

# Sequential Analysis of Myofibroblast Differentiation and Transforming Growth Factor- $\beta$ /Smad Pathway Activation in Murine Pulmonary Fibrosis

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## Abstract

Myofibroblasts play a critical role in tissue fibrosis. However, the intracellular signaling pathways in myofibroblast differentiation are poorly understood. Here, we studied the relationship between transforming growth factor- $\beta$  (TGF- $\beta$ )/Smad pathway activation and myofibroblast differentiation in both *in vivo* and *in vitro* experiments. In murine bleomycin-induced pulmonary fibrosis, nuclear localization of phosphorylated Smad2/3 (p-Smad2/3) was observed in pulmonary fibrotic lesions 7 days after bleomycin injection, whereas  $\alpha$ -smooth muscle actin (ASMA)-positive myofibroblasts appeared in the lesions at 14 days, when the cytoplasmic localization of p-Smad2/3 was observed. We also compared the effects of TGF- $\beta$ 1 on myofibroblast differentiation and on type I collagen expression in a murine lung fibroblast cell line (MLg2908). TGF- $\beta$ 1 induced rapid expression of p-Smad2/3 in nuclei, after which ASMA organization in the cytoplasm of fibroblasts was observed. However, TGF- $\beta$ 1 produced no effect on the quantity of ASMA, either in mRNA levels or protein levels, even after the phosphorylation of Smad2/3. In contrast, TGF- $\beta$ 1 upregulated the expression of type I collagen mRNA. These findings suggest that in pulmonary fibrosis the molecular mechanism of myofibroblast differentiation is complex and that the difference between ASMA expression and type I collagen expression is mediated by the TGF- $\beta$ /Smad pathway.

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**Key words:** myofibroblast differentiation, transforming growth factor- $\beta$ /Smad pathway, pulmonary fibrosis

## Introduction

Fibrotic disorders are characterized by an excessive deposition of extracellular matrix (ECM) during wound healing. In pulmonary fibrosis, intra-

alveolar fibrosis leads to the remodeling of alveolar structures. Because previous studies have shown that interventions in inflammatory processes do not improve the poor prognosis of idiopathic pulmonary fibrosis, direct interventions against fibrosis are needed. Among several cytokines affecting

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pulmonary fibrosis, transforming growth factor- $\beta$  (TGF- $\beta$ ) is a key mediator of fibrotic reactions<sup>12</sup>. TGF- $\beta$  activates ECM production by fibroblasts<sup>3</sup> and stimulates  $\alpha$ -smooth muscle actin (ASMA) expression in fibroblasts, leading to myofibroblast differentiation<sup>4,5</sup>. Myofibroblasts play an important role in pulmonary fibrosis<sup>6-8</sup>. Therefore, an understanding of the molecular mechanisms associated with myofibroblast differentiation mediated by TGF- $\beta$  may lead to new treatments for pulmonary fibrosis.

On cell surfaces, TGF- $\beta$  binds to its type I or type II receptors. The serine/threonine kinases of these receptors induce phosphorylation of Smad2 and Smad3 proteins, both of which are known as receptor-activated Smad proteins (R-Smad). Smad2 and Smad3 are structurally similar but functionally distinct<sup>9,10</sup>. These phosphorylated (p-) Smad2 and p-Smad3 proteins form heteromeric complexes with Smad4, and translocate into the nucleus, resulting in activation of several transcriptional factors. Among Smad proteins, Smad7 antagonizes the TGF- $\beta$ /Smad pathway by preventing phosphorylation of R-Smad. Although a role for the Smad signaling pathway in myofibroblast differentiation has been reported<sup>11,12</sup>, it remains a matter of debate<sup>13</sup>. The present study was designed to determine the role of the Smad pathway in myofibroblast differentiation and to compare it with its role in type I collagen production, using both a bleomycin-induced model of lung fibrosis and cultured mouse fibroblasts. In particular, we focused on ASMA expression as a marker of myofibroblast differentiation.

## Materials and Methods

### Antibodies and Other Reagents

Antibodies against the following compounds were used: TGF- $\beta$ 1 (R&D Systems, Minneapolis, MN, USA), p-Smad2/3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylated (p-) Smad2 (Cell Signaling Technology, Danvers, MA, USA), Smad7 (Santa Cruz Biotechnology) and ASMA (clone 1A4; Dako Japan, Kyoto). Human TGF- $\beta$ 1 was obtained from Becton, Dickinson and Company (Franklin Lakes, NJ, USA); actinomycin D from Sigma-Aldrich

(St. Louis, MO, USA); and bleomycin hydrochloride from Nippon Kayaku Co. (Tokyo).

### Bleomycin-induced Lung Fibrosis in Mice

Seven-week-old male C57BL/6 mice (Clea Japan, Inc., Tokyo) were treated with a single intravenous injection of bleomycin hydrochloride dissolved in normal saline at a dosage of 100 mg/kg body weight. Control mice received saline vehicle alone. One, 7, 14, and 28 days following the bleomycin treatment, mice were killed under ether anesthesia. The experimental protocols were approved by the Animal Care and Use Committee of Nippon Medical School.

### Histological Study

For morphological examination, the right lobes of the lungs were perfused via the trachea at a pressure of 20 cm H<sub>2</sub>O with a solution of 10% neutralized buffered formalin. The tissues were dehydrated and embedded in paraffin. Sections were stained with hematoxylin and eosin. For immunohistochemical studies, the sections were deparaffinized in xylene and rehydrated with a graded series of alcohol. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol. Slides were washed with 0.01 mol/L phosphate-buffered saline (PBS) and incubated for 10 minutes with diluted normal serum of the animals producing secondary antibodies. The slides were then incubated overnight at 4°C with primary antibodies against TGF- $\beta$ 1 (1 : 100), p-Smad2/3 (1 : 200), or ASMA (1 : 500). Slides were incubated with biotinylated secondary antibodies (Dako Japan) against goat IgG or mouse IgG2a and then with an avidin-biotin-peroxidase complex solution (Dako Japan). The bound peroxidase was visualized with diaminobenzidine and a hydrogen peroxide solution, followed by counterstaining with Meyer's hematoxylin. Histological observations were performed under a light microscope (AX80, Olympus Optical Co., Tokyo). Double immunostaining with the immunofluorescence method was performed with the same sections. To enhance the sensitivity of the immunoreaction, an avidin D-binding immunofluorescence kit (Vector Laboratories,

