Increased Expression of Integrin $\alpha v\beta 5$ Induces the Myofibroblastic Differentiation of Dermal Fibroblasts

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The biological effect of cytokines is mainly determined by the cytokine-receptor interaction, which is modulated by the concentration and the activity of cytokines and/or their receptors. Because αv -containing integrins can bind to and/or activate latent TGF- β , these integrins have been thought to be involved in the pathogenesis of fibrotic disorders. Our recent observations that $\alpha v\beta 5$ is up-regulated in scleroderma fibroblasts and that the transient overexpression of $\alpha v\beta 5$ increases the human $\alpha 2(I)$ collagen gene expression in normal fibroblasts suggest the involvement of $\alpha v\beta 5$ in the self-activation system in scleroderma fibroblasts. In this study, we established stable transfectants with $\alpha v\beta 5$ using normal dermal fibroblasts and demonstrated that such cells differentiated into myofibroblasts by the stimulation of autocrine TGF- β . This observation is explained by 1) $\alpha v\beta 5$ recruiting latent TGF- $\beta 1$ on the cell surface, 2) endogenous active TGF- β localizing on the cell surface, and 3) $\alpha v\beta 5$ interacting with TGF- β receptors. Furthermore, blockade of $\alpha v\beta 5$ reversed the myofibroblastic phenotype in scleroderma fibroblasts. These data identify a novel mechanism for the establishment of autocrine TGF- β signaling in dermal fibroblasts by the up-regulation of $\alpha v\beta 5$ and suggest the possibility of regulating fibrotic disorders, especially scleroderma, by targeting this integrin. (*Am J Pathol* 2006, 168:499–510; DOI: 10.2353/ajpath.2006.041306)

The transforming growth factor- β (TGF- β) superfamily mediates a broad range of biological responses.¹ One of the principal effects of TGF- β on mesenchymal cells is its stimulation of extracellular matrix (ECM) deposition, which is achieved by coordinating the expression of sev-

eral functionally distinct but biologically related gene products. TGF- β increases the expression of various ECM components, such as collagen types I, III, VI, VII, and X, fibronectin, and proteoglycans.^{2–8} It also inhibits the production of proteolytic enzymes that catalyze ECM degradation, while enhancing the expression of protease inhibitors.⁹ As a result of these multifaceted effects, TGF- β is believed to play a critical role in a variety of biological processes, such as wound healing and tissue remodeling.^{10,11} Studies of animal models and human clinical specimens strongly suggest that TGF- β is also important in the pathogenesis of several diseases, including fibrotic disorders. For example, it has been demonstrated that the dermal fibroblast activation in scleroderma may be a result of the stimulation by autocrine TGF- β .¹² However, the mechanism of the initiation and maintenance of autocrine TGF- β signaling in scleroderma fibroblasts remains to be clarified.

TGF- $\beta 1$ is normally secreted as a complex composed of three proteins, including the bioactive peptide of TGF- $\beta 1$, latency-associated peptide- $\beta 1$ (LAP- $\beta 1$), and latent TGF- β binding protein-1. TGF- $\beta 1$ forms a complex with LAP- $\beta 1$ noncovalently, forming the small latent complex (SLC), and in this configuration, TGF- $\beta 1$ is unable to bind to its receptors. SLC is joined by latent TGF- β binding protein-1, the N-terminal region of which is covalently cross-linked to ECM proteins by transglutaminase, and the complex of all three proteins is called the large latent complex.¹³ The constitutive secretion of latent TGF- $\beta 1$ by many cell types in culture suggests that there are extracellular mechanisms to control the activity of this potent cytokine. Although these processes are not fully understood, recent reports demonstrated that cell surface molecules or secreted extracellular molecules can activate latent TGF- $\beta 1$. Specifically, integrin $\alpha v\beta 6$ and throm-

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bospondin (TSP)-1 have been implicated in the activation of latent TGF- β 1 through nonproteolytic mechanisms.^{14–19} In addition, plasmin has been proposed to lead to the activation of latent TGF- β 1 through the proteolytic degradation of LAP- β 1.²⁰ Integrin α v β 8 has also been demonstrated to be able to activate latent TGF- β 1 by the membrane type 1-matrix metalloproteinase-dependent degradation of LAP- β 1.²¹ Thus, normal TGF- β function seems to be largely controlled by its interaction with extracellular or cell surface molecules.

LAP- β 1 contains an RGD motif that is recognized by α v-containing integrins, including α v β 1, α v β 3, α v β 5, α v β 6, and α v β 8. Although all α v-containing integrins bind to LAP- β 1, only α v β 6 and α v β 8 have been demonstrated to be able to activate SLC.^{14,21–23} In particular, the α v β 6-mediated activation of SLC was demonstrated to play an important role in response to tissue injury because epithelium-restricted β 6^{-/-} mice showed only a minor fibrotic response to bleomycin compared with wild-type mice. Although there have been no reports indicating the activation of SLC by other α v-containing integrins, such as α v β 1, α v β 3, and α v β 5, we recently demonstrated that α v β 5 is up-regulated in scleroderma dermal fibroblasts, and the transient overexpression of α v β 5 induces the increased transcriptional activity of human α 2(I) collagen (COL1A2) gene in normal dermal fibroblasts.²⁴ These findings imply the involvement of α v β 5 in the pathogenesis of scleroderma. The purpose of this study was to assess the hypothesis described above. To this end, we established stable transfectants with α v β 5 and investigated the detailed mechanism underlying the α v β 5-dependent phenotypical alteration of dermal fibroblasts.

Materials and Methods

Reagents

Recombinant human TGF- β 1 and SLC were obtained from R & D Systems (Minneapolis, MN). Pan-specific rabbit anti-TGF- β antibody (AB-100-NA), which has the ability to neutralize the biological activity of TGF- β 1, -2, -3, and -5, was also purchased from R & D Systems. Actinomycin D, cytochalasin D, and antibodies for β -actin and α -smooth muscle actin (α -SMA) were purchased from Sigma (St Louis, MO). Antibodies for β 5-subunit, α v-subunit, β 1-subunit, β 3-subunit, T β RI, T β RII, Smad2/3, Smad3, and clathrin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-serine antibody was purchased from Biomedex (Foster City, CA). Functional blocking antibody against α v β 5 (P1F6) was purchased from Chemicon (San Francisco, CA). FuGENE 6 was obtained from Roche Diagnostics (Indianapolis, IN). Synthetic RGD-containing peptides (GRGDSP) and RGE-containing peptide (GRGESp) were purchased from Sigma.

