Increased Expression Levels of Integrin $\alpha v \beta 5$ on Scleroderma Fibroblasts

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Integrin $\alpha v \beta 5$ is a receptor for vitronectin, a plasma glycoprotein that is also distributed in extracellular matrix of various tissues. Matrix-bound vitronectin has the potential to stabilize the active form of plasminogen activator inhibitor-1, resulting in the inhibition of the plasmin-mediated pericellular proteolytic cascade. In this study, we compared the levels of $\alpha v \beta 5$ and matrix-bound vitronectin between normal and scleroderma fibroblasts and investigated the association with fibrosis. We demonstrated that $\alpha v \beta 5$ was up-regulated on scleroderma fibroblasts. The up-regulated $\alpha v \beta 5$ contributed to the increase in vitronectin-binding ability in scleroderma fibroblasts, which led to the vitronectin-dependent activation of plasminogen activator inhibitor-1. In immunohistochemistry, the αv and $\beta 5$ subunits were stained strongly on scleroderma fibroblasts and the amount of vitronectin was increased in the pericellular matrix of those cells. The transient overexpression of $\alpha v \beta 5$ on normal fibroblasts enhanced the human $\alpha 2(I)$ collagen promoter activity through Sp-1 and Smad3 as well as the vitronectin-dependent plasminogen activator inhibitor-1 activity. This effect on the promoter activity was also observed in the absence of vitronectin and completely disappeared in the presence of anti- $\alpha v \beta 5$ antibody. These results indicate that the up-regulated $\alpha v \beta 5$ may contribute to the phenotypic alteration of scleroderma fibroblasts, while at the same time suppressing the plasmin-mediated pericellular proteolytic cascade. (*Am J Pathol* 2004, 164:1275–1292)

Systemic sclerosis or scleroderma is an acquired disorder that typically results in fibrosis of the skin and internal organs.¹ Although the pathogenesis of this disease is still unclear, it includes inflammation, autoimmune attack, and vascular damage, leading to the activation of fibroblasts and disturbed interactions with different components of the extracellular matrix (ECM).^{2,3} The reason for the presence of abnormal fibroblasts in scleroderma is not yet known, but it is possible that such fibroblasts develop from a subset of cells that have evaded normal control mechanisms.^{4,5}

ECM metabolism of fibroblasts is tightly regulated by multiple environmental influences, including soluble factors (ie, polypeptide growth factors and inflammatory cytokines) and adhesion to the ECM.⁶ The cell-ECM interaction is mediated through distinct receptors on the cell surface, mainly integrins. Integrins are heterodimeric receptors for cell surface counterreceptors as well as ECM proteins. Integrins not only participate in cell-ECM adhesion, but may also function as active receptors, capable of transducing signals to the cell interior via the cytoskeleton; they can thus induce gene expression, modulate the degree of cell differentiation, and interfere with the cell cycle.^{7,8} Evidence suggests that the abnormal expression of integrin receptors plays important roles in the pathogenesis of various diseases.⁹ Regarding scleroderma, a previous study investigated the expression levels of collagen receptors (integrin $\alpha 1 \beta 1$ and $\alpha 2 \beta 1$) on dermal fibroblasts, because these receptors have been shown to be used by fibroblasts for adhesion to and reorganization of type I collagen.¹⁰ In normal dermal fibroblasts cultured in three-dimensional collagen lattices, integrin $\alpha 1 \beta 1$ provides negative feedback for $\alpha 1(I)$ collagen gene expression, whereas integrin $\alpha 2 \beta 1$ stimulates collagenase gene expression.¹¹ Each receptor modulates the signaling activity of the other to coordinate the synthesis and remodeling of the matrix. Previous studies reported that the expression levels of both integrins are reduced on scleroderma fibroblasts and this finding might correlate with the up-regulated collagen gene expression and down-regulated collagenase gene expression of scleroderma fibroblasts.^{12,13} However, other reports demonstrated that there is no difference in the expression levels of integrin $\alpha 1 \beta 1$ and $\alpha 2 \beta 1$ on cultured dermal fibroblasts between scleroderma and controls.¹⁴ Thus, experimental data on the expression of these two receptors on scleroderma fibroblasts is limited and inconsistent.

Recently, reports have indicated the possibility that integrins are crucial to the pathogenesis of fibrotic disorders. In immunohistochemical analyses of pulmonary tissue sections from patients with idiopathic pulmonary fi-

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brosis, a strong reaction for integrin $\alpha 5 \beta 1$ was found in epithelial cells and mesenchymal cells in areas of intra-alveolar fibrosis.¹⁵ In progressive renal fibrosis, immunohistochemical analyses suggested that up-regulated expression of the integrin $\alpha 5$, $\beta 1$, and αv subunits on interstitial fibroblasts correlated with the fibrotic process.¹⁶ Recently, integrin $\alpha v \beta 6$ has been reported to serve as a receptor for latency-associated peptide, a specific component of the latent complex of transforming growth factor (TGF)- $\beta 1$, and be involved in the activation of latent TGF- $\beta 1$ by epithelial cells. Mice carrying a null mutation in the epithelium-restricted integrin $\beta 6$ subunit develop inflammation but are protected from pulmonary fibrosis after exposure to bleomycin.¹⁷

In this study, we focused on another integrin receptor, $\alpha v \beta 5$. Integrin $\alpha v \beta 5$ is a receptor for vitronectin, a 75-kD multifunctional plasma glycoprotein involved in the adhesion and spreading of cells and in the complement and coagulation pathways.¹⁸ Vitronectin is also found distributed in the ECM of various tissues *in vivo*, including dermal fibers in the skin.¹⁹ Many cultured cell types, including fibroblasts and endothelial cells, do not synthesize vitronectin and the exact nature by which vitronectin becomes deposited in and associated with the ECM *in vivo* has not been clearly defined.²⁰ Matrix-bound vitronectin binds glycosaminoglycans, collagen, plasminogen, and the urokinase-receptor, and also stabilizes the inhibitory conformation of plasminogen activator inhibitor-1 (PAI-1).^{18,21,22} Activated PAI-1 bound to vitronectin can inhibit urokinase-type plasminogen activator, thereby blocking the conversion of plasminogen to its active form, plasmin.²¹ Plasmin directly initiates collagen degradation by triggering the activation of cell-secreted matrix metalloproteinases (MMPs) such as MMP-1 and MMP-13.²³ Biochemical analyses have shown that these MMPs are synthesized and secreted by cells as inactive zymogens, proMMP-1 and proMMP-13,^{24,25} that can be activated by plasmin.²⁵⁻²⁷ Taken together, these previous findings suggest that matrix-bound vitronectin is a key molecule controlling the plasmin-dependent pericellular proteolytic cascade. The degradation of matrix-bound vitronectin is regulated by fibroblasts through integrin $\alpha v \beta 5$. The conformationally altered heparin binding form of vitronectin is internalized from the matrix by integrin $\alpha v \beta 5$ -mediated endocytosis and degraded via a lysosomal pathway.^{28,29} In contrast, the nonheparin binding, native vitronectin is not internalized by the cells but remains bound to the ECM.²⁸ These previous studies suggest that interstitial fibroblasts control the level of matrix-bound vitronectin through integrin $\alpha v \beta 5$ and subsequently organize ECM remodeling by regulating the plasmin-mediated pericellular proteolysis.

The notion that a disturbed pericellular proteolysis leads to aberrant ECM metabolism supports the hypothesis that abnormal expression of integrin $\alpha v \beta 5$ plays an important role in the pathogenesis of fibrotic disorders, including scleroderma. In this study, we determined the expression level of $\alpha v \beta 5$ integrin in scleroderma fibroblasts and investigated its association with fibrosis.

Materials and Methods

Reagents

Actinomycin D and antibody for β -actin were purchased from Sigma (St. Louis, MO). Antibodies for integrin αv and $\beta 5$ subunits, polyclonal anti-vitronectin antibody, anti-Smad2/3 antibody, and anti-Smad3 antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-serine antibody was purchased from Biomedica (Foster City, CA). Monoclonal antibody for vitronectin and blocking antibody for integrin $\alpha v \beta 5$ were obtained from Chemicon (Temecula, CA). Integrin αv cDNA was a gift from Dr. Joseph C. Loftus (Mayo Clinic, Scottsdale, AZ). Integrin $\beta 5$ cDNA was purchased from American Type Culture Collection (Manassas, VA). Purified vitronectin derived from human plasma was purchased from BD Bioscience (Bedford, MA).

