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Transforming Growth Factor-β2 Induces Bronchial Epithelial Mucin Expression in Asthma

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The transforming growth factor (TGF)-β family is important for tissue repair in pathological conditions including asthma. However, little is known about the impact of either TGF-β1 or TGF-β2 on asthmatic airway epithelial mucin expression. We evaluated bronchial epithelial TGF-β1 and TGF-β2 expression and their effects on mucin expression, and the role of TGF-β1 or TGF-β2 in interleukin (IL)-13-induced mucin expression. Epithelial TGF-β1, TGF-β2, and mucin expression were evaluated in endobronchial biopsies from asthmatics and normal subjects. The effects of TGF-β1 or TGF-β2 on mucin MUC5AC protein and mRNA expression, and the impact of IL-13 on epithelial TGF-β1, TGF-β2, and MUC5AC were determined in cultured bronchial epithelial cells from endobronchial brushings of both subject groups. In biopsy tissue, epithelial TGF-β2 expression levels were higher than TGF-β1 in both asthmatics and normals. TGF-β2, but not TGF-β1, was increased in asthmatics compared with normals, and significantly correlated with mucin expression. TGF-β2, but not TGF-β1, increased mucin expression in cultured epithelial cells from both subject groups. IL-13 increased the release of TGF-β2, but not TGF-β1, from epithelial cells. A neutralizing TGF-β2 antibody partially inhibited IL-13-induced mucin expression. These data suggest that TGF-β2 production by asthmatic bronchial epithelial cells may increase airway mucin expression. IL-13-induced mucin expression may occur in part through TGF-β2 up-regulation. *(Am J Pathol 2004, 165:1097–1106)*

Transforming growth factor (TGF)-β has been proposed as an important mediator in inflammatory and remodeling processes associated with many pathological conditions including asthma. At least five isoforms of TGF-β have been reported, of which TGF-β1 has been the most extensively studied. Increased TGF-β1 has been reported in asthmatic airways.[1,2] Although TGF-β1 has been intensively studied in fibrosis,[3,4] its role in airway epithelial cell biology remains unclear. Previous studies have suggested that TGF-β1 at a very high dose (10 ng/ml) may inhibit the proliferation and even induce apoptosis of bronchial epithelial cells.[5] However, the effects of TGF-β2, one of the TGF-β isoforms, in these systems are not known.

Goblet cell metaplasia/hyperplasia (GCM/H) in the airway epithelium is a common finding in pathological studies of asthma and other respiratory diseases and contributes to mucus hypersecretion.[6] GCM/H is also likely part of the epithelial repair process. Airway mucus consists of water, ions, serum protein transudates, and mucin glycoproteins (mucins). Because mucin is the major component of mucus, most research in mucus production has focused on mucin regulation. Factors that could increase mucin expression in asthma include both nonspecific stimuli (eg, bacteria and cigarette smoke) and specific stimuli such as Th2 cytokines [eg, interleukin (IL)-4, IL-13, and IL-9].[7] Although TGF-β has been widely studied in wound repair, the role of TGF-β isoforms in the process of GCM/H has not been well evaluated in human diseases, including asthma. Although TGF-β1 may be suppressive for GCM/H,[8] no systematic studies have been performed to address this phenomenon. On the other hand, TGF-β2 has been shown to induce mucin MUC4 mRNA and protein expression in human pancreatic tumor cells.[9] IL-13, a Th2 cytokine known to induce GCH/M, has also been shown to induce TGF-β2, but not TGF-β1, expression in cultured human bronchial epithelial cells from both asthmatic and nonasthmatic patients.[10,11] However, interactions between IL-13 and TGF-β2 in the control of mucin expression have not been studied.

We hypothesized that bronchial epithelial expression of TGF-β2, but not TGF-β1, would be increased in

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asthma, and that TGF-β2, but not TGF-β1, would induce mucin expression. It was further hypothesized that TGF-β2 could contribute to IL-13-induced mucin expression. To test these hypotheses, we evaluated bronchial epithelial TGF-β2 and TGF-β1 expression in endobronchial biopsy specimens from asthmatics of varying severity, as well as normal control subjects. Cultured primary human bronchial epithelial cells were used to evaluate the direct effects of TGF-β2 on mucin expression. In addition, the role of TGF-β2 in IL-13-induced mucin expression was determined.

