Dokkyo Journal of Medical Sciences
31(1) : 17～26, 2004

Originals

Airway Expression of Smad7, a TGF-β-inducible Inhibitory Molecule of TGF-β Signaling, Decreases after Repeated Airway Antigen Challenges

Mayumi Ota[1], Atsuhito Nakao[2], Kumiya Sugiyama[1], Gang Cheng[1], Kazumi Akimoto[3], Takenori Okada[1] and Hironori Sagara[1]

[1] *Department of Pulmonary Medicine and Clinical Immunology, Dokkyo University School of Medicine, Tochigi, Japan*
[2] *Department of Parasitology and Immunology, University of Yamanashi Faculty of Medicine, Yamanashi, Japan*
[3] *Laboratory of Molecular and Cellular Biology, Dokkyo University School of Medicine, Tochigi, Japan*

SUMMARY

Transforming growth factor-β (TGF-β) is a profibrogenic cytokine that is involved in airway remodeling largely associated with chronic asthma. Accordingly, regulators of TGF-β activity could also play some role in airway remodeling in asthma. In this study, we investigated expression of Smad7, a major intracellular inhibitor of TGF-β signaling, in the airways of mouse models of acute and chronic asthma. Sensitized, repeatedly (14 days) ovalbumin (OVA)-inhaled BALB/c mice exhibited evidence of airway remodeling including prominent subepithelial fibrosis associated with airway hyperresponsiveness (AHR) and airway inflammation (chronic asthma model) whereas sensitized, shortly OVA-inhaled BALB/c mice showed only AHR and airway inflammation (acute asthma model). Immunohistochemical analysis showed that Smad7 immunoreactivity in the airways was increased after the development of acute and chronic asthma models and mainly detected in bronchial epithelial cells. Interestingly, Smad7 immunoreactivity was significantly less in the airways of chronic asthma model than in those of acute asthma model, which was also confirmed by real-time PCR analysis of Smad7. In consistent with decreased Smad7 expression in the airways of chronic asthma model, phosphorylation of Smad2, a marker of active TGF-β signaling, was increased in bronchial epithelial cells of chronic asthma model when compared with acute asthma model. These results suggest that decreased Smad7 expression and Smad2 upregulation in bronchial epithelial cells might result in increased TGF-β activity and contribute to the development of airway remodeling seen in chronic asthma.

Key Words : Smad7, chronic asthma, bronchial epithelial cells, airway remodeling

INTRODUCTION

Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation causes an associated increase in airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment[1～3]. Chronic asthma is often associated with a variety of structural changes of airway wall ("airway remodeling"), including subepithelial and airway wall fibrosis, epithelial

Received November 6, 2003 ; accepted December 1, 2003
Reprint requests to : Mayumi Ota
Department of Pulmonary Medicine and Clinical Immunology, Dokkyo University School of Medicine, Mibu, Tochigi 321-0293, Japan

metaplasia and increased smooth muscle mass, that may contribute to airway hyperresponsiveness and to the fixed airflow obstruction[4, 5]. However, the pathology of airway remodeling in asthma has not been fully understood at molecular level[6].

TGF-β has been implicated in the development of airway remodeling based on several evidence ; 1) TGF-β is a potent stimulator for ECM production from fibroblasts[7], 2) TGF-β is expressed in asthmatic airways and its expression is well correlated with some features of airway remodeling such as subepithelial fibrosis[6, 8, 9]. We also recently showed that activation of Smad2, a major signaling mediator of TGF-β, was correlated with AHR and subepithelial fibrosis in chronic asthmatics[10]. Thus, it is possible that regulators of TGF-β activity also play some role in the development of airway remodeling in asthma via modulation of TGF-β activity.

Smad7 is a member of the Smad family of proteins which regulate signaling of TGF-β superfamily[11~13]. Smad7 has been identified as an inhibitory Smad that associates with the activated TGF-β receptors and interferes with the activation of TGF-β signaling mediators, 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 induces their degradation through proteasomal and lysosomal pathways. Thus, the expression level of Smad7 is a major determinant for TGF-β transcriptional responses[14].

In this study, we found that expression level of Smad7 was significantly decreased in the airways of a chronic asthma model when compared with an acute asthma model. Decreased Smad7 expression in the chronic asthma model was associated with increased phosphorylation of Smad2 in the airways. Thus, decreased Smad7 expression in the airways may result in increased TGF-β activity and contribute to the development of airway remodeling observed in chronic asthma.

MATERIALS AND METHODS

Mice

Specific pathogen-free male BALB/c mice (8 weeks old, Oriental Yeast Co., Ltd., Tsukuba, Japan) were used in all experiments. The study protocol was reviewed and approved by the Dokkyo University School of Medicine Committee on Animal Care and complies with National Institute of Health for animal care.

Immunization

BALB/c mice were immunized i.p. twice with 8 μg/mouse, of ovalbumin (OVA) (Grade V ; Sigma-Aldrich, St. Louis, MO) absorbed with aluminum hydroxide (alum) (Sigma-Aldrich, St. Louis, MO) at a 1-wk interval (day 0 and day 6).

