is thought to be a result of stimulation by autocrine TGF-β. This notion is supported by our previous findings that (i) SSc fibroblasts express elevated levels of TGF-β receptors, and this correlates with elevated levels of α2(I) collagen mRNA (Kawakami *et al.*, 1998) and (ii) the blockade of TGF-β signaling with anti-TGF-β antibodies or TGF-β1 antisense oligonucleotide (AS-TGF-β1) abolished the increased

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Abbreviations: LSc, localized scleroderma; SSc, systemic sclerosis; AS-TGF-β1, TGF-β1 antisense oligonucleotide; LAP-β1, latency-associated peptide-β1; SLC, small latent complex; α-SMA, α-smooth muscle actin

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expression of human α2(I) collagen mRNA in SSc fibroblasts (Ihn *et al.*, 2001).

TGF-β1 is normally secreted as a complex composed of three proteins, including the bioactive peptide of TGF-β1, a latency-associated peptide-β1 (LAP-β1), and a latent TGF-β-binding protein-1. TGF-β1 forms a complex with LAP-β1 noncovalently, which is called the small latent complex (SLC), and in this configuration TGF-β1 is unable to bind to its receptors. SLC is joined by a latent TGF-β-binding protein-1, the N-terminal region of which is covalently crosslinked to extracellular matrix proteins by transglutaminase, and the complex of all three proteins is called the large latent complex (Annes *et al.*, 2003). The constitutive secretion of latent TGF-β1 by many cell types in culture suggests that there are extracellular mechanisms to control the activity of this potent cytokine.

LAP-β1 contains an RGD motif that is recognized by αv-containing integrins, including αvβ1, αvβ3, αvβ5, αvβ6, and αvβ8 (Munger *et al.*, 1998; Munger *et al.*, 1999; Mu *et al.*, 2002; Ludbrook *et al.*, 2003). Though all of these αv-containing integrins bind to LAP-β1 and have the potential to modulate the localization and possibly activation of SLC, only αvβ6 and αvβ8, both of which are not expressed in dermal fibroblasts, have been demonstrated to be able to activate SLC (Munger *et al.*, 1999; Mu *et al.*, 2002). Though there have been no reports which indicate the activation of SLC by other αv-containing integrins (αvβ1, αvβ3, and αvβ5), we previously demonstrated that αvβ5 is upregulated in SSc dermal fibroblasts and the transient overexpression of αvβ5 induces the increased transcriptional activity of human α2(I) collagen gene in normal dermal fibroblasts (Asano *et al.*, 2004a). Furthermore, our recent report suggested that the upregulated expression of αvβ5 contributes to the establishment of autocrine TGF-β signaling in SSc fibroblasts (Asano *et al.*, 2005b, Asano *et al.*, 2006).

To clarify the similarity and the difference is a distinctively important process to further disclose the mechanism of fibrosis in LSc and SSc. We previously demonstrated that the expression levels of TGF-β receptors were elevated in LSc fibroblasts as well as in SSc fibroblasts by immunohistochemistry and *in situ* hybridization (Kubo *et al.*, 2001). In spite of the difference in autoimmune abnormalities (Takehara and Sato, 2005), these two disorders may share several aspects of fibrotic processes, including autocrine TGF-β signaling. The goal of this study is to investigate whether autocrine TGF-β signaling is involved in the self-activation system in LSc fibroblasts. In addition, we investigated the involvement of αvβ5 in the establishment of autocrine TGF-β signaling in LSc fibroblasts.

RESULTS

Expression levels of type I procollagen and MMP-1 in LSc fibroblasts

As an initial experiment, we compared the expression levels of type I procollagen and MMP-1 between normal and LSc fibroblasts by immunoblotting. As shown in Figure 1, the expression levels of type I procollagen protein were significantly elevated in LSc fibroblasts compared with normal fibroblasts (4.2-fold, $P<0.05$). By contrast, the expression levels of MMP-1 were significantly decreased in LSc fibroblasts compared with normal fibroblasts (0.07-fold, $P<0.05$). These results suggest that the increased synthesis and the decreased degradation of type I collagen contribute to the dermal fibrosis in LSc.

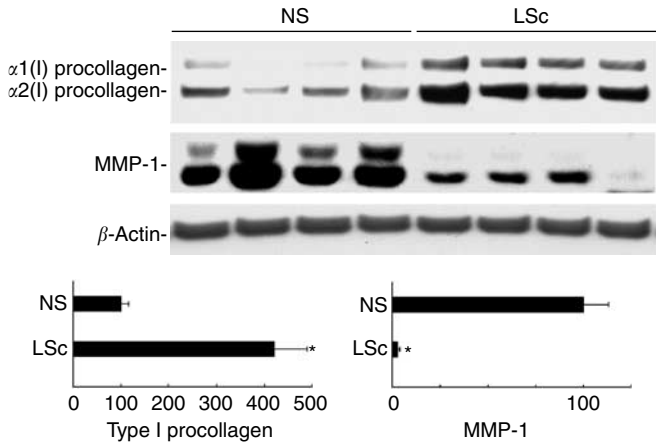

Figure 1. Comparison of the expression levels of type I procollagen and MMP-1 between normal and LSc fibroblasts. Whole-cell lysates were analyzed by immunoblotting using anti-type I collagen antibody, anti-MMP-1 antibody, or anti-β-actin antibody. One representative of five independent experiments is shown (upper panel). The protein levels quantitated by scanning densitometry and corrected for the level of β-actin in the same samples are shown relative to those in normal fibroblasts (100 arbitrary units (AU)) (lower panel). The levels of type I procollagen proteins were defined as the mean band density of α1(I) and α2(I) procollagen proteins. Data are expressed as the mean ± SD of five independent experiments. *$P<0.05$ versus normal fibroblasts. NS: normal skin fibroblasts. LSc: localized scleroderma fibroblasts.

