

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

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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

Abbreviation used

TGF- β : Transforming growth factor β

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 FEV₁.^{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.

Previous studies have suggested that a fibrogenic cytokine, transforming growth factor β 1 (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- β 1 expression in asthmatic airways by using immunohistochemistry with anti-TGF- β 1 antibody or in situ hybridization with a TGF- β 1 probe. However, because TGF- β 1 is secreted as latent complexes⁹ and anti-TGF- β 1 antibody and because detection of mRNA with a TGF- β 1 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- β 1), the actual activity of TGF- β 1 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.

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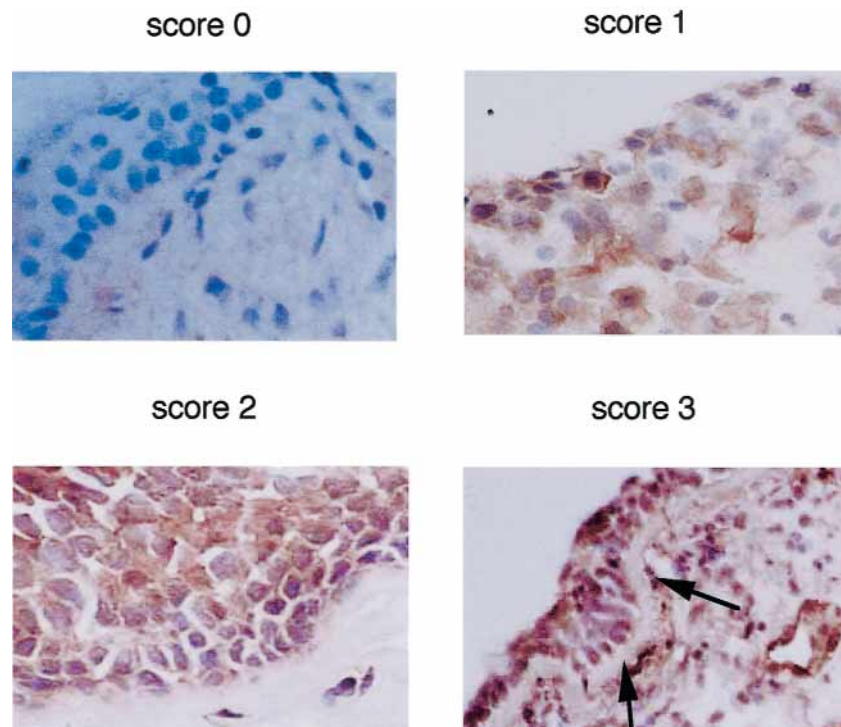


FIG 1. Photomicrographs show scoring for phosphorylated Smad2 staining in bronchial biopsy specimens from asthmatic and control subjects. Note the basement membrane thickness (arrows) in a sample derived from a severely asthmatic patient (score 3).

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- β 1 signaling was active in the process of airway remodeling.

METHODS

Subjects

Eight normal control subjects without asthma (mean $\pm$ SE] age, 43.0 ± 3.8 years) and 40 asthmatic subjects (20 with mild asthma, a mean $\pm$ SE] age of 41.4 ± 4.4 years, and a mean $\pm$ SE] FEV_{1.0} of 2441 ± 113.8 mL; 16 with moderate asthma, a mean $\pm$ SE] age of 43.0 ± 3.8 years, and a mean $\pm$ SE] FEV_{1.0} of 2518 ± 174.5 mL; and 4 with severe asthma, a mean $\pm$ SE] age of 54 ± 5.0 years, and a mean $\pm$ 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 Allergology¹¹) 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 ori-

gin 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 antibody¹³ (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% H₂O₂ 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