Table 1 Oligo DNA sequences for real-time RT-PCR

Target gene	Forward primer	Reverse primer	TaqMan probe
ASMA	GCCTCTGGACGTAC AACTGGTAT	TAGCCCTCATAGATAG GCACGTT	TGCTGGACTCTGGAGATG GTGTGACTCA
Smad2	TTGATGGCCGTCTT CAGGTT	AGAGCCGGCAATATA TAACATGTG	ACACCCGAAAGGGT
Smad3	TGGGCCTACTGTCC AATGTCA	TCCCAATGTGTCGCC TTGTA	TGCAGCCGTGGAAC
Smad7	CCATCAAGGCTTTT GACTATGAGA	CCATGGTTGCTGCAT GAACT	ACAGCCTGCAGCGGCC AA
$\alpha 2(I)$ collagen	CCCCAGCGAAGAA CTCATACA	TCAAACCTGGCTGCCA CCAT	AGGCCAACAAGCATGTCT GGTTAGGAGA

Burlingame, CA, USA) was used. Slides were incubated with an anti-p-Smad2/3 antibody (1 : 50), followed by the biotinylated secondary antibody and fluorescein isothiocyanate-labeled avidin-D. After the nonspecific binding of avidin or biotinylated products in tissues was blocked with avidin-biotin blocking solution (Vector Laboratories), the slides were incubated with an anti-ASMA antibody (1 : 100), followed by the biotinylated secondary antibody and Texas Red-labeled avidin-D. Sections were mounted with Vectashield medium containing 4,6-diamino-2-phenylindole (Vector Laboratories) and viewed under a fluorescent microscope (BX51, Olympus Optical Co.).

#### TGF- $\beta$ 1 Immunoassay

The left lobes of the lungs (stored at  $-80^{\circ}\text{C}$ ) were used for TGF- $\beta$ 1 immunoassay. Tissue was homogenized in radioimmunoprecipitation assay buffer. Total protein content in the supernatant was measured with the BCA protein assay kit (Pierce, Rockford, IL, USA). Samples with equal amounts of protein were used for TGF- $\beta$ 1 immunoassay with the Mouse/Rat/Porcine TGF- $\beta$ 1 Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc.) according to the manufacturer's instructions.

#### Cell Culture

A murine lung fibroblast cell line, MLg2908 (CCL-206, American Type Culture Collection, Manassas, VA, USA), originating from ddY mice was maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Immuno-Biological Laboratories, Co., Ltd., Takasaki) with 10% fetal calf

serum. Embryonic fibroblasts (3T3) were obtained from RIKEN BioResource Center (Tsukuba). Cells were grown at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  in a humidified atmosphere. Subconfluent cells were serum-starved overnight before treatment with human TGF- $\beta$ 1. The concentrations of TGF- $\beta$ 1 were 0.4 or 10 ng/mL, based on our previous study of the proliferation of MLg2908 cells and a study on the lung fibroblast proliferation stimulated by different concentrations of TGF- $\beta$ <sup>14,15</sup>. To inhibit *de novo* RNA synthesis, cells were incubated with actinomycin D (2.5  $\mu\text{g}/\text{mL}$ ) for 24 hours before stimulation with TGF- $\beta$ 1.

#### Real-time Reverse-transcriptase Polymerase Chain Reaction

Total RNA was extracted from cultured fibroblasts with a RiboPure RNA purification kit (Applied Biosystems/Ambion, Austin, TX, USA) and converted to complementary DNA, as previously described<sup>14</sup>. Real-time quantitative polymerase chain reaction (PCR) was performed with the TaqMan method, using GeneAmp 5700 Sequence Detection System (Applied Biosystems Japan, Ltd., Tokyo). The sequences of primer pairs and probes for ASMA, Smad2, Smad3, and  $\alpha 2(I)$  collagen (Table 1) were chosen with Primer Express software (Applied Biosystems Japan, Ltd.). As an internal control, murine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used. The set of primers and probes for murine GAPDH was purchased from Applied Biosystems Japan, Ltd. The relative amounts of each mRNA were normalized against GAPDH mRNA.

#### Western Blotting

Cells were washed twice with PBS, and then

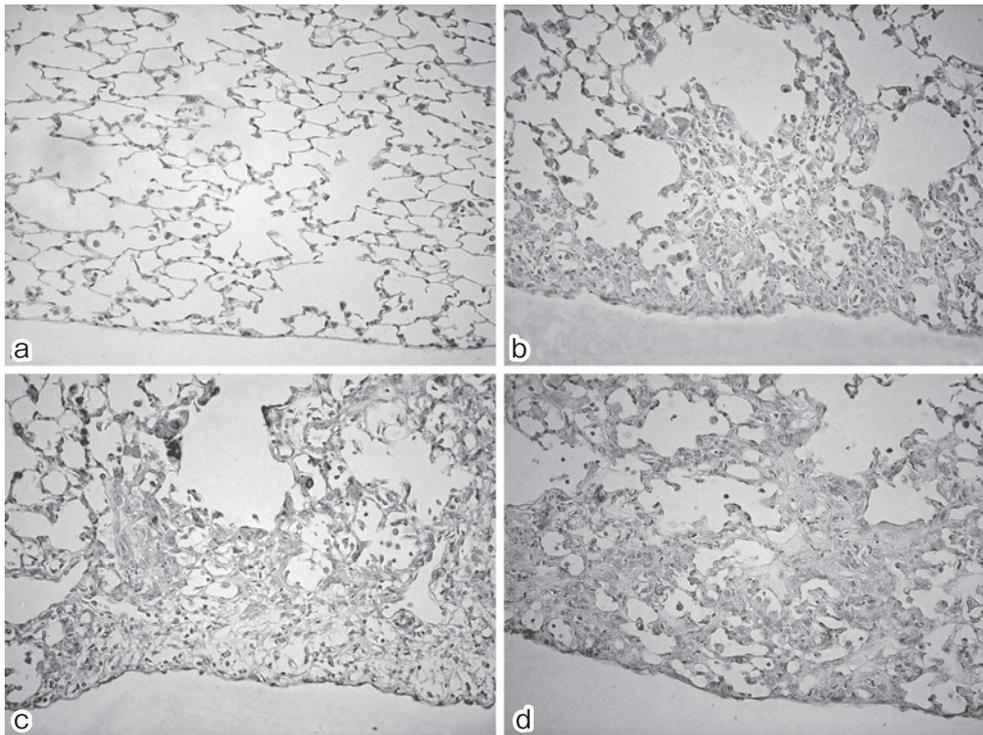


Fig. 1 Time course of immunohistochemistry for TGF- $\beta$ 1 in bleomycin-induced pulmonary fibrosis.