Acute or chronic model of asthma

Acute or chronic model of asthma was established by the short-term or long-term inhalation of OVA in OVA-sensitized mice based on the studies by Nakajima et al.[15] and Tanaka et al.[16] with some modification. Briefly, for establishing a model of acute asthma, the sensitized mice were given aerosolized OVA (10 mg/ml) dissolved in 0.9% saline two weeks after the second immunization (day 21) by a DeVilbiss 646 nebulizer (DeVilbiss, Somerset, PA) for 30 min (Fig. 1, method 1). OVA solution contained $<$ 1 ng/ml endotoxin. As a control, 0.9% saline alone was administered by the nebulizer. For establishing a model of chronic asthma, the sensitized mice were given aerosolized OVA (10 mg/ml) dissolved in 0.9% saline two weeks after the second immunization (day 10) 14 times at a 24-h interval as described above (Fig. 1, method 2). 3 days after the final antigen challenge, a sagittal block of right lung was excised, fixed in 10% buffered-formalin, and embedded in paraffin. The lung sections (3 μm thick) were stained with H & E, elastica van gieson and periodic acid-Schiff (PAS) solutions according to the standard protocols.

Airway Reactivity

3 days after the final OVA challenge in an acute or chronic model of asthma, airway reactivity to aerosolized methacholine (0-24 mg/ml) was measured using the Buxco whole body plethysmograph (Buxco Electronics, Sharon, CT) as described by Hamelmann et al.[17]. Airway reactivity was expressed as enhanced pause (Penh) values for each concentration of methacholine.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed 3 days after the last aerosol challenge and differential cell count was evaluated as previously described[18]. In short, sacri-

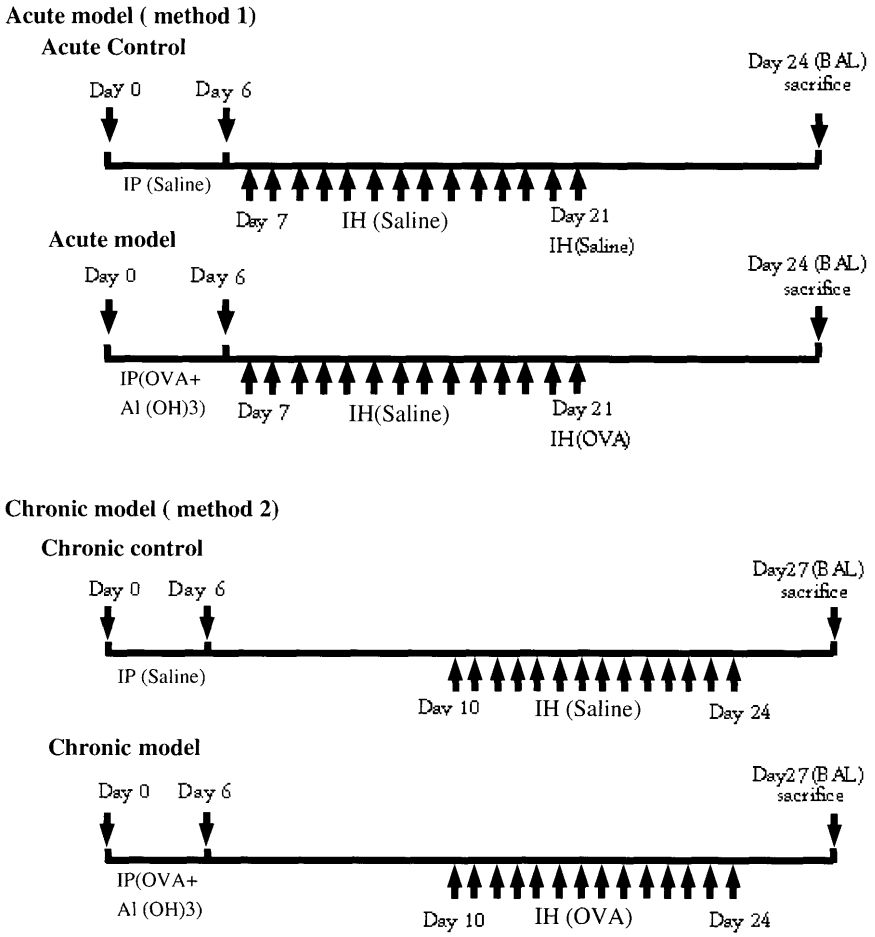

Fig. 1 Protocols used in this study. Acute or chronic model of asthma was established by the short-term or long-term inhalation of OVA in OVA-sensitized mice based on the studies. For establishing a model of acute asthma, the sensitized mice were given aerosolized OVA (10 mg/ml) dissolved in 0.9% saline two weeks after the second immunization (day 20) by a DeVilbiss 646 nebulizer (DeVilbiss, Somerset, PA) for 30 min (method 1). As a control, 0.9% saline alone was administered by the nebulizer. For establishing a model of chronic asthma, the sensitized mice were given aerosolized OVA (10 mg/ml) dissolved in 0.9% saline two weeks after the second immunization (day 20) 14 times at a 24-h interval (method 2).

ficed mouse lungs were cannulated with polyethylene tubing, lavaged with three 0.5 ml aliquots of saline, and total cell numbers were counted with a hemocytometer. Cytospin preparation of BAL cells were stained with Diff-Quik solution (Osaka, Japan) to determine the differential cell count and evaluated on the basis of at least 500 leukocytes.

