Brief communication

Activation of TGF-β/Smad2 signaling is associated with airway remodeling in asthma

Hironori Sagara, MD,[a] Takanori Okada, MD,[a] Ko Okumura, MD,[b] Hideoki Ogawa, MD,[b] Chisei Ra, MD,[b] Takeshi Fukuda, MD,[a] and Atsuhito Nakao, MD[b] *Tochigi and Tokyo, Japan*

Background: Transforming growth factor β (TGF-β) has been suggested to play an important role in the development of airway remodeling in asthma; this suggestion is based on evidence that expression levels of TGF-β are correlated with unique parameters of airway remodeling, such as thickness of basement membrane. However, the relevant studies were inconclusive because they were unable to demonstrate active signaling mediated by the cytokine in the airways of asthmatic individuals.

Objective: We sought to determine whether TGF-β signaling was active in the airways of asthmatic subjects and, if so, whether it was correlated with clinicopathologic features associated with the development of airway remodeling in asthma.

Methods: We examined the phosphorylation status of Smad2 in bronchial biopsy samples obtained from 40 asthmatic subjects as a marker of active TGF-β signaling, and we studied its correlation with basement membrane thickness.

Results: Expression levels of phosphorylated Smad2 in bronchial biopsy specimens from asthmatic subjects were higher than those in specimens from normal subjects, and they were correlated with basement membrane thickness in asthma.

Conclusion: The findings provide evidence that TGF-β signaling was active in asthmatic airways and that the activity was associated with the development of airway remodeling in asthma. (J Allergy Clin Immunol 2002;110:249-54.)

Key words: Transforming growth factor β, Smad, asthma, airway remodeling, subepithelial thickness

Chronic inflammation of the airways and airway tissue remodeling are the most common histopathologic features of bronchial asthma.[1] Airway remodeling is defined by a collection of chronic structural changes, including subepithelial fibrosis, myofibroblast hyperplasia, airway smooth muscle hypertrophy/hyperplasia, mucous gland and goblet cell hyperplasia, and epithelial disruption, and it is thought to lead to irreversible airway obstruction, which is one of the factors that makes treatment of asthmatic patients difficult.[2] Among the features of airway remodeling, subepithelial fibrosis that might result in basement membrane thickening has attracted much attention,[3] inasmuch as it has been suggested to be associated with disease severity and correlated with a decline of FEV_1.[4,5] Although the presence of airway remodeling in asthma is generally accepted, the cellular and molecular events underlying the remodeling process are poorly understood.

Abbreviation used
TGF-β: Transforming growth factor β

Previous studies have suggested that a fibrogenic cytokine, transforming growth factor β1 (TGF-β), might be a relevant molecule, given that TGF-β was shown to be expressed in the tissue of asthmatic airways[6,7] and its expression level is correlated with basement membrane thickness and fibroblast number/and or disease severity.[7,8] Most of the relevant studies evaluated the levels of TGF-β1 expression in asthmatic airways by using immunohistochemistry with anti–TGF-β1 antibody or in situ hybridization with a TGF-β1 probe. However, because TGF-β1 is secreted as latent complexes[9] and anti–TGF-β1 antibody and because detection of mRNA with a TGF-β1 probe is not generally able to discriminate the latent form from the active form (unless one specifically uses antibody against the active form of TGF-β1), the actual activity of TGF-β1 in asthmatic airways and its association with airway remodeling remain uncertain.

Recent identification of the Smad family of proteins has advanced our understanding how TGF-β signals from the membrane to the nucleus.[10] The activated TGF-β receptors induce phosphorylation of Smad2 and Smad3, which form hetero-oligomeric complexes with Smad4. The complexes then translocate to the nucleus and regulate transcriptional responses, together with DNA binding cofactors. Therefore, phosphorylation of Smad2 (or Smad3) is a key step in the initiation of TGF-β signal transduction and serves as an indicator of active TGF-β signaling.

From [a]the Department of Pulmonary Medicine and Clinical Immunology, Dokkyo University School of Medicine, Tochigi, and [b]the Atopy (Allergy) Research Center, Juntendo University School of Medicine, Tokyo.

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Reprint requests: Atsuhito Nakao, MD, Atopy (Allergy) Research Center, Juntendo University School of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo, 113-8421, Japan.

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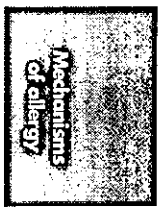

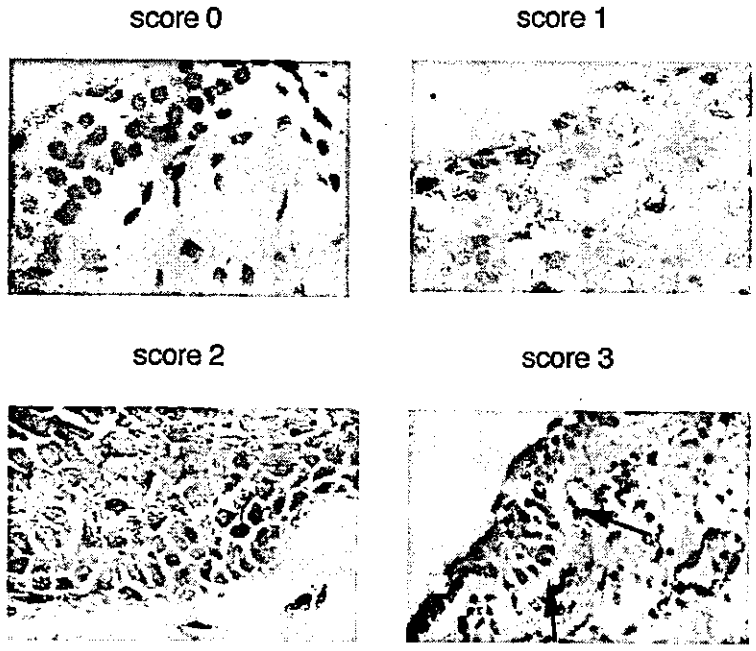

FIG 1. Photomicrographs show scoring for phosphorylated Smad2 staining in bronchial biopsy specimens from asthmatic and control subjects. Note the basement membrane thickness *(arrows)* in a sample derived from a severely asthmatic patient (score 3).

In this study, we examined the expression of phosphorylated Smad2 in the airways of asthmatic individuals and its correlation with basement membrane thickness in asthma to determine whether TGF-β1 signaling was active in the process of airway remodeling.

METHODS

Subjects

Eight normal control subjects without asthma (mean [± SE] age, 43.0 ± 3.8 years) and 40 asthmatic subjects (20 with mild asthma, a mean [± SE] age of 41.4 ± 4.4 years, and a mean [± SE] $FEV_{1.0}$ of 2441 ± 113.8 mL; 16 with moderate asthma, a mean [± SE] age of 43.0 ± 3.8 years, and a mean [± SE] $FEV_{1.0}$ of 2518 ± 174.5 mL; and 4 with severe asthma, a mean [± SE] age of 54 ± 5.0 years, and a mean [± SE] $FEV_{1.0}$ of 1990 ± 330.3 mL), as defined by a combination of asthma symptom grade and frequency of symptoms (based on the criteria of the Japanese Society of Allergology[11]) were studied (Table I). The investigation was approved by the Ethics Committee of Dokkyo University School of Medicine, and all subjects gave written informed consent. The thickness of total basement membrane in each asthmatic subject and in each control subject was assessed as previously described.[4] Airway responsiveness was measured as the minimal cumulative dose of acetylcholine at which respiratory resistance began to increase during continuous inhalation of acetylcholine in stepwise incremental concentrations.[12]

Bronchial biopsy

Tissue samples of asthmatic patients were taken from subcarina between the right lower lobe and middle lower lobe bronchi (the origin of the right B6 bronchus) through use of standard forceps under fiberoptic bronchoscopic examination, as previously described.[4] Each biopsy specimen was placed immediately in OCT medium, snap-frozen in liquid nitrogen, and stored at –80°C until cryostat sectioning.