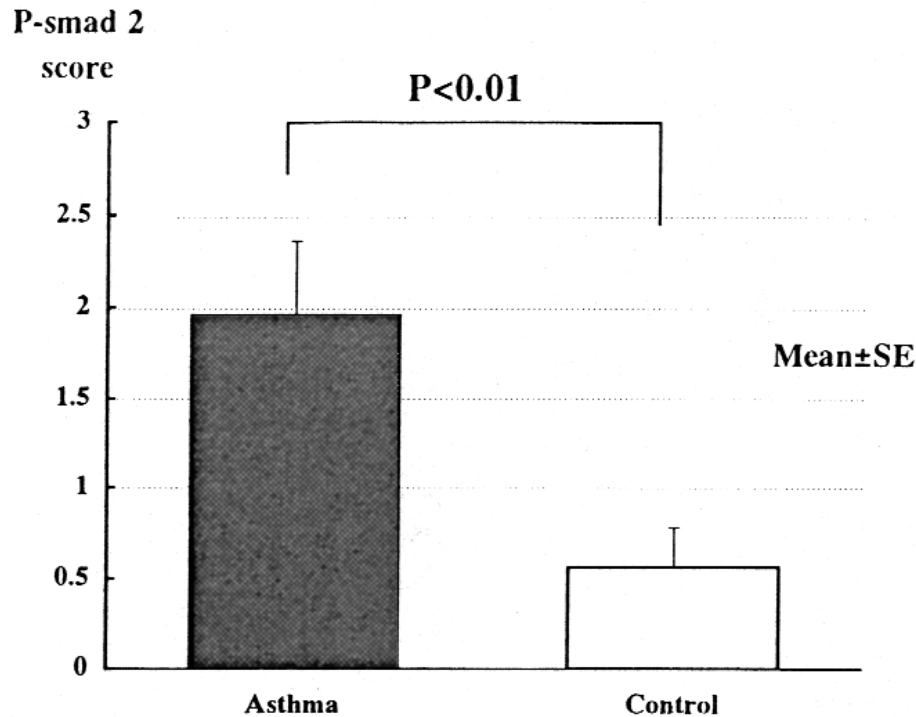


FIG 2. Higher scoring for phosphorylated Smad2 in bronchial biopsy samples from asthmatic subjects than in samples from normal subjects.

TABLE I. Characteristics of asthmatic subjects studied

	Subjects with asthma			Healthy subjects
	Mild	Moderate	Severe	
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 hematoxylin-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 con-