Establishment of Stable Transfectants with α v β 5

Normal human dermal fibroblasts, scleroderma fibroblasts, and the expression vector of β 5-subunit were

prepared as described previously.²⁴ A normal human dermal fibroblast was transfected with either this construct or empty vector (pcDNA3) by electroporation. Stable transfectants were selected in the presence of the neomycin analog G418 (0.25 mg/ml; Sigma).

Immunoblotting

Protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (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).

Immunoprecipitation

Protein extracts (1 μ g) were pre-adsorbed with protein G-agarose beads (Santa Cruz) and incubated with 2 μ g of appropriate antibodies and then with protein G-agarose beads. The precipitated proteins were subjected to immunoblotting.

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.

Cell Adhesion Assays

Ninety-six-well plates were coated with vitronectin or type I collagen (0.3, 1, 3, 10, and 30 μ g/ml). Additional wells were coated with 1% bovine serum albumin (BSA) in PBS (minimal adhesion-negative control) and 1 mg/ml of poly-D-lysine (maximal adhesion-positive control). Cells were detached by trypsin-EDTA, washed once in Eagle's minimum essential medium (MEM) with trypsin inhibitor, and then washed three times with serum-free medium (MEM plus 0.1% BSA). Thereafter, the cells were resuspended in serum-free medium and rocked for 1 hour at 37°C. The cells were then plated onto coated wells (3×10^4 cells/200 μ l/well) and incubated for 1 hour at 37°C. Nonadherent cells were removed by centrifugation (top side down) at $48 \times g$ for 5 minutes. The attached cells were fixed with 1% formaldehyde, stained with 0.5% crystal violet overnight, washed with PBS, and lysed in buffer containing 0.1% NaOH and 0.1% SDS. The absorbance (Abs) at 595 nm was measured. The percentage of cells attached to each well was calculated from formula as follows:

$$\frac{\text{Abs(experimental well)} - \text{Abs(mean of BSA-coated wells)}}{\text{Abs(mean of poly-D-lysine well)}}$$

$$- \text{Abs(mean of BSA-coated wells)}$$

For inhibition experiments, cells were incubated for 1 hour with or without increasing dilutions (1/200, 1/100, or 1/50) of anti- $\alpha v\beta 5$ antibody, plated on to wells coated with vitronectin or type I collagen (5 $\mu\text{g/ml}$), and incubated for 1 hour at 37°C. The percentage of cells attached to each well was calculated from the following formula:

$$\frac{\text{Abs(experimental well with anti-}\alpha v\beta 5 \text{ monoclonal Ab)}}{\text{Abs(mean of control wells without anti-}\alpha v\beta 5 \text{ monoclonal Ab)}}$$

$$- \text{Abs(mean of BSA-coated wells)}$$

$$- \text{Abs(mean of BSA-coated wells)}$$

RNA Preparation and Northern Blot Analysis

Total RNA (2 μg) was extracted and subjected to electrophoresis on a 1% agarose/formaldehyde gels and blotted onto a nylon filter. The filter was hybridized with DNA probes for COL1A2, TGF- $\beta 1$, and glyceraldehyde-3-phosphate dehydrogenase genes, or RNA probes for αv - and $\beta 5$ -subunit genes. The membrane was then washed and exposed to X-ray film.

Immunofluorescence

Cells grown in 4-well Lab-Tek chambers (Nunc, Naperville, Illinois) were fixed with 3.7% formaldehyde, permeabilized, and blocked with 10% fetal bovine serum in PBS containing 0.5% Triton X-100. Cells were stained with anti- α -SMA antibody, washed, and incubated with fluorescein isothiocyanate-conjugated secondary antibody (Sigma).

DNA Affinity Precipitation

Two double-stranded oligonucleotides, 3xCAGA oligo and 3xCAGA-M oligo, containing biotin on the 5'-nucleotide of the sense strand were prepared as described previously.²⁵ 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 oligonucleotides. 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 3xCAGA oligo was confirmed by experiments using 3xCAGA-M oligo. The binding of Smad3 with 3xCAGA-M oligo was not observed in the presence or absence of TGF- $\beta 1$ (data not shown).

Plasmid Construction

A -353COL1A2/CAT construct was generated as previously described.²⁶ Plasmids were purified twice on CsCl

gradients. At least two different plasmid preparations were used for each experiment.

Transient Transfection

Cells were grown to 50% confluence in 100-mm dishes, transfected with the indicated constructs (2 μg) along with pSV- β -galactosidase (1 μg) using FuGENE6, and incubated for 48 hours. Extracts, normalized for protein content, were incubated with butyl-CoA and [¹⁴C]-chloramphenicol for 90 minutes at 37°C. Butylated chloramphenicol was extracted using an organic solvent (a 2:1 mixture of tetramethylpentadecane and xylene) and quantitated by scintillation counting.

Measurement of TGF- $\beta 1$ Levels

Cells were grown to confluence in 6-well plates. The culture medium was removed, cells were washed with MEM to remove excess TGF- $\beta 1$, and medium was replaced with serum-free medium. After 24 hours, the levels of active and total TGF- $\beta 1$ in the supernatants were measured using a TGF- $\beta 1$ enzyme-linked immunosorbent assay system (Amersham Pharmacia Biotech).²⁷ This system is designed to measure active TGF- $\beta 1$. Total TGF- $\beta 1$ was assayed after acid activation by the addition of 1 N HCl to samples.

Latent TGF- $\beta 1$ Binding Assay

The binding of cells to SLC was determined as described previously with minor modification.^{28,29} Cells were incubated at 4°C for 1 hour in binding buffer (1 ml of MEM containing 200 mmol/L HEPES [pH 7.4] and 25 mg/ml BSA) supplemented with ³⁵S-labeled SLC, which was synthesized *in vitro* from TGF- $\beta 1$ cDNA by using the T_NT Coupled Reticulocyte Lysate System (Promega, Madison, WI), and then washed twice with PBS containing 0.9 mmol/L CaCl₂, 0.5 mmol/L MgCl₂, and 0.1% BSA. Cell surface-bound SLC was removed by incubating once with extraction buffer containing 150 mmol/L NaCl, 0.1% acetic acid, and 2 mol/L urea, at 4°C for 3 minutes, and extracted ³⁵S-labeled SLC was quantitated by scintillation counting. To determine RGD-specific binding, we constructed mutant SLC, in which the RGD site in LAP- $\beta 1$ is changed to RGE, and performed the binding assay using this construct under the same condition. We also performed the same experiments in the presence of excessive cold SLC (R and D system) to determine the nonspecific binding of ³⁵S-labeled SLC. Specific ³⁵S-labeled SLC binding was calculated by subtracting nonspecific binding (measured in the presence of excessive cold SLC) from total binding (measured in the absence of unlabeled SLC).