Immunohistochemistry

Sections of respiratory mucosa from asthmatic subjects were stained with affinity-purified antiphosphorylated Smad2 antibody[13] (kindly provided by Drs Peter ten Dijke and Carl-Henrik Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden) through use of ABC kits (Vector Laboratories, Burlingame, Calif) on 3-μm consecutive serial sections. Briefly, slides were quenched in 3% H_2O_2 for 10 minutes to block endogenous peroxidase and then washed in PBS. Next, sections were incubated with the primary antibody for 1 hour and then with biotinylated secondary antibody followed by ABC reagents. Color development was achieved by incubating diaminobenzidine as a substrate. Slides were counterstained with Mayer's hematoxylin. Preincubation of the primary antibody with specific blocking peptides or substitution of the primary antibody with an irrelevant IgG served as negative controls. Phosphorylated Smad2-positive cells were counted in at least 6 high power fields in each sample by 3 independent observers (H.S., T.O., and A.N.). A minimum of 500 cells were counted. In each instance, the percentage of positive cells was calculated as follows:

$$\frac{\text{no. of positive cells}}{\text{total no. of cells}} \times 100\%$$

Scoring was as follows: 0, no staining found; 1, staining seen in 0% to 40% of cells; 2, staining seen in 40% to 70% of cells; 3, staining seen in >70% of cells. The average of the scores of the 3 investigators for each sample was calculated; these averages were used as

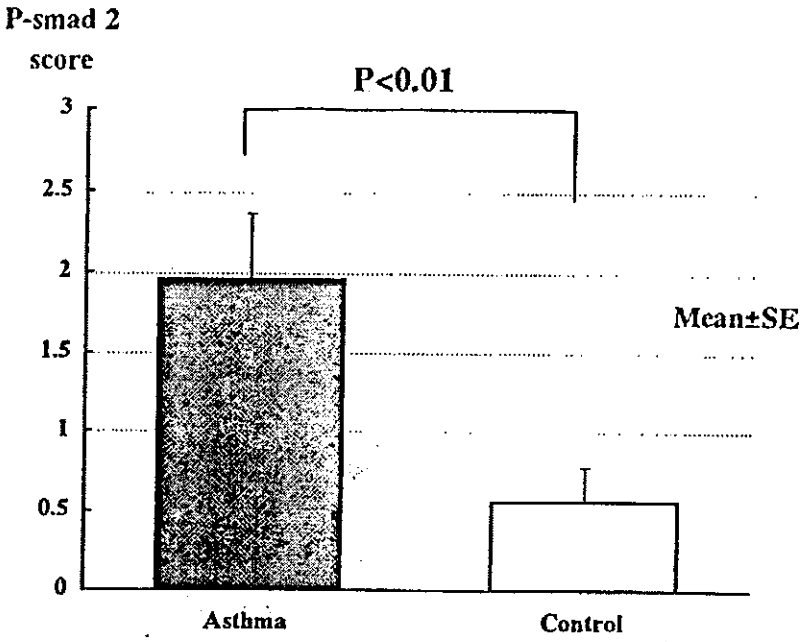

FIG 2. Higher scoring for phosphorylated Smad2 in bronchial biopsy samples from asthmatic subjects than in samples from normal subjects.

TABLE I. Characteristics of asthmatic subjects studied

	Subjects with asthma			
	Mild	Moderate	Severe	Healthy subjects
n	20	16	4	6
Age (y)*	41 ± 4.4	43 ± 3.8	54 ± 5.0	59 ± 16
Age at onset (y)*	34.0 ± 4.7	32.5 ± 5.1	27.5 ± 7.4	57.2 ± 16.4
$FEV_{1.0}$ (mL)*	2241 ± 113.8	2518 ± 174.5	1990 ± 330.3	2410 ± 690.0

Values are means ± SEs.

data. Sections used in immunohistochemistry series were stained with hematoxlyin-eosin.

Data analysis

Data are summarized as means ± SEs. Statistical analysis of the results was based on the amount of variance; we used Fisher's least significant difference test for multiple comparisons. Relationships were estimated through use of the Spearman rank correlation coefficient (rs). A P value of $<.05$ was considered significant.

RESULTS

To determine whether TGF-β signaling was active in the airways of asthmatic individuals, we examined the phosphorylation status of Smad2 in bronchial biopsy samples obtained from 40 asthmatic subjects and 6 control subjects by immunohistochemistry. Smad2 is a downstream effector for TGF-β and thus serves as indicator of active TGF-β signaling in situ.

Bronchial biopsy samples derived from healthy control subjects showed little immunoreactivity of phosphorylated Smad2. In contrast, biopsy samples derived from asthmatic subjects showed clearly positive immunoreactivity of phosphorylated Smad2 in bronchial epithelial cells, fibroblastlike cells, and vascular endothelial cells. Although substantial proportions (30%) of infiltrating cells, including mononuclear cells, also appeared to be positively stained, accurate quantification (scoring) of the staining was difficult because of their scattered presence. We confirmed that the immunoreactivity was efficiently blocked by a 10-fold excess of the phosphorylated Smad2 peptide used to pro-

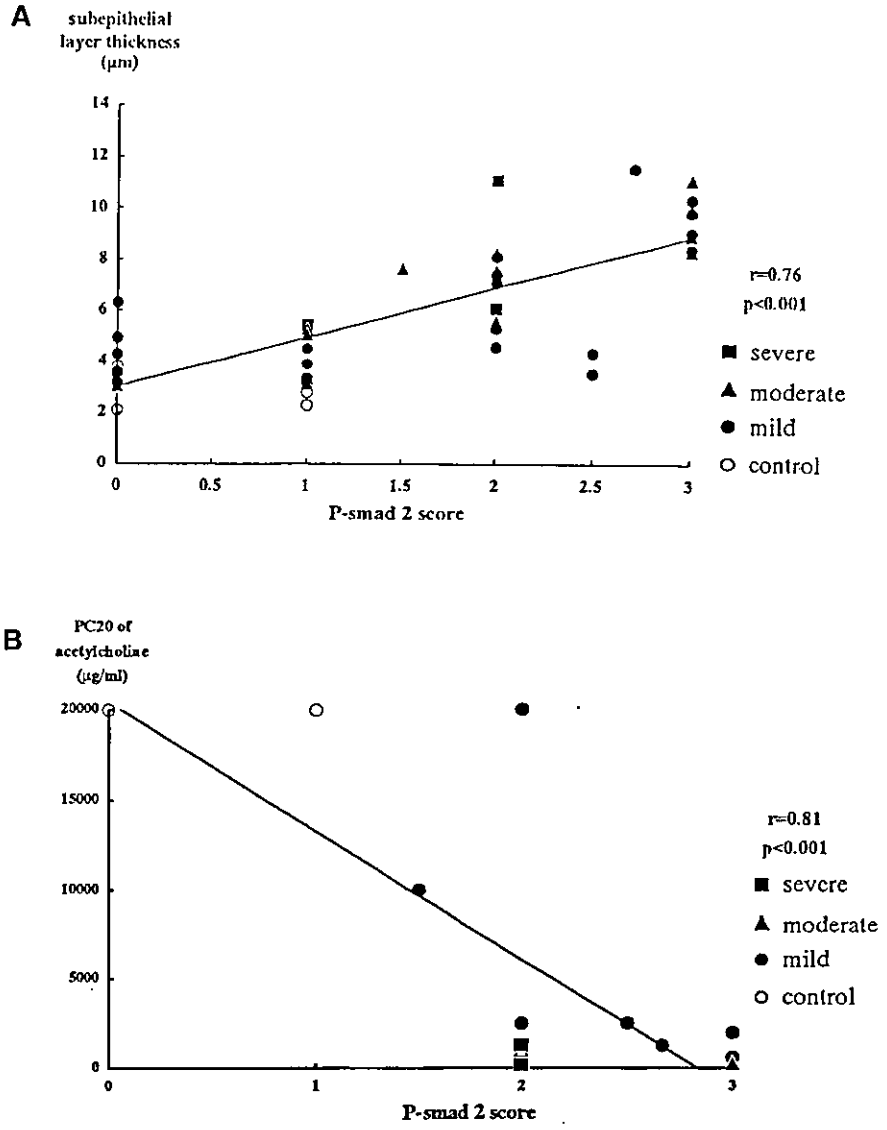

FIG 3. Correlation of expression of phosphorylated Smad2 with basement membrane thickness and airway hyperresponsiveness in the airways of subjects with mild to severe asthma. Expression of phosphorylated Smad2 was scored as described in the Methods section; its correlation with basement membrane thickness (A) and hypersensitivity to acetylcholine (B) in the airways of asthmatic individuals was analyzed.

duce the antisera[13] but not by a similar amount of non-phosphorylated Smad2 peptide (data not shown).