Materials and Methods

Study Participants

Asthmatic patients met the American Thoracic Society criteria for asthma. Mild asthmatics were defined as patients with an FEV_1 of >80% predicted on β-agonist alone. Moderate asthmatics had an FEV_1 of ≤80% predicted, were on β-agonists and low to moderate doses of inhaled corticosteroids, without a history of urgent health care or oral corticosteroid use. Severe asthmatics were patients referred to National Jewish Medical and Research Center for severe, oral corticosteroid-dependent asthma, with a history of frequent hospitalizations and/or emergency room visits, evidence for ongoing severe airflow limitation, and oral or high-dose inhaled corticosteroid use. None of the asthma patients currently smoked or had a history of smoking >5 pack years. Normal controls had no history of respiratory disease, viral illness, or tobacco use and were on no medications. The protocol was approved by the institutional review board and all patients gave informed consent.

Biopsy Tissue Processing and Immunostaining

Bronchoscopy with endobronchial biopsy was performed on 40 patients (mild asthma = 9, moderate asthma = 5, severe asthma = 16, normal = 10). Four to six biopsies were obtained from the lower lobe subcarinae, fixed in acetone, and embedded in glycol-methacrylate resin. Serial 2-μm sections were immunostained using rabbit polyclonal antibodies against TGF-β1 and TGF-β2 (1 μg/ml working solution; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Bronchial epithelial TGF-β staining was analyzed using a NIH Scion image analysis program (National Institutes of Health, Bethesda, MD) in the intact epithelium. Both the TGF-β1 or TGF-β2 epithelial staining area and the total area of the epithelium were measured. The result was expressed as percentage of total area of epithelium stained for TGF-β1 or TGF-β2. Bronchial epithelial general mucin expression was evaluated by Alciar Blue/periodic Acid Schiff (AB/PAS) staining[12] and analyzed as for TGF-β1 or TGF-β2.

Primary Bronchial Epithelial Cell Culture

The effects of TGF-β1, TGF-β2, or IL-13 on mucin expression in cultured human primary bronchial epithelial cells (from endobronchial brushings) were evaluated in a subpopulation of 13 patients (5 normal, 3 mild/moderate, and 5 severe asthma patients). Bronchial brushings were performed as previously described using a standard sterile single-sheathed nylon cytology brush (Olympus BC9C-26101, Olympus, Tokyo, Japan).[11] A total of four to eight brushings were obtained and cells were placed into 10 ml of ice-cold phosphate-buffered saline (PBS), centrifuged, washed in ice-cold F12 medium, and resuspended in 1 ml of serum-free hormonally supplemented bronchial epithelial growth medium (Clonetics, San Diego, CA) containing 50 μg/ml of gentamicin and 50 μg/ml of amphotericin. After counting the cells, the cell concentration was brought to 3×10^4/ml in bronchial epithelial growth medium. A total of 9×10^4 cells were seeded into 60-mm tissue culture dishes coated with rat tail type I collagen (BD Discovery Labware, Bedford, MA). Cells were cultured at 37°C in a 5% CO_2 environment. When the cells reached 70 to 80% confluence, they were dissociated with trypsin/ethylenediaminetetraacetic acid and used for cell stimulation experiments.

Analysis of the Effects of TGF-β1 and TGF-β2 on Intracellular Mucin Expression by Bronchial Epithelial Cells under the Immersed Culture System

Primary bronchial epithelial cells at passage one were seeded into 24-well plates at a density of 1×10^4cells/well and grown to 70 to 80% confluence. The cells were then treated in triplicate in the absence and presence of active TGF-β1 (100 pg/ml; R&D Systems, Minneapolis, MN) and TGF-β2 (100 pg/ml, R&D Systems). This TGF-β dose was selected based on our preliminary experiments showing maximal intracellular mucin expression at 100 pg/ml. This dose is also within the range of active TGF-β1 levels in bronchoalveolar lavage fluid of asthmatics.[13] After 48 hours, the cells were collected for mucin MUC5AC protein and mRNA evaluation. The selection of 48 hours of treatment with cytokines in our study was based on a previous study by Wen and co-workers[10] who used the immersed epithelial cell culture to evaluate the effects of IL-13 on epithelial TGF-β2 protein release levels at 6 hours, 12 hours, 24 hours, and 48 hours and found maximal induction of TGF-β2 at 48 hours. Immersed culture was used for the aforementioned experiments because it is easier to perform, as compared to air-liquid interface (ALI) culture, and two previous studies used this system to evaluate the effects of IL-13 on primary human bronchial epithelial cell TGF-β2 expression.[10,11]

To detect intracellular MUC5AC protein expression, dot-blot immunoassay was performed by applying 100 μg of protein per culture condition to nitrocellulose membranes, which were then probed with a mouse monoclonal anti-human MUC5AC antibody (clone 45M1; NeoMarkers Inc., Fremont, CA), detected with an anti-mouse peroxidase-conjugated antibody, and developed with the enhanced chemiluminescence system on film.[14] The film was scanned and the intensity of the MUC5AC protein signal was measured by densitometry. To validate the

dot-blot immunoassay for MUC5AC protein, an asthmatic-induced sputum supernatant sample was spotted with five series of twofold dilutions that represented the total protein levels for each blot at 200, 100, 50, 25, and 12.5 μg, respectively. A linear relationship was observed between the MUC5AC intensity and the total protein levels ($r = 0.98$, $P = 0.001$), suggesting that the MUC5AC dot-blot immunoassay was appropriate for qualifying MUC5AC protein expression levels.