Representative results 1, 7, 14, and 28 days after intravenous injection (a, b, c, and d, respectively). (a) One day after bleomycin treatment, scattered positive signals for TGF- $\beta$ 1 were observed in the cytoplasm of alveolar macrophages and type II alveolar epithelial cells. (b) After 7 days, alveolar macrophages and type II alveolar epithelial cells in pulmonary fibrosis showed a positive reaction for TGF- $\beta$ 1 (c and d). After 14 and 28 days, positive staining in the cytoplasm and extracellular matrices was also observed; however, the reaction was more obvious at the peripheral areas of fibrotic lesions. Original magnification,  $\times 200$ .

radioimmunoprecipitation assay buffer was added. Protein concentrations in cleared cell lysates were measured with a protein assay kit. Total protein (10 to 100  $\mu$ g) was subjected to 10% sodiumdodecylsulfate-polyacrylamide gel electrophoresis under reducing conditions and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% skim milk and incubated with primary antibodies against p-Smad2 (1 : 1,000), Smad7 (1 : 500), or ASMA (1 : 1,000). An anti- $\beta$ -actin antibody was used to confirm equal protein loading. After incubation with horseradish peroxidase-conjugated secondary antibodies, immunoreactive bands were detected with the ECL Plus Western blotting detection system (Amersham Biosciences, Piscataway, NJ), according to the

manufacturer's instructions. Protein levels were quantified with scanning densitometry and the software program ImageJ 1.42 (National Institutes of Health, Bethesda, MD, USA).

#### Immunocytochemical Studies

Cells were seeded on 8-well chamber slides (Lab-Tek Chamber Slide, Nalge Nunc International, Rochester, NY, USA) at  $1 \times 10^4$  cells/slide in RPMI-1640 medium. Serum-starved subconfluent cells were incubated with or without TGF- $\beta$ 1 and then fixed with 4% paraformaldehyde in PBS. Double immunostaining was performed with similar procedures to those used for immunohistochemical studies. Slides were viewed under a fluorescent microscope. Cells positive for ASMA were counted with ImageJ 1.42.

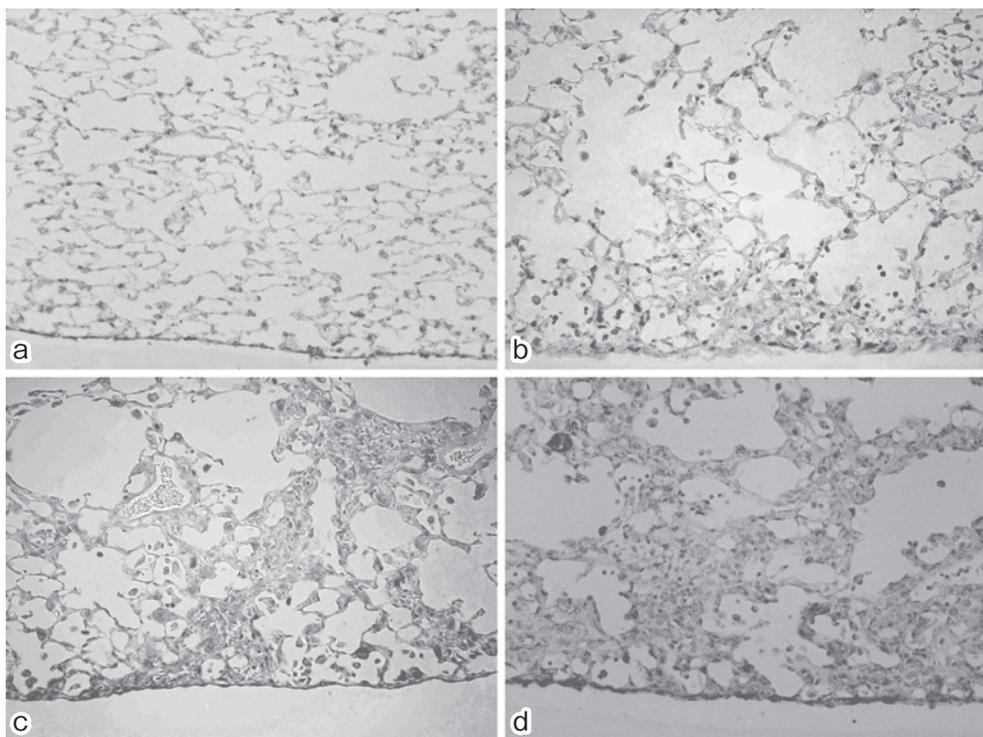


Fig. 2 Time course of immunohistochemistry for p-Smad2/3 in bleomycin-induced pulmonary fibrosis.

Representative results 1, 7, 14, and 28 days after intravenous injection (a, b, c, and d, respectively). (a) One day after bleomycin treatment, scattered positive signals for p-Smad2/3 were observed in the cytoplasm of type II alveolar epithelial cells. (b) After 7 days, many types of cell, including alveolar macrophages, type II alveolar epithelial cells, and fibroblast-like mesenchymal cells, showed strong nuclear reactivity for p-Smad2/3. (c) After 14 days, the reactivity was localized mainly in the cytoplasm of cells. (d) After 28 days, only a few cells were weakly reactive for p-Smad2/3 in pulmonary fibrosis. Original magnification,  $\times 200$ .

### Statistical Analysis

Data are expressed as means  $\pm$  SD. Student's *t*-test was used to determine the significance of differences between data, with differences with *p*-values less than 0.05 considered significant. To compare positive ratios between groups, the chi-square test was used.

### Results

#### Histopathological Assessment

Sequential histopathological changes detected with immunohistochemical studies for TGF- $\beta$ 1 (**Fig. 1**), p-Smad2/3 (**Fig. 2**), and ASMA (**Fig. 3**) are shown. Seven days after bleomycin injection, early fibrotic

changes were observed with fibroblasts migrating into alveolar spaces and mild collagen deposition in alveoli. Fibroblast-like mesenchymal cells and several other types of cell, including alveolar macrophages and type II alveolar epithelial cells, showed strong nuclear reactivity for p-Smad2/3 (**Fig. 2b**). Myofibroblast differentiation determined with ASMA expression was not obvious at this stage (**Fig. 3b**). Fourteen days after bleomycin injection, pulmonary fibrosis was more obvious with ECM deposition. Fibroblast-like mesenchymal cells in fibrotic lesions were positive for ASMA (**Fig. 3c**), suggesting myofibroblast differentiation. At this stage, the immunoreactivity for p-Smad2/3 was distributed mainly in the cytoplasm of cells in the lesions (**Fig. 2c**). These immunoreactive cells included type II alveolar epithelial cells, alveolar