The percentages of phosphorylated Smad2-positive cells were similar in mild, moderate, and severe asthma for the different cell types. We therefore used the scoring of bronchial epithelial cells as representative in each sample. An example of phosphorylated Smad2 scoring focusing on bronchial epithelial cells is shown in Fig 1. We found significantly higher scoring for phosphorylated Smad2 in biopsy specimens from asthmatic subjects than in specimens from normal subjects (Fig 2).

We then examined the relationships between the scoring and clinicopathologic features associated with airway remodeling. As shown in Fig 3, *A*, the scoring of phosphorylated Smad2 was significantly correlated with basement membrane thickness in asthmatic subjects. Interestingly, phosphorylated Smad2 scoring was also correlated with airway hypersensitivity to acetylcholine in asthmatic subjects (Fig 3, *B*). These findings indicate that TGF-β signaling was active in asthmatic airways, and the activity was associated with the degree of airway remodeling and airway hyperresponsiveness in asthma.

DISCUSSION

In this study, we showed that expression levels of phosphorylated Smad2 in bronchial biopsy samples were clearly correlated with basement membrane thickness and airway hypersensitivity to acetylcholine in asthmatic individuals. The findings suggest that TGF-β signaling was active in asthmatic airways, and the activity was associated with the extent of airway remodeling and airway hyperresponsiveness in asthma.

Immunoreactivity of phosphorylated Smad2 in bronchial samples derived from healthy control subjects was weak or very weak in contrast to that in samples derived from asthmatic subjects. In previous studies it had been reported that TGF-β1 was expressed in bronchial biopsy specimens or bronchoalveolar lavage fluid in normal subjects as well as in asthmatic subjects.[14,15] Our findings thus suggest that though TGF-β1 was expressed in the airways of normal subjects, it might not be the active form of TGF-β1 and did not mediate signaling in situ. Inflammation is a factor in conversion of the latent form of TGF-β1 to the active form[9] and might thus contribute to activation of TGF-β1 at the site of asthmatic airways.

We found that immunoreactivity of phosphorylated Smad2 was similar in mild, moderate, and severe asthma for different cell types—bronchial epithelial cells, fibroblastlike cells, and endothelial cells. This was consistent with recent findings by Rosendahl et al[16] in a murine model of asthma. They showed that expression of phosphorylated Smad2 in bronchial epithelial cells, fibroblasts, endothelial cells, and inflammatory cells was dramatically induced by inhalation of ovalbumin in the airways of ovalbumin-sensitized Balb/c mice. Little expression of phosphorylated Smad2 was observed without the inhaled challenge. Thus, TGF-β signaling might become active in almost all residential and inflammatory cells in the airways on allergen challenge and subsequent airway inflammation in asthma.

TGF-β affects functions of airway residential cells, such as bronchial epithelial cells, lung fibroblasts, and smooth muscle cells, and it might contribute to several features of airway remodeling, including subepithelial fibrosis and smooth muscle cell hyperplasia.[2] Our results suggest that active TGF-β signaling indeed occurs in these cell types, though activity of TGF-β signaling in smooth muscle cells remains unknown.

It is not yet clear whether TGF-β signaling was active in infiltrating cells in our study (in contrast to what was seen in a study involving an acute model of asthma conducted by Rosendahl et al[16]). Substantial fractions of infiltrating cells appeared to show positive immunoreactivity for phosphorylated Smad2, but most of the cells appeared to show negative staining. TGF-β inhibits immune cell functions in general; we thus speculate that inflammatory cells might express inhibitory molecules for TGF-β signaling and escape from the suppression by TGF-β in situ. Abnormal expression of Smad7 (an intracellular antagonist of TGF-β signaling) in the mucosa in inflammatory bowel disease was recently reported; this might result in the deterioration of TGF-β signaling.[17]

In summary, we have provided evidence that TGF-β signaling was active in respiratory mucosa in asthmatic patients and that the activity correlated well with basement membrane thickness, a feature of airway remodeling. To our knowledge, this is the first description of intracellular molecular events underlying the remodeling process in asthma. Smad2 might become a therapeutic target for airway remodeling in asthma.

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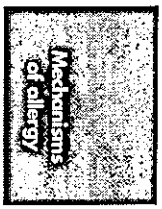

Expression of Smad7 in bronchial epithelial cells is inversely correlated to basement membrane thickness and airway hyperresponsiveness in patients with asthma

Atsuhito Nakao, MD,[a] Hironori Sagara, MD,[b] Yasuhiro Setoguchi, MD,[c] Takenori Okada, MD,[b] Ko Okumura, MD,[a] Hideoki Ogawa, MD,[a] and Takeshi Fukuda, MD[b] *Tokyo and Tochigi, Japan*

Background: Smad7 is an intracellular antagonist of transforming growth factor β (TGF-β) signaling, which could determine the intensity or duration of the TGF-β signal. Because TGF-β has been implicated in the development of airway remodeling in asthma on the basis of its strong capacity to induce extracellular matrix production, it is possible that Smad7 also plays some roles in the regulation of the process.
Objective: We sought to determine the relationships between Smad7 expression in bronchial biopsy samples from asthmatic subjects and clinicopathologic features.
Methods: Bronchial biopsy specimens were obtained from 40 asthmatic subjects and 6 healthy control subjects. Expression levels of Smad7 on a histologic section were estimated by immunohistochemical staining. In addition, the roles of Smad7 in TGF-β–mediated transcriptional responses were studied by in vitro studies.
Results: Smad7 immunoreactivity was detected mainly in bronchial epithelial cells in control and asthmatic subjects. Interestingly, asthmatic subjects exhibited less Smad7 immunoreactivity in bronchial epithelial cells than normal subjects. Expression levels of Smad7 in bronchial epithelial cells were inversely correlated with basement membrane thickness and airway hyperresponsiveness in asthmatic subjects. In addition, abrogation of endogenous Smad7 expression through use of an antisense oligonucleotide enhanced transcriptional responses to TGF-β, whereas overexpression of Smad7 inhibited TGF-β–induced plasminogen activator inhibitor 1production in a human bronchial epithelial cell line, BEAS2B cells.
Conclusion: These findings suggest that Smad7 is a key molecule that defines the susceptibility of bronchial epithelial cells to TGF-β action and that regulation of Smad7 expression in bronchial epithelial cells might be related to the development of airway remodeling and airway hyperresponsiveness in asthma. (J Allergy Clin Immunol 2002;110:873-8.)

From [a]Atopy (Allergy) Research Center and [c]the Department of Respiratory Medicine, Juntendo University School of Medicine, Tokyo; and [b]the Department of Pulmonary Medicine and Clinical Immunology, Dokkyo University School of Medicine, Tochigi.

