

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

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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 1 production 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.)

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 FEV₁.

A fibrogenic cytokine, transforming growth factor (TGF) β 1, has been suggested to play an important role in the development of airway remodeling in asthma because (1) TGF- β 1 was produced by epithelial cells, eosinophils, and fibroblasts in the airways of asthmatic patients^{2,3}; (2) TGF- β 1 was detected in higher than normal quantities in asthmatic BAL fluid⁴; and (3) TGF- β 1 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-

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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 Allergy⁸ 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

Tissue samples of asthmatic patients were taken from the subcarina between the right lower lobe and middle lower lobe bronchi (the origin of right B6 bronchus) by using standard forceps during fiberoptic bronchoscopic examination, as previously described.⁹ 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 patients were stained with affinity-purified anti-Smad7 antibody (KAF¹¹; 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% H₂O₂ 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% CO₂ atmosphere at 37°C. Rested BEAS2B cells (1×10^6 /well) were transiently transfected with p3TP-Lux¹² or (CAGA)₁₂MLP-Luc¹³ by using the lipofectin method (Fugene TM, 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- β 1 (R&D; 10 ng/mL) for 24 hours. The Smad7 sense and antisense oligonucleotides were phosphorothioate single-stranded oligonucleotides matching region 107-128 (5'-GCT-GCGGGGAGAAGGGGCGAC-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- β 1 (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 $\pm$ 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-

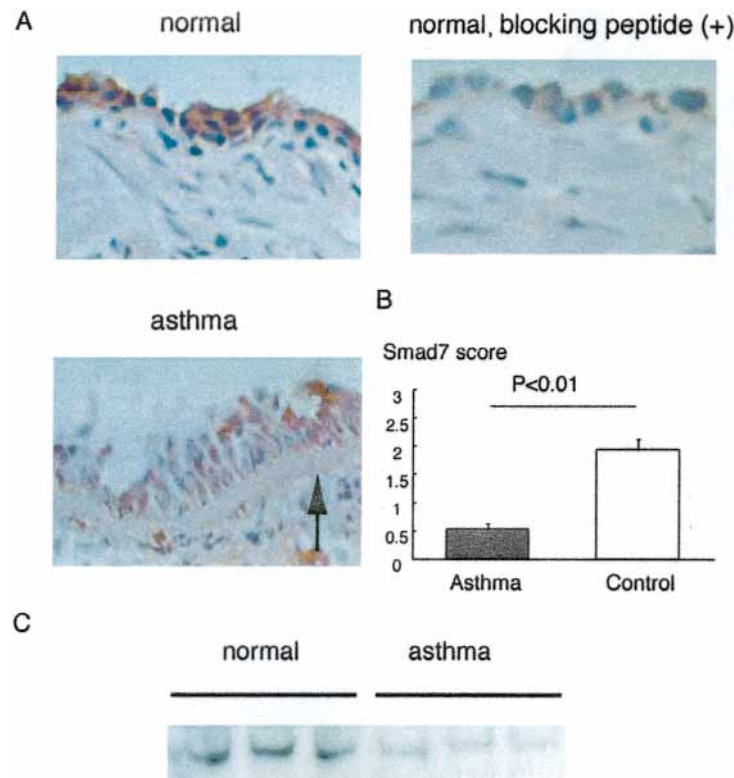


FIG 1. **A**, Immunostaining of Smad7 in bronchial biopsy specimens from control (with or without Smad7 blocking peptide) and asthmatic subjects. Relatively decreased Smad7 immunoreactivity was detected in bronchial epithelial cells obtained from asthmatic subjects. Representative pictures are shown. *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 $\pm$ 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 antisera¹¹ 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-