MUC5AC mRNA expression by epithelial cells was determined by reverse transcription, followed by real-time quantitative polymerase chain reaction. Total RNA was extracted using TRIzol reagent and treated with DNase I. Reverse transcription was performed using 1 μg of total RNA and random hexamers in a 50-μl reaction. The MUC5AC primers and probes were those previously reported,[6] and the housekeeping gene GAPDH was used as a control. Real-time polymerase chain reaction was performed on the ABI Prism 7700 sequence detection system (PE Applied Biosystems, Foster City, CA). The threshold cycle (CT) was recorded and the relative MUC5AC mRNA expression levels were obtained using comparative CT methods as previously described.[15]

Analysis of the Effects of TGF-β1 and TGF-β2 on Intracellular Mucin Expression by Bronchial Epithelial Cells under the ALI Culture System

To extend previous studies using the immersed epithelial cell culture and to mimic *in vivo* bronchial epithelial cells, ALI culture of bronchial epithelial cells was performed from a subset of seven human subjects including three normal subjects and four asthmatic patients (one mild and three severe asthmatics). As previously reported,[16] epithelial cells at passage one were plated at 4×10^4 cells/cm^2 onto collagen-coated polyester Transwell inserts of 12 mm in diameter (pore size, 0.4 μm; Corning Inc., Corning, NY). After a week in the immersed culture condition, epithelial cells reached 100% confluence and were shifted to an ALI condition by removing the apical medium. From day 0 of ALI, cells were stimulated in triplicate every 48 hours with addition of TGF-β1 (100 pg/ml), TGF-β2 (100 pg/ml), or PBS (as a negative control) into the lower chamber. Because it is reported to take 10 days for epithelial cells to demonstrate mucociliary differentiation,[17] ALI cells on day 10 were collected in TRIzol for MUC5AC mRNA detection by real-time polymerase chain reaction or fixed in 4% paraformaldehyde and embedded in glycol methacrylate for mucin protein staining. The embedded cells were sectioned at 2-μm thickness, and evaluated for general mucin by AB/PAS staining or for MUC5AC by immunocytochemical staining as previously reported.[18] Mucin protein expression by epithelial cells was quantified by measuring the mucin staining area and the total epithelial area. The results were presented as mucin area/total epithelial area (%).

Analysis of the Effects of IL-13 on TGF-β2 and TGF-β1 Release and the Effects of a TGF-β2-Neutralizing Antibody on IL-13-Induced Mucin Production

Similar to the experiments with TGF-β, bronchial epithelial cells in immersed culture were treated in the absence and presence of IL-13 (10 ng/ml), IL-13 (10 ng/ml) plus a specific goat anti-human TGF-β2-neutralizing antibody (1 μg/ml, R&D Systems), or TGF-β2-neutralizing antibody alone (1 μg/ml) as the control antibody. After 48 hours in culture, supernatants were collected for TGF-β1 and TGF-β2 protein measurement, and cells were collected for MUC5AC protein and mRNA detection. Levels of released total (both latent and active) TGF-β1 and TGF-β2 proteins were measured in acidified cell supernatants by enzyme-linked immunosorbent assay using antibodies and standards from R&D Systems. Levels of active TGF-β1 and TGF-β2 were obtained by analyzing non-acidified cell supernatants. The detection range of TGF-β1 and TGF-β2 enzyme-linked immunosorbent assay was 16 to 2000 pg/ml. Treatment of cells with goat IgG (1 μg/ml) alone did not have significant effects on expression of mucin, TGF-β1, or TGF-β2 protein.

The effects of IL-13 on TGF-β2 and TGF-β1 release and intracellular mucin expression were also evaluated in the ALI culture system in which IL-13 at 10 ng/ml was added into the lower chamber from day 0 for a total of 10 days, as previously described for TGF-β stimulation. Additionally, TGF-β2-neutralizing antibody experiments were performed to determine the effects of TGF-β2 in IL-13-induced mucin expression. In those experiments, cells were treated with a TGF-β2-neutralizing antibody (1 μg/ml) every 48 hours for a total of 10 days.