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Reprint requests: Atsuhito Nakao, MD, Atopy (Allergy) Research Center, Juntendo University School of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo, 113-8421, Japan.

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Key words: Transforming growth Factor β, Smad7, asthma, airway remodeling

Airway remodeling in asthma is characterized by several structural changes of airway walls, including subepithelial fibrosis and hyperplasia-hypertrophy of submucosal glands and bronchial smooth muscle cells, and is thought to be associated with airflow obstruction and airway hyperresponsiveness in asthmatic patients.[1] Among the features of airway remodeling, subepithelial fibrosis that might result in basement membrane thickness attracts much attention because it has been suggested to be associated with disease severity and correlated with a decrease of FEV_1.

A fibrogenic cytokine, transforming growth factor (TGF) β1, has been suggested to play an important role in the development of airway remodeling in asthma because (1) TGF-β1 was produced by epithelial cells, eosinophils, and fibroblasts in the airways of asthmatic patients[2,3]; (2) TGF-β1 was detected in higher than normal quantities in asthmatic BAL fluid[4]; and (3) TGF-β1 expression was correlated with the disease activity or subepithelial fibrosis that resulted in basement membrane thickness.[5] It is therefore possible that regulators of TGF-β activity might also play some role in the modulation of the airway remodeling process in asthma.

Identification of Smad proteins has advanced our understanding of how TGF-β signals from membrane to nucleus.[6] The activated TGF-β receptors induce phosphorylation of Smad2 and Smad3, which form heterooligomeric complexes with a common mediator, Smad4. The complexes then translocate to the nucleus and regulate transcriptional responses together with DNA-binding cofactors. Recent studies have identified several molecules controlling the signal transduction of TGF-β, thereby regulating TGF-β activity.[7] Smad7 is one of these molecules, which inhibits TGF-β–induced transcriptional responses. Smad7 associates with the activated TGF-β receptors and interferes with the activation of signal-transducing Smads (ie, Smad2 and Smad3) by preventing their receptor interaction and phosphorylation. In addition, Smad7 interacts with ubiquitin ligases, termed Smurf. After recruitment of the Smad7-Smurf complex to the activated TGF-β receptors, Smurf induced their degradation through proteasomal and lyso-

Abbreviations used
CMV: Cytomegalovirus
ECM: Extracellular matrix
PAI: Plasminogen activator inhibitor
TGF: Transforming growth factor

somal pathways. Thus the expression level of Smad7 is a major determinant for TGF-β transcriptional responses.

In this study we sought to determine whether Smad7 played some role in the development of airway remodeling in asthma. Our findings suggest that regulation of Smad7 expression in bronchial epithelial cells might be associated with the development of airway remodeling and airway hyperresponsiveness in asthma.

METHODS

Subjects

Forty asthmatic subjects (20 with mild asthma, aged 41.4 ± 4.4 years with an $FEV_{1.0}$ of 2441 ± 113.8 mL; 16 with moderate asthma, aged 43.0 ± 3.8 years with an $FEV_{1.0}$ of 2518 ± 174.5 mL; and 4 with severe asthma, aged 54 ± 5.0 years with an $FEV_{1.0}$ of 1990 ± 330.3 mL) whose disease severity was defined by using a combination of asthma symptom grade and the frequency of the symptoms on the basis of the criteria of the Japanese Society of Allergology[8] and 6 normal control subjects without asthma (aged 59.0 ± 16 years) were studied (Table I). None of the subjects was a current smoker, and none had smoked during the previous 2 years. No subjects had any bronchial or respiratory tract infections during the month preceding the test. The study was approved by the Ethics Committee of Dokkyo University School of Medicine, and all subjects provided written informed consent. The thickness of total basement membrane in each asthmatic and control subject was assessed as previously described.[9] Airway responsiveness was measured as the minimal cumulative dose of acetylcholine at which respiratory resistance began to increase during continuous inhalation of acetylcholine in stepwise incremental concentrations.[10]

Bronchial biopsy

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Immunohistochemistry

Sections of respiratory mucosa from asthmatic patients were stained with affinity-purified anti-Smad7 antibody (KAF[11]; kindly provided by Drs Peter ten Dijke and Carl-Henrik Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden) on 3-μm consecutive serial sections by using ABC kits (Vector Laboratories). Briefly, slides were quenched in 3% H_2O_2 for 10 minutes to block endogenous peroxidase and washed in PBS. Sections were then incubated with the primary antibody for 1 hour and then with biotinylated secondary antibody, followed by ABC reagents. Color development was achieved by incubating diaminobenzidine as a substrate. Slides were counterstained with Mayer hematoxylin. Preincubation of the primary antibody with specific blocking peptides or substitution of the primary antibody with an irrelevant IgG served as negative controls. Smad7-positive cells were counted in at least 6 high-power fields in each sample by 3 independent observers (H.S., A.N., and T.O.). A minimum of 500 cells was counted. Percentages of positive cells among bronchial epithelial cells were calculated as the number of positive cells divided by the total number of cells times 100% and scored as follows: 0, no staining found; 1, staining seen in 0% to 40% of cells; 2, staining seen in 40% to 70% of cells; and 3, staining seen in greater than 70% of cells. Average scoring by each investigator for each sample was first calculated, and then average scoring in each sample by 3 investigators was calculated and presented as data. Sections in series of immunohistochemistry were stained with hematoxylin and eosin stain.

Luciferase assay

Human bronchial epithelial cell line BEAS2B cells were cultured in F12 medium with 10% heat-inactivated FCS (GIBCO) in a humidified 5% CO_2 atmosphere at 37°C. Rested BEAS2B cells (1×10^6/well) were transiently transfected with p3TP-Lux[12] or $(CAGA)_{12}$MLP-Luc[13] by using the lipofectin method (Fugene TM,[6] Boehringer Mannheim), according to the manufacturer's recommendations. Twenty-four hours after the transfection, cells were incubated in media with Smad7 sense or antisense oligonucleotide for 24 hours, followed by stimulation with recombinant human TGF-β1 (R&D; 10 ng/mL) for 24 hours. The Smad7 sense and antisense oligonucleotides were phosphorothioate single-stranded oligonucleotides matching region 107-128 (5′-GCTGCGGGGAGAAGGGGCGAC-3′) of the human Smad7 cDNA sequence (GenBank accession no. AF010193) synthesized in the sense and antisense orientations, as previously described.[14] In each experiment equal total amounts of DNA were transfected, and luciferase activity was measured as previously described.[15] The values were normalized for transfection efficiency by using the β-gal reporter gene under the control of the cytomegalovirus (CMV) promoter. Results shown are representative of at least 3 experiments.

Adenovirus vector construction and virus purification

The recombinant E1-deleted adenoviral vectors carrying mouse Smad7 or LacZ cDNA under CMV promoters (ie, AdCMV-Smad7 or AdCMV-LacZ) were generated, purified, and transfected as previously described.[16,17] Infection of recombinant adenoviruses was performed at a multiplicity of infection of 2×10^2 plaque-forming units per cell in 1 mL of serum-free F12 medium for 60 minutes. BEAS2B cells infected with the adenovirus solutions were cultured in media for 24 hours and thereafter stimulated with TGF-β1 (10 ng/mL) for 24 hours.

Western blotting

Immunoblotting with anti-PAI-1 antibody (Santa Cruz), anti-Flag M2 antibody (Sigma), or anti-Smad7 antibody (KAF) for BEAS2B cell lysates or lung tissue specimens was performed as previously described.[15]

Data analysis

Data are summarized as means ± SD or SE. The statistical analysis of the results was performed by using the amount of variance with the Fisher least-significant-difference test for multiple comparisons. Relationships were estimated by using the Spearman rank correlation coefficient. *P* values of less than .05 were considered significant.