Immunohistochemistry

Parafin sections of lung tissues were deparaffinized and hydrated by submersion in xylene followed by reagent-grade alcohol. The sections were rinsed for 5 min and incubated with 0.3% H_2O_2 for 30 min to quench endogenous peroxidase activity. After washing three times in TBS for 15 min, the sections were incubated with goat anti-human/mouse Smad7 antibody (sc-9183; SantaCruz Biotechnology, Santa Cruz, CA), goat anti-human/mouse phosphorylated Smad2 antibody (sc-11769; SantaCruz Biotechnology, Santa Cruz, CA), or a goat IgG as a control overnight. Then, the sections were washed three times in TBS for 15 min, and a secondary antibody Biotinylated Rabbit Anti-Goat Immunoglobulines (E0466; DAKO Japan, Kyoto, Japan) was applied for 30 min. After washing, the sections were incubated

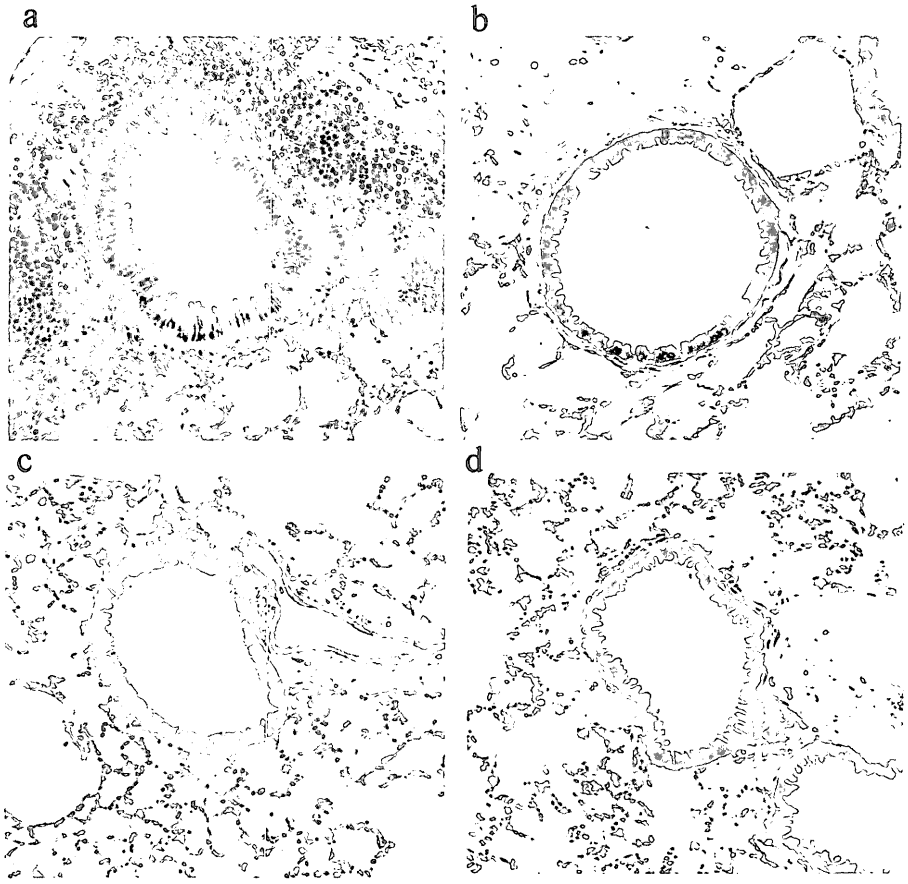

Fig. 2 Development of an acute or a chronic model of asthma.
Membranous bronchioles obtained from mouse lungs challenged with OVA for 14 days (chronic model) (a), or challenged with saline for 14 days (b), or received a single OVAchallenge (acure model) (c), or received a single saline challenged (d). The airway wall challenged with OVA for 14 days was markedly thickened.

with streptavidin peroxidase reagent for 30 min. The sections were washed again and stained with peroxidase substrate solution until the desired intensity was reached. After rinsing in running water, the sections were counterstained with hematoxylin. The used reagents were derived from commercially available DAKO labeled streptoavidin-biotin kits.

Evaluation of Smad 7-or P-Smad 2-positive cells

The immunohistochemically-stained sections were observed under a Olympus BX51 camera microscope equipped with condenser (Olympus, Tokyo, Japan). Digital images around bronchioles of each section were randomly captured through a DP70 digital camera (Olympus, Tokyo, Japan) and quantitative analysis of Smad 7-positive or phosphorylated Smad 2-positive cells were performed by the calculation of the area of positive immunoreactivity around the bronchioles with Win Roof image analysis software (Mitani Co., Fukui, Japan) as described previously by Sugimoto et al.[19]. The results were expressed as a mean ± SE of 4 randomly-selected tissue sections.

To avoid fluctuations in the strength of immunoreactivity among experiments, tissue sections for digital image were prepared at the same time under the same conditions.

Real-time PCR

Total RNA was extracted from the mouse lung tissues 3 days after the final OVA challenge in acute and chronic asthma models by using Isogen solution (Nippon Gene, Japan) and complementary DNA (cDNA) was synthesized from 3 mg of total RNA using Takara RNA PCR kit (AMV) Ver. 2.1 (Takara Bio Inc., Siga, Japan).