Cell Culture

Human dermal fibroblasts were obtained by skin biopsy from the affected areas (dorsal forearm) of five patients with diffuse cutaneous scleroderma and <2 years of skin thickening.³⁰ Control fibroblasts were obtained by skin biopsy from five healthy donors. Institutional approval and informed consent from all patients were obtained. Control donors were matched with each scleroderma patient for age, sex, and biopsy site, and control samples were processed in parallel. Primary explant cultures were established in 75-cm² culture flasks in modified Eagle's medium (MEM) supplemented with 10% fetal calf serum (FCS), 2 mmol/L L-glutamine, and 50 μ g/ml of amphotericin. Fibroblast cultures independently isolated from different individuals were maintained as monolayers at 37°C in 95% air, 5% CO₂, and studied between the third and sixth subpassages.

Cell Lysis and Immunoblotting

Cells were cultured to confluence in MEM supplemented with 10% FCS. After incubation for 24 hours in serum-free medium (MEM plus 0.1% bovine serum albumin) cells were washed with phosphate-buffered saline (PBS) at 4°C and solubilized in lysis buffer (1% Triton X-100 in 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 3 mmol/L MgCl₂, and 1 mmol/L CaCl₂ containing 10 μ g/ml of leupeptin, pepstatin, and aprotinin, and 1 mmol/L phenylmethyl sulfonyl fluoride). The lysates were incubated for 30 minutes at 4°C and then centrifuged for 15 minutes at 4°C. Protein concentrations of lysates were determined using Bio-Rad (Hercules, CA) protein assay reagent. Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes as described previously.³¹ Membranes were incubated overnight with anti-integrin αv , $\beta 5$, or β -actin antibodies, washed, and incubated for 60 minutes with secondary antibodies. After further washing, visualization was achieved using enhanced chemiluminescence (Amersham Life Science, Buckingham-

shire, UK) according to the manufacturer's recommendations. The densities of bands were measured with a densitometer.

Biotinylation and Immunoprecipitation

Cells were cultured to confluence in MEM supplemented with 10% FCS. After incubation for 24 hours in serum-free medium, cells were washed with PBS at 4°C. Then, they were incubated with membrane-impermeant NHS-LC-biotin (Pierce, Rockford, IL) dissolved at 0.5 mg/ml in PBS at 4°C for 30 minutes. The cells were washed with cold PBS and harvested into lysis buffer as described above. The integrin $\alpha v \beta 5$ receptor was immunoprecipitated using antibody against integrin $\beta 5$. Immune complexes were collected using protein A-agarose and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blots were prepared as described previously.³² The blots were probed with streptavidin coupled to horseradish peroxidase (Amersham) and visualized as described above.

RNA Preparation and Northern Blot Analysis

Cells were grown to confluence in MEM supplemented with 10% FCS and then incubated for 24 hours in serum-free medium before addition of the reagent. Total RNA (2 μ g) was subjected to electrophoresis on 1% agarose/formaldehyde gels and blotted onto nylon filters (Roche, Indianapolis, IN). The filters were UV cross-linked, prehybridized, and sequentially hybridized with a DNA probe for GAPDH and RNA probes for the integrin αv and $\beta 5$ subunits as described previously.³³ The membrane was then washed and exposed to X-ray film. The X-ray films were scanned as described above.

The Reversible Biotinylation Assay for Endocytosis of Integrin $\alpha v \beta 5$

The endocytosis of integrin $\alpha v \beta 5$ was investigated by biotinylation with sulfo-NHS-SS-biotin, which can be released by reduction with glutathione.³⁴ The confluent quiescent fibroblasts were biotinylated at 4°C with sulfo-NHS-SS-biotin (0.5 mg/ml) using the procedure described above. The cells were then incubated at 37°C in MEM containing 10% FCS for various periods of time, after which they were placed on ice and rinsed once with ice-cold PBS containing 0.1 mmol/L CaCl_2 and 1 mmol/L MgCl_2 (PBS-CM). Any surface biotin was then removed by a 45-minute incubation with a glutathione-containing solution. The solution consisted of 50 mmol/L glutathione, 90 mmol/L NaCl, 1 mmol/L MgCl_2 , and 0.1 mmol/L CaCl_2 , to which NaOH (60 mmol/L final concentration) and FCS (10% final concentration) were added just before use. Control cells were incubated in buffer without glutathione. The cells were then rinsed once with PBS-CM, and free sulfhydryl groups were quenched by rinsing twice with 1 ml of iodoacetamide (5 mg/ml). After a final rinse with PBS-CM, the cells were solubilized in 1 ml of cell lysis

buffer, and immunoprecipitation and immunoblotting were performed as described above.

Preparation of Vitronectin-Depleted FCS

A polyclonal anti-vitronectin affinity column was prepared to deplete vitronectin from FCS as described previously.³⁵ Purified polyclonal anti-vitronectin antibody, which was confirmed to interact with vitronectin derived from bovine as well as human as described below, was dialyzed against 0.1 mol/L Hepes buffer, pH 7.5, and adjusted to a concentration of 1 mg/ml using bovine serum albumin as standard. Antibody was conjugated to Affigel 10 (Bio-Rad) at a rate of 1 ml of antibody per 1 ml of packed gel. FCS was applied to the affinity column and the absence of vitronectin was verified by Western blotting. Only preparations with undetectable levels of vitronectin were used in serum-stripping experiments. All samples were filtered through 0.2- μ m filters before use in tissue culture.

The Determination of Cytoskeleton-Associated Vitronectin Levels in Cultured Fibroblasts

Triton X-100-soluble and -insoluble components were prepared as described previously.^{36,37} Cells were cultured to confluence in MEM supplemented with 10% vitronectin-depleted FCS, and serum-starved for 24 hours. Confluent quiescent fibroblasts were treated with serum-free medium with or without 10 μ g/ml of human vitronectin for 60 minutes. Then cells were washed twice with cold PBS and solubilized in Triton X-100 lysis buffer (100 mmol/L Tris-HCl, pH 7.4, 10 mmol/L EGTA, 2% Triton X-100, 1 μ g/ml of leupeptin, 1 μ g/ml of aprotinin, 1 μ g/ml of pepstatin, and 1 mmol/L phenylmethyl sulfonyl fluoride). The lysates were incubated for 30 minutes at 4°C, centrifuged for 15 minutes at 4°C, and then the supernatant was preserved as the Triton X-100-soluble fraction (TS). The pellet was re-extracted with the extraction buffer to remove any remaining detergent-soluble proteins, pelleted, resuspended, and boiled in 0.5 vol of the extraction buffer with 1 $\times$ sodium dodecyl sulfate sample buffer. The samples were next centrifuged for 15 minutes at 4°C and the supernatants (the Triton X-100-insoluble fraction, TIS) were subjected to immunoblotting using polyclonal anti-vitronectin antibody.

Assay for PAI-1 Activity

To determine the PAI-1 activity in cultured normal and scleroderma fibroblasts, a specific kit for measuring PAI-1 activity (COATEST PAI; Chromogenix, Milano, Italy) was used according to the manufacturer's instructions with minor modification.³⁸ Normal and scleroderma fibroblasts were plated in six-well tissue culture plates and cultured to confluence with MEM supplemented with 10% vitronectin-depleted FCS. After a 24-hour culture in serum-free medium, the indicated concentration of vitronectin and 10 μ g/ml of anti-integrin $\alpha v \beta 5$ antibody or

preimmune mouse IgG were added to the medium and cells were cultured for an additional 60 minutes. Then, cells were washed with serum-free medium three times at 37°C to remove excessive vitronectin that does not interact with cells and incubated for 60 minutes in serum-free medium without phenol red supplemented with 40 IU/ml of tissue plasminogen activator. To determine the levels of tissue plasminogen activator remaining in medium, which does not interact with activated PAI-1, 20 μ l of each medium was transferred to a 96-well plate, and 20 μ l of reagent containing 2.4 μ g of plasminogen and 16 μ g of plasmin substrate (S-2403) as well as 10 μ l of stimulator solution containing 6 μ g of human fibrinogen fragments, was added to each well. During an additional 50-minute incubation at 37°C, the residual tissue plasminogen activator changes plasminogen into plasmin, and plasmin releases *p*-nitroaniline from substrate S-2403. The level of *p*-nitroaniline released was determined from the absorbance at 405 nm. Standard samples were included in each test. One arbitrary unit (AU) of PAI-1 is defined as the amount that inhibits 1 IU of tissue plasminogen activator. The PAI-1 activity (AU/ml) was calculated from the standard curve, and normalized to the number of cells.