TGF- β Bioassay

To determine the TGF- β activation, TMLC cells were co-cultured with either transfectant as described previously

with minor modifications.^{14,21} TMLC and test cells were mixed at a ratio of 1:1 and suspended at 1×10^6 cells/ml in MEM containing 10% fetal bovine serum. These cells were plated at 200 μ l/well in 12-well plates and allowed to attach for 1 hour. Medium was replaced with 200 μ l/well of new medium with or without antibodies and cultured for 24 hours. Cell lysates were prepared using the Reporter Lysis buffer (Promega), and the luciferase activity was determined using the Promega luciferase assay system. Similar co-cultures were performed in 24-well plates with inserts designed for attachment-dependent cell culture (Millicell-PCF 3- μ m filter, Millipore, Bedford, MA), but 1.5×10^5 TMLC and test cells were added to upper and lower chambers, respectively. In some experiments, assays were performed in the presence of anti- α v β 5 antibody, preimmune mouse IgG, anti-TGF- β antibody, or preimmune rabbit IgG.

Statistical Analysis

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

Results

Generation and Characterization of Stable α v β 5-Transfectants

We transfected the β 5-subunit expression vector or pcDNA3 (empty vector) into four strains of normal dermal fibroblasts. The β 5-positive transfectants (β 5-transfectants) were screened for increased expression of β 5 mRNA (Figure 1A). Twenty positive clones were isolated that expressed higher levels of β 5 mRNA than normal dermal fibroblasts or pcDNA3-transfected control cells (mock transfectants). The increased β 5 mRNA expression was accompanied by increased cell surface expression of α v β 5 (Figure 1B). The levels of cell surface α v-subunit dimerized with β 5-subunit also increased in β 5-transfectants, but there was no significant difference in the levels of α v mRNA between these three groups (Figure 1C). This is consistent with the notion that α v-subunit is excessively expressed and cell surface expression levels of α v-containing integrins are regulated by the levels of β -subunits.³⁰ In contrast, the levels of α v β 1 and α v β 3 were unchanged in β 5-transfectants (Figure 1, D and E), excluding the possibility that the increased β 5 expression might compete with other β -subunits for dimerizing with the α v-subunit. Consistent with previous reports, the expression of β 6 and β 8 was not observed in normal dermal fibroblasts (data not shown). Two mock transfectants and two β 5-transfectants were selected from each of four normal fibroblast strains, and all of the following experiments were performed using these eight mock transfectants and eight β 5-transfectants. To prove the functionally active state of the newly expressed α v β 5 in β 5-transfectants, we assayed the attachment of either

Figure 1. Confirmation of the establishment of β 5-transfectants. Total RNAs were extracted from confluent quiescent cells, and the levels of β 5 mRNA (A) or α v mRNA (C) were determined by Northern blotting. Cell surface proteins were labeled with biotin, and whole-cell lysates were subjected to immunoprecipitation using goat anti- β 5 antibody (B, left panel), goat anti- β 1 antibody (D), or goat anti- β 3 antibody (E). Precipitated proteins were subjected to SDS-PAGE, and Western blots were prepared. The blots were probed with streptavidin coupled to horseradish peroxidase. A parallel immunoprecipitation performed with preimmune goat IgG failed to show integrin immunoreactivities (B, right panel). The bands in immunoprecipitation were confirmed to be the indicated subunits by a reprobing analysis using specific antibodies to α v-, β 1-, β 3-, or β 5-subunits (data not shown), whereas the identity of α 1, α 2, α 3, and α 5 was determined by the size on a gel. Experiments were repeated five times with similar results.

transfectant to vitronectin and type I collagen. β 5-Transfectants strongly attached to vitronectin compared with mock transfectants, whereas the attachment of either transfectant to type I collagen was at a similar level (Figure 2A). The specificity of adhesion was confirmed by the application of anti- α v β 5 antibody (Figure 2B). Thus, the newly expressed α v β 5 was functionally active.

β 5-Transfectants Display the Myofibroblastic Differentiation

To assess the phenotypical alteration of β 5-transfectants, we compared the expression of the COL1A2 gene between mock and β 5-transfectants. The levels of COL1A2 mRNA and the activity of the -353COL1A2 promoter were significantly elevated in β 5-transfectants (Figure 3, A and B). Furthermore, the level of α -smooth muscle actin (α -SMA) protein, a specific marker of myofibroblasts, was also markedly elevated in β 5-transfectants (Figure 3C),

Figure 2. Assay of mock and $\beta 5$ -transfectant attachment to vitronectin or type I collagen. **A:** Cells were incubated at 37°C for 1 hour in the wells coated with vitronectin or type I collagen. After centrifugation, attached cells were stained, and the absorbance of 595 nm was measured. **B:** Cells were preincubated at 37°C for 1 hour with anti- $\alpha\text{v}\beta 5$ antibody or preimmune IgG, and cell attachment was determined. The mean from five separate experiments is shown.

indicating the myofibroblastic differentiation of $\beta 5$ -transfectants. This finding was confirmed by immunofluorescence of $\beta 5$ -transfectants, which showed the morphological changes of cellular hypertrophy and well-formed α -SMA fibers, specific features of myofibroblasts previously described³¹ (Figure 3D).