LSc fibroblasts may be activated by the stimulation of autocrine TGF-β. To clarify whether the activation of LSc fibroblasts is the result of the stimulation by autocrine TGF-β, we investigated the effect of AS-TGF-β1 on the expression levels of type I procollagen and MMP-1. As shown in Figure 2a, the treatment of AS-TGF-β1 significantly reduced the expression levels of type I procollagen protein and increased the expression levels of MMP-1 protein to a similar extent to those observed in normal fibroblasts. These results indicate that LSc fibroblasts may be activated by the stimulation of autocrine TGF-β1. To further confirm this point, we next focused on the activation state of TGF-β signaling. In our previous report, we demonstrated that the phosphorylation level of Smad3, determined by immunoprecipitation, and the DNA-binding ability of Smad3, determined by DNA affinity precipitation, were significantly elevated in SSc fibroblasts and that the phosphorylation level of Smad3 was completely correlated with the DNA-binding ability of Smad3 (Asano *et al.*, 2004c; Asano *et al.*, 2005b). Since DNA affinity precipitation is highly sensitive to detect the DNA-binding ability of Smad3, we used this method to determine the activation state of TGF-β signaling in LSc fibroblasts. As shown in the upper panel in Figure 2b, the DNA–Smad3 binding was marginal in normal fibroblasts. In contrast, the

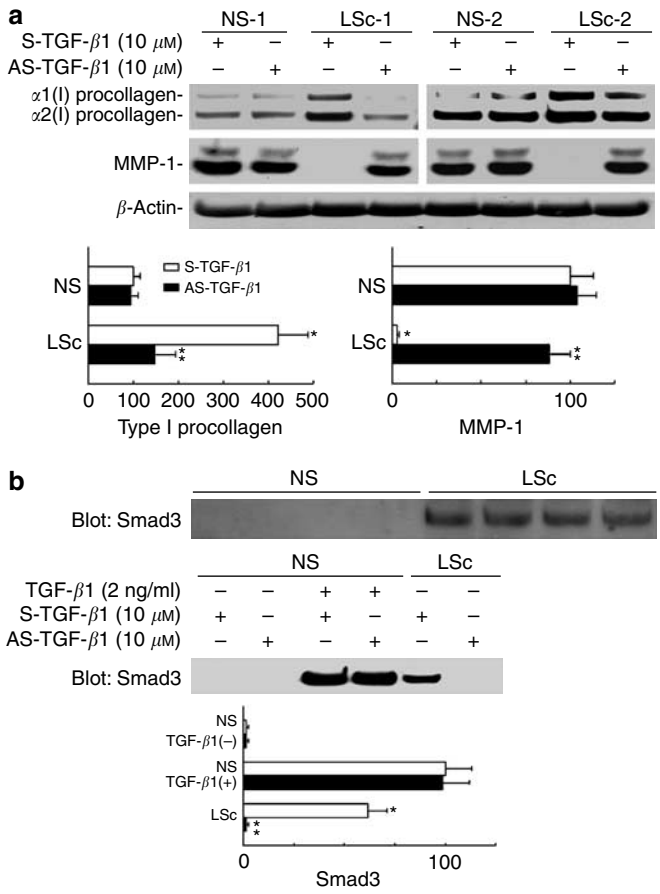

Figure 2. LSc fibroblasts may be activated by the stimulation of autocrine TGF-β. (**a**) Cells were treated with the indicated reagent for 48 hours. Whole-cell lysates were analyzed by immunoblotting using indicated antibodies. One representative of five independent experiments is shown (upper panel). The protein levels quantitated by scanning densitometry and corrected for the level of β-actin in the same samples are shown relative to those in normal fibroblasts (100 AU) (lower panel). (**b**) Cells were treated with TGF-β1 antisense oligonucleotide (AS-TGF-β1) or TGF-β1 sense oligonucleotide (S-TGF-β1) for 48 hours. In some experiments, cells were treated with TGF-β1 for the last 3 hours. Nuclear extracts were incubated with biotin-labeled oligonucleotides. Proteins bound to these nucleotides were isolated with streptavidin-agarose beads, and Smad3 was detected by immunoblotting. One representative of five independent experiments is shown in the upper and middle panels. In the lower panels, relative Smad3 levels quantitated by scanning densitometry are shown relative to those in normal fibroblasts treated with 10 μM of S-TGF-β1 and 2 ng/ml of TGF-β1 (100 AU). The mean and SD from 5 separate experiments are shown. *$P<0.05$ versus normal fibroblasts treated with S-TGF-β1. **$P<0.05$ versus LSc fibroblasts treated with S-TGF-β1.

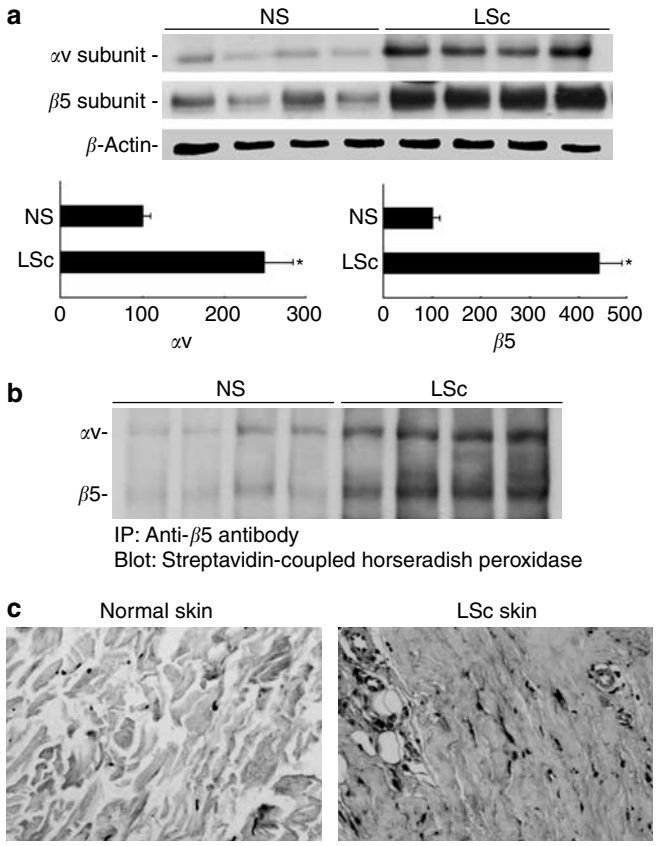

Figure 3. Comparison of the expression levels of αvβ5 between normal and LSc fibroblasts. (**a**) Whole-cell lysates were analyzed by immunoblotting using indicated antibodies. One representative of five independent experiments is shown (upper panel). The protein levels quantitated by scanning densitometry and corrected for the level of β-actin in the same samples are shown relative to those in normal fibroblasts (100 AU) (lower panel). Data are expressed as the mean ± SD of five independent experiments. *$P<0.05$ versus normal fibroblasts. (**b**) Cell surface proteins were labeled with biotin and cell lysates were immunoprecipitated using anti-β5 antibody. One representative of five independent experiments is shown. (**c**) Paraffin sections of normal and LSc dermal tissue were subjected to immunohistochemical analysis with anti-αvβ5 antibody. The immunoreactivity was visualized by diaminobenzidine. The sections were counterstained with hematoxylin. Original magnification, × 200.