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.³⁹ Two- μ m-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 integrin α v, integrin β 5, or vitronectin (monoclonal antibody) diluted 100-fold in PBS overnight at 4°C. The immunoreactivity was visualized using diaminobenzidine. The sections were then counterstained with hematoxylin. We used the following grading system: +, slight staining; + + +, strong staining; and + +, staining between + and + + +.

Plasmid Construction

The generation of a -772 COL1A2/CAT construct consisting of the human collagen α 2(I) gene fragment (+58 to -772 bp relative to the transcription start site) linked to the chloramphenicol acetyltransferase (CAT) reporter gene and COL1A2/CAT constructs used for deletion analysis were done as previously described.⁴⁰ Substitution mutations were introduced into all three GC boxes (located between bp -304 and -299, bp -294 and -289, and bp -277 and -272), a Smad3 binding site (located between bp -263 and -258), or an AP-1 binding site (located between bp -256 and -250) of the -353 COL1A2/CAT construct using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as described previously.⁴¹⁻⁴³ Integrin β 5 cDNA (full length) was digested with *Eco*RI and ligated with

pcDNA3 digested by *Eco*RI (Invitrogen, San Diego, CA). The orientation was confirmed to be correct by sequencing and Western blot analysis was performed to determine the expressed proteins. Plasmids used in transient transfection assays were purified twice on CsCl gradients. At least two different plasmid preparations were used for each experiment.

Transient Transfection

Fibroblasts were grown to 50% confluence in 100-mm dishes in MEM with 10% FCS. The medium was replaced with serum-free medium, and after 4 hours of incubation the cultures were transfected with 2 μ g of each deletion construct, along with 2 or 4 μ g of the integrin β 5 expression vectors or corresponding empty constructs, using FuGENE6 (Roche) as described previously.³¹ To control for minor variations in transfection efficiency, 1 μ g of pSV- β -galactosidase vector (Promega, Madison, WI) was included in all transfections. After 48 or 72 hours of incubation, cells were harvested in 0.25 mol/L of Tris-HCl (pH 8) and fractured by freeze-thawing. Extracts, normalized for protein content as measured with the Bio-Rad reagent, 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. Each experiment was performed in duplicate.

Immunoprecipitation for Smad3

Fibroblasts were transfected with 4 μ g of the β 5 expression vectors or corresponding empty constructs as described above and incubated for 72 hours. In some experiments, cells were stimulated with 2 ng/ml of TGF- β 1 for the last 24 hours. Whole cells lysates were prepared using lysis buffer (1% Nonidet P-40 in 10 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L Na₃VO₄, 50 mmol/L NaF, containing 10 μ g/ml of leupeptin, pepstatin, and aprotinin, and 1 mmol/L phenylmethyl sulfonyl fluoride). Each extract was precleared with 10 μ l of protein G-Sepharose for 1 hour with rotation. The beads were pelleted, the supernatant was transferred to a new tube, and 10 μ l of protein G-Sepharose beads conjugated to anti-Smad3 antibody was added. Immunoprecipitation was performed overnight at 4°C with rotation, after which the immunoprecipitates were washed four times with lysis buffer. After the last wash, the beads were resuspended in 30 μ l of sample buffer, and boiled for 5 minutes. Proteins were subjected to immunoblotting using anti-phospho-serine antibody. After the development, the membrane was stripped and reprobed with anti-Smad2/3 antibody to determine the total levels of Smad3.

Statistical Analysis

Statistical analysis was performed with the Mann-Whitney test for comparison of means. *P* values <0.05 were considered significant.

Figure 1. Expression levels of integrin αv and $\beta 5$ subunit proteins in normal and scleroderma fibroblasts. **A:** Whole cell lysates were electrophoresed through a 4/20% gradient polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with anti-integrin αv , anti-integrin $\beta 5$, or anti- β -actin antibodies. One representative of three independent experiments is shown. **Lanes 1 to 4,** normal fibroblasts; **lanes 5 to 8,** scleroderma fibroblasts. **B:** Quantitative analysis of integrin αv and $\beta 5$ subunit protein levels. Values represent the band density relative to the mean value for normal dermal fibroblasts, which was set at 100. *, $P < 0.05$ versus normal fibroblasts.

Results

Expression Levels of Integrin αv and $\beta 5$ Subunit Proteins in Cultured Normal and Scleroderma Fibroblasts

As an initial experiment, we compared the expression levels of αv and $\beta 5$ subunit proteins between normal and scleroderma dermal fibroblasts using whole cell lysates by Western blot analysis. As shown in Figure 1, the expression levels of integrin αv subunit protein were ~2.5 times higher in scleroderma dermal fibroblasts than normal dermal fibroblasts. The expression levels of integrin $\beta 5$ subunit protein were also approximately three times higher in scleroderma dermal fibroblasts than normal dermal fibroblasts.

Figure 2. Expression levels of integrin $\alpha v\beta 5$ receptor on the surface of normal and scleroderma fibroblasts. **A:** Cell surface proteins were labeled with biotin and cell lysates were immunoprecipitated using anti-integrin $\beta 5$ subunit antibody. One representative of three independent experiments is shown. **Lanes 1 to 4,** normal fibroblasts; **lanes 5 to 8,** scleroderma fibroblasts. **B:** After the detection of biotinylated proteins (**lane 1**), the same membrane was stripped and reprobed with anti-integrin αv antibody (**lane 2**) or anti-integrin $\beta 5$ antibody (**lane 3**). One representative of three independent experiments is shown.

Expression Levels of the Integrin $\alpha v\beta 5$ Receptor on the Surface of Normal and Scleroderma Fibroblasts

To function as a receptor transducing extracellular signals to intracellular messenger proteins, integrins have to be present on the cell surface as dimers.⁸ Therefore, we determined the expression levels of the integrin $\alpha v\beta 5$ receptor on the surface of normal and scleroderma dermal fibroblasts by immunoprecipitation. As shown in Figure 2A, expression levels were markedly elevated on scleroderma dermal fibroblasts compared with normal dermal fibroblasts. These bands were confirmed to be the integrin αv or $\beta 5$ subunit by immunoblotting with anti-integrin αv or $\beta 5$ subunit antibody (Figure 2B).

Expression Levels of Integrin αv and $\beta 5$ Subunit mRNA in Normal and Scleroderma Fibroblasts

The expression levels of integrin αv and $\beta 5$ subunit mRNA in normal and scleroderma fibroblasts were also determined by Northern blot analysis. As shown in Figure 3, levels of integrin αv mRNA were ~2.1 times higher in scleroderma dermal fibroblasts than normal dermal fibroblasts. Similarly, levels of integrin $\beta 5$ mRNA were ~4.2

Figure 3. Expression levels of integrin αv and $\beta 5$ subunit genes in normal and scleroderma fibroblasts. **A** and **B**: Total RNA (2 μ g) was subjected to electrophoresis on 1% agarose/formaldehyde gels, blotted onto nylon filters, and hybridized with a DNA probe for GAPDH and RNA probes for integrin αv (**A**) and $\beta 5$ (**B**) subunits. One representative of three independent experiments is shown. **Lanes 1 to 4**, normal fibroblasts; **lanes 5 to 8**, scleroderma fibroblasts. **C**: Quantitative analysis of integrin αv and $\beta 5$ subunit mRNA levels. Values represent the band density relative to the mean value for normal dermal fibroblasts, which was set at 100. *, $P < 0.05$ versus normal fibroblasts.

times higher in scleroderma dermal fibroblasts than normal dermal fibroblasts.