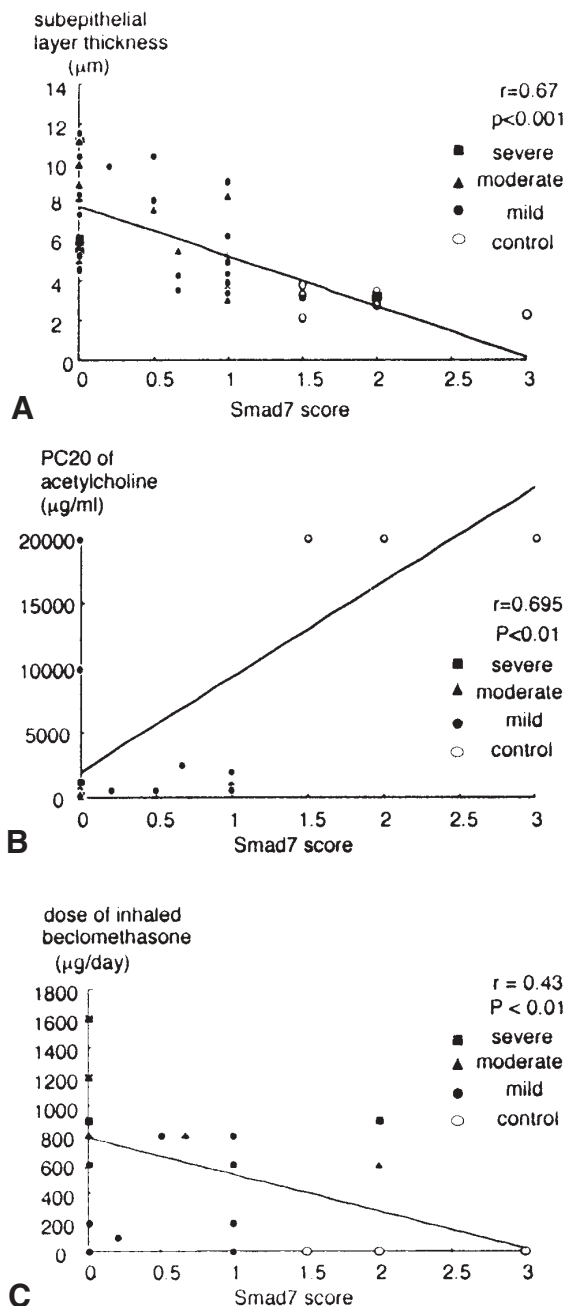


FIG 2. Inverse correlation of Smad7 expression levels in bronchial epithelial cells with basement membrane thickness (**A**), hyper-sensitivity to acetylcholine (**B**), and inhaled dosage of beclomethasone (**C**) in the airways of patients with mild-to-severe asthma or control subjects. Expression 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.

$\beta 1$ when compared with that seen with sense Smad7 oligonucleotide.

The inhibitory effect of endogenous Smad7 in transcriptional activation by TGF- $\beta 1$ in BEAS2B cells was

further confirmed by using another established reporter construct, (CAGA)₁₂MLP-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)₁₂MLP-Luc luciferase by TGF- $\beta 1$ 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- $\beta 1$ -mediated PAI-1 induction in BEAS2B cells.^{18,19} TGF- $\beta 1$ -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- $\beta 1$ -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-

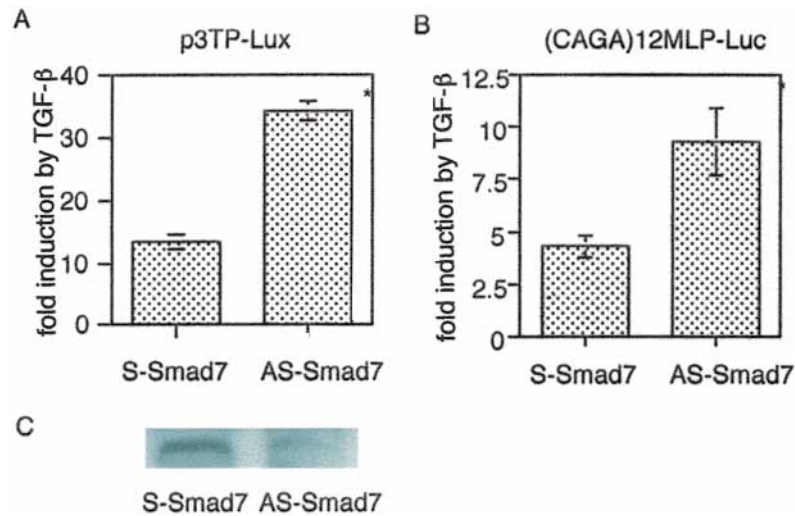


FIG 3. Luciferase activity of prepared cell lysates from BEAS2B cells that were cotransfected with p3TP-Lux (A) or (CAGA)₁₂MLP-Luc (B) reporter construct and Smad7 sense (*S-Smad7*) or antisense (*AS-Smad7*) oligonucleotide and stimulated with or without TGF-β1 for 24 hours. Values are averages of triplicate 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.

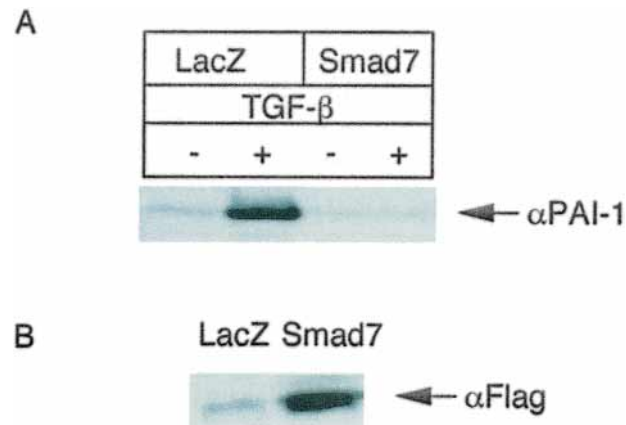


FIG 4. Western blot analysis of prepared cell lysates from BEAS2B cells overexpressing Flag-tagged Smad7 or a control LacZ stimulated with or without TGF-β1 for 24 hours. Detection with anti-PAI-1 (A) or anti-Flag (B) antibody.

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 remodeling⁵ 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.

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