Statistical Analyses

Normally distributed data (TGF-β1 and TGF-β2 levels in cultured epithelial supernatants) were presented as means ± SEM with intergroup comparison by analysis of variance. Nonparametric data (rest of the data) were expressed as medians with interquartile (25 to 75%) ranges with intergroup comparison by Kruskal-Wallis analysis. Correlations were performed by using Spearman's rank correlation coefficients. A P value of ≤ 0.05 was regarded as statistically significant.

Results

Study Participant Characteristics

The characteristics of the study participants are given in Table 1 for both endobronchial biopsy and brushing studies. The age and gender of normal control and asthma patients were well matched. The baseline FEV_1% predicted was significantly lower in asthmatics than that in normal control subjects.

Table 1. Study Participant Characteristics

Group	*n*	Age (year)	Gender (Female/Male)	FEV1%
Endobronchial biopsy study				
Normal controls	10	31 (25–39)*	5/5	105 (100–107)
Mild asthmatics	9	35 (28–45)	5/4	84 (80–99)†
Moderate asthmatics	5	33 (23–50)	3/2	73 (44–84)†‡
Severe asthmatics	16	29 (21–43)	9/7	50 (41–64)†‡
Endobronchial brushing study				
Normal controls	5	23 (23–38)	2/3	105 (90–112)
Asthmatics	8	21 (20–47)	5/3	68 (48–96)†

*Values expressed as medians with 25% to 75% range.
†$P < 0.05$, compared with normal controls.
‡$P < 0.05$, compared with mild asthmatics.

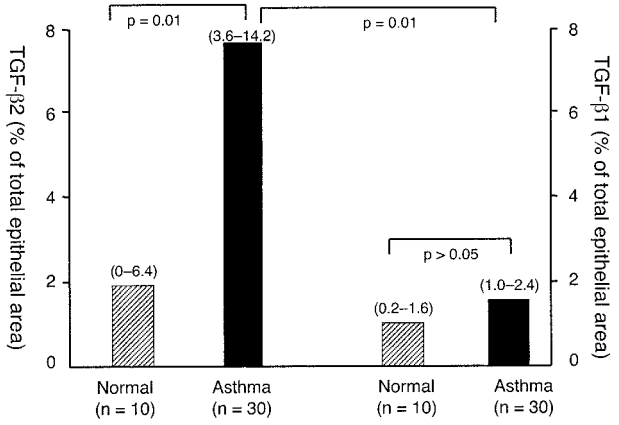


Figure 1. TGF-β1 and TGF-β2 protein expression by bronchial epithelial cells in endobronchial biopsy specimens from asthmatic and normal subjects. Data expressed as medians [interquartile (25 to 75%) range].

TGF-β2, TGF-β1, and Mucin Expression in Bronchial Epithelium of Biopsy Specimens

As shown in Figures 1 and 2, asthmatic patients demonstrated significantly higher levels of TGF-β2 protein expression than normal subjects, whereas no differences were seen for TGF-β1. Epithelial TGF-β2 levels were significantly higher than TGF-β1 levels in asthmatics. In normal subjects, epithelial TGF-β2 levels tended to be higher than TGF-β1 ($P = 0.12$).

Similar to TGF-β2 expression, general mucin levels (Figure 3, A and B) in bronchial epithelia were significantly higher in asthmatic patients than in normal controls. Mucin expression levels in both asthmatics and normal controls significantly correlated with expression of epithelial TGF-β2 (Figure 4). However, there was no sig-