Comparison of mRNA Stability of Integrin αv and $\beta 5$ between Normal and Scleroderma Fibroblasts

The steady-state level of mRNA can be affected by the level of gene transcription and/or the stability of mRNA. To determine whether the up-regulated expression of integrin αv and $\beta 5$ subunit mRNAs takes place at the transcriptional level or posttranscriptional level, fibroblasts were treated with actinomycin D for 4 hours or 8 hours before RNA extraction. As shown in Figure 4, there was no difference in the stability of integrin αv and $\beta 5$ subunit mRNAs between normal and scleroderma dermal fibroblasts. These results indicate that the expression of the integrin αv and $\beta 5$ subunits is up-regulated at the transcriptional level in scleroderma dermal fibroblasts.

Comparison of Endocytosis of Integrin $\alpha v\beta 5$ between Normal and Scleroderma Fibroblasts

The previous finding that integrin $\alpha v\beta 5$ is endocytosed after the activation of protein kinase C in human skin

fibroblasts⁴⁴ suggests that the cell surface level of integrin $\alpha v\beta 5$ may be controlled by endocytosis as well as gene expression. To clarify this point, we compared the rate of integrin $\alpha v\beta 5$ internalization using a reversible biotinylation technique. Confluent quiescent fibroblasts were biotinylated at 4°C to label cell surface integrin $\alpha v\beta 5$ and rewarmed. At the indicated time points, cells were chilled again and treated with an impermeant reducing agent, which cleaves the disulfide bond joining the biotin moiety with the protein-binding NHS ester. Thus, at time 0 most of the label should be removed, and if internalization occurs, at later time points the biotin label should be protected. Using this method, we indeed observed the internalization of integrin $\alpha v\beta 5$. As shown in Figure 5A, the cell surface integrin $\alpha v\beta 5$ was well labeled (lanes 1 and 5), and the treatment of cells with reducing agents efficiently removed biotin from the cell surface at time 0 (lanes 2 and 6). However, 3 or 6 hours after biotinylation, some of the biotinylated integrin $\alpha v\beta 5$ was unavailable to impermeable reducing agents (lanes 3 and 7 or lanes 4 and 8, respectively), indicating that it had been endocytosed. Sensitivity to cleavage by impermeant reducing agents was determined from these experiments and the percentage of endocytosed integrin $\alpha v\beta 5$ was compared. As shown in Figure 5B, there was no significant difference in the rate of $\alpha v\beta 5$ endocytosis between normal and scleroderma fibroblasts. These results indicate

Figure 4. Time course of integrin αv and $\beta 5$ subunit mRNA degradation in normal and scleroderma fibroblasts. Actinomycin D (400 ng/ml) was added to stop all *de novo* RNA transcription. Cells were harvested at the times indicated after actinomycin D treatment and analyzed for integrin αv , $\beta 5$, and GAPDH mRNA by Northern blotting. Plots show the linear regression of the percentage of remaining integrin αv subunit mRNA (**A**) and integrin $\beta 5$ subunit mRNA (**B**) relative to time 0 and after normalization to the GAPDH mRNA. Shown is the mean $\pm$ SE of five experiments.

that the rate of endocytosis does not contribute to the increased cell surface levels of integrin $\alpha\text{v}\beta 5$ in scleroderma fibroblasts.

Comparison of Cytoskeleton-Associated Vitronectin Levels and Plasminogen Activator Inhibitor-1 Activity between Normal and Scleroderma Fibroblasts

Vitronectin is one of the major ligands for the integrin $\alpha\text{v}\beta 5$ receptor. Therefore, we investigated the levels of cytoskeleton-associated vitronectin in cultures of normal and scleroderma fibroblasts. For the experiments, we first prepared vitronectin-depleted FCS as described in Materials and Methods. As shown in Figure 6A, the polyclonal anti-vitronectin antibody used in this study interacts with bovine vitronectin, and vitronectin was efficiently depleted from FCS using this method. Cells were cultured to confluence in MEM supplemented with 10% vitronectin-depleted FCS, and after serum starvation

Figure 5. Comparison of the rate of endocytosis of integrin $\alpha\text{v}\beta 5$ between normal and scleroderma fibroblasts. **A:** Confluent quiescent fibroblasts were biotinylated at 4°C with sulfo-NHS-SS-biotin (0.5 mg/ml) and rewarmed. At the time points indicated, cells were treated with an impermeant reducing agent to remove the remaining biotin. Whole cell lysate was subjected to immunoprecipitation using anti-integrin $\beta 5$ antibody and the Western blots were probed with streptavidin coupled to horseradish peroxidase. **Lanes 1 and 5,** cells untreated with reducing agent at time 0; **lanes 2 and 6,** cells treated with reducing agent at time 0; **lanes 3 and 7,** cells treated with reducing agent at 3 hours; **lanes 4 and 8,** cells treated with reducing agent at 6 hours. **B:** Quantitative analysis of the rate of endocytosis of integrin $\alpha\text{v}\beta 5$. Data are expressed as percent cell surface integrin $\alpha\text{v}\beta 5$ at time 0. Shown is the mean $\pm$ SE of five experiments.

for 24 hours, cells were cultured for an additional 60 minutes in serum-free medium with or without 10 $\mu\text{g}/\text{ml}$ of vitronectin. The levels of cytoskeleton-associated vitronectin were determined by Western blotting using Triton X-100-soluble and -insoluble fractions. As shown in Figure 6B, vitronectin was not detected in either of the fractions derived from cells cultured in serum-free medium without vitronectin, which is consistent with the finding that fibroblasts do not synthesize vitronectin.²⁰ In cells cultured in serum-free medium supplemented with vitronectin, vitronectin was detected in the Triton X-100-insoluble fraction derived from normal and scleroderma fibroblasts, indicating the cytoskeletal association of vitronectin in these cells. Levels of vitronectin in the Triton X-100-insoluble fraction were significantly elevated in scleroderma fibroblasts compared with normal fibroblasts (3.7-fold increase, $P < 0.05$). Treatment with a functional blocking antibody against integrin $\alpha\text{v}\beta 5$ de-

Figure 6. Comparison of levels of cytoskeleton-associated vitronectin between normal and scleroderma fibroblasts. **A:** FCS was applied to the polyclonal anti-vitronectin affinity column. A 5- μ l aliquot of control FCS and of vitronectin-depleted FCS was subjected to immunoblotting using polyclonal anti-vitronectin antibody. **Lane 1**, control FCS; **lane 2**, vitronectin-depleted FCS. Vn, vitronectin. **B:** Cells were cultured to confluence in MEM supplemented with 10% vitronectin-depleted FCS, and after serum starvation for 24 hours, were cultured for an additional 60 minutes in serum-free medium with or without 10 μ g/ml of vitronectin. In some experiments, cells were cultured with vitronectin in the presence of anti-integrin α v β 5 antibody. Then, Triton X-100-soluble and -insoluble fractions (TS and TIS, respectively) were prepared and subjected to immunoblotting using anti-vitronectin antibody or anti- β -actin antibody. **Lane 1**, TS derived from normal fibroblasts cultured without vitronectin; **lane 2**, TIS derived from normal fibroblasts cultured without vitronectin; **lane 3**, TS derived from normal fibroblasts cultured with 10 μ g/ml of vitronectin; **lane 4**, TIS derived from normal fibroblasts cultured with 10 μ g/ml of vitronectin; **lane 5**, TIS derived from normal fibroblasts cultured with 10 μ g/ml of vitronectin in the presence of anti-integrin α v β 5 antibody; **lane 6**, TS derived from scleroderma fibroblasts cultured without vitronectin; **lane 7**, TIS derived from scleroderma fibroblasts cultured without vitronectin; **lane 8**, TS derived from scleroderma fibroblasts cultured with 10 μ g/ml of vitronectin; **lane 9**, TIS derived from scleroderma fibroblasts cultured with 10 μ g/ml of vitronectin; **lane 10**, TIS derived from scleroderma fibroblasts cultured with 10 μ g/ml of vitronectin in the presence of anti-integrin α v β 5 antibody. **C:** Quantitative analysis of cytoskeleton-associated vitronectin levels. Values represent the band density relative to the mean value of TIS derived from normal fibroblasts cultured with 10 μ g/ml of vitronectin, which was set at 100. *, $P < 0.05$ versus normal fibroblasts under the same conditions.