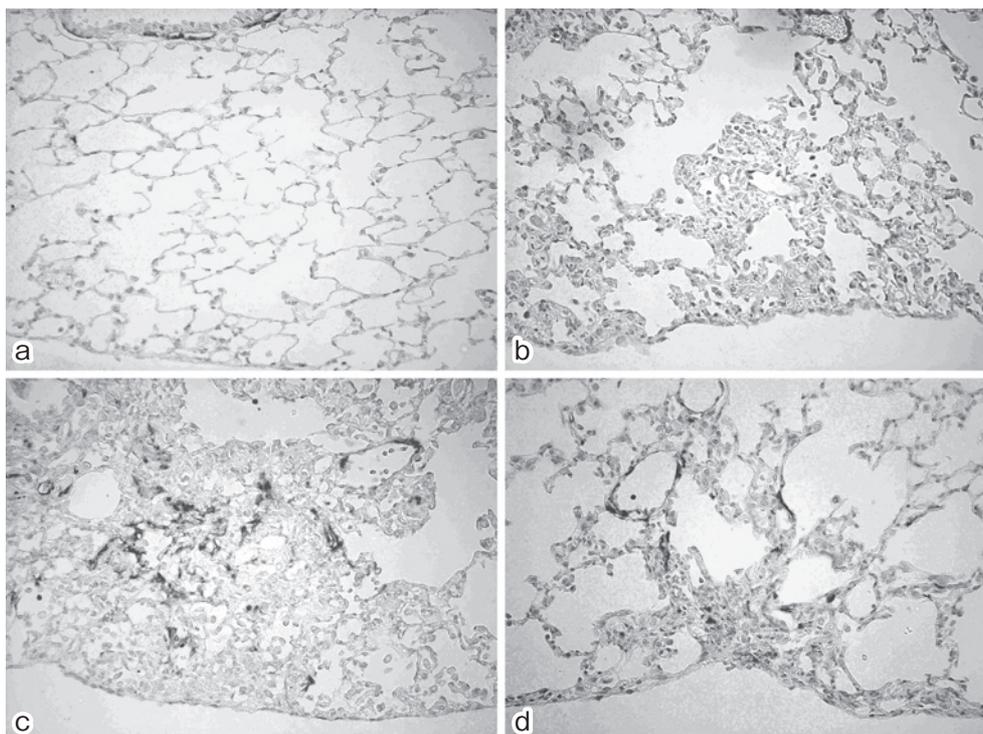


Fig. 3 Time course of immunohistochemistry for ASMA in bleomycin-induced pulmonary fibrosis.

Representative results 1, 7, 14, and 28 days after intravenous injection (a, b, c, and d, respectively). After 1 day (a) and 7 days (b) after bleomycin treatment, only vascular and airway smooth muscle cells were positive for ASMA. (c) After 14 days, several fibroblast-like mesenchymal cells were positive in fibrotic lesions, suggesting myofibroblast differentiation. (d) After 28 days, fibroblast-like mesenchymal cells in areas of fibrous scars accompanied by pleural indentations showed reactivity for ASMA. Original magnification,  $\times 200$ .

macrophages, and fibroblast-like mesenchymal cells. The number of cells with nuclear immunoreactivity for p-Smad2/3 was significantly lower at 14 days (18.9%) than at 7 days (47.3%;  $p < 0.001$ ). Double immunostaining showed that most fibroblast-like mesenchymal cells expressing ASMA did not show nuclear reactivity for p-Smad2/3 (Fig. 4).

#### TGF- $\beta$ 1 Content in Lung Tissue

Concentrations of TGF- $\beta$ 1 in homogenized lung tissue were significantly higher 7, 14, or 28 days after bleomycin injection than on day 1 (Fig. 5). The TGF- $\beta$ 1 concentrations were highest 7 or 14 days after bleomycin injection.

#### Morphological Change of MLg2908 Stimulated by TGF- $\beta$ 1

We examined the association between

myofibroblast differentiation and the activation of Smad proteins *in vitro* using the cultured mouse lung fibroblast cell line (MLg2908) stimulated with TGF- $\beta$ 1. Compared with unstimulated cells (Fig. 6a), TGF- $\beta$ 1-stimulated cells showed higher percentages of elongated and spindle-shaped cells after 1 hour (Fig. 6b). Twenty-four hours after TGF- $\beta$ 1 stimulation, cultured cells showed hypertrophy in a greater degree with a greater percentage of cells with cytoplasm expressing ASMA (Fig. 6c). The incorporation of ASMA in stress fibers was also increased. The percentages of cells positive for ASMA in untreated group, groups 1 hour and 24 hours after treatment were respectively 15%, 25% and 33%, showing significant increase following TGF- $\beta$ 1 stimulation.

Double immunostaining for ASMA and p-Smad2/3 was then performed to study the effect of Smad

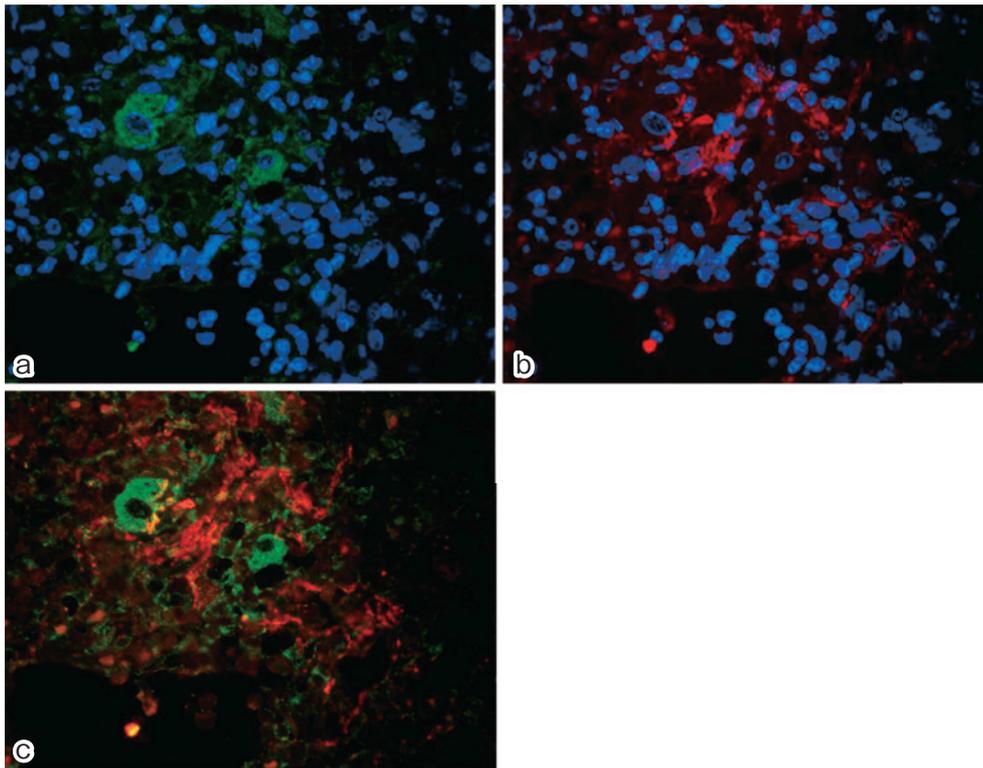


Fig. 4 Double immunostaining for p-Smad2/3 and ASMA at 14 days in bleomycin-induced pulmonary fibrosis.  
 (a) Alveolar macrophages showed cytoplasmic immunoreactivity for p-Smad2/3 (green).  
 (b) Some fibroblast-like mesenchymal cells were positive for ASMA (red). (c) A merged image shows that the nuclei of most ASMA-positive mesenchymal cells were negative for p-Smad2/3. Original magnification,  $\times 600$ .