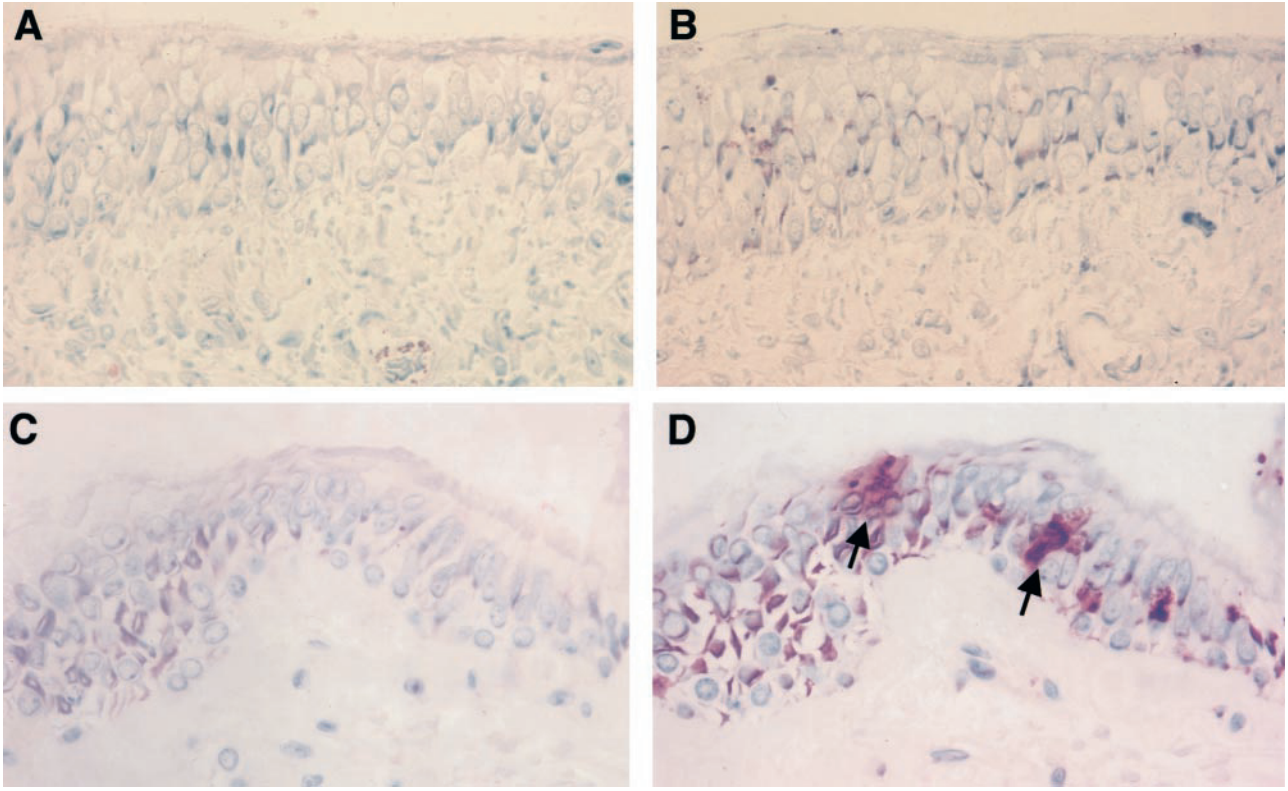


Figure 2. Representative photomicrographs of bronchial epithelial TGF-β1 (**A**, normal subject; **C**, asthmatic) and TGF-β2 immunostaining (**B**, normal subject; **D**, asthmatic). Original magnifications, ×400.

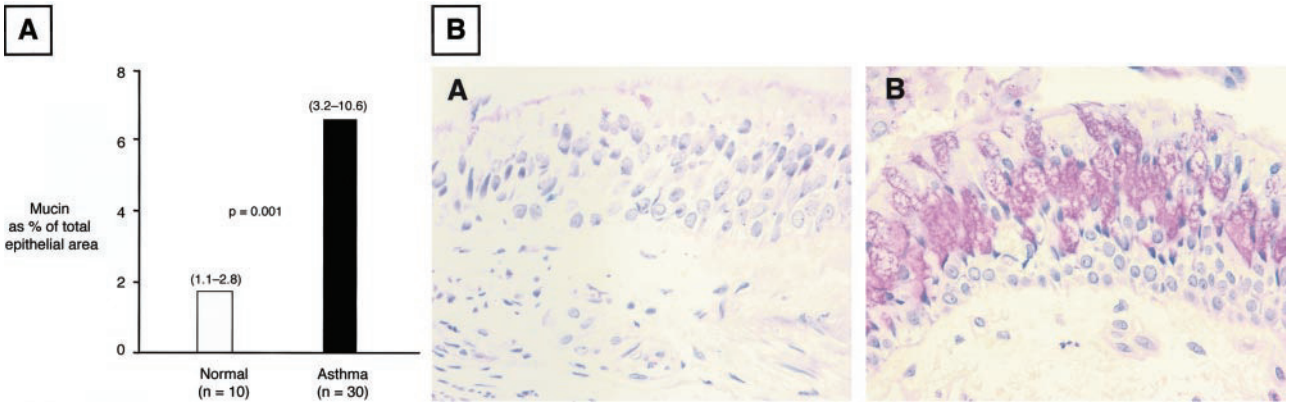


Figure 3. A: Bronchial epithelial mucin expression levels in normal and mild to severe asthmatic patients. Data expressed as medians [interquartile (25 to 75%) range]. **B:** Representative photomicrographs of bronchial epithelial mucin AB/PAS staining in a normal (**A**) and a severe asthmatic patient (**B**). Original magnifications, ×400.

nificant correlation between expression levels of TGF-β1 and mucin in bronchial epithelium ($\rho = 0.17$, $P = 0.28$).