creased the levels of vitronectin by $\sim 50\%$ in scleroderma fibroblasts, whereas the same treatment only slightly lowered the levels of vitronectin in normal fibroblasts. These results indicate that the up-regulated expression of integrin α v β 5 contributes to the increased ability of scleroderma fibroblasts to interact with vitronectin. Because PAI-1 associated with vitronectin is stabilized in its active form, we next investigated the activity of PAI-1 in normal and scleroderma fibroblasts cultured in the presence or absence of vitronectin. As shown in Figure 7, there was no significant difference in PAI-1 activity between normal and scleroderma fibroblasts cultured in serum-free medium without vitronectin. However, the addition of 10 μ g/ml of vitronectin significantly increased the activity in scleroderma fibroblasts (2.5-fold increase, $P < 0.05$), whereas the same treatment did not affect the PAI-1 activity in normal fibroblasts. In the presence of anti-integrin α v β 5 antibody, the increase in PAI-1 activity in scleroderma fibroblasts was reduced $\sim 40\%$. These re-

sults indicate that the enhanced ability to interact with vitronectin, which is partially attributed to the up-regulated expression of integrin α v β 5, leads to the increase in PAI-1 activity in scleroderma fibroblasts.

Distribution of Integrin α v and β 5 Subunit Proteins in Normal and Scleroderma Skin Sections

To investigate the distribution of the integrin α v and β 5 subunits *in vivo*, immunohistochemical staining was performed. Representative results are shown in Figure 8, A to D, and the results are summarized in Table 1. Regarding the epidermis, blood vessels, and smooth muscles, there were no differences in immunoreactivity for the anti-integrin α v subunit and β 5 subunit antibodies between normal and scleroderma skin sections. The expression of the α v subunit protein was moderate in the

Figure 7. Comparison of PAI-1 activity between normal and scleroderma fibroblasts. Cells were cultured to confluence in MEM supplemented with 10% vitronectin-depleted FCS. After serum starvation for 24 hours, the indicated concentration of vitronectin was added to medium and cells were cultured for an additional 60 minutes. Then, the medium was changed to serum-free medium without phenol red supplemented with 40 IU/ml of tissue plasminogen activator and the cells were incubated for 60 minutes. The level of tissue plasminogen activator remaining in the medium was determined using COATEST PAI as described in Materials and Methods. The PAI-1 activity was calculated from a standard curve, and normalized to the number of cells. In some experiments, cells were cultured in the presence of anti-integrin $\alpha\beta 5$ antibody. The mean and SEM from five separate experiments are shown. *, $P < 0.05$ versus normal fibroblasts under the same conditions. AU, arbitrary unit.

blood vessels, and weak in the epidermis and smooth muscles. The expression of the $\beta 5$ subunit 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 integrin α and $\beta 5$ subunits in scleroderma skin sections, whereas those in normal skin sections were weakly stained. These results were consistent with the results for cultured fibroblasts described above. Interestingly, integrin α and $\beta 5$ subunit epitopes were also observed scattered between thickened collagen bundles in all scleroderma skin sections.

Distribution of Vitronectin in Normal and Scleroderma Skin Sections

To investigate the distribution of vitronectin, a ligand for the integrin $\alpha\beta 5$ receptor, immunohistochemical staining was performed in normal and scleroderma skin sections. Representative results are shown in Figure 8; E to H. In normal skin samples, vitronectin immunoreactivity was strongly detected in the superficial reticular dermis. In middle and deep dermis, vitronectin immunoreactivity was detected only weakly or not at all. There was no immunostaining around spindle-shaped cells between collagen bundles. In scleroderma skin samples, the vitronectin immunoreactivity in superficial reticular dermis was similar to that in normal skin samples. However, scleroderma skin samples demonstrated strong immunoreactivity around spindle-shaped cells between thickened collagen bundles in middle and deep dermis. Several perivascular regions were also strongly stained in scleroderma dermis. There was no immunostaining in the

epidermis and dermal-epidermal junction area in normal or scleroderma skin sections.

Transient Overexpression of Integrin $\alpha\beta 5$ Receptor on Normal Dermal Fibroblasts Induced Increased Human $\alpha 2(I)$ Collagen Gene Expression

To study the effect of integrin $\alpha\beta 5$ expression on ECM metabolism by fibroblasts, we transiently overexpressed integrin $\alpha\beta 5$ receptors on normal dermal fibroblasts. As an initial experiment, we studied the expression levels of the integrin $\alpha\beta 5$ receptor on the surface of transfectants using immunoprecipitation. As shown in Figure 9, A and B, levels were elevated in a time- and dose-dependent manner, suggesting that overexpression of the integrin $\beta 5$ subunit is sufficient to induce the expression of the integrin $\alpha\beta 5$ receptor on the cell surface. Next, we compared the COL1A2 promoter activity between $\beta 5$ transfectants and mock transfectants. As shown in Figure 9C, at 48 hours after transfection, transfectants with 2 μ g or 4 μ g of integrin $\beta 5$ expression vector showed a significant increase in COL1A2 promoter activity (1.4-fold increase, $P < 0.05$; 2.0-fold increase, $P < 0.05$, respectively). Furthermore, the COL1A2 promoter activities of $\beta 5$ transfectants were markedly increased at 72 hours of incubation compared with those of mock transfectants (2.0-fold increase, $P < 0.05$; 3.8-fold increase, $P < 0.01$, respectively). These results indicate that the transient overexpression of the integrin $\alpha\beta 5$ receptor on normal dermal fibroblasts significantly increases the COL1A2 promoter activity in a time- and dose-dependent manner.

Vitronectin-Dependent Increase of PAI-1 Activity in $\beta 5$ Transfectants

Because the up-regulated expression of integrin $\alpha\beta 5$ contributes to the vitronectin-dependent inhibition of the plasmin-mediated pericellular proteolytic cascade in scleroderma fibroblasts, we next investigated whether the proteolytic cascade is altered in $\beta 5$ transfectants. First, we compared the levels of cytoskeleton-associated vitronectin between mock and $\beta 5$ transfectants. As shown in Figure 10A, vitronectin was not detected in cells cultured in medium without vitronectin. In cells cultured in medium with vitronectin, however, the levels of vitronectin in the Triton X-100-insoluble fraction of $\beta 5$ transfectants were significantly elevated compared with those of mock transfectants (2.1-fold increase, $P < 0.05$). Treatment with a functional blocking antibody against integrin $\alpha\beta 5$ decreased the levels of cytoskeleton-associated vitronectin by ~45% in $\beta 5$ transfectants, whereas the same treatment only slightly decreased the levels of cytoskeleton-associated vitronectin in mock transfectants (Figure 10B). These results confirmed that the increased vitronectin-binding ability of $\beta 5$ transfectants completely depends on integrin $\alpha\beta 5$. Next, we investigated the PAI-1 activity in mock and $\beta 5$ transfectants. As shown

Figure 8.

Table 1. Results of Immunohistochemical Staining for Integrin α v and β 5 Subunits

Subjects	Age/sex	Disease duration, years	Epidermis		Blood vessel		Smooth muscle		Fibroblasts		Between collagen bundle	
			αv	β5	αv	β5	αv	β5	αv	β5	αv	β5
Patients												
1	57/F	1	+	+	++	+++	+	+++	+++	+++	+++	+++
2	61/F	0.5	+	+	++	+++	+	+++	+++	+++	+++	+++
3	31/F	1	+	+	++	+++	+	+++	+++	+++	+++	+++
4	32/F	1	+	+	++	+++	+	+++	+++	+++	+++	+++
5	27/F	1	+	+	++	+++	+	+++	+++	+++	+++	+++
Controls												
1	57/F		+	+	++	+++	+	+++	+	+	—	—
2	61/F		+	+	++	+++	+	+++	+	+	—	—
3	31/F		+	+	++	+++	+	+++	+	+	—	—
4	32/F		+	+	++	+++	+	+++	+	+	—	—
5	27/F		+	+	++	+++	+	+++	+	+	—	—

in Figure 10C, there was no significant difference in the activity levels between mock and β 5 transfectants cultured in medium without vitronectin. Moreover, treatment with 10 μ g/ml of vitronectin significantly increased the PAI-1 activity of β 5 transfectants (twofold increase, $P < 0.05$), while the same treatment had no effect on the level in mock transfectants. These results indicate that the transient overexpression of integrin α v β 5 activates the vitronectin-dependent inhibition of the plasmin-mediated pericellular proteolytic cascade.