Constitutive Activation of TGF- β Signaling by Endogenous TGF- β Stimulation in $\beta 5$ -Transfectants

We next investigated the effects of anti-TGF- β antibody or TGF- $\beta 1$ antisense oligonucleotide (5'-GAGGGCGGCATGGGGAGG-3', AS-TGF- $\beta 1$), which has been found to be sufficient to block TGF- $\beta 1$ transcription *in vitro*³² and *in vivo*,³³ in relation to the COL1A2 promoter activity. Mock transfectants treated with anti-TGF- β antibody showed little reduction in -353COL1A2 promoter activity, whereas treated $\beta 5$ -transfectants showed a marked, dose-dependent reduction (Figure 4A). The treatment with AS-TGF- $\beta 1$ showed similar results to those obtained by anti-TGF- β antibody (Figure 4B). These results indicate that constitutive activation of $\beta 5$ -transfectants depends on stimulation by autocrine TGF- β . The TGF- β isoform that was primarily responsible for the activation of $\beta 5$ -transfectants was TGF- $\beta 1$ because AS-TGF- $\beta 1$ de-

Figure 3. Myofibroblastic differentiation of $\beta 5$ -transfectants. **A:** mRNA levels of $\alpha 2(I)$ collagen and GAPDH genes were analyzed by Northern blotting. DNA probes against $\alpha 2(I)$ collagen hybridized to cellular transcripts of 5.2 and 5.7 kb. One representative of five independent experiments is shown. **B:** The -353COL1A2 promoter activities were determined by CAT assays. Values represent the CAT activity relative to that of mock transfectants (100 arbitrary units [AU]). The means $\pm$ SD of five independent experiments are shown. * $P < 0.05$ versus mock transfectants. The expression of α -SMA protein was determined by immunoblotting (**C**) and by immunofluorescence (**D**). Experiments were repeated five times with similar results.

creased the promoter activity to a similar extent as that achieved with anti-TGF- β antibody. This notion was confirmed by immunoprecipitation and DNA affinity precipitation, which showed constitutive phosphorylation of Smad3 and increased DNA binding ability of Smad3 in $\beta 5$ -transfectants, respectively (Figure 4, C and D).

Myofibroblastic Differentiation of $\beta 5$ -Transfectants Requires Cell Adhesion Signals and Stimulation by Autocrine TGF- β

As described above, myofibroblastic differentiation of $\beta 5$ -transfectants may be attributed to the stimulation by autocrine TGF- β . Because a previous report demonstrated that myofibroblastic differentiation is dependent on cell adhesion signaling, such as integrin/FAK, as well as TGF- β stimulation,³⁴ we investigated the activation state of focal adhesion kinase (FAK). As shown in Figure 5A, the phosphorylation levels of FAK on Tyr-397 were elevated in $\beta 5$ -transfectants compared with mock transfectants. Treatment with PP-2, which is a pharmacological inhibitor of FAK/Src, or transient overexpression of a kinase-deficient FAK mutant, mutated by substituting Tyr-397 with Phe, markedly decreased the expression levels of α -SMA in $\beta 5$ -transfectants (Figure 5, B and C, respectively). Furthermore, the treatment of AS-TGF- $\beta 1$ sup-

Figure 4. Constitutive activation of TGF- β /Smad signaling by endogenous TGF- β stimulation in β 5-transfectants. Cells were transfected with 2 μ g of -353COL1A2 promoter in the presence of anti-TGF- β antibody (anti-TGF- β) or preimmune IgG (**A**) or in the presence of TGF- β 1 antisense oligonucleotide (AS-TGF- β 1) or TGF- β 1 sense oligonucleotide (S-TGF- β 1) (**B**). Values represent the CAT activity relative to that of mock transfectants treated with preimmune IgG (**A**) or treated with S-TGF- β 1 (**B**) (100AU). The means $\pm$ SD of five independent experiments are shown. * P < 0.05 versus β 5-transfectants treated with preimmune IgG (**A**) or S-TGF- β 1 (**B**). Whole-cell lysates derived from multiple clones of each transfectant were subjected to immunoprecipitation using anti-Smad3 antibody, and phospho-Smad3 was detected by immunoblotting using anti-phospho-serine antibody (**C**). The same membrane was stripped and reprobed with anti-Smad2/3 antibody (**left panel**). A parallel immunoprecipitation performed with preimmune mouse IgG failed to show Smad3 immunoreactivity (**right panel**). **D**: Nuclear extracts derived from multiple clones of each transfectant were incubated with biotin-labeled oligonucleotides. Proteins bound to these nucleotides were isolated with streptavidin-agarose beads, and Smad3 was detected by immunoblotting. All blots show one representative of five independent experiments.

pressed the expression of α -SMA and the phosphorylation of FAK on Tyr-397 (Figure 5, D and E, respectively). Cumulatively, these results support the previous finding that myofibroblastic differentiation by TGF- β stimulation is dependent on cell adhesion signaling.

Latent TGF- β 1 Is Recruited on the Cell Surface by the Interaction of LAP- β 1 with α v β 5 in β 5-Transfectants

We next compared the expression levels of TGF- β 1 between mock and β 5-transfectants. There was no significant difference in mRNA levels (Figure 6A). However, the levels of total TGF- β 1 proteins (active + latent) in conditioned media were significantly decreased in β 5-transfectants (Table 1, 0.284 ± 0.075 ng/ml versus 0.497 ± 0.105 ng/ml, P < 0.05), but there was no significant

Figure 5. Myofibroblastic differentiation of β 5-transfectants is dependent on both TGF- β signaling and integrin/FAK signaling. **A**: Whole-cell lysates derived from multiple clones of each transfectant were subjected to immunoblotting using anti-397Y-phospho-FAK antibody. The same membrane was stripped and reprobed with anti-FAK antibody. **B**: Quiescent fibroblasts were treated with the indicated reagent for 48 hours. Whole-cell lysates were subjected to immunoblotting using anti- α -SMA antibody and anti- β -actin antibody. **C**: Cells were transfected with 2 μ g of expression vector of Y397F-FAK or corresponding empty vector. After a 48-hour incubation, whole-cell lysates were subjected to immunoblotting using anti- α -SMA antibody and anti- β -actin antibody. **D**: Quiescent cells were treated with 10 μ M/L TGF- β 1 antisense oligonucleotide (AS-TGF- β 1) or TGF- β 1 sense oligonucleotide (S-TGF- β 1) for 48 hours. Whole-cell lysates were subjected to immunoblotting using anti- α -SMA antibody and anti- β -actin antibody. **E**: Quiescent cells were treated with 10 μ M/L TGF- β 1 antisense oligonucleotide (AS-TGF- β 1) or TGF- β 1 sense oligonucleotide (S-TGF- β 1) for 48 hours. Whole-cell lysates were subjected to immunoblotting using anti-397Y-phospho-FAK antibody. The same membrane was stripped and reprobed with anti-FAK antibody. All experiments were performed using multiple clones of each transfectant, and one representative is shown. In **right panels** of **B** through **D**, the protein levels quantitated by scanning densitometry are shown relative to those in mock transfectants treated with dimethylsulfoxide (DMSO), empty vector, or S-TGF- β 1, respectively (1 arbitrary unit [AU]). Data are expressed as the means $\pm$ SD of five independent experiments. * P < 0.05 versus mock transfectants under the same condition. ** P < 0.05 versus β 5-transfectants treated with DMSO, empty vector, or S-TGF- β 1.