TGF-β2 and TGF-β1 Expression in Cultured Primary Bronchial Epithelial Cells

Because bronchial epithelial TGF-β2 levels in endobronchial biopsy tissues from both asthmatics and normal subjects were higher than TGF-β1, we determined if TGF-β2 protein levels were similarly higher in cultured epithelial cells. Similar to the biopsy data, total TGF-β2 protein levels in supernatants from unstimulated epithelial cells of both asthmatic and normal subjects (515 ± 77 pg/ml) were approximately fivefold higher ($P < 0.01$) than those of TGF-β1 (95 ± 13 pg/ml). Active TGF-β2 and TGF-β1 represented <8% of total TGF-β. Although active TGF-β1 was below the detection limit (16 pg/ml), active TGF-β2 levels in supernatants from unstimulated epithelial cells of both asthmatic and normal subjects were at 39 ± 4 pg/ml. Unlike tissue results, total TGF-β2 levels in unstimulated cell supernatants were not different between asthma and normal control patients (normal patients, 633 ± 129 pg/ml; asthmatics, 555 ± 102 pg/ml; $P = 0.66$). The

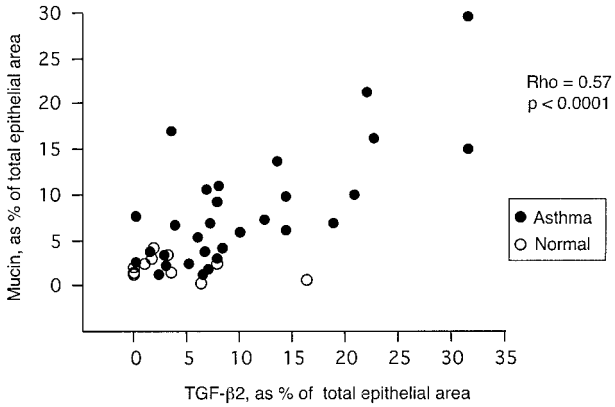


Figure 4. Correlations between bronchial epithelial TGF-β2 expression and mucin expression in mild to severe asthmatic and normal subjects.

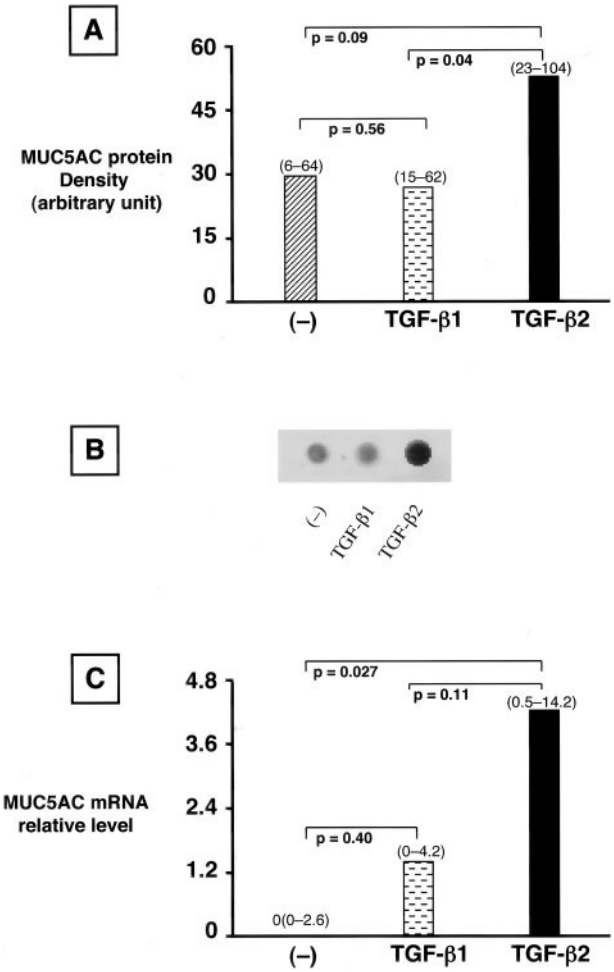


Figure 5. Effects of TGF-β on mucin expression in the immersed cell culture system. Epithelial cells were incubated for 48 hours in the absence and presence of TGF-β1 (100 pg/ml) and TGF-β2 (100 pg/ml). **A:** TGF-β2, but not TGF-β1, tended to increase intracellular mucin MUC5AC protein expression. **B:** Representative image of mucin MUC5AC protein dot-blot immunoassay of cultured bronchial epithelial cells from a severe asthmatic patient. **C:** TGF-β2, but not TGF-β1, increased mucin MUC5AC mRNA expression levels. Data expressed as medians [interquartile (25 to 75%) range].