The Increased COL1A2 Promoter Activity in β 5 Transfectants Is Independent of the Interaction between Vitronectin and Integrin α v β 5

Next, we investigated the requirement of the interaction between integrin α v β 5 and vitronectin for the increased COL1A2 promoter activity in β 5 transfectants. To this end, we performed transient transfection assays using medium with or without vitronectin. As shown in Figure 11A, the level of COL1A2 promoter activity was significantly elevated in β 5 transfectants compared with mock transfectants in the absence of vitronectin (3.8-fold increase, $P < 0.01$). The addition of 5, 10, or 25 μ g/ml of vitronectin had no significant effect on the COL1A2 activity in mock and β 5 transfectants. Taken together with the finding that 10 μ g/ml of vitronectin is sufficient for a significant increase in PAI-1 activity in β 5 transfectants, these results indicate that the increase in COL1A2 promoter activity in β 5 transfectants is independent of the vitronectin-dependent effect on the plasmin-mediated proteolytic cascade. To clarify the requirement of integrin α v β 5 for the induction of COL1A2 in β 5 transfectants, we next investigated the effect of the anti-integrin α v β 5 antibody on the COL1A2 promoter activity. As seen in Figure 11B, mock transfectants treated with the anti-integrin

α v β 5 antibody showed little reduction in COL1A2 promoter activity compared with those treated with preimmune mouse IgG. In contrast, β 5 transfectants treated with the anti- α v β 5 antibody showed a marked, dose-dependent reduction in COL1A2 promoter activity compared with those treated with preimmune mouse IgG. These results suggest that the interaction of integrin α v β 5 with ligands other than vitronectin plays a central role in the induction of the COL1A2 promoter activity in β 5 transfectants.

Functional Mapping of the Response Element of β 5 Transfectants in the α 2(I) Collagen Promoter

To further analyze the transcriptional regulation of the collagen gene in β 5 transfectants, we tested a series of 5'-deletions of the COL1A2 promoter in transient transfection assays. Cells were transfected with 2 μ g of reporter gene, along with 4 μ g of β 5 expression vector or corresponding empty vector. After the transfection, cells were cultured for 72 hours before cell extraction. As shown in Figure 12, the deletion of Sp1-binding sites (three GC boxes) between bp -353 and -264 significantly decreased the basal promoter activity. Subsequent deletion to bp -186 had no additional effect on the activity, whereas deletion to bp -148 increased the basal promoter activity to approximately twofold compared with deletion to bp -186. These results further corroborate the location of the repressor site between bp -164 and -159. Subsequent deletion to bp -108 caused a decrease in promoter activity to ~10% of that of the -353 COL1A2/CAT construct. This dramatic effect on basal promoter activity most likely results from the removal of the previously identified positive constitutive *cis*-element containing a TCCTCC motif located between bp -128

Figure 8. Integrin α v and β 5 subunit protein expression and matrix-bound vitronectin levels in normal and scleroderma skin sections. Paraffin sections of normal and scleroderma dermal tissue were subjected to immunohistochemical analysis with anti-integrin α v, anti-integrin β 5, and anti-vitronectin antibodies, as described in Materials and Methods. Integrin α v and β 5 immunoreactivity was weakly detected on normal dermal fibroblasts (**A** and **C**, respectively). Integrin α v and β 5 immunoreactivity was strongly detected on scleroderma fibroblasts and between thickened collagen bundles (**B** and **D**, respectively). Vitronectin immunoreactivity was not detected around spindle-shaped cell and perivascular lesions in normal dermal tissues (**E** and **G**, respectively). In scleroderma dermal tissues, vitronectin immunoreactivity was strongly detected around spindle cells between thickened collagen bundles and several perivascular lesions (**F** and **H**, respectively). Original magnifications: $\times 200$ (**A–D**); $\times 400$ (**E**, **F**); $\times 1000$ (**G**, **H**).

Figure 9. Effect of integrin $\alpha 5$ overexpression on the transcriptional activity of the human $\alpha 2(I)$ collagen gene in normal dermal fibroblasts. Normal dermal fibroblasts were transfected with 2 μ g of the -772 COL1A2/CAT construct, along with 2 μ g or 4 μ g of the integrin $\beta 5$ or corresponding empty construct. **A:** The cell surface levels of integrin $\alpha 5$ at the time points indicated were determined by biotinylation and immunoprecipitation. One representative of five independent experiments is shown. **Lanes 1 to 3,** normal fibroblasts transfected with 4 μ g of empty vector, 2 μ g of empty vector and 2 μ g of integrin $\beta 5$ expression vector, or 4 μ g of integrin $\beta 5$ expression vector for 48 hours, respectively. **Lanes 4 to 6,** normal fibroblasts transfected with 4 μ g of empty vector, 2 μ g of empty vector and 2 μ g of integrin $\beta 5$ expression vector, or 4 μ g of integrin $\beta 5$ expression vector for 72 hours, respectively. **B:** Quantitative analysis of integrin $\alpha 5$ levels. Values represent the band density relative to that in mock transfectants, which was set at 100. The mean and SEM results from five separate experiments are shown. *, $P < 0.05$ and **, $P < 0.01$, versus mock transfectants under the same conditions. **C:** Values represent the $\alpha 2(I)$ promoter activity relative to that of mock transfectants, which was set at 100. The mean and SEM from five separate experiments are shown. *, $P < 0.05$ and **, $P < 0.01$, versus mock transfectants under the same conditions. CAT, chloramphenicol acetyltransferase.

and -123 in this promoter region. In $\beta 5$ transfectants, the -772 COL1A2/CAT construct responded at the highest level, and the deletion to bp -353 did not significantly alter the level of inducibility. However, the deletion of promoter sequences between residues -353 and -264 led to a significant reduction in the level of inducibility (3.8 ± 0.5 versus 2.5 ± 0.2 , $P < 0.01$). Moreover, additional deletions to position -186 led to a further signifi-

cant reduction in the level of inducibility (2.5 ± 0.3 versus 1.3 ± 0.2 , $P < 0.01$). In contrast, $\beta 5$ transfectants failed to stimulate either the -186 COL1A2/CAT or the -108 COL1A2/CAT construct. These results suggest that the response of COL1A2 gene expression in $\beta 5$ transfectants is mediated by *cis*-acting elements located in the COL1A2 promoter between nucleotides -353 and -264 and between nucleotides -264 and -186.

Figure 10. The levels of cytoskeleton-associated vitronectin and PAI-1 activity in mock and $\beta 5$ transfectants. **A:** Cells were cultured in MEM supplemented with 10% vitronectin-depleted FCS, and transfected with 4 μg of the integrin $\beta 5$ or corresponding empty construct. After a 72-hour incubation, cells were cultured for an additional 60 minutes in serum-free medium with or without 10 $\mu\text{g/ml}$ of vitronectin. Then, Triton X-100-soluble and -insoluble fractions (TS and TIS, respectively) were prepared and subjected to immunoblotting using anti-vitronectin antibody or anti- β -actin antibody. **Lane 1,** TS derived from mock transfectants cultured without vitronectin; **lane 2,** TIS derived from mock transfectants cultured without vitronectin; **lane 3,** TS derived from mock transfectants cultured with 10 $\mu\text{g/ml}$ of vitronectin; **lane 4,** TIS derived from mock transfectants cultured with 10 $\mu\text{g/ml}$ of vitronectin; **lane 5,** TIS derived from mock transfectants cultured with 10 $\mu\text{g/ml}$ of vitronectin in the presence of anti-integrin $\alpha\beta 5$ antibody; **lane 6,** TS derived from $\beta 5$ transfectants cultured without vitronectin; **lane 7,** TIS derived from $\beta 5$ transfectants cultured without vitronectin; **lane 8,** TS derived from $\beta 5$ transfectants cultured with 10 $\mu\text{g/ml}$ of vitronectin; **lane 9,** TIS derived from $\beta 5$ transfectants cultured with 10 $\mu\text{g/ml}$ of vitronectin; **lane 10,** TIS derived from $\beta 5$ transfectants cultured with 10 $\mu\text{g/ml}$ of vitronectin in the presence of anti-integrin $\alpha\beta 5$ antibody. **B:** Quantitative analysis of cytoskeleton-associated vitronectin levels. Values represent the band density relative to the mean value of TIS derived from mock transfectants cultured in 10 $\mu\text{g/ml}$ of vitronectin, which was set at 100. The mean and SEM results from five separate experiments are shown. *, $P < 0.05$ versus mock transfectants under the same conditions. **C:** After a 48-hour incubation, transfected cells were cultured for an additional 60 minutes in serum-free medium with or without 10 $\mu\text{g/ml}$ of vitronectin. Then, the medium was changed to serum-free medium without phenol red supplemented with 40 IU/ml of tissue plasminogen activator and incubation continued for 60 minutes. The level of tissue plasminogen activator remaining in the medium was determined using COATEST PAI as described in Materials and Methods. The PAI-1 activity was calculated from the standard curve, and normalized to the number of cells. In some experiments, cells were cultured in the presence of anti-integrin $\alpha\beta 5$ antibody. The mean and SEM from five separate experiments are shown. *, $P < 0.05$ versus mock transfectants under the same conditions. AU, arbitrary unit.