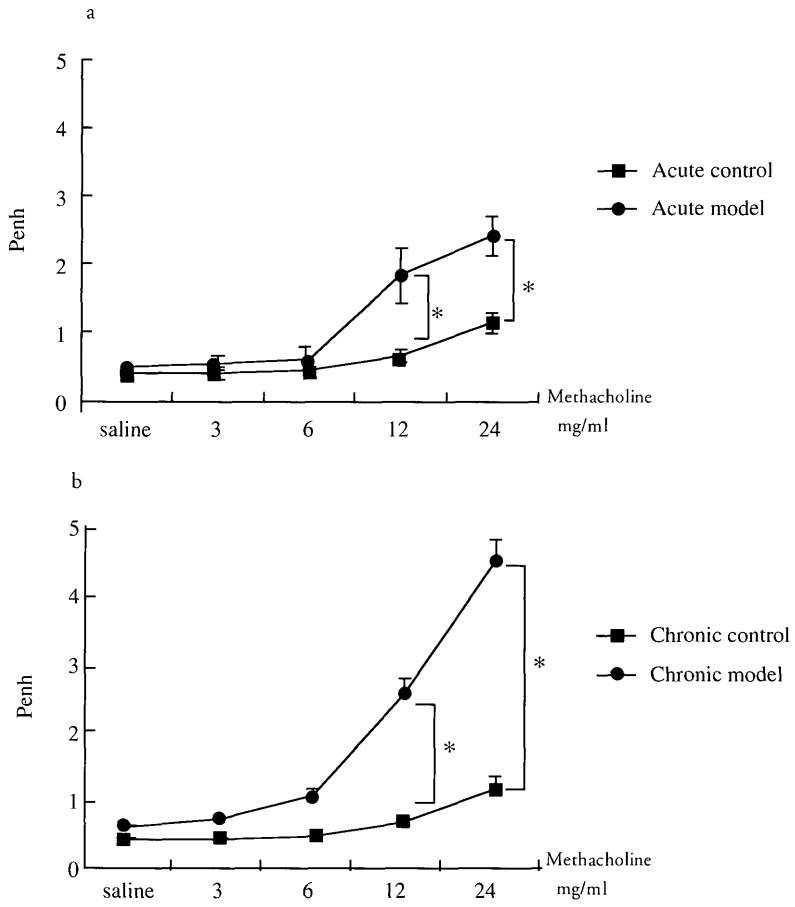

Fig. 3 Airway hyperresponsiveness to methacholine in the acute (a) or chronic (b) model of asthma. Data are expressed as means ± SE (n = 7) and are representative of 7 independent experiments. * $p < 0.01$.

Real-time PCR was performed in Gene Amp 5700 (Applied Biosystems, Tokyo, Japan) using Taq man universal PCR master mix (Applied Biosystems, Chiba, Japan) and the following probes and primers ; Smad7 probe (5′-TGTGGGTTTACAACCGCAGCA-3′) Smad7 primers (5′-CTGACGCGGGAAGTGGAT-3′and 5′-TGGCGGACTTGATGAAGAAGATG-3′) ; TGF-β_1 probe (5′-CGTGGAAATCAACGGGATCAGCCC-3′) TGF-β_1 primers (5′-AGCGCTCACTGCTCTGTGA-3′and 5′-GGTCGCCCCGACGTTT-3′) ; GAPDH probe and primers were obtained from Taq man Rodent GAPDH control reagent (VIC probe) (Applied Biosystems, Tokyo, Japan).

PCR amplification was performed as described below ; 50℃ for 2 minutes, 95℃ for 10minutes, 40 cycles at 95℃ for 15 seconds, 60℃ for 1 minute.

Data analysis

Data are summarized as mean ± SE. The statistical analysis of the results was performed by the unpaired t test. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

Development of airway remodeling in chronic, but not acute, model of asthma

Consistent with previous reports on a mouse model of chronic asthma [16], sensitized, repeatedly (14 days) ovalbumin (OVA)-exposed BALB/c mice exhibited evidence of airway remodeling including prominent subepithelial fibrosis and smooth muscle cell hyperplasia (Fig. 2 A-a). There was no evidence of airway remodeling in the airways of sensitized, shortly OVA-exposed BALB/c mice (Fig. 2 A-c). The extent of AHR and airway inflammation was largely comparable between the acute and chronic mouse models of asthma (Fig. 3-a, 3-b), although the relative ratio of macrophages, lymphocytes, or eosinophils models in BALF was significantly different