RESULTS

Expression levels of Smad7 in bronchial epithelial cells are inversely correlated with basement membrane thickness and airway hyperresponsiveness in asthmatic subjects

To determine whether Smad7 played some role in the development of airway remodeling in asthma, we exam-

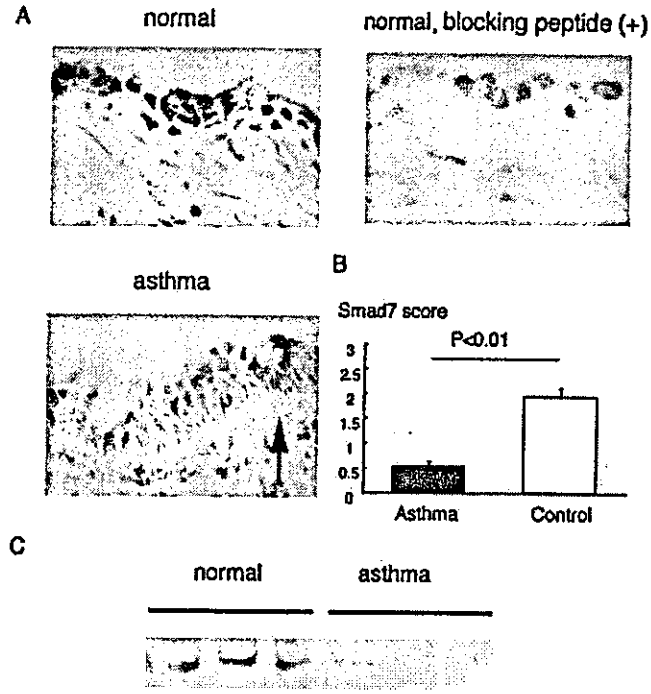

FIG 1. A, Immunostaining of Smad7 in bronchial biopsy specimens from control (with or without Smad7 blocking peptide) and asthmatic subjects. Relatively decreased Smad7 immunoreactivity was detected in bronchial epithelial cells obtained from asthmatic subjects. Representative pictures are shown. *Arrows* indicate basement membrane thickness. B, Higher scoring for Smad7 in bronchial biopsy samples from healthy subjects than that seen in asthmatic subjects. Expression of Smad7 in bronchial epithelial cells was scored as described in the "Methods" section. Data are summarized as means ± SE. C, Western blotting of lung biopsy samples from control subjects or subjects with severe asthma with anti-Smad7 antibody. Three representative whole-tissue samples for each group were examined.

ined the expression of Smad7 in bronchial biopsy samples obtained from 40 asthmatic and 6 normal subjects by means of immunohistochemistry. Strong Smad7 immunoreactivity was observed mainly in bronchial epithelial cells in the bronchial biopsy samples, which was efficiently blocked by a 10-fold excess of the Smad7 peptide used to produce the antisera[11] but not by a similar amount of non-Smad7 peptide (Fig 1, *A*). Intermediate staining of Smad7 was observed in vascular endothelial cells and fibroblast-like cells. Subcellular localization of Smad7 immunoreactivity was both cytoplasmic and nuclear. Interestingly, relatively strong staining of Smad7 in bronchial epithelial cells was observed in healthy control subjects compared with that seen in asthmatic subjects. In fact, scoring for Smad7 immunoreactivity in bronchial epithelial cells showed significantly higher scoring in biopsy specimens from normal subjects than in those from asthmatic subjects (Fig 1, *B*). Western blotting with the Smad7 antibody also confirmed relatively higher expression of Smad7 in normal subjects than in subjects with severe asthma (Fig 1, *C*).

We then examined the relationships between the Smad7 scoring and the clinicopathologic features associated with airway remodeling. As shown in Fig 2, *A*, the scoring of Smad7 was inversely correlated with basement membrane thickness in asthmatic subjects. Smad7 scoring was also inversely correlated with airway hypersensitivity to acetylcholine in asthmatic subjects (Fig 2, *B*). Furthermore, the scoring of Smad7 was inversely correlated with the inhaled dosage of beclomethasone in asthmatic subjects (Fig 2, *C*). These findings strongly suggested that expression levels of Smad7 in bronchial epithelial cells were associated with the development of airway remodeling and airway hyperresponsiveness in asthma.

Smad7 regulates TGF-β–mediated transcriptional responses in BEAS2B cells

To investigate the roles of endogenous Smad7 in bronchial epithelial cell function, we examined the effect of Smad7 antisense oligonucleotide on TGF-β–mediated transcriptional responses in the human bronchial epithelial cell line BEAS2B. We transfected the TGF-β–inducible luciferase p3TP-Lux reporter construct, which contained the TGF-β–inducible PAI-1 promoter,[12] into BEAS2B cells in the presence of Smad7 sense or antisense oligonucleotide. As shown in Fig 3, *A*, incubation of the cells with antisense Smad7 oligonucleotide enhanced the induction of p3TP-Lux luciferase by TGF-

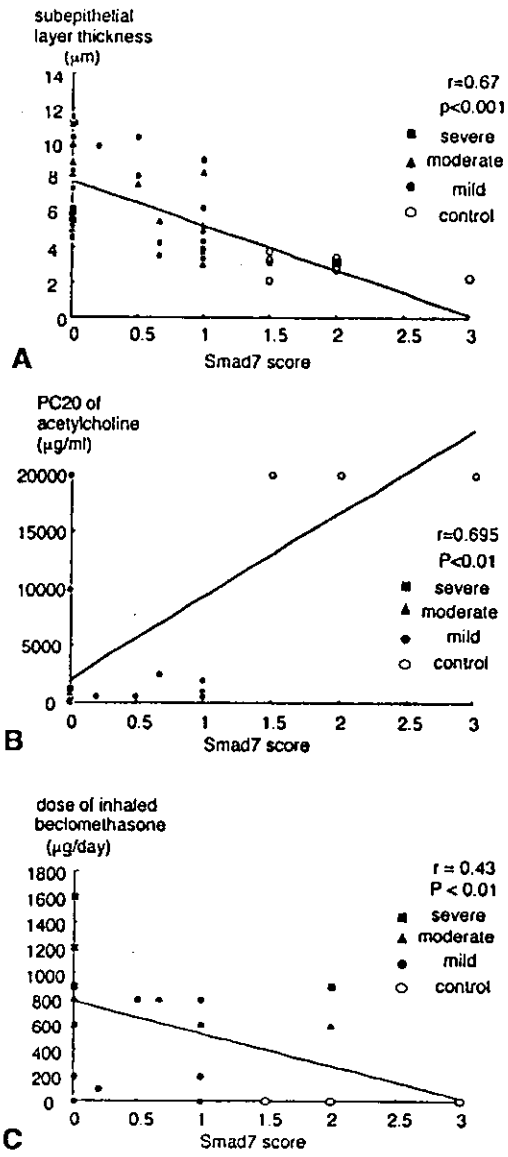

FIG 2. Inverse correlation of Smad7 expression levels in bronchial epithelial cells with basement membrane thickness (A), hypersensitivity to acetylcholine (B), and inhaled dosage of beclomethasone (C) in the airways of patients with mild-to-severe asthma or control subjects. Expression of Smad7 in bronchial epithelial cells was scored as described in the "Methods" section, and its correlation with basement membrane thickness in the airways of asthmatic patients was examined. Forty-six spots (subjects) were blotted in each scattergram, and several spots overlapped.

β1 when compared with that seen with sense Smad7 oligonucleotide.

The inhibitory effect of endogenous Smad7 in transcriptional activation by TGF-β1 in BEAS2B cells was further confirmed by using another established reporter construct, $(CAGA)_{12}$MLP-Luc, which contained a promoter consisting of 12 tandem copies of the Smad-binding element (CAGA) designed to monitor Smad-dependent transcriptional activity.[13] Consistent with the results of p3TP-Lux, antisense Smad7 oligonucleotide enhanced induction of $(CAGA)_{12}$MLP-Luc luciferase by TGF-β1 when compared with sense Smad7 oligonucleotide (Fig 3, *B*). We confirmed that endogenous Smad7 expression was suppressed by antisense Smad7 oligonucleotide in BEAS2B cells 24 hours after incubation with the antisense oligonucleotide (Fig 3, *C*).