difference in the levels of active TGF- β 1. Preincubation with anti- α v β 5 antibody restored the levels of total TGF- β 1 proteins in β 5-transfectants (Table 1), whereas the same treatment had no effect on the levels of TGF- β 1 mRNA (data not shown). Because LAP- β 1 binds α v β 5,²² these results suggest that SLC is recruited on the cell surface of β 5-transfectants through the interaction of LAP- β 1 with α v β 5. To confirm this hypothesis, we compared binding to SLC between mock and β 5-transfectants (Figure 6B). SLC binding ability was significantly elevated in β 5-transfectants (5.0-fold increase, P < 0.05). By the addition of anti- α v β 5 antibody, the increased SLC binding in β 5-transfectants was significantly reduced to a

Figure 6. SLC is recruited and activated by its interaction with $\alpha\text{v}\beta 5$ in $\beta 5$ -transfectants. **A:** Northern blotting was performed with DNA probes for TGF- $\beta 1$ and GAPDH genes. Experiments were repeated five times with similar results. **B:** Confluent quiescent cells were incubated at 4°C for 1 hour with ^{35}S -labeled SLC or mutated SLC (mSLC) in the presence or absence of cold SLC. Cell surface-bound SLC was removed by extraction buffer and quantitated by scintillation counting. In some experiments, assays were performed in the presence of RGD peptide (10 $\mu\text{g/ml}$), RGE peptide (10 $\mu\text{g/ml}$), anti- $\alpha\text{v}\beta 5$ antibody (anti- $\alpha\text{v}\beta 5$, 10 $\mu\text{g/ml}$), or preimmune mouse IgG (IgG, 10 $\mu\text{g/ml}$). Values represent the scintillation count relative to that of mock transfectants treated with SLC (100AU). * $P < 0.05$ versus mock transfectants under the same condition. **C:** Cells were cultured in MEM containing 10 $\mu\text{mol/L}$ AS-TGF- $\beta 1$ for 48 hours. Then, the medium was changed to serum free-medium containing 10 $\mu\text{mol/L}$ AS-TGF- $\beta 1$, and cells were transfected with 2 μg of -353COL1A2 promoter. Cells were stimulated with the indicated concentration of TGF- $\beta 1$ or SLC for the last 24 hours, and the CAT activities were determined. In some experiments, SLC stimulation was performed in the presence of RGD peptide (10 $\mu\text{g/ml}$), RGE peptide (10 $\mu\text{g/ml}$), anti- $\alpha\text{v}\beta 5$ antibody (anti- $\alpha\text{v}\beta 5$, 10 $\mu\text{g/ml}$), or preimmune mouse IgG (IgG, 10 $\mu\text{g/ml}$). Values represent the CAT activity relative to that of mock transfectants treated with S-TGF- $\beta 1$ (100AU). The means $\pm$ SD of five independent experiments are shown. * $P < 0.05$ versus $\beta 5$ -transfectants treated with AS-TGF- $\beta 1$. * $P < 0.05$ versus $\beta 5$ -transfectants treated with AS-TGF- $\beta 1$, SLC, and RGE peptide. * $P < 0.05$ versus $\beta 5$ -transfectants treated with AS-TGF- $\beta 1$, SLC, and preimmune mouse IgG.

level similar to that observed in mock transfectants. Because previous reports demonstrated that LAP- $\beta 1$ binds to αv -containing integrins, such as $\alpha\text{v}\beta 6$ and $\alpha\text{v}\beta 8$,

through its RGD motif,^{14,21} we further investigated whether LAP- $\beta 1$ interacts with $\alpha\text{v}\beta 5$ through its RGD motif. To this end, we generated mutant SLC, in which the RGD motif was changed to RGE, and performed binding assay. As expected, there was no significant difference in the binding of mutant SLC between mock and $\beta 5$ -transfectants. We further performed binding assays in the presence of RGD peptide or RGE peptide and demonstrated that RGD peptide, but not RGE peptide, significantly decreased SLC binding in $\beta 5$ -transfectants, whereas both peptides showed no effect in mock transfectants. Cumulatively, these results indicate that SLC is recruited on the cell surface of $\beta 5$ -transfectants through the interaction of RGD motif of LAP- $\beta 1$ with $\alpha\text{v}\beta 5$.

The Inducibility of the COL1A2 Promoter Activity against Exogenous SLC Stimulation Is Increased in $\beta 5$ -Transfectants

We next investigated the effect of exogenous SLC stimulation on -353COL1A2 promoter activity in mock and $\beta 5$ -transfectants (Figure 6C). To suppress the endogenous TGF- $\beta 1$ production, cells were pretreated with 10 $\mu\text{mol/L}$ AS-TGF- $\beta 1$ for 48 hours before transfection. AS-TGF- $\beta 1$ almost completely diminished the increased promoter activity in $\beta 5$ -transfectants, whereas it showed little reduction in mock transfectants. Exogenous TGF- $\beta 1$ stimulation showed significant increase in the promoter activity in both transfectants pretreated with AS-TGF- $\beta 1$. Exogenous SLC stimulation also showed significant increase in the promoter activity in $\beta 5$ -transfectants in a dose-dependent manner, whereas the same treatment revealed no significant effect in mock transfectants. In addition, RGD peptide, but not RGE peptide, significantly decreased SLC-induced promoter activity in $\beta 5$ -transfectants, whereas both peptides showed no effect on the promoter activity in mock transfectants treated with SLC. Furthermore, in the presence of anti- $\alpha\text{v}\beta 5$ antibody, the effect of SLC in $\beta 5$ -transfectants was completely diminished. These results indicate that the inducibility of the COL1A2 promoter activity against exogenous SLC stimulation is increased in $\beta 5$ -transfectants by the $\alpha\text{v}\beta 5$ -dependent manner.