constitutive DNA–Smad3 binding was detected in LSc fibroblasts. These results indicate that TGF-β signaling is constitutively activated in LSc fibroblasts. Next, we investigated the effect of AS-TGF-β1 on the DNA–Smad3 binding in LSc fibroblasts. As shown in the middle panel in Figure 2b, the DNA–Smad3 binding was markedly elevated in normal fibroblasts treated with TGF-β1 and the treatment of AS-TGF-β1 showed no effect on the DNA–Smad3 binding in normal fibroblasts. In contrast, the same treatment almost completely abolished the DNA–Smad3 binding in LSc fibroblasts. These results confirmed the notion that LSc fibroblasts may be activated by the stimulation of autocrine TGF-β.

Expression levels of αv and β5 subunit proteins in cultured normal and LSc fibroblasts

To investigate whether αvβ5 is involved in the establishment of the autocrine TGF-β signaling in LSc fibroblasts, we initially compared the expression levels of αv and β5 subunit proteins between cultured normal and LSc fibroblasts by immunoblotting. As shown in Figure 3a, the expression levels of αv and β5 subunit proteins were significantly higher in LSc fibroblasts than normal fibroblasts (2.5- and 4.5-fold increase, respectively).

Expression levels of αvβ5 on the surface of cultured normal and LSc fibroblasts

To function as active receptors, integrins have to be present on the cell surface as dimers. Therefore, we next determined the cell surface levels of αvβ5 in normal and LSc fibroblasts. To this end, cell surface proteins were labeled with biotins and immunoprecipitation was performed using anti-β5 antibody. As shown in Figure 3b, cell surface levels of αvβ5 were markedly elevated in LSc fibroblasts compared with normal fibroblasts. These bands were confirmed to be αv or β5 by a reprobing analysis using anti-αv or β5 antibodies (data not shown).

Distribution of αvβ5 protein in normal and LSc dermal sections

To investigate the distribution of αvβ5 protein *in vivo*, immunohistochemical staining was performed using dermal section derived from four LSc patients and four normal controls. Representative results are shown in Figure 3c. Regarding the epidermis, blood vessels, and smooth muscles, there was no difference in immunoreactivity for the anti-αvβ5 antibody between normal and LSc dermal sections. The expression of the αvβ5 protein was strong in the blood vessels and smooth muscles, and weak in the epidermis. However, the spindle-shaped cells, especially those between thickened collagen bundles in the middle and deep dermis, demonstrated strong immunoreactivity for the αvβ5 protein in LSc dermal sections, whereas those in normal skin sections were weakly stained. These results were consistent with the results for cultured fibroblasts described above. Unexpectedly, the epitope of the αvβ5 protein was also observed scattered between thickened collagen bundles in all LSc dermal sections.

The treatment of anti-αvβ5 antibody partially reversed the expression levels of type I procollagen and MMP-1 by reducing the constitutive DNA–Smad3 binding in LSc fibroblasts. We next investigated the effects of anti-αvβ5 antibody on the expression levels of type I procollagen and MMP-1. As shown in Figure 4a, this antibody partially, but significantly, reversed the expression levels of type I procollagen and MMP-1 in LSc fibroblasts. We also investigated the effect of anti-αvβ5 antibody on the DNA–Smad3 binding levels in LSc fibroblasts. As shown in Figure 4b, the pretreatment of anti-αvβ5 antibody did not affect the DNA–Smad3 binding levels in normal fibroblasts either treated or untreated with TGF-β1. In contrast, the treatment of anti-αvβ5 antibody partially, but significantly, reduced the DNA–Smad3 binding levels in LSc fibroblasts. These results indicate that the upregulation of αvβ5 may be involved in the activation of TGF-β signaling in LSc fibroblasts.

Exogenous SLC stimulation induces the expression of type I procollagen in LSc fibroblasts

Next, we compared the ability to activate SLC between normal and LSc fibroblasts. To this end, we investigated the effect of exogenous SLC stimulation on the expression levels of type I procollagen protein. To suppress the endogenous TGF-β1 production, cells were pretreated with 10 μM of AS-TGF-β1 for 48 hours prior to the stimulation by active TGF-β1 or SLC. As shown in the upper panels of Figure 5a, AS-TGF-β1 significantly decreased the expression levels of type I procollagen protein in LSc fibroblasts to levels similar to those in normal fibroblasts. Exogenous active TGF-β1 stimulation showed a significant increase in the expression levels of type I procollagen protein in normal and LSc fibroblasts pretreated with AS-TGF-β1. In contrast, exogenous SLC stimulation showed a significant increase in the expression levels of type I procollagen protein in LSc fibroblasts treated with AS-TGF-β1, while the same treatment revealed no significant effect in normal fibroblasts treated with AS-TGF-β1. Furthermore, as shown in the lower panels