Finally, we examined the effect of Smad7 overexpression on TGF-β1–mediated PAI-1 induction in BEAS2B cells.[18,19] TGF-β1–mediated PAI-1 induction was abolished in BEAS2B cells overexpressing Smad7 after infection with adenovirus-carrying, Flag-tagged Smad7 cDNA when compared with BEAS2B cells expressing a control LacZ plasmid (Fig 4, *A*). Expression of exogenous Flag-tagged Smad7 after adenoviral infection in BEAS2B cells was confirmed by means of Western blotting with anti-Flag antibody (Fig 4, *B*). These findings indicated that Smad7 negatively regulated TGF-β1–induced transcriptional responses in BEAS2B cells depending on the Smad pathway and also inhibited TGF-β–induced PAI-1 production in BEAS2B cells.

DISCUSSION

In this study we showed that expression levels of Smad7 in bronchial epithelial cells were inversely related to basement membrane thickness and airway hyperresponsiveness in asthmatic patients (Fig 2). The in vitro findings with BEAS2B cells suggested that Smad7 functioned as an inhibitor for TGF-β–mediated transcriptional responses in bronchial epithelial cells (Figs 3 and 4). Taken together with numerous evidence that TGF-β is involved in airway remodeling in asthma, the results suggest that Smad7 in bronchial epithelial cells might act as an anti-remodeling molecule in asthma, in part through inhibition of TGF-β–mediated transcriptional responses.

What are the possible major target molecules of Smad7 inhibition in bronchial epithelial cells for anti-remodeling? PAI-1 is a candidate because it is an inhibitor of enzymes that degrade the extracellular matrix (ECM), such as collagenase and stromelysin,[20] and decrease of TGF-β–induced PAI-1 expression by Smad7 (Figs 3 and 4) would reduce ECM deposition. It is also possible that Smad7 might act as an anti-remodeling molecule by preventing synthesis of ECM components consisting of subepithelial fibrosis, such as tenascin and collagens.[21,22] Indeed, bronchial epithelial cells produce tenascin on TGF-β stimulation.[19] Recent advances in DNA microarray or proteomics techniques will help reveal identification of major targets of Smad7 in bronchial epithelial cells.

Altered epithelial repair responses caused by various factors present in the microenvironment are also considered to be important for the development of airway remodeling in asthma.[23] Increased production of TGF-β by inflamma-

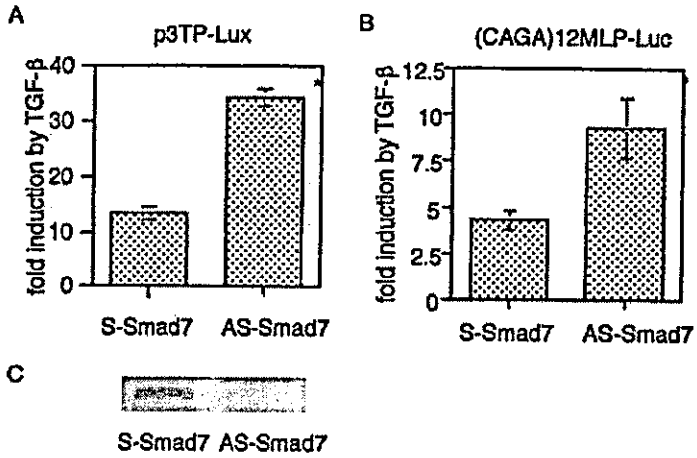

FIG 3. Luciferase activity of prepared cell lysates from BEAS2B cells that were cotransfected with p3TP-Lux (A) or $(CAGA)_{12}$MLP-Luc (B) reporter construct and Smad7 sense *(S-Smad7)* or antisense *(AS-Smad7)* oligonucleotide and stimulated with or without TGF-β1 for 24 hours. Values are averages of triplicate determinations ± SD. *A significant difference ($P < .05$) from the mean value of the corresponding control response. C, Immunoblotting of BEAS2B cell lysates with anti-Smad7 antibody after 24 hours' culture with Smad7 sense or Smad7 antisense.

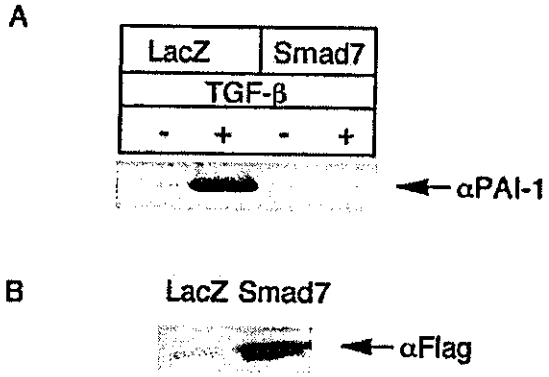

FIG 4. Western blot analysis of prepared cell lysates from BEAS2B cells overexpressing Flag-tagged Smad7 or a control LacZ stimulated with or without TGF-β1 for 24 hours. Detection with anti-PAI-1 (A) or anti-Flag (B) antibody.

TABLE I. Subject characteristics

	Patients with mild asthma	Patients with moderate asthma	Patients with severe asthma	Control subjects
No. of subjects	20	16	4	6
Male	11	10	1	3
Female	9	6	3	3
Age (y)	41 ± 4.4	43 ± 3.8	54 ± 5.0	59 ± 16
Onset (age [y])	34 ± 4.7	32.5 ± 5.1	27.5 ± 7.4	
Duration (y)	7.7 ± 3.8	10.2 ± 2.3	26.8 ± 7.4	
FEV1.0 (mL)	2441 ± 113.8	2518 ± 174.5	1990 ± 330.3	2410 ± 690.0
FEV1.0 (%)	82.3 ± 2.8	59.0 ± 20.2	52.9 ± 4.5	104.4 ± 5.7
IgE (IU/mL)	663.0 ± 192.0	775.0 ± 401.0	165.0 ± 53.4	25.5 ± 7.2
Inhaled corticosteroid (μg/d)	366.6 ± 76.1	800.0 ± 70.4	1150.0 ± 165.8	
Theophylline (mg/d)	325.0 ± 51.4	375.0 ± 41.4	500.0 ± 100.0	

tory cells in asthmatic airways might inhibit epithelial cell proliferation and prevent normal repair of the epithelium. Smad7 in bronchial epithelial cells might also affect this process, accelerating normal epithelial repair.

Expression of Smad7 was detected not only in bronchial epithelial cells but also in vascular endothelial and fibroblast-like cells in bronchial biopsy samples. Because fibroblasts were implicated in the development of airway remodeling[5] and were thought to be a major cell that produces ECM in response to TGF-β through the Smad pathway,[24,25] it would be interesting to examine the relationships of Smad7 levels in fibroblasts with basement membrane thickness. However, in this study the scattered presence of fibroblast-like cells in bronchial biopsy samples made it difficult to quantify expression levels of Smad7 in these cells. This issue therefore remains to be investigated in the future.

Smad7 expression in bronchial epithelial cells was also inversely correlated with inhaled dosage of beclomethasone (Fig 2, *C*). The findings suggest the possibility that inhaled corticosteroids might affect Smad7 expression in bronchial epithelial cells. This issue is currently under investigation.

In summary, we showed that Smad7 expression in bronchial epithelial cells was inversely correlated with basement membrane thickness and airway hyperresponsiveness in asthmatic patients, suggesting that Smad7 might act as an anti-remodeling molecule in the airways of asthmatic patients. Thus modulation of Smad7 expression might have therapeutic potential for airway remodeling in asthma.