Effect of Substitution Mutations in All Three GC-Boxes, a Smad3 Binding Site, or an AP-1 Binding Site on the Increased COL1A2 Promoter Activity in $\beta 5$ Transfectants

Previous reports demonstrated that three Sp1 binding sites are located between bp -353 and -264 and both a Smad3 binding site and an AP-1 binding site lie between bp -264 and -186. Furthermore, it has been demonstrated that three Sp1 binding sites, a Smad3 binding site, and an AP-1 binding site functionally contribute to the basal activity and responsiveness of the COL1A2 promoter. Taken together, the findings described above imply that the up-regulation of COL1A2 promoter activity caused by the up-regulation of integrin $\alpha\beta 5$ expression is mediated by *trans*-acting factors including Sp1,

Smad3, and/or AP-1. To analyze the involvement of Sp1, Smad3, or AP-1 in the increase in COL1A2 promoter activity in $\beta 5$ transfectants, we introduced substitution mutations into all three GC-boxes (GGGCGG was changed to GTTCGG, designated -353-Sp1-M COL1A2/CAT), a Smad3 binding site (CAGACA was changed to TACATA, designated -353-Smad3-M COL1A2/CAT), or an AP-1 binding site (CGAGTCA was changed to CCAGTGA, designated -353-AP-1-M COL1A2/CAT) using the -353 COL1A2/CAT construct, and performed a transient transfection assay (Figure 13). Though the promoter activities of -353-Sp1-M, -353-Smad3-M, and -353-AP-1-M COL1A2/CAT constructs were also significantly elevated in $\beta 5$ transfectants compared with mock transfectants (1.9 ± 0.12 -fold increase, $P < 0.05$; 2.0 ± 0.10 -fold increase, $P < 0.05$; $3.7 \pm$

Figure 11. The effect of vitronectin and anti-integrin $\alpha 5 \beta 5$ antibody on the up-regulated COL1A2 promoter activity of $\beta 5$ transfectants. Normal dermal fibroblasts were transfected with 2 μ g of the -772 COL1A2/CAT construct, along with 4 μ g of the integrin $\beta 5$ or corresponding empty construct. After a 24-hour incubation, the indicated concentration of vitronectin (**A**) or anti-integrin $\alpha 5 \beta 5$ antibody (**B**) was added to the medium. Cells were cultured for an additional 48 hours and the COL1A2 promoter activity was measured. Values represent the $\alpha 2(I)$ promoter activity relative to that of mock transfectants without vitronectin treatment or treated with preimmune mouse IgG, which was set at 100. The mean and SEM from five separate experiments are shown. *, $P < 0.05$ versus mock transfectants under the same conditions. CAT, chloramphenicol acetyltransferase.

0.19-fold increase, $P < 0.05$; respectively), the induction in $\beta 5$ transfectants relative to mock transfectants was significantly decreased in the -353-Sp1-M and -353-Smad3-M COL1A2/CAT constructs, but not in the -353-AP-1-M COL1A2/CAT construct, compared with the wild-type -353 COL1A2/CAT construct. These results indicate that Sp1 and Smad3, but not AP-1, are involved in the enhancement of the COL1A2 promoter activity in $\beta 5$ transfectants.

Constitutive Phosphorylation of Smad3 in $\beta 5$ Transfectants

We finally investigated the phosphorylation level of Smad3 in $\beta 5$ transfectants. As shown in Figure 14, the phosphorylation level of Smad3 was below the detectable level in mock transfectants without TGF- $\beta 1$ stimulation, whereas strong phosphorylation of Smad3 was observed in those stimulated with TGF- $\beta 1$. In contrast, Smad3 was constitutively phosphorylated in $\beta 5$ transfectants, but the phosphorylation level of Smad3 was weaker in $\beta 5$ transfectants compared with mock transfectants

stimulated with TGF- $\beta 1$. These results confirm the finding described above in the CAT assays.

Discussion

The ECM fulfills a variety of vital functions. In addition to providing the structural framework for tissues, it mediates cell-cell and cell-ECM interactions and participates in cellular signaling by acting as a ligand binding site as well as a signal itself.^{8,45} It has become apparent that in addition to collagens, other components of the ECM are deposited in excessive amounts in scleroderma. The list of ECM components that are overexpressed in scleroderma includes several collagens (eg, types I, III, V, VI, and VII), as well as glycosaminoglycans and noncollagenous glycoproteins, such as fibronectin, laminin, and tenascin.^{4,46-54} The excessive deposition of matrix components not only results in the characteristic tissue fibrosis of scleroderma but also may play an integral role in the pathogenesis of the disease. However, to date, no information has been available regarding expression lev-

Figure 12. Functional mapping of the response element of $\beta 5$ transfectants in the $\alpha 2(I)$ collagen promoter. Normal dermal fibroblasts were transfected with 2 μg of each 5'-deletion COL1A2/CAT constructs, along with 4 μg of the integrin $\beta 5$ or corresponding empty construct. After a 72-hour incubation, the CAT activity was measured. Values represent the $\alpha 2(I)$ promoter activity relative to that of mock transfectants with the -353 COL1A2/CAT construct, which was set at 100. The mean and SEM from five separate experiments are shown. *, $P < 0.05$ versus mock transfectants under the same conditions. CAT, chloramphenicol acetyltransferase.

els of integrin, a major mediator of cell-ECM interactions, in scleroderma fibroblasts except for collagen receptors such as integrin $\alpha 1\beta 1$ and $\alpha 2\beta 1$.^{10-14,55,56} The present study demonstrated the up-regulated expression of integrin $\alpha\beta 5$ receptors on scleroderma fibroblasts and an association with alteration of the plasmin-mediated pericellular proteolytic cascade and an increase in $\alpha 2(I)$ collagen gene expression. To our knowledge, this is the first report to indicate the significance of integrin $\alpha\beta 5$ in the pathogenesis of scleroderma.