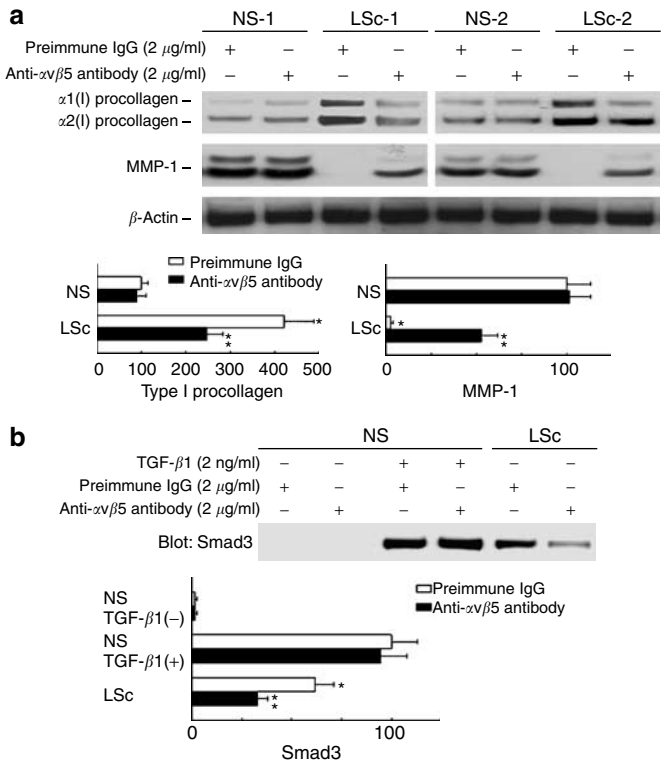

Figure 4. Anti-αvβ5 antibody partially reverses the expression levels of type I procollagen and MMP-1 and the DNA–Smad3 binding levels in LSc fibroblasts. (**a**) Cells were treated with the indicated reagents for 48 hours. Whole-cell lysates were analyzed by immunoblotting using indicated antibodies. One representative of five independent experiments is shown (upper panel). The protein levels quantitated by scanning densitometry and corrected for the level of β-actin in the same samples are shown relative to those in normal fibroblasts treated with preimmune IgG (100 AU) (lower panel). (**b**) Cells were treated with the indicated antibodies for 48 hours. In some experiments, cells were treated with TGF-β1 for the last 3 hours. Nuclear extracts were incubated with biotin-labeled oligonucleotides. Proteins bound to these nucleotides were isolated with streptavidin-agarose beads, and Smad3 was detected by immunoblotting. One representative of five independent experiments is shown in the upper panels. In the lower panels, relative Smad3 levels quantitated by scanning densitometry are shown relative to those in normal fibroblasts treated with preimmune IgG and TGF-β1 (100 AU). The mean and SD from five separate experiments are shown. $*P<0.05$ versus normal fibroblasts treated with preimmune IgG. $**P<0.05$ versus LSc fibroblasts treated with preimmune IgG.

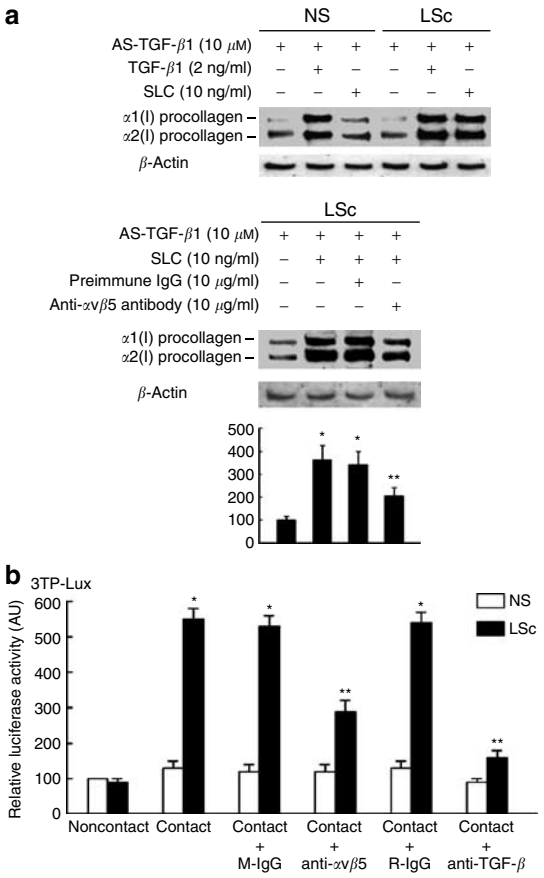

Figure 5. Endogenous latent TGF-β is activated on the cell surface of LSc fibroblasts in the αvβ5-dependent manner. (**a**) Cells treated with AS-TGF-β1 (10 μM, for 72 h) were stimulated with indicated reagents (TGF-β1 or SLC) for the last 24 hours. In some experiments, SLC stimulation was performed in the presence of anti-αvβ5 antibody or preimmune IgG. Whole-cell lysates were analyzed by immunoblotting using indicated antibodies. One representative of five independent experiments is shown (upper and middle panels). The protein levels quantitated by scanning densitometry and corrected for the level of β-actin in the same samples are shown relative to those in LSc fibroblasts treated with AS-TGF-β1 (100 AU) (lower panel). Data are expressed as the mean ± SD of five independent experiments. *$P<0.05$ versus LSc fibroblasts treated with AS-TGF-β1. **$P<0.05$ versus LSc fibroblasts treated with AS-TGF-β1, SLC, and preimmune IgG. (**b**) Either normal or LSc fibroblasts were mixed with TMLC cells at a ratio of 1:1 and cocultured in confluence. In experiments without cell–cell contact, either normal or LSc fibroblasts were cocultured with TMLC cells separately by using inserts. After 24-hour incubation, the luciferase activities were determined. In some experiments, assays were performed in the presence of anti-αvβ5 antibody (anti-αvβ5, 10 μg/ml), preimmune mouse IgG (M-IgG, 10 μg/ml), anti-TGF-β antibody (anti-TGF-β, 10 μg/ml), or preimmune rabbit IgG (R-IgG, 10 μg/ml). Values represent the luciferase activity relative to that of TMLC cells cocultured with normal fibroblasts in the absence of contact (100 AU). The mean and SD from five separate experiments are shown. *$P<0.05$ versus normal fibroblasts under the same condition. **$P<0.05$ versus LSc fibroblasts treated with preimmune mouse IgG (a control against anti-αvβ5 antibody) or preimmune rabbit IgG (a control against anti-TGF-β antibody).

of Figure 5a, this effect of SLC in LSc fibroblasts was significantly reduced by the pretreatment of anti-αvβ5 antibody. These results suggest that the upregulated expression of αvβ5 contributes to the SLC activation in LSc fibroblasts.