We thank Drs Hiroko Ushio, Keiko Maeda, Chiharu Nishiyama, Toshiro Takai, and Yutaka Kanamaru for technical assistance and helpful discussion and Peter ten Dijke and Carl-Henrik Heldin for providing the anti-Smad7 antibody.

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Research News

Smad7: a new key player in TGF-β-associated disease

Atsuhito Nakao, Ko Okumura and Hideoki Ogawa

Smad7 is a major inhibitory regulator of transforming growth factor (TGF)-β signaling. Smad7 expression is induced by TGF-β itself and other signaling pathways, indicating a key role for Smad7 in feedback or cross-talk control of TGF-β signaling. Recent reports have implicated Smad7 as a crucial regulator of TGF-β activity in human disease; aberrant expression of Smad7 is involved in inflammatory bowel disease and scleroderma. Thus, modulation of Smad7 expression could provide a novel therapeutic basis for TGF-β-associated disorders.

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TGF-β is a multifunctional cytokine, capable of regulating the growth, differentiation and apoptosis of virtually all cell types, and has been implicated in numerous disease states including atherosclerosis, immune-mediated diseases, fibrotic diseases, angiogenic diseases and cancer [1]. In general, abnormal production of TGF-β or genetic mutations in TGF-β signaling components, resulting in dysregulation of TGF-β activity, have been linked to disease states. Now, a new example of dysregulated TGF-β signaling associated with disease states has been revealed by two publications showing that aberrant expression of Smad7, an intracellular regulator of TGF-β signaling, resulting in uncontrolled TGF-β activity, is involved in the pathology of inflammatory bowel disease (IBD) and sclerosis [2,3]. Regulation of Smad7 expression is thus critical for balanced TGF-β activity, and dysregulated Smad7 activity can lead to the development of TGF-β-associated human disease in certain cases.

Smad7 is a major inhibitory regulator for TGF-β/Smad signaling

The identification of Smad proteins has advanced our understanding of how TGF-β signals from the membrane to the nucleus [4]. The activated TGF-β receptors phosphorylate Smad2 and Smad3, which form heteromeric complex with Smad4 and enter the nucleus, bind to DNA in a sequence-specific manner, and regulate gene transcription in cooperation with various transcriptional factors and coactivators and/or corepressors.

Recent studies have identified several molecules that control the signal transduction of TGF-β, thereby regulating TGF-β activity [4–6]. Smad7 is one of these molecules and inhibits TGF-β-induced transcriptional responses [7–9]. Smad7 associates with the activated TGF-β receptor and interferes with the activation of Smad2 and Smad3 by preventing their receptor interaction and phosphorylation. In addition, Smad7 interacts with a group of ubiquitin ligases, termed Smurf [5]. After recruitment of the Smad7–Smurf1 complex to the activated TGF-β receptors, Smurf1 induces their degradation through proteasomal and lysosomal pathways. Thus, the expression level of Smad7 is a major determinant for TGF-β transcriptional responses, which could regulate intensity and/or duration of TGF-β signals.

The mechanisms that regulate the expression of Smad7 are not fully understood. Smad7 expression is strongly and rapidly induced by TGF-β itself [9],

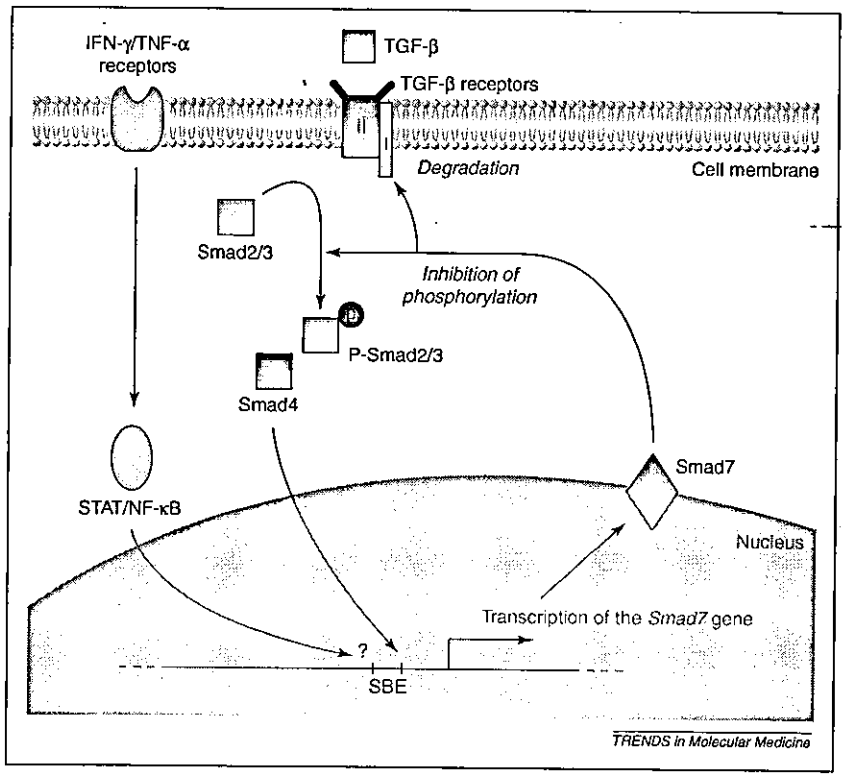

Fig. 1. Smad7 is an inhibitory regulator in feedback or cross-talk control of transforming growth factor (TGF)-β signal transduction. TGF-β receptors consist of type I and type II receptors with intrinsic serine/threonine kinase activity. Activated TGF-β receptors phosphorylate Smad2 and Smad3, leading to hetero-oligomeric complexes with Smad4. The complexes then translocate to the nucleus, where they regulate the transcription of target genes including the Smad7 gene. Smad7 associates with the activated type I receptor and interferes with the activation of Smad2 and Smad3 by competing with their receptor interaction. In addition, Smad7 interacts with ubiquitin ligases, termed Smurf, and binds to the activated TGF-β receptors, inducing degradation of the receptors, through proteasomal and lysosomal pathways. Other signaling pathways, such as interferon (IFN)-γ/tumor necrosis factor (TNF)-α pathways, are reported to induce Smad7 expression in certain cell types. Thus, the expression level of Smad7 is a major determinant for TGF-β transcriptional responses. Abbreviations: NF-κB, nuclear factor κB; SBE, Smad binding element.

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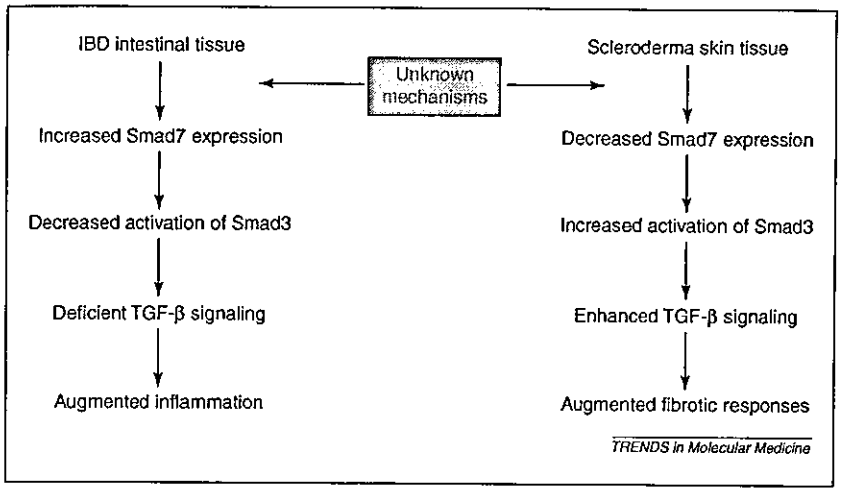

Fig. 2. Effect of aberrant expression of Smad7 in the intestinal lesions of inflammatory bowel disease (IBD) or skin lesions of scleroderma. Transforming growth factor (TGF)-β has two properties in inflammation: anti-inflammatory and pro-fibrotic. Aberrant Smad7 expression occurs at the target sites of IBD or sleroderma by as yet undefined mechanisms. The increased or decreased Smad7 expression in IBD or scleroderma, respectively, leads to decreased or increased activity of a major signal transducing Smad, Smad3, respectively, and then suppresses or enhances TGF-β signaling, respectively. As a consequence, aberrant TGF-β activity results in sustained inflammation in IBD or enhanced fibrotic responses in sclerosis.

and the promoter region of the Smad7 gene contains a consensus Smad3–Smad4 binding element (SBE), a palindromic sequence of GTCTAGAC, to which the Smad3–Smad4 complex binds [10–12]. Efficient expression of Smad7 appears to require cooperation of Smad, Sp1, and AP-1 transcription factors [13].