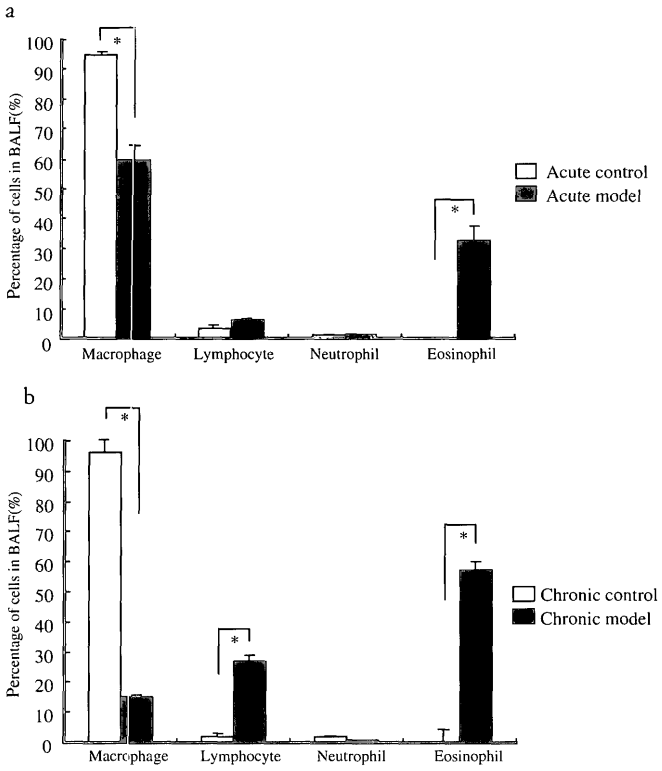

Fig. 4 Differential cell counts in BALF in the acute (a) or chronic (b) model of asthma. Data are expressed as means ± SE (n = 7) and are representative of 7 independent experiments. * $p < 0.01$.

between the acute and chronic (Fig. 4-a, 4-b).

Decreased Smad7 expression in the airways of chronic asthma

To investigate association between Smad7 and the development of airway remodeling in vivo, we examined expression of Smad7 in the lung tissues obtained from acute (Fig. 5-a) or chronic model (Fig. 5-b) of asthma. Immunohistochemical staining showed that immunoreactivity of Smad7 was significantly increased in the airways of OVA-sensitized BALB/c mice after exposure of OVA. The immunoreactivity of Smad7 was mainly detected in bronchial epithelial cells. Interestingly, Smad7 immunoreactivity in the airways was significantly less in sensitized, repeatedly (14 days) OVA-exposed BALB/c mice than shortly OVA-exposed ones (acute model 50.21 ± 1.51, chronic model 24.56 ± 0.78, $p < 0.01$) (Fig. 5-c).

To further confirm decreased Smad7 expression in the airways of chronic model of asthma, we examined Smad7 mRNA expression in the lungs by using quantitative real-time PCR method. As shown in Fig. 6, mRNA expression of Smad7 was significantly less in sensitized, repeatedly (14 days) OVA-exposed BALB/c mice than sensitized, shortly OVA-exposed ones (acute model 4.59 ± 0.09, chronic model 2.73 ± 0.18, $p < 0.01$), which was compatible with immunohistochemical data.

Increased phosphorylation of Smad2

Smad7 inhibits TGF-β-induced phosphorylation of Smad2, resulting in suppression of transcriptional responses by TGF-β[11~14]. We thus examined phosphorylation of Smad2 in the airways of acute (Fig. 7-a) and chronic model (Fig. 7-b) of asthma. Immunohistochemical analysis using anti-phosphorylated Smad2 antibody showed that immunoreactivity of phosphorylated Smad2 was significantly increased in the airways of OVA-sensitized BALB/c mice after exposure of OVA. The immunoreactivity of phosphorylated Smad2 was also mainly detected in bronchial epithelial cells. In contrast to Smad7, immunoreactivity of phosphorylated Smad2 in the airways was significantly higher in sensitized, repeatedly (14 days) OVA-exposed BALB/c mice than sensitized, shortly OVA-exposed ones (acute model 30.0 ±