Integrin $\alpha\beta 5$ is widely expressed on epithelial cells including keratinocytes, airway epithelial cells, fibroblasts, osteoclasts, and monocytes. Integrin $\alpha\beta 5$ has been suggested to play important roles in activation-dependent cell migration,^{57,58} in promoting adenovirus-mediated gene delivery,^{59,60} and in cutaneous wound healing.^{57,61,62} Regarding fibroblasts, integrin $\alpha\beta 5$ mediates the degradation of matrix-bound vitronectin by endocytosis. Matrix-bound vitronectin is a key protein controlling the plasmin-mediated pericellular proteolytic cascade. PAI-1 bound to vitronectin is stabilized in its active form and regulates plasmin-dependent proteolysis, a major pericellular proteolytic cascade controlling the activity of MMP-1 and MMP-13. Increasing evidence indicates the importance of the vitronectin-related pericellular proteolytic cascade in tissue remodeling and

Figure 13. Effect of substitution mutations in all three GC-boxes, a Smad3 binding site, or an AP-1 binding site on the increased COL1A2 promoter activity in $\beta 5$ transfectants. Normal dermal fibroblasts were transfected with 2 μg of the indicated COL1A2/CAT constructs, along with 4 μg of the integrin $\beta 5$ or corresponding empty construct. After a 72-hour incubation, the CAT activity was measured. Values represent the $\alpha 2(I)$ promoter activity relative to that of mock transfectants with the wild-type -353 COL1A2/CAT construct, which was set at 100. The mean and SEM from five separate experiments are shown. *, $P < 0.05$ versus mock transfectants under the same conditions. CAT, chloramphenicol acetyltransferase. WT, wild-type -353 COL1A2/CAT construct. Sp1-M, -353-Sp1-M COL1A2/CAT construct. Smad3-M, -353-Smad3-M COL1A2/CAT construct. AP-1-M, -353-AP-1-M COL1A2/CAT construct.

pathophysiological processes. Accelerated skin wound healing was observed in PAI-1-deficient mice,⁶³ whereas plasminogen-deficient mice showed impaired wound healing.⁶⁴ In an immunofluorescence study of rheumatoid arthritic synovia, strong immunoreactivity for vitronectin was detected. Plasmin-generating activity was detected in the cultured adherent cells derived from rheumatoid arthritic synovia, and this activity was increased by addition of anti-vitronectin antibodies.³⁸ These previous findings imply that the plasmin-mediated pericellular proteolytic cascade is indispensable for ECM remodeling and the vitronectin-dependent inhibition of this cascade might lead to disease processes. In this study, we demonstrated that the up-regulated expression of integrin $\alpha\beta 5$ contributes to the increase in cytoskeleton-associated vitronectin levels in scleroderma fibroblasts. Furthermore, the level of PAI-1 activity in scleroderma fibroblasts was increased in the presence of vitronectin. These results suggest that the altered plasmin-mediated pericellular proteolytic cascade contributes to the excessive deposition of ECM proteins in scleroderma dermal tissue. This notion is strongly supported by the previous finding that the activity of MMP-1 is decreased in cultured scleroderma fibroblasts, because the generation of plasmin, one of the major pro-

A

B

Figure 14. Constitutive phosphorylation of Smad3 in β 5 transfectants. **A:** Normal fibroblasts were transfected with 4 μ g of β 5 expression vector or corresponding empty vector and incubated for 72 hours. In some experiments, mock transfectants were stimulated with TGF- β 1 (2 ng/ml) for the last 24 hours. Whole cell lysates were immunoprecipitated with anti-Smad3 antibody and immunoprecipitates were subjected to immunoblotting using anti-phospho-serine antibody. After stripping, total levels of Smad3 were determined by immunoblotting using anti-Smad2/3 antibody. One representative of five independent experiments is shown. **Lane 1**, normal fibroblasts transfected with 4 μ g of empty vector. **Lane 2**, normal fibroblasts transfected with 4 μ g of empty vector and stimulated with 2 ng/ml of TGF- β 1. **Lane 3**, normal fibroblasts transfected with 4 μ g of integrin β 5 expression vector. **B:** Quantitative analysis of phospho-Smad3 levels. Values represent the band density of phospho-Smad3, which is normalized by total Smad3 levels, relative to mean value of mock transfectants stimulated with 2 ng/ml of TGF- β 1, which was set at 100.

teases activating cell-secreted pro-MMP-1, is reduced by the activation of PAI-1.⁶⁵

It is becoming increasingly clear that pericellular proteolytic activity is regulated by the anchoring of proteases to the cell membrane, thereby targeting the catalytic activity to specific substrates within the pericellular space. In recent years, specific integrin-MMP interactions have been reported, such as the interaction of gelatinase-A (MMP-2) with integrin α v β 3 and collagenase-1 (MMP-1) with integrin α 2 β 1. The expression of α v β 3 on

cultured melanoma cells enabled them to bind MMP-2 in a proteolytically active form, facilitating cell-mediated collagen degradation.⁶⁶ Pro-MMP-1 specifically binds the α 2 β 1 integrin on keratinocytes migrating on type I collagen.⁶⁷ Our present results demonstrated the importance of integrin α v β 5 in regulating the plasmin-mediated pericellular proteolytic cascade through control of the levels of cytoskeleton-associated vitronectin. Collectively, these findings have established the significance of integrins in controlling pericellular proteolysis by anchoring proteases in the pericellular region.

Previous reports have indicated that specific signaling from cell adhesion receptors could change the gene expression of ECM proteins,¹¹ the MMP family,^{11,68–70} cytokines,^{71,72} and cytokine receptors.⁷³ Furthermore, it has been demonstrated that the overexpression of integrins directly affects cell behavior.⁷⁴ Taken together with these previous reports, the present results suggest that the up-regulated expression of integrin α v β 5 contributes to the phenotypical changes in scleroderma fibroblasts. To confirm this point, we investigated the effect of integrin α v β 5 on the gene expression of collagen in a transient transfection assay of normal dermal fibroblasts with the β 5 integrin. Interestingly, the transient overexpression of integrin α v β 5 on normal dermal fibroblasts induced a significant increase in the activity of the human α 2(I) collagen gene promoter. We further demonstrated that the increased COL1A2 activity in β 5 transfectants was independent of the interaction between vitronectin and integrin α v β 5 and treatment with anti-integrin α v β 5 antibody completely diminished the increased COL1A2 promoter activity of β 5 transfectants. These results suggest that the interaction of the up-regulated integrin α v β 5 with ligands other than vitronectin contributes to the phenotypical alteration in scleroderma dermal fibroblasts.

To further understand the mechanism up-regulating the COL1A2 promoter activity of β 5 transfectants, we performed a functional analysis of the promoter. We demonstrated that both Sp1 and Smad3 are involved in the enhancement of COL1A2 promoter activity in β 5 transfectants. Furthermore, we revealed that Smad3 is constitutively phosphorylated in β 5 transfectants. Taken together with the finding that Sp1 and Smad3 are major trans-acting factors involved in the up-regulation of COL1A2 promoter activity by TGF- β , the present results suggest that the enhancement of COL1A2 gene expression caused by the up-regulated integrin α v β 5 expression is a result of stimulation with TGF- β 1. It has been demonstrated that α v-containing integrins can bind to latent-TGF- β 1 through the RGD motif in latency-associated peptide- β 1 and among them, α v β 6 and α v β 8 activate latent TGF- β 1 by a nonproteolytic and proteolytic mechanism, respectively.^{17,75} Although no relationship between integrin α v β 5 and latent-TGF- β 1 activation has been reported, the present observations imply that the activation of latent TGF- β 1 by integrin α v β 5 may contribute to the establishment of autocrine TGF- β signaling in scleroderma fibroblasts. To test this hypothesis, further experiments are needed using stable transfectants expressing α v β 5.

A recent study demonstrated that mice lacking the integrin $\beta 5$ subunit develop, grow, and reproduce normally. There was also no abnormality in wound healing or adenoviral infection, two major biological processes that integrin $\alpha v \beta 5$ participates in.⁷⁶ These results suggest that any role of integrin $\alpha v \beta 5$ can be compensated for by other $\alpha v \beta 5$ -independent pathways. This functional redundancy in the integrin $\alpha v \beta 5$ receptor makes the pharmacological interference of $\alpha v \beta 5$ functions a promising approach to the treatment of diseases. Using a functional blocking reagent for the integrin $\alpha v \beta 5$ receptor, the levels of cytoskeleton-associated vitronectin may be reduced and vitronectin-dependent inhibition of the plasmin-mediated pericellular proteolytic cascade may be restored. Furthermore, inhibition of the interaction of integrin $\alpha v \beta 5$ with ligands may contribute to a reduction in COL1A2 promoter activity in scleroderma fibroblasts. To assess the possibility of establishing a therapy for scleroderma by targeting this integrin, further studies are needed to clarify the significance of this receptor in the pathogenesis of scleroderma.

In summary, we established the notion that excess $\alpha v \beta 5$ levels on scleroderma fibroblasts interfere with the balance of collagen metabolism in those cells, shifting the balance toward excess production, while at the same time potentially suppressing collagen-degrading MMP activity.

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