trol 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-

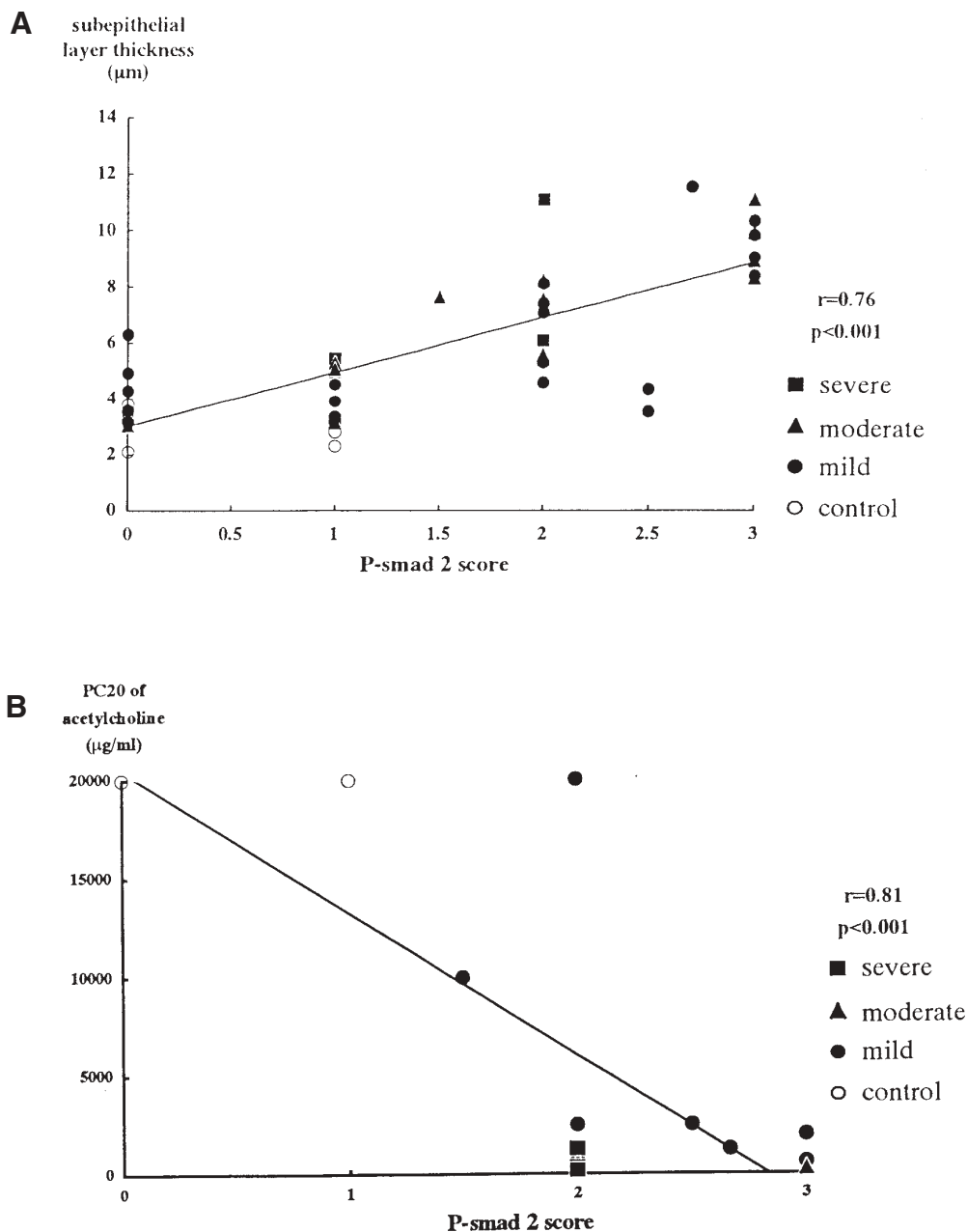


FIG 3. Correlation of expression of phosphorylated Smad2 with basement membrane thickness and airway hyperresponsiveness in the airways of subjects with mild to severe asthma. Expression of phosphorylated Smad2 was scored as described in the Methods section; its correlation with basement membrane thickness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

duce the antisera¹³ 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- β 1 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- β 1 was expressed in the airways of normal subjects, it might not be the active form of TGF- β 1 and did not mediate signaling in situ. Inflammation is a factor in conversion of the latent form of TGF- β 1 to the active form⁹ and might thus contribute to activation of TGF- β 1 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 al¹⁶ 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 al¹⁶). 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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REFERENCES

1. Holgate ST. What does inflammation and airway remodeling mean? *Clin Exp Allergy Rev* 2001;1:59-61.
2. Elias JA, Zhu Z, Chupp G, Homer RJ. Airway remodeling in asthma. *J Clin Invest* 1999;104:1001-6.
3. Roche WR, Easley R, Williams J, Holgate ST. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* 1989;1:520-4.
4. Chetta A, Forese A, Donno MD, Bertorelli G, Pesci A, Olivieri D. Airway remodeling is a distinctive feature of asthma and is related to severity of disease. *Chest* 1997;111:852-7.
5. Hoshino M, Nakamura Y, Sim J, Shimojo J, Isogai S. Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation. *J Allergy Clin Immunol* 1998;102:783-8.
6. Ohno I, Nitta Y, Yamauchi K, Hoshi H, Honma M, Woolley K, et al. Transforming growth factor- β 1 gene expression by eosinophils in asthmatic airway inflammation. *Am J Respir Cell Mol Biol* 1996;15:404-9.
7. Minshall EM, Leung DY, Martin RJ, Song YL, Cameron L, Ernst P, et al. Eosinophil-associated TGF- β 1 mRNA expression and airways fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* 1997;17:326-33.
8. Vignola A, Chanez P, Chiappara G, Merendino A, Pace E, Rizzo A, et al. Transforming growth factor- β expression in mucosal biopsies in asthma and chronic bronchitis. *Am J Respir Crit Care Med* 1997;156:591-9.
9. Massague J. The transforming growth factor- β family. *Annu Rev Cell Biol* 1990;6:597-641.
10. Moustakas A, Souchelnytskyi S, Heldin CH. Smad regulation in TGF- β signal transduction. *J Cell Sci* 2001;114:4359-69.
11. Makino S, Furusho K, Miyamoto T, Ohta K, editors. Asthma prevention and management guidelines 1998, Japan (JGL1998). Report of the research group for asthma prevention and management guidelines, supported by the Immunology & Allergy Research Study Project, Ministry of Health and Welfare. *Jpn Int Arch Allergy Immunol* 2000;121(Suppl 1):4-14.
12. Makino S, Ikemori R, Fukuda T, Motojima S, Yukawa T, Yamada G, et al. Clinical evaluation of standard method of acetylcholine inhalation test in bronchial asthma. *Jpn J Allergol* 1984;33:167-75.
13. Brodin GP, ten Dijke P, Funa K, Heldin CH, Landstrom M. Increased Smad expression and activation are associated with apoptosis in normal and malignant prostate after castration. *Cancer Res* 1999;59:2731-8.

14. Redington AE, Roche WR, Holgate ST, Howarth PH. Co-localization of immunoreactive TGF- β 1 and decorin in bronchial biopsies from asthmatics and normal subjects. *J Pathol* 1998;186:410-5.
15. Redington AE, Madden J, Frew AJ, Djukanovic R, Roche WR, Holgate ST, et al. Transforming growth factor- β 1 in asthma. Measurement in bronchoalveolar lavage fluid. *Am J Respir Crit Care Med* 1997;156:642-7.
16. Rosendahl A, Checchin D, Fehniger TE, ten Dijke P, Heldin CH, Sideras P. Activation of the TGF- β /activin-Smad2 pathway during allergic airway inflammation. *Am J Respir Cell Mol Biol* 2001;25:60-8.
17. Fiocchi C. TGF- β /Smad signaling defects in inflammatory bowel disease: mechanisms and possible novel therapies for chronic inflammation. *J Clin Invest* 2001;108:523-6.