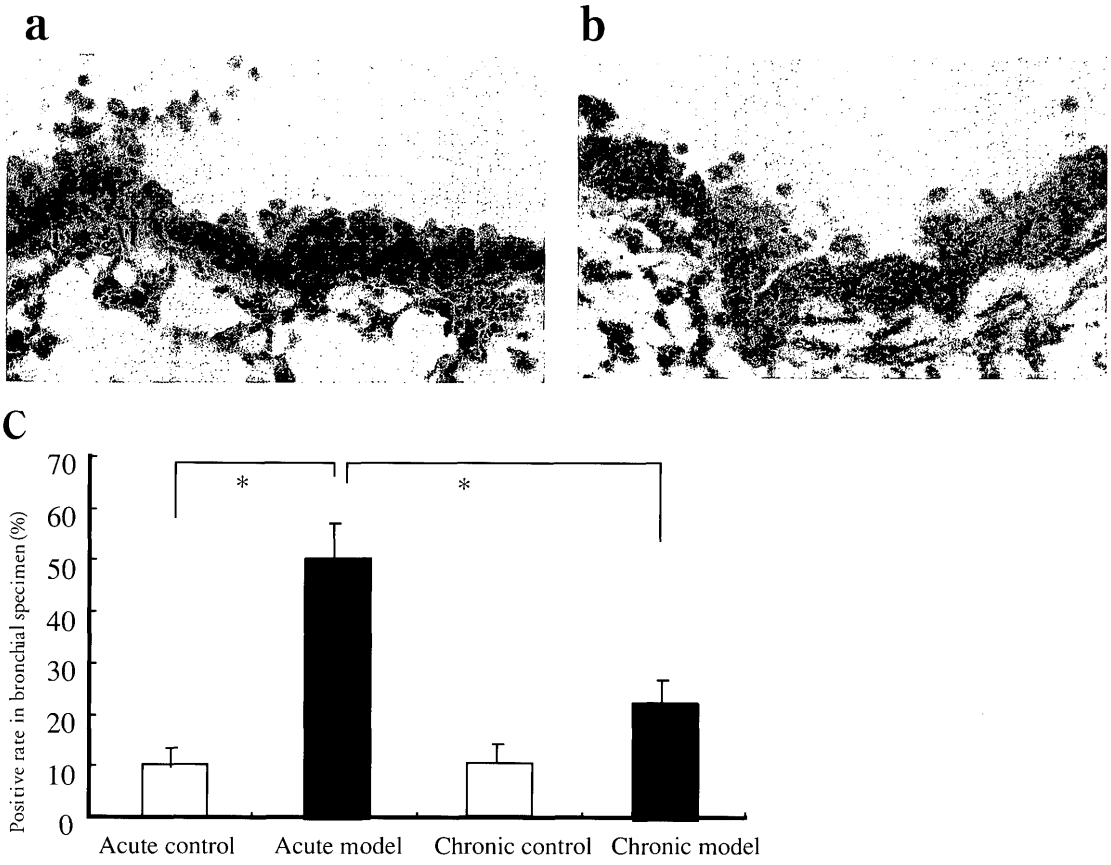

Fig. 5 Decreased Smad7 expression in the airways of a chronic model of asthma.
(A) Representative pictures showing decreased Smad7 immunoreactivity in bronchial epithelial cells in lung tissues obtained from the mice challenged with OVA for 14 days (chronic model) (b) or from the mice received a single OVA challenge (acute model) (a). (B) Quantitative analysis of Smad7 immunoreactivity in lung specimens from acute or chronic model of asthma (c). Data are summarized as means ± SE. 7 representative tissue samples for each group were examined. * $p < 0.01$.

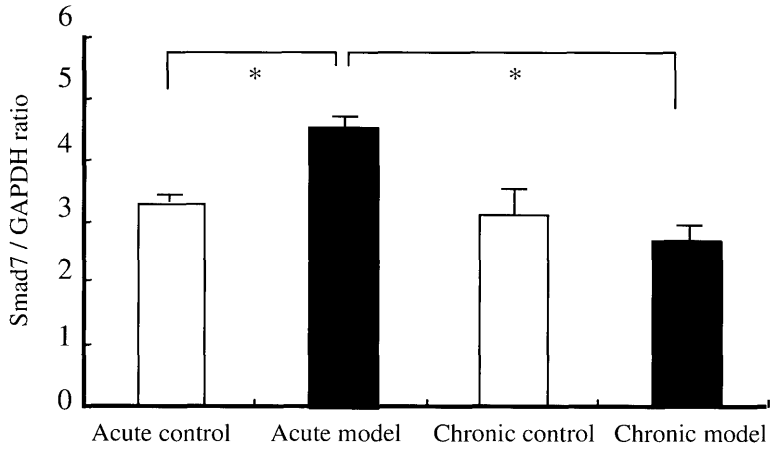

Fig. 6 Real-time PCR analysis of Smad7 in lung specimens from acute and chronic models of asthma. Real-time PCR was performed as described in the text using lung tissues obtained from the mice challenged with OVA for 14 days (chronic model) or from the mice received a single OVA challenge (acute model). Data are summarized as means ± SE ($n = 7$). * $p < 0.01$.