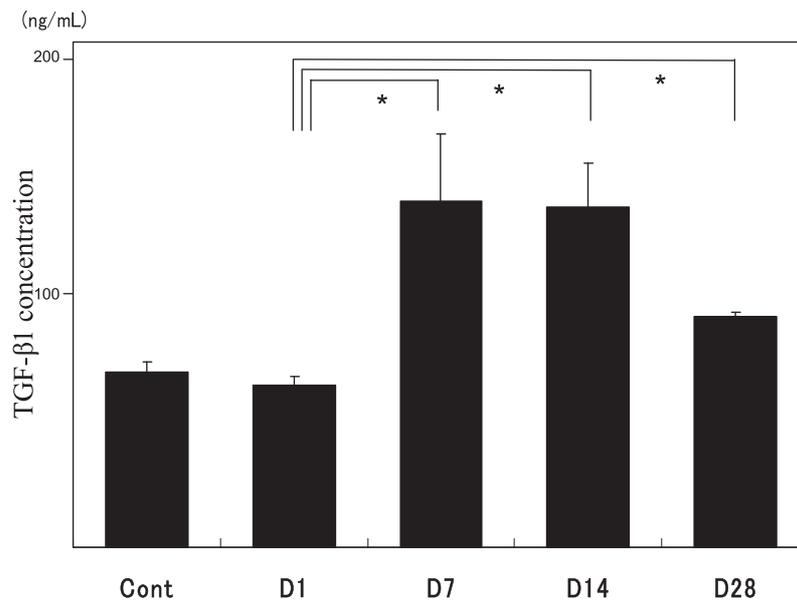


Fig. 5 TGF-β1 concentrations in homogenized lung tissue after bleomycin treatment.  
 Data are expressed as the means  $\pm$  SD of 5 mice in each group.  
 \* $p < 0.05$  versus day 1.

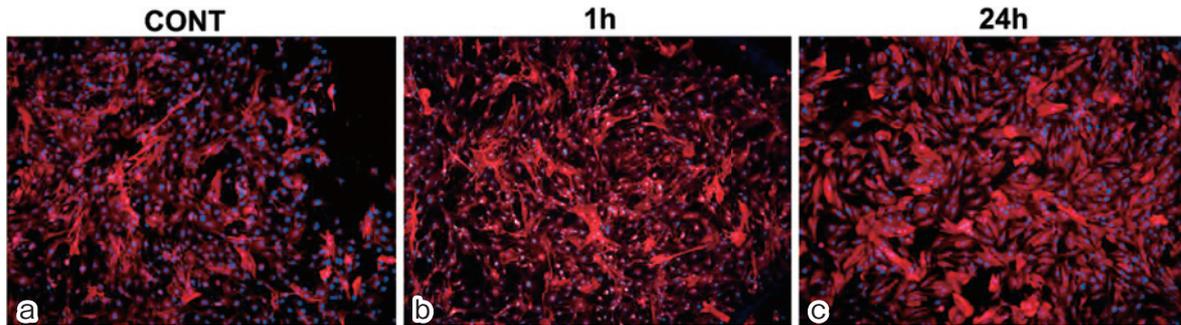


Fig. 6 Morphological features of mouse lung fibroblasts (MLg2908) stimulated with TGF- $\beta$ 1, associated with ASMA expression.

(a) A small number of untreated MLg2908 cells showed reactivity for ASMA (red). The cells seemed to be spindle-shaped with poor formation of ASMA-positive fibers. (b) One hour after TGF- $\beta$ 1 (10 ng/mL) treatment, the number of ASMA-positive cells was increased and was associated with obvious formation of ASMA-positive fibers in the cytoplasm. (c) Twenty-four hours after TGF- $\beta$ 1 treatment, most cells showed development of ASMA-positive fibers, associated with altered cellular size and shape.

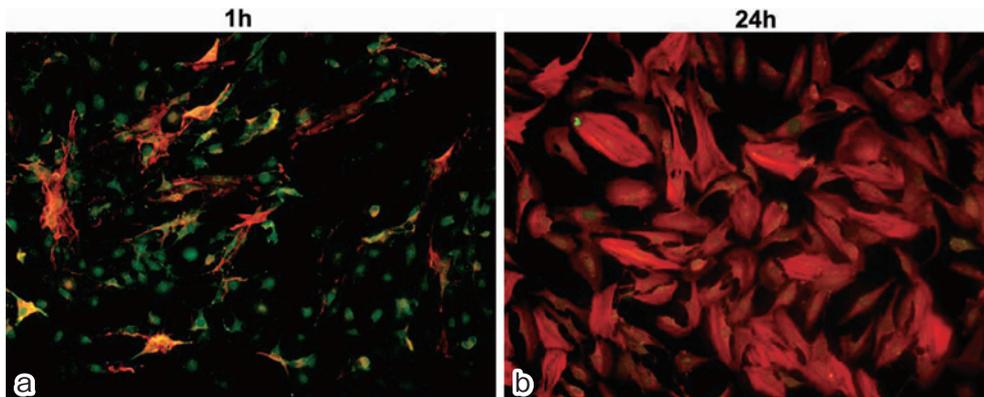


Fig. 7 Double immunostaining for p-Smad2/3 and ASMA in mouse lung fibroblasts (MLg2908) stimulated with TGF- $\beta$ 1.