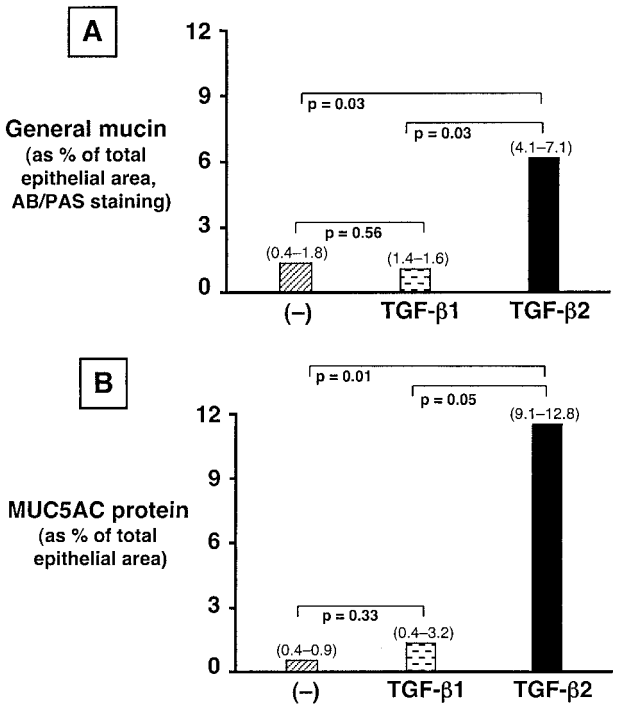


Figure 6. Effects of TGF-β on mucin expression in the ALI culture system. Epithelial cells were incubated for 10 days in the absence and presence of TGF-β1 (100 pg/ml) and TGF-β2 (100 pg/ml). TGF-β2, but not TGF-β1, significantly increased intracellular general mucin (**A**) and MUC5AC (**B**) protein expression. Data expressed as medians [interquartile (25 to 75%) range].

active TGF-β2 levels in unstimulated cell supernatants were also similar between the two subject groups (normal, 43 $\pm$ 8 pg/ml; asthma, 36 $\pm$ 3 pg/ml; $P = 0.47$).

Effects of TGF-β2 and TGF-β1 on Epithelial Mucin Expression in Vitro

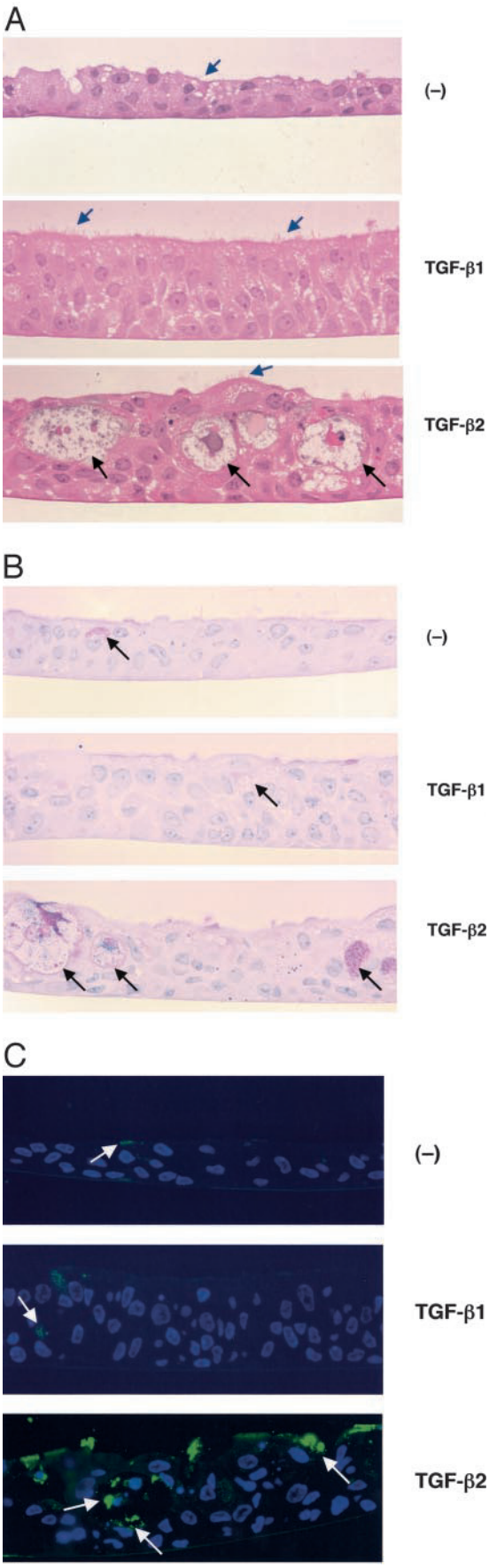
Under the immersed culture system, intracellular MUC5AC protein levels, as evaluated by dot-blot immunoassay, in unstimulated first passage cells were similar between normal [median (interquartile range), 38 arbitrary units (15 to 55)], and asthmatic [35 arbitrary units (3 to 101); $P = 0.95$] patients. After TGF-β2 stimulation, MUC5AC protein expression increased in 9 of 13 subjects (normal and asthmatic patients) ($P = 0.09$) (Figure 5, A and B), and there were no differences between the two groups. However, TGF-β1 did not increase MUC5AC protein expression (Figure 5, A and B). MUC5AC mRNA expression levels were low in unstimulated epithelial cells in both subject groups. As shown in Figure 5C, TGF-β2, but not TGF-β1, significantly increased MUC5AC mRNA expression levels.