Latent TGF-β is activated on the cell surface of LSc fibroblasts through αvβ5-dependent pathway

We next investigated the localization of active TGF-β in normal and LSc fibroblasts by coculture assay. In this assay, normal or LSc fibroblasts were cocultured with TMLC cells (Figure 5b). Since the luciferase activity of TMLC cells is hypersensitive to the stimulation of TGF-β, this assay can determine the localization of active TGF-β in normal and LSc fibroblasts. In the situation that active TGF-β is localized on the cell surface of fibroblasts, the luciferase activity of TMLC cells cocultured in the presence of cell contact will be significantly elevated compared with that cocultured in the absence of cell contact. By contrast, in the situation that active TGF-β is freely diffusible in cell culture medium, there will be no significant difference in the luciferase activity between TMLC cells cocultured in the presence of cell contact and those cocultured in the absence of cell contact. The luciferase activity was significantly elevated in TMLC cells cocultured with LSc fibroblasts compared with those with normal fibroblasts (about 5.5-fold increase, $P<0.05$). This increase was significantly reduced by anti-αvβ5 antibody (about 50% reduction) and almost completely abolished by anti-TGF-β antibody, while preimmune mouse or rabbit IgG showed no effect. We also carried out coculture assays with inserts to separate TMLC cells and normal or LSc fibroblasts while allowing soluble molecules to pass. In the absence of contact, LSc fibroblasts showed no significant induction of luciferase activity. These results indicate that SLC is activated on the cell surface of LSc fibroblasts and suggest that this activation process was partially attributed to αvβ5-dependent pathway.

Blockade of αvβ5 reverses the myofibroblastic phenotype of LSc fibroblasts

Finally, we performed immunofluorescence using anti-α-smooth muscle actin (SMA) antibody to determine the effect of anti-αvβ5 antibody on the expression levels of α-SMA and the morphology of cells (Figure 6). In LSc fibroblasts, about 80% of cells showed the morphological changes of cellular hypertrophy and well-formed α-SMA fibers, which are characteristics of myofibroblasts. However, after the treatment of anti-αvβ5 antibody, the percentage of cells with these features was reduced to about 30%. In contrast, in normal fibroblasts, cells with these features were less than 5% in the presence or absence of functional blocking antibody. These results indicate that anti-αvβ5 antibody reverses the myofibroblastic phenotype of LSc fibroblasts.

DISCUSSION

This study was undertaken to clarify the mechanism of the activation process in LSc fibroblasts. We initially demonstrated the following three evidences: (i) The expression levels of type I procollagen protein are elevated and those of MMP-1 protein were decreased in LSc fibroblasts. (ii) Constitutive DNA–Smad3 binding is observed in LSc fibroblasts. (iii) The treatment of AS-TGF-β1 reverses the expression levels of type I procollagen protein and MMP-1 protein in LSc fibroblasts to levels similar to those observed in normal

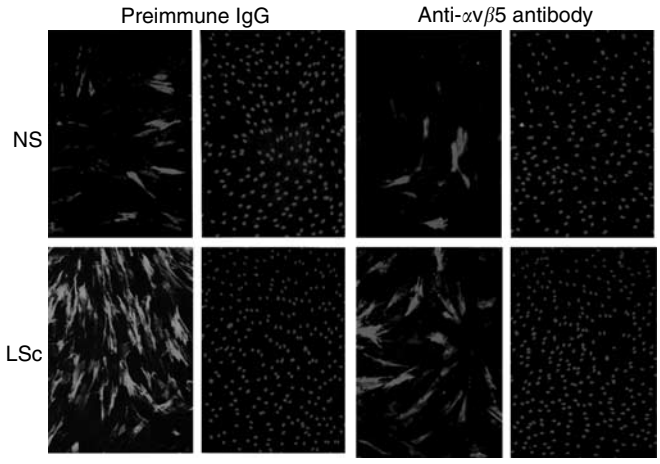

Figure 6. Effect of blocking antibodies against αvβ5 on the morphological features in normal and LSc fibroblasts. Cells were treated with indicated antibodies for 48 hours. Cells were stained with anti-α-smooth muscle actin antibody, washed, and incubated with FITC-conjugated rabbit anti-mouse IgG (green). The nuclei were counterstained with DAPI (blue).

fibroblasts and almost completely diminishes the constitutive DNA–Smad3 binding in LSc fibroblasts. These results indicate that LSc fibroblasts may be activated by the stimulation of autocrine TGF-β. In addition, the evidence that blockade of endogenous TGF-β1 did not completely reverse the myofibroblastic phenotype of LSc fibroblasts suggests that there are other fibrogenic cytokines in addition to TGF-β1 that participate in the fibrogenesis and generation of the myofibroblast phenotype in those cells. Although the detailed mechanism of autocrine TGF-β signaling remains to be clarified, Kawakami *et al* (1998) previously demonstrated that the upregulated expression of TGF-β receptors could establish the autocrine TGF-β signaling in dermal fibroblasts. Since the expression levels of TGF-β receptors are elevated in LSc fibroblasts *in vivo* (Kubo *et al.*, 2001), such abnormalities may contribute to the establishment of autocrine TGF-β signaling in LSc fibroblasts. To further elucidate the mechanism of dermal fibroblast activation in LSc, we next focused on αvβ5, which is recently demonstrated to be involved in the establishment of autocrine TGF-β signaling in SSc fibroblasts (Asano *et al.*, 2005b), and verified the following three evidences: (i) The expression levels of αvβ5 are elevated in LSc fibroblasts *in vivo* and *in vitro*. (ii) Anti-αvβ5 antibody partially, but significantly, reverses the expression levels of type I procollagen protein and MMP-1 protein and reduces the constitutive DNA–Smad3 binding in LSc fibroblasts. (iii) SLC is activated on the cell surface of LSc fibroblasts through αvβ5-dependent pathway. These results indicate that the upregulated expression of αvβ5 may contribute to the establishment of autocrine TGF-β signaling in LSc fibroblasts through activating endogenous latent TGF-β1. To our knowledge, this is the first report which demonstrates the similarity in the self-activation system between SSc and LSc fibroblasts.