(a) One hour after TGF- $\beta$ 1 (10 ng/mL) treatment, the nuclei of most cells were positive for p-Smad2/3 (green). Most ASMA (red)-positive cells were negative for p-Smad2/3. (b) Twenty-four hours after TGF- $\beta$ 1 treatment, in contrast, in most cells the nucleus was negative for p-Smad2/3, and the cytoplasm was positive for ASMA.

signals on the expression of ASMA. One hour after TGF- $\beta$ 1 stimulation, we observed a large number of cells with nuclear reactivity for p-Smad2/3. However, less than 10% of p-Smad2/3-positive cells were also positive for ASMA (**Fig. 7a**). Twenty-four hours after TGF- $\beta$ 1 stimulation, the percentage of cells with nuclear reactivity for p-Smad2/3 was markedly decreased; however, more than 50% of stimulated cells were positive for ASMA (**Fig. 7b**).

#### Expression of ASMA mRNA in MLg2908 and 3T3 Cell Stimulated by TGF- $\beta$

Real-time reverse-transcriptase (RT)-PCR showed

that the expression of ASMA mRNA by MLg2908 cells was not significantly affected by TGF- $\beta$ 1 stimulation at different concentrations (**Fig. 8a**). Even 48 hours after TGF- $\beta$ 1 stimulation, the level of ASMA mRNA remained constant. We also found similar results from an experiment with 3T3 cells stimulated with TGF- $\beta$ 1 (**Fig. 8b**), suggesting that the response was not cell lineage specific.

To confirm the transcriptional effect of TGF- $\beta$ 1 on ASMA expression, we treated cells with actinomycin-D to inhibit *de novo* RNA synthesis before stimulation with TGF- $\beta$ 1. Three hours after TGF- $\beta$ 1 stimulation, real-time RT-PCR showed no

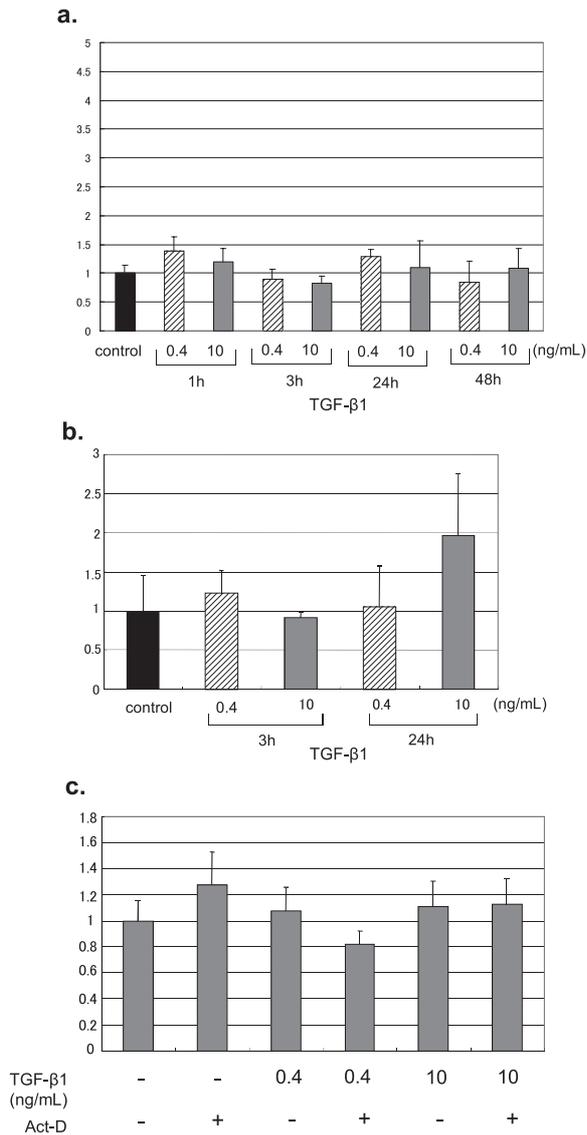


Fig. 8 Effect of TGF-β1 on ASMA mRNA expression in mouse fibroblast cell lines (MLg2908 or 3T3). Treatment with TGF-β1 (0.4 or 10 ng/mL) did not significantly affect ASMA mRNA expression in either MLg2908 cells (a) or 3T3 cells (b). (c) ASMA mRNA expression at 3 hours following TGF-β1 stimulation after the inhibition of *de novo* RNA synthesis by pretreatment with actinomycin-D (2.5 μg/mL) in MLg2908 cells. Data are expressed as the means ± SD of 3 independent experiments.

significant change in ASMA mRNA expression even after treatment with actinomycin-D. Cells not treated with TGF-β1 also showed steady expression of ASMA mRNA after treatment with actinomycin-D (Fig. 8c).

### Expression of Smad2, Smad3, Smad7, and Type I Collagen mRNA in MLg2908

Real-time RT-PCR showed that the levels of Smad2 and Smad3 mRNA were slightly upregulated 1 hour after treatment with TGF-β1 (Fig. 9a, b). However, 3 hours or more after TGF-β1 treatment, Smad3 expression was significantly inhibited. In contrast, Smad7 mRNA showed a more dynamic change: expression 1 hour after TGF-β1 stimulation was markedly greater than in untreated cells but decreased from 3 to 24 hours and increased again at 48 hours (Fig. 9c). The expression of α2(I) collagen mRNA was increased from 1 hour to 24 hours after TGF-β1 stimulation (Fig. 9d).

### Expression of ASMA, p-Smad2, and Smad7 Protein in MLg2908

Western blotting showed that ASMA protein expression was not affected by TGF-β1 stimulation (Fig. 10). In contrast, p-Smad2 protein expression was increased by TGF-β1 stimulation at 3 and 24 hours. After treatment with 10 ng/mL TGF-β1, Smad 7 protein expression was significantly increased at 3 hours but was decreased again at 24 hours.

### Discussion

In the present study, we have demonstrated histologically the activation of the Smad pathway and myofibroblast differentiation in a bleomycin-induced model of fibrosis. To our knowledge, this report is the first to show with a double immunostaining method both phosphorylated R-Smad and myofibroblast differentiation in lung tissue. Corresponding to the TGF-β1 content in the lung, measured with ELISA, p-Smad2/3 was observed in cell nuclei 7 days after bleomycin injection. Even though we could not separate the active form of TGF-β1 from its inactive form by means of the ELISA kit we used, the nuclear localization of p-Smad2/3 suggests activation of the Smad pathway, stimulated by TGF-β. Seven days after bleomycin injection, early fibrosis was accompanied by nuclear localization of p-Smad2/3 in various types of cell, including epithelial cells,