However, in some cell types, Smad7 expression is induced by other signaling pathways, for example, by the Jak1/Stat1 pathway following stimulation with IFN-γ [14], by activated nuclear factor (NF)-κB following stimulation with tumor necrosis factor (TNF)-α [15], and by fluid shear stress acting on endothelial cells [8]. How Smad7 expression is differentially regulated dependent on cell type remains to be determined. In any case, the resulting surge in Smad7 expression levels interferes with activation of Smad2 and Smad3 or accelerates degradation of TGF-β receptors, inhibiting TGF-β/Smad signaling. Thus, Smad7 has a potential central role as an effector in an autoregulatory feedback loop in TGF-β/Smad signaling and as a mediator of inhibitory signaling cross-talk between various pathways and the TGF-β–Smad pathway (Fig. 1).

Aberrant expression of Smad7 impairs efficient TGF-β signaling in IBD and scleroderma

Recent studies have implicated Smad7 as an important molecule for regulating TGF-β activity in human disease (Fig. 2). Monteleone *et al.* reported that Smad7 was overexpressed in IBD mucosa and purified mucosal T cells [2]. Both whole tissue and isolated cells exhibited defective TGF-β signaling as measured by phospho-Smad3 immunoreactivity. Importantly, antisense oligonucleotides for Smad7 restored TGF-β signaling and enabled TGF-β to inhibit pro-inflammatory cytokine production such as interferon (IFN)-γ and TNF-α in cells isolated from IBD patients and also in inflamed tissue explants from patients with Crohn's disease. Thus, Smad7 blockade of TGF-β signaling helps maintain the chronic production of pro-inflammatory cytokines that drives the inflammatory process in IBD.

In a separate study, Dong *et al.* reported deficient Smad7 expression in the skin lesions of scleroderma [3]. TGF-β induces fibroblast growth and stimulates the synthesis of extracellular-matrix proteins including collagen. It has been shown that fibroblasts from skin lesion of patients with scleroderma show enhanced responses to TGF-β [16]. Dong *et al.* showed that basal level and TGF-β-inducible expression of Smad7 were selectively decreased both in scleroderma skin and in explanted scleroderma fibroblasts in culture. They also showed enhanced TGF-β signaling in scleroderma skin as judged by increased phospho-Smad3 immunoreactivity. Importantly, *in vitro* adenoviral gene transfer of Smad7 restored normal TGF-β signaling in scleroderma fibroblasts. Thus, in the case of scleroderma, suppression of Smad7 expression appeared to be a key to enhanced responses to TGF-β in fibroblasts and development of the sclerotic skin lesions.

In both reports, it remains unclear how aberrant expression of Smad7 occurs at the site of the diseases. It is possible that pro-inflammatory cytokines such as IFN-γ and TNF-α, usually elevated in IBD tissue [2], upregulate Smad7 expression, but this has not been demonstrated. Because little is known about the negative transcriptional regulation of the Smad7 gene, how Smad7 expression decreases in scleroderma skin remains unexplained at the moment. Most recently, another example of the effect of decreased Smad7 expression was shown by Wang *et al.* in a cardiac infarction model in rats [17]. Thus, positive and negative regulation of Smad7 expression needs to be thoroughly investigated in future studies.

TGF-β has been shown to effectively suppress inflammation in several animal models including IBD [18,19], which could be in contrast to the human study [2]. In general, animal studies deal with acute models of inflammation. It might therefore require a chronic inflammatory state to impair regulatory mechanisms of Smad7 expression and to cause abnormal responses to TGF-β observed in human IBD [2]. It is possible that persistent production of TGF-β or pro-inflammatory cytokines at the site of chronic inflammation induces Smad7 expression, making disease cells unresponsive to TGF-β.

Perspectives

Many *in vitro* and *in vivo* animal studies have implicated Smad7 as a major inhibitory regulator of the TGF-β–Smad pathway. It is well known that signal-transduction pathways have their own intracellular regulators and there is much cross-talk signaling regulation through regulatory molecules such as Smad7 [4]. This regulatory role for Smad7 is supported by the findings in the IBD and scleroderma studies in humans [2,3].

Upregulation of TGF-β has been documented in certain inflammatory disorders such as IBD and asthma, and it seems paradoxical that elevated TGF-β levels fail to control the diseases through its anti-inflammatory activity [2,20]. As seen in the IBD study, overexpression of

Smad7 ininflammatory cells can dampen their responses to TGF-β produced at the site ofchronic inflammation. Thus, Smad7 could be a key molecule for the mechanisms of chronic inflammation in these disorders, and downregulation of Smad7 expression (e.g. by the use of antisenseSmad7 oligonucleotides) might restore normal control of TGF-β signaling in inflammatory cells and be beneficial for the treatment of chronic inflammatory disorders associated with high TGF-β production and its resistance.

Th observed reduced expression of Smad7 in scleroderma should encourage us to analyze the transcriptional regulation of the promoter region of the Smad7 gene in more detail; for example, by searching for single nucleotide polymorphisms (SNPs) that might affect Smad7 transcription in patients with scleroderma. Such studies might also provide clues as to why certain population are susceptible to tissue fibrosis or scar formation after skin injury. In addition, exploring mechanisms of Smad7 degradation should be another important area of investigation for scleroderma study.

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Atsuhito Nakao*
Ko Okumura
Hideoki Ogawa
Atopy (Allergy) Research Center, Juntendo University School of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo, 113-8421, Japan.
*e-mail: anakao@med.juntendo.ac.jp

Techniques & Applications

Encapsulation of pancreatic islets for transplantation in diabetes: the untouchable islets

Paul de Vos and Piero Marchetti

The aim of encapsulation of pancreatic islets is to transplant in the absence of immunosuppression. It is based on the principle that transplanted tissue is protected from the host immune system by an artificial membrane. Encapsulation allows for application of insulin-secreting cells of animal or other surrogate sources, to overcome human islet shortage. The advantages and pitfalls of the approaches developed so far are discussed and compared, together with some recent progress, in view of applicability in clinical islet transplantation.

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Recent successes [1] have increased the optimism and interest in clinical application of pancreatic islet transplantation in type 1 diabetes on a large scale. Unfortunately, present approaches involve the use of high-dose and strict immunosuppressive protocols, which is associated with serious side-effects. Therefore, it is still doubtful whether clinical islet transplantation in combination with immunosuppression will ever be a sound alternative to insulin therapy for the majority of diabetic patients [2]. By encapsulation (i.e. immunoisolation) of the islets, chronic administration of immunosuppressants can be eliminated, as the hostile host-immune system cannot reach the physically protected pancreatic islet cells.

Immunoisolation is based on the principle that transplanted cells are separated from the host immune system by a biocompatible, semipermeable membrane [2,3]. The membrane does not allow the entry of inflammatory cells and large molecules such as antibodies,

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