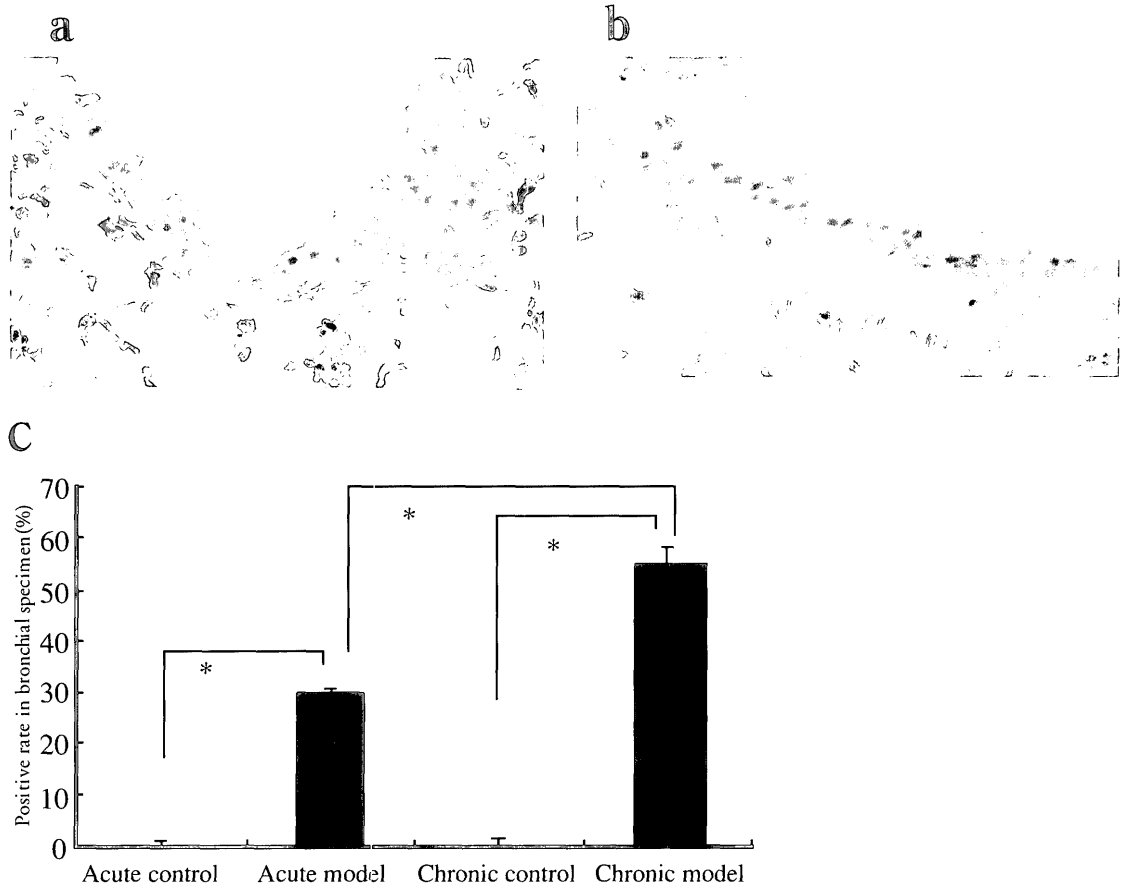

Fig. 7 Increased expression of phosphorylated Smad2 in the airways of a chronic model of asthma.
(A) Representative pictures showing increased phosphorylated Smad2 immunoreactivity in bronchial epithelial cells in lung tissues obtained from the mice challenged with OVA for 14 days (chronic model) (b) or from the mice received a single OVA (acute model) (a). (B) Quantitative analysis of phosphorylated Smad2 immunoreactivity in lung specimens from acute or chronic model of asthma (c). Data are summarized as means ± SE. 7 representative tissue samples for each group were examined. * $p < 0.01$.

0.01, chronic model 55.3 ± 0.03, $p < 0.01$) (Fig. 7-c).

Comparable level of lung TGF-β between acute and chronic models of asthma

Increased immunoreactivity of phosphorylated Smad2 in the airways of chronic asthma may be due to increased TGF-β production, but not to decreased Smad7 expression, in the chronically, inflamed airways. Therefore, we checked expression of TGF-β_1 mRNA in the lungs by using quantitative real-time PCR method. As shown in Fig. 8, TGF-β_1 mRNA expression was comparable between chronic and acute model of asthma.

DISCUSSION

In this study, we showed that Smad7 was significantly decreased in the airways of a chronic asthma model developing airway remodeling when compared with an acute asthma model and decreased Smad7 was associated with increased phosphorylation of Smad2 in the airways (Fig. 7). It is generally believed that increased TGF-β expression in the airways plays an important role in airway remodeling in asthma[6, 9]. Because lung TGF-β levels were comparable between acute and chronic asthma models in our system (Fig. 8), the current data may provide a novel biological mechanism on airway remodeling in asthma : decreased Smad7 expression in chronically antigen-exposed airways might result in increased TGF-β sensitivity in the airways and contribute to the development of airway remodeling through upregulation of TGF-β activity.

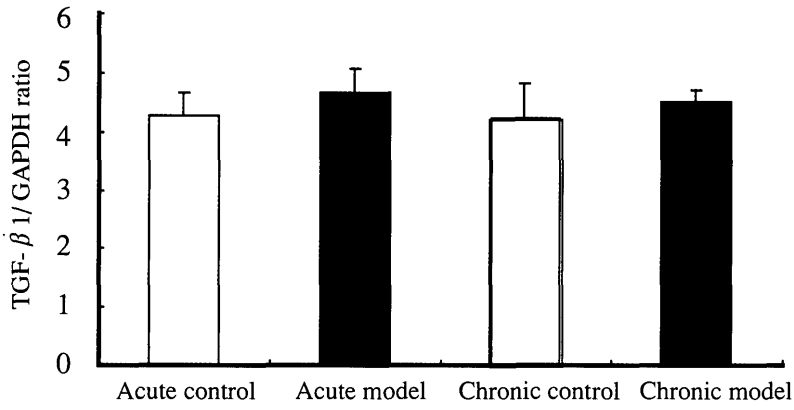

Fig. 8 Real-time PCR analysis of TGF-β_1 in lung specimens from acute and chronic models of asthma. Real-time PCR was performed as described in the text using lung tissues obtained from the mice challenged with OVA for 14 days (chronic model) or from the mice receive a single OVA challenge with (acute model). Data are summarized as means ± SE. (n = 7)

Decreased Smad7 expression at the site of chronic inflammatory diseases was recently reported in human scleroderma, a mouse model of scleroderma, and cardiac fibrosis in the infracted rat heart[20~22]. Therefore, chronic, but not acute, inflammation might contribute to selective downregulation of Smad7 through unknown mechanisms.

Recent intense investigations shed light on mechanisms of Smad7 protein degradation[23,24]. Smad7 binds to ubiquitin ligases, termed Smurf, and, then, Smurf induces Smad7 degradation through proteasomal and lysosomal pathways. Furthermore, most recently, Gronroos et al showed control of Smad7 stability by competition between acetylation and ubiquitination[25]. Thus, it will be interesting to investigate ubiquitination or acetylation of Smad7 or Smurf expression in the airways of chronic asthmatics to explore the mechanisms on decreased Smad7 expression.