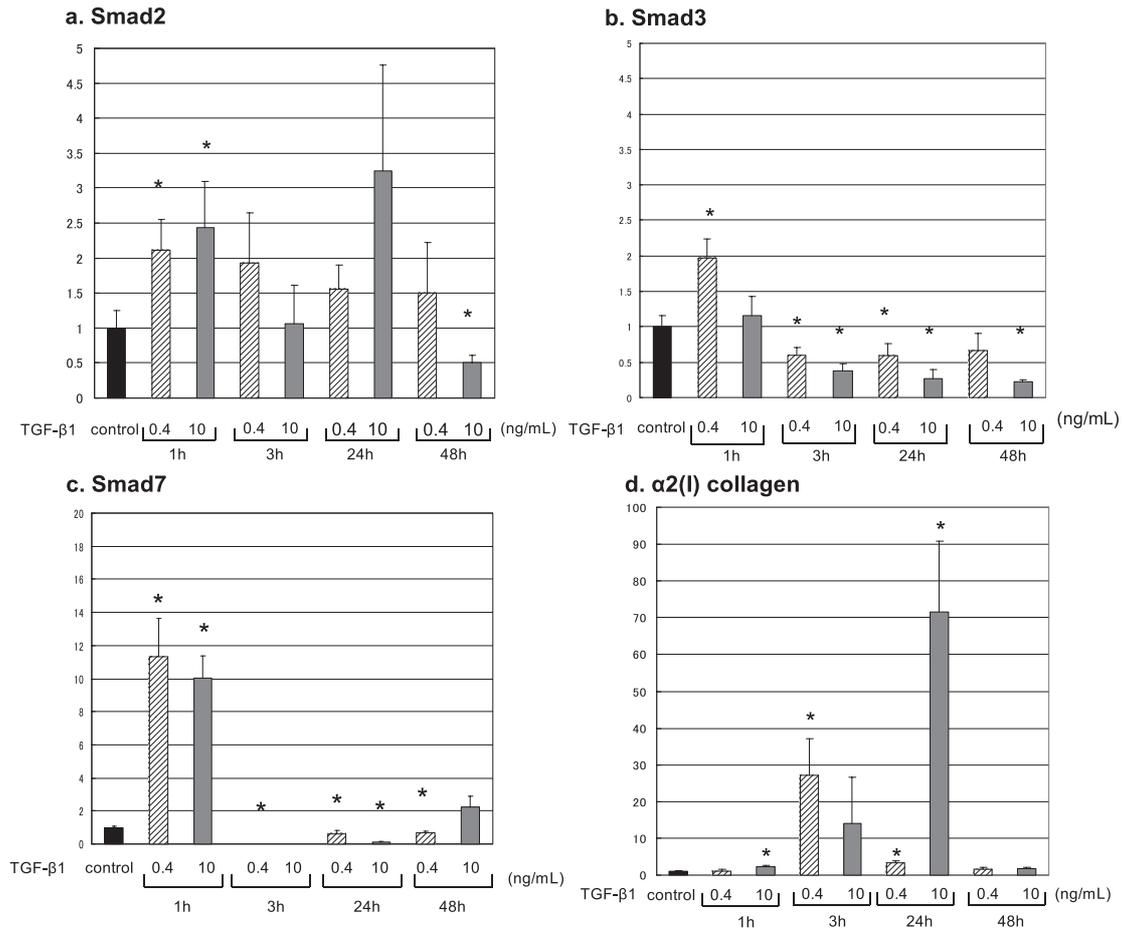


Fig. 9 Effect of TGF- $\beta$ 1 on expression of mRNA for Smad2, Smad3, Smad7, and  $\alpha$ 2(I) collagen in mouse lung fibroblasts (MLg2908).

Cells were treated with TGF- $\beta$ 1 (0.4 or 10 ng/mL). The amounts of each mRNA were estimated with real-time quantitative PCR. Data are expressed as the means  $\pm$  SD of 3 independent experiments. \* $p$  < 0.05 versus untreated cells (control).

macrophages, and mesenchymal cells. However, there was little reactivity for p-Smad2/3 in nonfibrotic areas of the lung. These findings suggest that the concentrated active form of TGF- $\beta$ 1 simultaneously activates the Smad signaling pathway in fibroblasts as well as in other types of cell in areas of fibrosis. Consistent with previous reports on the role of the Smad pathway in pulmonary fibrosis, our results indicate that ECM deposition in the lung is dependent on the Smad pathway<sup>16</sup>. Fourteen days after bleomycin injection, p-Smad2/3 was located mainly in the cytoplasm, perhaps due to the proteasomal degradation of activated Smad proteins, as previously reported<sup>17,18</sup>, and indicates inactivation of the Smad pathway at this stage.

We observed no ASMA expression in fibroblast-

like mesenchymal cells 7 days after bleomycin injection. If myofibroblast differentiation with ASMA expression is directly dependent on the Smad pathway, ASMA expression should also be observed after about 7 days, corresponding to the TGF- $\beta$ 1 content and p-Smad2/3 expression in the lung. In contrast to fibrotic changes in alveoli, ASMA-positive fibroblast-like mesenchymal cells were initially recognized after 14 days, when the immunoreactivity for p-Smad2/3 was observed only in the cytoplasm of cells, including fibroblast-like mesenchymal cells and inflammatory cells. These results suggest that the regulatory mechanism for ASMA expression differs from that inducing the deposition of ECM derived from fibroblasts. The results from cultured fibroblasts stimulated with TGF- $\beta$ 1 *in vitro* support our hypothesis. We have

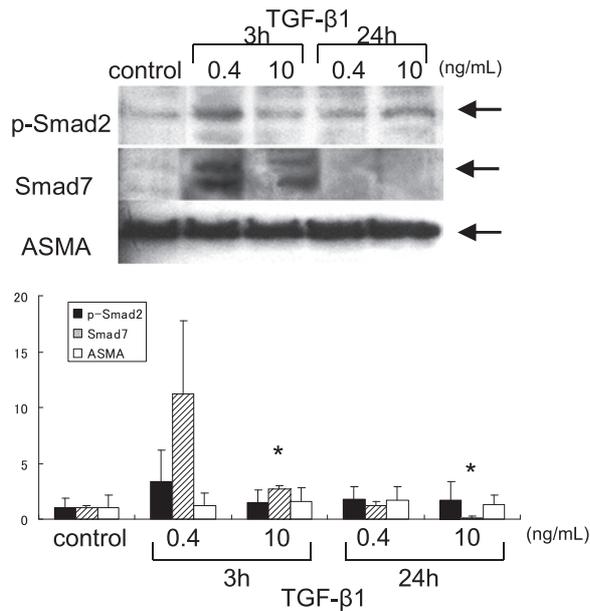


Fig. 10 Effects of TGF- $\beta$ 1 on expression of proteins of p-Smad2, Smad7, and ASMA in mouse lung fibroblasts (MLg2908).