Previous reports demonstrated that all of αv-containing integrins bind to LAP-β1 and have the potential to modulate the localization and possibly activation of SLC (Munger *et al.*, 1998; Munger *et al.*, 1999; Mu *et al.*, 2002; Ludbrook *et al.*, 2003). Taken together with this notion, the present findings indicate that αvβ5 may recruit and activate SLC on the cell surface of LSc fibroblasts. To our best knowledge, the following two mechanisms have been proposed in the activation process of SLC: (i) the proteolysis of LAP and (ii) the conformational change of LAP. The former mechanism is attributed to proteases such as plasmin, metalloproteases, aspartic proteases, cysteine proteases, and serine proteases, resulting in the release of active TGF-β from LAP (Lyons *et al.*, 1990; Schultz-Cherry *et al.*, 1994; Munger *et al.*, 1998). The latter mechanism is well-studied in thrombospondin-1 and αvβ6. SLC binds to thrombospondin-1 through the N-terminus of LAP and such interaction induces a conformational change and a subsequent activation of SLC, although the active TGF-β molecules remain bound to thrombospondin-1 (Schultz-Cherry *et al.*, 1994; Crawford *et al.*, 1998). SLC also interacts with αvβ6 through the C-terminus of LAP, but such interaction is not sufficient for its activation. Following the binding, αvβ6 requires the interaction with actin cytoskeleton to activate bound SLC (Munger *et al.*, 1999). Since the previous finding that only β5 has a cytoplasmic domain highly homologous to those of β6 subunits among β1, β3, β5, and β8, which can interact with αv subunit (Ylänne, 1998), αvβ5 may activate SLC by a nonproteolytic pathway. Further studies are required to disclose this point.

In general, the biological effect of cytokines, including TGF-β1, is mainly determined by the occurrence of cytokine–receptor interaction, which is modulated by the concentration and the activity of cytokines and/or their receptors. Therefore, the concentration and/or the activity of TGF-β1 as well as the expression levels of its receptors are important aspects in the pathogenesis of SSc and LSc. Since we previously demonstrated that the expression levels of TGF-β receptors were elevated in SSc and LSc fibroblasts (Kawakami *et al.*, 1998; Ihn *et al.*, 2001; Kubo *et al.*, 2001; Asano *et al.*, 2004a), our next interest had been focused on the molecules which recruit and/or activate latent TGF-β1 in the pericellular region. In this study, we demonstrated that one of the molecules that mediate this process in LSc may be αvβ5 as well as in SSc and this finding strongly supports the further understanding of the establishment of autocrine TGF-β signaling in LSc fibroblasts.

Previous reports demonstrated that TGF-β1-null animals developed massive autoimmune inflammation affecting multiple organs because TGF-β is one of the major regulators of immune system (Shull *et al.*, 1992). In addition, expression of kinase-deficient TβRII selectively in fibroblasts leads to paradoxical ligand-dependent activation of TGF-β signaling and causes skin and lung fibrosis in transgenic mice (Denton *et al.*, 2003). These previous data suggest that TGF-β receptors cannot be a target for developing the treatment of fibrotic disorders. In such a situation, the molecules that can modulate the activation state of latent TGF-β and/or its interaction with TGF-β receptors, such as αvβ5, can be the next new target for the treatment of fibrotic disorders. The present observation that the blockade of αvβ5 reverses the myofibroblastic phenotype in LSc fibroblasts strongly

supports this notion. A previous study demonstrated that β5-knockout mice develop, grow, and reproduce normally and show no abnormality in wound healing and adenovirus infection, which are the major biological processes αvβ5 participates in (Huang *et al.*, 2000). These previous observations suggest that most roles of αvβ5 can be compensated for by other αvβ5-independent pathways. Taken together, this functional redundancy in αvβ5 makes the pharmacological interference of αvβ5 functions a promising approach to the treatment of LSc.

In summary, this study demonstrated that LSc fibroblasts might be activated by the stimulation of autocrine TGF-β and the upregulated expression of αvβ5 might be implicated in this process. These present findings reinforce the notion that SSc and LSc share the pathogenesis of dermal fibrosis. Although *in vivo* studies using animal models are required in the future, the present data suggest that this integrin can be a promising target in developing the treatment of fibrotic disorders, especially SSc and LSc.

MATERIALS AND METHODS

Reagents

Recombinant human TGF-β1 and SLC were obtained from R&D systems (Minneapolis, MN). Antibodies for β-actin and α-SMA were purchased from Sigma (St Louis, MO). Antibodies for β5 subunit (E-19) and αv subunit (Q-20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Functional blocking antibodies against αvβ5 (P1F6) and antibody against MMP-1 were purchased from Chemicon (San Francisco, CA). Anti-Smad2/3 antibody (S66220) was obtained from Transduction laboratories (Lexington, KY).

Cell cultures

Human dermal fibroblasts were obtained by skin biopsy from the affected areas, which developed within 1 year, of four patients with LSc. All of the patients were diagnosed by clinical appearance and histologic findings, and none of them had any other collagen disease, including SSc. Four patients were classified into the following three subgroups: one patient with generalized morphea (sex and biopsy site; female, right thigh), one with linear scleroderma (female, right upper arm), and two with morphea (female, abdomen; male, abdomen) as described previously (Sato *et al.*, 1994). Control fibroblasts were obtained by skin biopsy from four healthy donors. Ideal control in this study should be the adjacent skin, not the totally different individuals. However, skin biopsies from uninvolved areas were not obtained in those patients. Informed consent was obtained from all subjects. Control donors were matched with each LSc patient for age, sex, and biopsy site, and control and patient samples were processed in parallel. Primary explant cultures were established in 25 cm^2 culture flasks in minimum essential medium (MEM) with 10% FCS, 2 mM L-glutamine, and 50 μg/ml of amphotericin. Fibroblast cultures were maintained as monolayers at 37°C in 95% air, 5% CO_2, and studied between the third and sixth subpassages. TMLC cells, which were kindly provided by Dr Daniel B. Rifkin, were cultured in MEM with 10% FCS until assayed. In all experiments, cells were cultured in serum-free medium for at least 24 hours before the addition of any reagents. All described studies were approved by the Graduate School of Medicine, University of Tokyo and The University of Tokyo Hospital. This study was conducted according to the Declaration of Helsinki principles.

Immunoblotting

Protein extracts were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated overnight with primary antibody, washed, and incubated for 1 hour with secondary antibody. After washing, visualization was performed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Biotinylation and immunoprecipitation

Confluent quiescent cells were incubated with membrane-impermeant NHS-LC-biotin (Pierce, Rockford, IL) dissolved at 0.5 mg/ml. in phosphate-buffered saline (PBS) at 4°C for 30 minutes. Immunoprecipitation was performed using whole-cell lysates. Each immunoprecipitate was subjected to SDS-PAGE, and Western blots were prepared. The blots were probed with streptavidin coupled to horseradish peroxidase and visualized by enhanced chemiluminescence.