In summary, we showed decreased Smad7 expression in the airways of a chronic model of asthma developing airway remodeling. Because lung TGF-β levels were comparable between our acute and chronic asthma models, the results might provide a novel mechanism that underlies the development of airway remodeling ; downregulation of Smad7 in the airways would increase airway TGF-β sensitivity, resulting in airway remodeling. Smad7 expression level in the airways might thus determine susceptibility to airway remodeling in patients with asthma.

REFERENCES

1) Chung KF, Adcock I. : Asthma : application of cell and molecular biology techniques to unravel causes and pathophysiological mechanisms. In : Chung KF and Adcock I, editors. Asthma : Mechanisms and protocols. Totowa, NJ : Humana Press Inc., 1-29, 2000.
2) Busse WW, Lemanske RF Jr. : Asthma. N Engl J Med., **344** : 350-62, 2001.
3) Kay AB. : Allergy and allergic diseases. N Eng J Med, **344** : 30-37, 2001.
4) Holgate ST. : Asthma : more than an inflammatory disease. Curr Opin Allergy Clin Immunol, **2** : 27-29, 2002.
5) Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola A. : Asthma : From bronchoconstriction to airways inflammation and remodeling. Am J Respir Crti Care Med, **161** : 1720-1745, 2000.
6) Elias JA, Zhu Z, Chupp G, Homer RJ. : Airway remodeling in asthma J. Clin. Invest., **104** : 1001-100, 1999.
7) Verrecchia F, Mauvil A : Transforming growth factor-β signaling through the Smad pathway : role in extracellular matrix gene expression and regulation. J Invest Dermatol, **118** : 211-215, 2002.
8) Ohno, I, Nitta Y, Yamauchi K, et al : Transforming growth factor-β1 gene expression by eosinophils in asthmatic airway inflammation. Am J Respir Cell Mol Biol, **15** : 404-409, 1996.
9) Vignola AM, Chanez P, Chiappara G, et al : Transforming growth factor-β expression in mucosal

biopsies in asthma and chronic bronchitis. Am J Respir Crit Care Med., **156** : 591-9, 1997.

10) Sagara H, Okada T, Okumura K, et al : Activation of TGF-β/Smad2 signaling is associated with airway remodeling in asthma. J Allergy Clin Immunol, **110** : 249-254, 2002.
11) Heldin CH, Miyazono K, ten Dijke P. : TGF-β signaling from cell membrane to nucleus through SMAD proteins. Nature, **390** : 465-471, 1997.
12) Massague J. : How cells read TGF-β signals. Nat Rev Mol Cell Biol, **1** : 169-178, 2000.
13) Attisano L, Warana JL. : Smads as transcriptional co-modulators. Curr Opin Cell Biol, 12 : 235-243, 2000.
14) Nakao A, Okumura K, Ogawa H. : Smad7 : a new key player in TGF-β-associated disease. Trends Mol Med, **8** : 361-363, 2002.
15) Nakajima H, Iwamoto I, Tomoe S, et al : CD4 T lymphocytes and interleukin-5 mediate antigen-induced eosinophil infiltration into the mouse trachea. Am Rev Respir Dis, **146** : 374-377, 1992.
16) Tanaka H, Matsuda T, Tokuoka S, et al : The effect of allergen-induced airway inflammation on airway remodeling in a murine model of allergic asthma. Inflamm Res, **50** : 616-624, 2001.
17) Hamelmann E, Schwartz J, Takeda K, et al : Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. Am J Respir Crit Care Med, **156** : 766-775, 1997.
18) Kumano K, Nakao A, Nakajima H, et al : Interleukin-18 enhances antigen-induced eosinophil recruitment into the mouse airways. Am J Respir Crit Care Med, **160** : 873-878, 1999.
19) Sugimoto M, Tsuji S, Kuwahara M, et al : Shear-dependent functions of the interaction between soluble von Willebrand factor and platelet glycoprotein Ib in mural thrombus formation on a collagen surface. Int J Hematol, **69** : 48-54, 1999.
20) Dong D, Zhu S, Wang T, et al : Deficient Smad7 expression : a putative molecular defect in scleroderma. Proc Nat Acad Sci, **99** : 3908-3913, 2002.
21) Takagawa S, Lakos G, Mori Y, et al : Sustained activation of fibroblast transforming growth factor-β/Smad signaling in a murine model of scleroderma. J Invest Dermatol, **121** : 41-50, 2003.
22) Wang B, Hao J, Jones SC, et al : Decreased Smad7 expression contribute to cardiac fibrosis in the infracted rat heart. Am J Physiol Heart Circ Physiol, **282** : H1685-H1696, 2002.
23) Ebisawa T, Fukuchi M, Murakami G, et al : Smurf1 interacts with transforming growth factor-β type I receptor through Smad7 and induces receptor degradation. J Biol Chem, **276** : 12477-12480, 2001.
24) Kavsak P, Rasmussen RK, Causing CG, et al : Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF-β receptor for degradation. Mol Cell, **6** : 1365-1375, 2000.
25) Gronroos E, Hellman U, Heldin CH, et al : Control of Smad7 stability by competition between acetylation and ubiquitination. Mol Cell, **10** : 483-493, 2002.