Endogenous Active TGF- β Is Localized on the Cell Surface of $\beta 5$ -Transfectants

To investigate the localization of active TGF- β , we co-cultured either transfectant with TMLC cells, a mink lung

Table 1. Levels of Total (Active + Latent) and Active TGF- $\beta 1$ Secreted by Either Transfectant into Culture Supernatants

	Total TGF- $\beta 1$ (ng/ml)	Active TGF- $\beta 1$ (ng/ml)
Mock transfectants without any treatments	0.497 $\pm$ 0.105	0.071 $\pm$ 0.015
Mock transfectants treated with anti- $\alpha\text{v}\beta 5$ antibody	0.525 $\pm$ 0.112	0.080 $\pm$ 0.019
$\beta 5$ -Transfectants without any treatments	0.284 $\pm$ 0.075*	0.063 $\pm$ 0.015
$\beta 5$ -Transfectants treated with anti- $\alpha\text{v}\beta 5$ antibody	0.501 $\pm$ 0.106	0.070 $\pm$ 0.017

Values are means $\pm$ SD.
 * $P < 0.05$.

Figure 7. SLC is activated on the cell surface of $\beta 5$ -transfectants. **A:** Transfectant and TMLC cells were mixed at a ratio of 1:1 and co-cultured in confluence. In experiments without cell-cell contact, either transfectant or TMLC cells were co-cultured separately by using inserts. After a 24-hour incubation, the luciferase activities were determined. In some experiments, assays were performed in the presence of anti- $\alpha\beta 5$ antibody (anti- $\alpha\beta 5$, 10 $\mu\text{g/ml}$), preimmune mouse IgG (M-IgG, 10 $\mu\text{g/ml}$), anti-TGF- β antibody (anti-TGF- β , 10 $\mu\text{g/ml}$), or preimmune rabbit IgG (R-IgG, 10 $\mu\text{g/ml}$). Values represent the luciferase activity relative to that of TMLC cells co-cultured with mock transfectants in the absence of contact (100AU). * $P < 0.05$ versus mock transfectants under the same condition. **B:** Mock or $\beta 5$ -transfectants were transfected with 2 μg of -353COL1A2 promoter in the presence of appropriate reagents. Values represent the CAT activity relative to that of $\beta 5$ -transfectants treated with DMSO (100AU). The means $\pm$ SD of five independent experiments are shown.

epithelial reporter cell line stably expressing a portion of the plasminogen activator inhibitor 1 promoter³⁵ (Figure 7A). The luciferase activity was significantly elevated in TMLC cells co-cultured with $\beta 5$ -transfectants compared with those co-cultured with mock transfectants (about 10-fold increase, $P < 0.05$). This increase was almost completely abolished by antibodies against TGF- β or $\alpha\beta 5$, whereas preimmune mouse or rabbit IgG showed no effect. We also performed co-culture assays with inserts to separate TMLC cells and either transfectant while allowing soluble molecules to pass. In the absence of contact, $\beta 5$ -transfectants showed no significant induction of luciferase activity. These results indicate that activated endogenous TGF- β is localized on the cell surface of $\beta 5$ -transfectants. We next tested whether the activation of SLC in $\beta 5$ -transfectants was occurring through previously described mechanisms.^{20,22,36,37} Plasmin inhibitor (aprotinin), mannose-6-phosphate, metalloprotease inhibitor (GM6001), aspartic protease inhibitor (leupeptin, pepstatin A), cysteine protease inhibitor (leupeptin and E64), serine protease inhibitor (phenylmethylsulfonyl fluoride, aprotinin, and leupeptin), and functional blocking antibody against TSP-1 (monoclonal Ab 133) did not affect the COL1A2 promoter activity in $\beta 5$ -transfectants (Figure

Figure 8. Interaction between $\alpha\beta 5$ and TGF- β receptors. **A:** Whole-cell lysates were subjected to immunoprecipitation using anti-clathrin antibody, and $\alpha\beta$, $\beta 5$, T β RI, T β RII, or clathrin were detected by immunoblotting. **B:** Whole-cell lysates were subjected to immunoprecipitation using anti-clathrin antibody (CL) or anti-caveolin antibody (CV), and $\beta 5$, clathrin, or caveolin levels in precipitated proteins were determined by immunoblotting (**left panels**). Residual (unprecipitated) proteins were also subjected to immunoblotting using anti-clathrin antibody or anti-caveolin antibody to confirm the efficiency of immunoprecipitation (**right panels**). **C:** Immunoprecipitation was performed using anti- $\beta 5$ antibody, and $\beta 5$, T β RI, or T β RII was detected by immunoblotting. All blots show one representative of five independent experiments.

7B). These results indicate that the activation of SLC in $\beta 5$ -transfectants does not require other known activators of SLC.

Interaction between $\alpha\beta 5$ and TGF- β Receptors

Previous reports demonstrated that TGF- β receptors and $\beta 5$ -subunit were located in clathrin-coated membranes.^{28,38,39} Furthermore, a recent report proposed a novel model of TGF- β signaling regulation in which clathrin-dependent internalization promotes TGF- β signaling by sequestering the complex of TGF- β and its receptors from the Smad7-Smurf-mediated degradation pathway, which is mediated by the caveolin-dependent internalization.⁴⁰ Taken together, these results suggest that $\alpha\beta 5$ promotes the interaction of endogenous TGF- $\beta 1$ with its receptors in clathrin-coated membranes, subsequently resulting in efficient activation of TGF- β signaling through the acceleration of the clathrin-dependent internalization. To confirm this point, we performed immunoprecipitation. Consistent with previous reports, anti-clathrin antibody precipitated detectable levels of $\alpha\beta$ -subunit, $\beta 5$ -subunit, T β RI, and T β RII in both transfectants (Figure 8A). Furthermore, as shown in immunoprecipitation assays using anti-clathrin antibody or anti-caveolin antibody, which ef-

Figure 9. The treatment of anti- $\alpha\text{v}\beta 5$ antibody reverses the myofibroblastic phenotype in scleroderma fibroblasts. **A:** Whole-cell lysates prepared from quiescent normal and scleroderma fibroblasts were subjected to immunoblotting using anti- α -SMA antibody and anti- β -actin antibody. **B:** Quiescent normal and scleroderma fibroblasts were treated with anti- $\alpha\text{v}\beta 5$ antibody (10 $\mu\text{g}/\text{ml}$) or preimmune mouse IgG (10 $\mu\text{g}/\text{ml}$) for 48 hours. Whole-cell lysates were subjected to immunoblotting using anti- α -SMA antibody and anti- β -actin antibody. Experiments were repeated five times with similar results.

fectively precipitated these proteins (Figure 8B, right panels), $\beta 5$ -subunits predominantly interacted with clathrin rather than with caveolin (Figure 8B, left panels). Moreover, the interaction of $\beta 5$ -subunits with TGF- β receptors was markedly elevated in $\beta 5$ -transfectants, whereas such interaction was marginal in mock transfectants (Figure 8C), suggesting that the levels of interaction between $\alpha\text{v}\beta 5$ and TGF- β receptors depends on the levels of $\alpha\text{v}\beta 5$. These results support the hypothesis described above.