Cells were treated with TGF- $\beta$ 1 (0.4 or 10 ng/mL). The immunoblots shown are representative results. Signal intensities were quantified with densitometry, and the results compared with those of untreated cells from at least 3 independent experiments are shown in the lower graph. Data are expressed as the means  $\pm$  SD of 3 independent experiments. \* $p$  < 0.05 versus untreated cells (control).

shown that TGF- $\beta$ 1 rapidly upregulates  $\alpha$ 2(I) collagen expression, which depends on the Smad pathway<sup>19,20</sup>. In contrast, TGF- $\beta$ 1 induced a morphological change with ASMA organization in the cytoplasm within 1 hour. However, there were no significant increases in ASMA mRNA or protein levels. Twenty-four hours after TGF- $\beta$ 1 stimulation, when most fibroblasts were morphologically similar to myofibroblasts, the ASMA mRNA and protein levels were unchanged. The experiment with actinomycin-D treatment suggests that in MLg2908 cells ASMA expression is constitutional and that this morphological change can be induced without an increase in the quantity of ASMA. Similarly, in cultured glomerular mesangial cells, TGF- $\beta$  has been reported to induce rapid cytoskeletal rearrangement with ASMA organization within 15 minutes<sup>21</sup>. Such changes might be induced without a transcriptional effect. If myofibroblasts must be accompanied with

newly expressed ASMA by definition, these fibroblasts with ASMA organization might be the intermediate between protomyofibroblasts with stress fibers<sup>22</sup> and myofibroblasts. Collectively, the molecular mechanisms leading to myofibroblast differentiation may be distinct from those leading to type I collagen production.

Our experiments had several limitations. Although our results are from experiments with specific kinds of cultured fibroblasts, we cannot rule out a possible role of the Smad pathway in other types of fibroblast. In previous reports, the first increase in ASMA expression was observed around 3 days after TGF- $\beta$  stimulation<sup>23</sup>. Our *in vitro* experiment did not examine this stage. We must also consider the effects of stimulation of the culture system on the induction of ASMA expression. The cell culture itself might induce the differentiation of fibroblasts to myofibroblasts without TGF- $\beta$ . To confirm the effect of the TGF- $\beta$ /Smad pathway on myofibroblast differentiation, experiments using inhibitors of the Smad pathways are required. The ASMA mRNA alteration in myofibroblast-like mesenchymal cells *in vivo* also remains for further investigation.

Several signaling pathways of TGF- $\beta$  have been reported. In Smad3-deficient mice treated with bleomycin, pulmonary fibrosis is attenuated, but ASMA expression in lung tissue is not completely inhibited<sup>24</sup>. During stimulation with TGF- $\beta$ , there may be crosstalk between Smad-dependent and Smad-independent pathways. In the regulation of ASMA gene expression, the additional involvement of several transcription factors, such as CCAAT/enhancer-binding protein  $\beta$ <sup>25</sup>, gut-enriched Kruppel-like factor<sup>26</sup>, Sp1/Sp3<sup>27</sup>, c-myb<sup>28</sup>, and the downstream effector component of Notch signaling<sup>29</sup>, has been documented. Several signaling pathways have also been shown to contribute to myofibroblast differentiation by TGF- $\beta$ , such as mitogen-activated protein kinase<sup>30-32</sup> and Rho-kinase<sup>33</sup>. An experiment with ASMA-deficient mice has shown an inhibitory effect of ASMA on renal fibrosis<sup>34</sup>. Further investigation is needed to clarify the complex mechanisms of myofibroblast differentiation and collagen production.

In bleomycin-treated mice, ASMA-positive

myofibroblast-like mesenchymal cells were observed 7 days after the TGF- $\beta$ 1 concentration peaked, a finding that seems to conflict with the results of our *in vitro* study showing a prompt effect of ASMA organization by TGF- $\beta$ 1. The difference might be explained by the following 2 possibilities. First, in an animal model, factors other than TGF- $\beta$  might induce or inhibit ASMA expression. Mechanical stress is an essential factor<sup>35</sup>. Found in inflammatory zone 1, mechanical stress is a candidate for inducing myofibroblast differentiation in a TGF- $\beta$ -independent manner in bleomycin-induced pulmonary fibrosis<sup>36</sup>. Epigenetic regulation has also been reported in the liver<sup>37</sup>. As previously described, signaling pathways other than the Smad pathway are possibly involved in ASMA gene expression after TGF- $\beta$  treatment. Second, ASMA-positive myofibroblasts *in vivo* might be derived from cells other than lung-residing interstitial fibroblasts. Cells originating in the bone marrow or circulating fibrocytes have been reported to differentiate to myofibroblasts<sup>38,39</sup>. Epithelial cells in the lung have also been shown to differentiate into fibroblast-like mesenchymal cells (i.e., epithelial-mesenchymal transition)<sup>40,41</sup>. As TGF- $\beta$  has been implicated in all these processes, myofibroblast-like mesenchymal cells in the fibrotic lung might originate from heterogeneous cell populations.

In the present experiment, we have shown physiological changes in endogenous Smad expression after TGF- $\beta$ 1 stimulation of cultured lung fibroblasts. The expression of both Smad2 and Smad3 mRNA was slightly upregulated 1 hour after TGF- $\beta$ 1 treatment but was downregulated after 3 hours or more. In contrast, Smad7 mRNA expression was markedly upregulated after 1 hour and was markedly downregulated after 3 hours or more. The results from Western blotting support these transcriptional alterations. It seems that the difference in expression between the protein and mRNA of Smad7 3 hours after TGF- $\beta$ 1 treatment is due to the time for protein translation. Consistent with our results, it has been reported by Chen et al that in normal skin fibroblasts TGF- $\beta$  inhibits Smad3 mRNA expression, with a maximal reduction by 48 hours<sup>42</sup>. These authors also found the rapid induction of Smad7 by TGF- $\beta$ , with a maximal increase by 2

hours and a return to control levels by 24 hours. Although the regulatory mechanism of endogenous Smad expression is not fully understood, an immediate induction of Smad7 implies that Smad7 is a direct target of TGF- $\beta$  for terminating the Smad signaling pathways. Our immunocytochemical experiments have also shown the rapid phosphorylation of R-Smad after TGF- $\beta$ 1 stimulation. Therefore, the activation of the Smad pathway by TGF- $\beta$  may depend mainly on the phosphorylation of endogenous R-Smad, and not on an increase in R-Smad proteins.

### Conclusions

Our present observations suggest the complexity of myofibroblast differentiation in bleomycin-induced pulmonary fibrosis. The molecular mechanism of ASMA induction in fibroblasts is possibly different from that of type I collagen production, in regards to the role of the TGF- $\beta$ /Smad pathway.

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