Immunohistochemical stainings

Immunohistochemical staining of paraffin-embedded sections was performed using a Vecstain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions as described previously (Asano *et al.*, 2004b). 4-μm thick sections were mounted on silane-coated slides, then deparaffinized with xylene and rehydrated through a graded series of solutions of ethyl alcohol and PBS. The sections were then incubated with antibody against αvβ5 diluted 100-fold in PBS overnight at 4°C. The immunoreactivity was visualized using diaminobenzidine. The sections were then counterstained with hematoxylin.

DNA affinity precipitation

Two oligonucleotides containing biotin on the 5′-nucleotide of the sense strand were prepared as described previously (Yagi *et al.*, 2002). Poly(dI-dC) competitor (5 μg) was incubated with 500 μg of nuclear protein for 30 minutes at 4°C, followed by 1 hour incubation with 500 pmol of each oligonucleotide. Then, 65 μl of streptavidin-agarose (Sigma) was added to the reaction and incubated at 4°C for overnight. The precipitated proteins were subjected to immunoblotting with anti-Smad2/3 antibody. The specific binding of Smad3 with 3 × CAGA oligo was confirmed by the experiments using 3 × CAGA-M oligo. The binding of Smad3 with 3 × CAGA-M oligo was not observed in the presence or absence of TGF-β1 (data not shown), as described previously (Asano *et al.*, 2004c).

TGF-β bioassay

To determine the TGF-β activation, TMLC cells, mink lung epithelial reporter cells stably expressing a portion of the plasminogen activator inhibitor 1 promoter (Abe *et al.*, 1994), were cocultured with test cells as described previously (Asano *et al.*, 2005b, c; Asano *et al.*, 2006). TMLC cells are highly responsive to TGF-β and produce a very low background of TGF-β activation. TMLC cells can thus be used in coculture with other cell lines or cell-free fractions to test for the presence of active TGF-β using luminescence as a readout. TMLC and test cells were mixed in a ratio of 1:1 and suspended at 1×10^6 cells/ml in MEM containing 10% FBS. These cells were

plated at 200 μl/well in 12-well plates and allowed to attach for 1 hour. The medium was replaced with 200 μl/well of new serum-free medium and cultured for 24 hour. Cell lysates were prepared using the Reporter Lysis Buffer (Promega, Madison, WI) and the luciferase activity was determined using the Promega luciferase assay system. Similar cocultures were done in 24-well plates with inserts designed for attachment-dependent cell culture (Millicell-PCF 3 μm filter, Millipore, Bedford, MA), but 1.5×10^5 of TMLC and test cells were added to the upper or lower chambers. In some experiments, cocultures were performed in the presence of anti-αvβ5 antibody, preimmune mouse IgG, anti-TGF-β antibody, or preimmune rabbit IgG.

Immunofluorescence

Cells grown in four-well LAB TEK chambers (Nunc, Naperville, IL) were treated with 10 μg/ml of anti-αvβ5 antibody (P1F6) or preimmune mouse IgG for 48 hours. Then, cells were fixed with 3.7% formaldehyde, permeabilized with 0.5% Triton X-100 in PBS, and blocked with 10% FCS in PBS containing 0.5% Triton X-100. Cells were stained with anti-α-SMA antibody, washed, and incubated with FITC-conjugated rabbit anti-mouse IgG (Sigma). Myofibroblast was defined as the cell with features such as cellular hypertrophy and well-formed α-SMA fibers. To determine the percentage of cells which differentiate into myofibroblasts, hundred cells per each cell strain were examined microscopically.

Statistical analysis

Data presented as bar graphs are the means ± SD of at least five independent experiments. Statistical analysis was performed using the Mann–Whitney *U*-test ($P < 0.05$ was considered significant).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

Abe M, Harpel JG, Metz CN, Nunes I, Loskutoff DJ, Rifkin DB (1994) An assay for transforming growth factor-β using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. *Anal Biochem* 216:276–84

Annes JP, Munger JS, Rifkin DB (2003) Making sense of latent TGF-β activation. *J Cell Sci* 116:217–24

Asano Y, Ihn H, Yamane K, Jinnin M, Mimura Y, Tamaki K (2004a) Phosphatidylinositol 3-kinase is involved in α2(I) collagen gene expression in normal and scleroderma fibroblasts. *J Immunol* 172:7123–35

Asano Y, Ihn H, Yamane K, Kubo M, Tamaki K (2004b) Impaired Smad7-Smurf-mediated negative regulation of TGF-β signaling in scleroderma fibroblasts. *J Clin Invest* 113:253–64

Asano Y, Ihn H, Yamane K, Kubo M, Tamaki K (2004c) Increased expression levels of integrin αvβ5 on scleroderma fibroblasts. *Am J Pathol* 164:1275–92

Asano Y, Ihn H, Yamane K, Jinnin M, Mimura Y, Tamaki K (2005a) Differential effects of the immunosuppressant FK-506 on human α2(I) collagen gene expression and transforming growth factor β signaling in normal and scleroderma fibroblasts. *Arthritis Rheum* 52:1237–47

Asano Y, Ihn H, Yamane K, Jinnin M, Mimura Y, Tamaki K (2005b) Involvement of αvβ5 integrin-mediated activation of latent transforming growth factor β1 in autocrine transforming growth factor β signaling in systemic sclerosis fibroblasts. *Arthritis Rheum* 52:2897–905

Asano Y, Ihn H, Yamane K, Jinnin M, Mimura Y, Tamaki K (2005c) Increased expression of integrin αvβ3 contributes to the establishment of autocrine TGF-β signaling in scleroderma fibroblasts. *J Immunol* 175:7708–18

Asano Y, Ihn H, Yamane K, Jinnin M, Tamaki K (2006) Increased expression of integrin αvβ5 induces the myofibroblastic differentiation of dermal fibroblasts. *Am J Pathol* 168:499–510

Buckingham RB, Prince RK, Rodnan GP, Taylor F (1978) Increased collagen accumulation in dermal fibroblast cultures from patients with progressive systemic sclerosis (scleroderma). *J Lab Clin Med* 92:5–21