The Treatment of Anti- $\alpha\text{v}\beta 5$ Antibody Reversed the Myofibroblastic Phenotype in Scleroderma Fibroblasts

Finally, we investigated whether $\alpha\text{v}\beta 5$ was involved in the self-activation system in scleroderma fibroblasts, which might be activated by the stimulation of autocrine TGF- β . To this end, we examined the effect of anti- $\alpha\text{v}\beta 5$ antibody on the expression levels of α -SMA in scleroderma fibroblasts (Figure 9). The expression levels of α -SMA were significantly elevated in scleroderma fibroblasts compared with normal fibroblasts. However, the treatment of anti- $\alpha\text{v}\beta 5$ antibody significantly reduced the expression levels of α -SMA in scleroderma fibroblasts, whereas the treatment of preimmune IgG showed no effect. In contrast, neither anti- $\alpha\text{v}\beta 5$ antibody nor preimmune IgG affected the expression levels of α -SMA in normal fibroblasts. These results indicate that the up-regulated expression of $\alpha\text{v}\beta 5$ contributes to the establishment of myofibroblastic phenotype in scleroderma fibroblasts.

Discussion

This study was undertaken to clarify the mechanism underlying the $\alpha\text{v}\beta 5$ -mediated myofibroblast differentiation

of dermal fibroblasts. First, we demonstrated that stable transfection of $\beta 5$ -subunit into dermal fibroblasts resulted in the myofibroblast differentiation through the establishment of autocrine TGF- β signaling. This whole process was dependent on FAK activation, consistent with the notion that stable expression of the myofibroblast phenotype requires both TGF- $\beta 1$ and integrin/FAK signals. Next, we showed that $\beta 5$ -transfectants recruited SLC on the cell surface through the interaction of $\alpha\text{v}\beta 5$ with RGD motif in LAP and that endogenous active TGF- β was localized on the cell surface of $\beta 5$ -transfectants. Furthermore, we revealed that $\alpha\text{v}\beta 5$ interacted with TGF- β receptors and that the complex of $\alpha\text{v}\beta 5$ with TGF- β receptors was markedly elevated in $\beta 5$ -transfectants. Collectively, $\alpha\text{v}\beta 5$ may promote a ternary complex formation of endogenous TGF- β and TGF- β receptors, leading to the establishment of autocrine TGF- β signaling. Finally, we elucidated that the blockade of $\alpha\text{v}\beta 5$ reversed the myofibroblastic phenotype in scleroderma fibroblasts. To our knowledge, this is the first report to demonstrate the detailed mechanism underlying the $\alpha\text{v}\beta 5$ -mediated myofibroblast differentiation of dermal fibroblasts.

In general, the biological effect of cytokines 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. For example, in scleroderma fibroblasts, the up-regulated expression of TGF- β receptors contributes to the increased biological effect of autocrine TGF- β , resulting in excessive ECM deposit.¹² The present observations that $\alpha\text{v}\beta 5$ recruits SLC on cell surface and complexes with TGF- β receptors indicate that $\alpha\text{v}\beta 5$ is a novel molecule that enhances the occurrence of the interaction between endogenous TGF- β and its receptors. In support of this concept, $\alpha\text{v}\beta 5$ is markedly elevated in scleroderma fibroblasts,²⁴ and the blockade of $\alpha\text{v}\beta 5$ reverses the myofibroblastic phenotype of scleroderma fibroblasts.

Because TGF- β is secreted in its latent form, normal TGF- β function is thought to be largely controlled by its activation from the latent state. So far, two mechanisms have been reported in the activation of SLC. One is the proteolysis of LAP, which results in the release of active TGF- β from SLC. Proteases such as plasmin, metalloproteases, aspartic proteases, cysteine proteases, and serine proteases have been reported to be involved in this process.^{20,22,37} In addition, $\alpha\text{v}\beta 8$ activates SLC via a membrane type 1-matrix metalloproteinase-dependent proteolytic pathway.²¹ The other mechanism is the conformational change of LAP leading to the activation of SLC. This nonproteolytic process is thought to be dependent on an intrinsic ability of LAP to adopt different conformations.⁴¹ TSP-1 and $\alpha\text{v}\beta 6$ have been demonstrated to be involved in this process.^{14,17,37} SLC binds to TSP-1 through the N-terminus of LAP and such interaction induces a conformational change and subsequent activation of SLC, although the active TGF- β molecules remain bound to TSP-1. SLC also interacts with $\alpha\text{v}\beta 6$ through the C-terminus of LAP, but such interaction is not sufficient for its activation. After binding, $\alpha\text{v}\beta 6$ requires interaction with actin cytoskeleton to activate bound SLC. In the present study, we demonstrated that the up-regulated

expression of $\alpha v\beta 5$ induced myofibroblast differentiation by the establishment of autocrine TGF- β signaling, suggesting that $\alpha v\beta 5$ activates SLC. Because $\beta 5$ has a cytoplasmic domain highly homologous to that of $\beta 6$ -subunit,⁴² we made a hypothesis that $\alpha v\beta 5$ activates SLC by the nonproteolytic pathway. To clarify this point, we performed TGF- β bioassay. $\beta 5$ -Transfectants significantly increased the luciferase activity of TMLC cells in the presence of contact, suggesting that endogenous active TGF- β is localized on the cell surface of $\beta 5$ -transfectants. By contrast, $\beta 5$ -transfectants showed no significant effect on the luciferase activity of TMLC cells in the absence of cell contact. Consistently, there was no significant difference in the levels of active TGF- $\beta 1$ in cultured media between mock and $\beta 5$ -transfectants. Furthermore, any known activators of SLC did not inhibit the luciferase activity of TMLC cells co-cultured with $\beta 5$ -transfectants. These observations suggest that SLC is activated on the cell surface of $\beta 5$ -transfectants via a nonproteolytic pathway. However, Munger et al¹⁴ previously demonstrated that TMLC cells co-cultured with $\beta 6$ -transfectants showed significantly increased luciferase activity compared with those co-cultured with mock transfectants even in the absence of cell contact, which is not consistent with the present observation in $\beta 5$ -transfectants. There are two possible explanations against this discrepancy. The first is that $\alpha v\beta 5$ does not activate SLC but promotes formation of a ternary complex between endogenous TGF- β and the TGF- β receptors, which would result in the activation of downstream pathways in a TGF- β -dependent fashion. The second is that SLC is activated by $\alpha v\beta 5$ on the cell surface and is rapidly exposed to and consumed by TGF- β receptors in $\beta 5$ -transfectants, which would lead to the low levels of freely diffusible TGF- β in cultured media. Although we cannot determine which hypothesis is likely, the interaction of $\alpha v\beta 5$ with TGF- β receptors may play a central role in the establishment of autocrine TGF- β signaling in $\beta 5$ -transfectants.