Crawford SE, Stellmach V, Murphy-Ullrich JE, Ribeiro SH, Lawler J, Hynes RO *et al.* (1998) Thrombospondin-1 is a major activator of TGF-β1 *in vivo*. *Cell* 93:1159–70

Denton CP, Zheng B, Evans LA, Shi-wen X, Ong VH, Fisher I *et al.* (2003) Fibroblast-specific expression of a kinase-deficient type II transforming growth factor β (TGFβ) receptor leads to paradoxical activation of TGFβ signaling pathways with fibrosis in transgenic mice. *J Biol Chem* 278:25109–19

Falanga V, Tiegs SL, Alstadt SP, Roberts AB, Sporn MB (1987) Transforming growth factor-β: selective increase in glycosaminoglycan synthesis by cultures of fibroblasts from patients with progressive systemic sclerosis. *J Invest Dermatol* 89:100–4

Higley H, Persichitte K, Chu S, Waegell W, Vancheeswaran R, Black C (1994) Immunocytochemical localization and serologic detection of transforming growth factor β1. Association with type I procollagen and inflammatory cell markers in diffuse and limited systemic sclerosis, morphea, and Raynaud's phenomenon. *Arthritis Rheum* 37:278–88

Huang X, Griffiths M, Wu J, Farese RV Jr, Sheppard D (2000) Normal development, wound healing, and adenovirus susceptibility in β5-deficient mice. *Mol Cell Biol* 20:755–9

Ihn H, Yamane K, Kubo M, Tamaki K (2001) Blockade of endogenous transforming growth factor β signaling prevents up-regulated collagen synthesis in scleroderma fibroblasts: association with increased expression of transforming growth factor β receptors. *Arthritis Rheum* 44:474–80

Jablonska S, Rodnan GP (1979) Localized forms of scleroderma. *Clin Rheum Dis* 5:215–41

Jelaska A, Arakawa M, Broketa G, Korn JH (1996) Heterogeneity of collagen synthesis in normal and systemic sclerosis skin fibroblasts: increased proportion of high collagen-producing cells in systemic sclerosis fibroblasts. *Arthritis Rheum* 39:1338–46

Kahaleh B (1993) Immunologic aspects of scleroderma. *Curr Opin Rheumatol* 5:760–5

Kawakami T, Ihn H, Xu W, Smith E, LeRoy C, Trojanowska M (1998) Increased expression of TGF-β receptors by scleroderma fibroblasts: evidence for contribution of autocrine TGF-β signaling to scleroderma phenotype. *J Invest Dermatol* 110:47–51

Kubo M, Ihn H, Yamane K, Tamaki K (2001) Up-regulated expression of transforming growth factor β receptors in dermal fibroblasts in skin sections from patients with localized scleroderma. *Arthritis Rheum* 44:731–4

LeRoy EC (1974) Increased collagen synthesis by SSc skin fibroblasts *in vitro*: a possible defect in the regulation or activation of the scleroderma fibroblast. *J Clin Invest* 54:880–9

LeRoy EC, Smith EA, Kahaleh MB, Trojanowska M, Silver RM (1989) A strategy for determining the pathogenesis of systemic sclerosis. Is transforming growth factor β the answer? *Arthritis Rheum* 32:817–25

Ludbrook SB, Barry ST, Delves CJ, Horgan CMT (2003) The integrin αvβ3 is a receptor for the latency-associated peptides of transforming growth factors β1 and β3. *Biochem J* 369:311–8

Lyons RM, Gentry LE, Purchio AF, Moses HL (1990) Mechanism of activation of latent recombinant transforming growth factor-β1 by plasmin. *J Cell Biol* 110:1361–7

Massague J (1990) Transforming growth factor-β family. *Ann Rev Cell Biol* 6:597–641

Mu D, Cambier S, Fjellbirkeland L, Baron JL, Munger JS, Kawakatsu H *et al.* (2002) The integrin αvβ8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-β1. *J Cell Biol* 157:493–507

Munger JS, Harpel JG, Giancotti FG, Rifkin DB (1998) Interactions between growth factors and integrins: latent forms of transforming growth factor-β are ligands for the integrin αvβ1. *Mol Biol Cell* 9:2627–38

Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J *et al.* (1999) The integrin αvβ6 binds and activates latent TGF-β1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell* 96:319–28

Peltonen J, Kahari L, Uitto J, Jimenez SA (1990) Increased expression of type VI collagen genes in systemic sclerosis. *Arthritis Rheum* 33:1829–35

Raghow R, Postlethwaite AE, Keski-Oja J, Moses HL, Kang AH (1987) Transforming growth factor-β increases steady state levels of type I procollagen and fibronectin messenger RNAs posttranscriptionally in cultured human dermal fibroblasts. *J Clin Invest* 79:1285–8

Rudnicka L, Varga J, Christiano AM, Iozzo RV, Jimenez SA, Uitto J (1994) Elevated expression of type VII collagen in the skin of patients with systemic sclerosis. Regulation by transforming growth factor β. *J Clin Invest* 93:1709–15

Sato S, Fujimoto M, Ihn H, Kikuchi K, Takehara K (1994) Clinical characteristics associated with antihistone antibodies in patients with localized scleroderma. *J Am Acad Dermatol* 31:567–71

Schultz-Cherry S, Lawler J, Murphy-Ullrich JE (1994) The type 1 repeats of thrombospondin 1 activate latent transforming growth factor-β. *J Biol Chem* 269:26783–8

Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M *et al.* (1992) Targeted disruption of the mouse transforming growth factor-β1 gene results in multifocal inflammatory disease. *Nature* 359:693–9

Takehara K, Sato S (2005) Localized scleroderma is an autoimmune disorder. *Rheumatology* 44:274–9

Wahl SM (1994) Transforming growth factor β: the good, the bad, and the ugly. *J Exp Med* 180:1587–90

Xu WD, LeRoy EC, Smith EA (1991) Fibronectin release by systemic sclerosis and normal dermal fibroblasts in response to TGF-β. *J Rheumatol* 18:241–6

Yagi K, Furuhashi M, Aoki H, Goto D, Kuwano H, Sugamura K *et al.* (2002) c-myc is a downstream target of the Smad pathway. *J Biol Chem* 277:854–61

Ylänne J (1998) Conserved functions of the cytoplasmic domains of integrin β subunits. *Front Biosci* 3:877–86