Numerous previous findings indicate that integrin-mediated adhesion regulates transmission of growth factor receptor signals.⁴³ Regarding TGF- β signaling, Thannickal et al³⁴ demonstrated that stable expression of the myofibroblast phenotype requires both TGF- $\beta 1$ and adhesion-dependent signals, whereas TGF- β receptor(s)-mediated signaling of Smad2 phosphorylation does not appear to require simultaneous integrin activation. In the present study, we demonstrated that $\beta 5$ -transfectants differentiate into myofibroblasts, and pharmacological inhibition of FAK or expression of kinase-deficient FAK reduced the expression of α -SMA in those cells. The treatment of AS-TGF- $\beta 1$ reduced the phosphorylation levels of 397-FAK and the expression levels of α -SMA in $\beta 5$ -transfectants, suggesting that activation of integrin/FAK signaling as well as stimulation of autocrine TGF- β is also required in myofibroblastic differentiation of $\beta 5$ -transfectants. Alternatively, adhesion-dependent signals may induce the expression of other proteins modifying the TGF- β /Smad pathway. Further studies regarding the detailed mechanism of the interaction between TGF- β signaling and integrin-dependent adhesion signaling may give valuable clues for the treatment of fi-

brotic diseases characterized by persistent myofibroblast activation.

The novel finding in the present study is the interaction of $\alpha v\beta 5$ with TGF- β receptors. To date, the interaction of integrins with receptor tyrosine kinases, such as epidermal growth factor receptor, platelet-derived growth factor receptor β , insulin-like growth factor receptor, and vascular endothelial growth factor receptor-2, have been well studied. The epidermal growth factor receptor forms a complex with $\beta 1$ integrins and antibodies against the $\beta 1$ integrin subunit cause autophosphorylation of this receptor, even in the absence of the ligand stimulation.⁴⁴ $\alpha v\beta 3$ has been shown to associate with receptor tyrosine kinases such as platelet-derived growth factor receptor β ,^{45–47} insulin-like growth factor receptor,⁴⁶ and vascular endothelial growth factor receptor-2⁴⁷ in the presence of ligand stimulation, and such interactions result in a synergistic signaling effect. In contrast, the interaction of integrins with nonreceptor tyrosine kinases, such as those for TGF- β , has been poorly understood. However, Scaffidi et al⁴⁸ recently demonstrated that $\alpha v\beta 3$ interacts with T β RII, and such interaction potentiates the proliferative effects of TGF- $\beta 1$ in human lung fibroblasts. In the present study, TGF- $\beta 1$ antisense oligonucleotide or anti-TGF- $\beta 1$ antibody almost completely diminished the increased COL1A2 promoter activity in $\beta 5$ -transfectants. This observation excludes the possibility that the interaction of $\alpha v\beta 5$ with TGF- β receptors causes autophosphorylation of these receptors even in the absence of ligand but strongly supports the concept that such interaction potentiates the fibrogenic effects of TGF- $\beta 1$ on human dermal fibroblasts. Collectively, the interaction of integrins with growth factor receptors may be a major form of cross-talk as well as the sharing of intracellular signaling molecules. The interaction of integrins with TGF- β receptors may give new insight into the cooperation of these two signaling pathways to enhance cellular responses.

A previous report demonstrated that epithelium-restricted $\beta 6^{-/-}$ mice exhibited exaggerated lung inflammation and protection from pulmonary fibrosis by the treatment of bleomycin.¹⁴ This observation is explained by the loss of paracrine effect of SLC, which is recruited and activated on the cell surface of lung and bronchial epithelium by $\alpha v\beta 6$. The current data that the up-regulated expression of $\alpha v\beta 5$ enhances the COL1A2 gene expression in normal dermal fibroblasts indicate that autocrine effect is biologically critical in the $\alpha v\beta 5$ -dependent activation of TGF- β signaling in dermal fibroblasts. This notion is strongly supported by the present result that the up-regulated $\alpha v\beta 5$ interacts with TGF- β receptors in dermal fibroblasts. Considering that clathrin-dependent internalization sequesters activated TGF- β receptor complex from Smad7-Smurf-mediated degradation,⁴⁰ the predominant interaction of $\alpha v\beta 5$ with clathrin rather than with caveolin may also contribute to the establishment of autocrine TGF- β signaling through the acceleration of clathrin-dependent internalization. Because the cell surface expression of $\alpha v\beta 5$ is controlled by the levels of $\beta 5$ -subunit and a major cytokine that increases the expression of $\beta 5$ -subunit is TGF- β ,^{30,49} it is postulated that $\alpha v\beta 5$ is one of candidates that play important roles in positive feedback of TGF- β signaling.

A previous study demonstrated that mice lacking integrin $\beta 5$ -subunit develop, grow, and reproduce normally. There was also no abnormality in wound healing and adenovirus infection, which are major biological processes in which $\alpha v\beta 5$ participates.⁵⁰ These results suggest that most roles of $\alpha v\beta 5$ can be compensated for by other $\alpha v\beta 5$ -independent pathways. This functional redundancy in $\alpha v\beta 5$ makes the pharmacological interference of $\alpha v\beta 5$ functions a promising approach to the treatment of fibrotic diseases. It would be of interest to determine whether there is a defect of TGF- β activation in these mice. Alternatively, to our knowledge, investigations of TGF- β activity in animal models treated with $\alpha v\beta 5$ blocking antibody have not been performed.

In summary, we demonstrated that the up-regulated expression of $\alpha v\beta 5$ induces myofibroblastic differentiation of dermal fibroblasts. The interaction of $\alpha v\beta 5$ with TGF- β receptors is a novel finding that can explain the establishment of autocrine TGF- β signaling in $\beta 5$ -transfectants. This evidence suggests the possibility of regulating fibrotic disorders, especially scleroderma, by targeting this integrin.

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