To further evaluate mucin induction by TGF-β2, epithelial cell ALI culture was performed in seven patients (three normal subjects, four asthmatics). Both general mucin (as evaluated by AB/PAS staining) and specific mucin MUC5AC levels were significantly higher in cells stimulated with TGF-β2, but not TGF-β1 (Figure 6, A and B) as compared to unstimulated cells. Figure 7, A to C, further illustrates mucin induction by TGF-β2, but not TGF-β1. Goblet cells under TGF-β2 stimulation appear to be larger than unstimulated or TGF-β1-stimulated cells. An increase of cell layer depth appears to occur in cells stimulated with both isoforms of TGF-β. As compared to unstimulated cells, MUC5AC mRNA expression levels increased approximately twofold in TGF-β2-stimulated cells [relative expression levels: unstimulated cells, 1.1 (0.7 to 2.2); TGF-β2-stimulated cells, 2.1 (1.5 to 3.5); $P = 0.005$], whereas no increase was seen in TGF-β1-stimulated cells ($P = 0.24$).

Induction of TGF-β2, but Not TGF-β1, by IL-13: in Vitro *Association with Mucin Expression*

Under immersed culture conditions, IL-13 stimulation consistently increased total TGF-β2 levels in the epithelial supernatants (Figure 8). Similarly, IL-13 increased active TGF-β2 levels (unstimulated cells, 39 $\pm$ 4 pg/ml; IL-13-stimulated cells, 75 $\pm$ 12 pg/ml; $P = 0.007$). In contrast, IL-13 had no effect on total TGF-β1 release (unstimulated cells, 95 $\pm$ 13 pg/ml; IL-13-stimulated cells, 96 $\pm$ 15 pg/ml; $P = 0.85$). Active TGF-β1 in IL-13-stimulated cell supernatants was below the detection limit. Intracellular MUC5AC protein levels in IL-13-stimulated cells from both normal and asthmatic subjects were marginally higher than those in nonstimulated cells ($P = 0.10$; Figure 9A). However, MUC5AC mRNA expression was significantly increased after IL-13 stimulation ($P = 0.046$, Figure 9B). When cells were treated with IL-13 in the presence of a TGF-β2-neutralizing antibody, intracellular MUC5AC protein and mRNA tended to decrease (Figure 9, A and B). There was a significant inverse correlation between TGF-β2 levels in IL-13-stimulated cells and MUC5AC protein levels in epithelial cells treated with IL-13 plus a TGF-β2-neutralizing antibody ($\rho = -0.78$, $P = 0.008$). This suggests that the inhibition of intracellular MUC5AC protein expression by the TGF-β2-neutralizing antibody in IL-13-stimulated cells may be more effective in those epithelial cells with high levels of TGF-β2 expression induced by IL-13. As compared to unstimulated epithelial cells, the TGF-β2-neutralizing antibody alone did not significantly alter MUC5AC expression (Figure 9, A and B).

Similar to the immersed cell culture system, addition of IL-13 into the ALI culture system significantly increased both total and active TGF-β2 release (Table 2). Unlike the immersed cell culture system, IL-13 slightly, but significantly reduced TGF-β1 release in the ALI culture system (Table 2). Additionally, as shown in Figure 10, A and B, IL-13 significantly increased mucin protein expression. IL-13 stimulation increased MUC5AC mRNA expression by approximately threefold [relative levels: unstimulated cells, 1.1 (0.7 to 2.2), IL-13-stimulated cells, 3.2 (1.7 to 5.5); $P = 0.05$]. Addition of a TGF-β2-neutralizing antibody reduced IL-13-induced intracellular protein mucin expression by $\sim$10-fold and mRNA expression by approximately twofold. Figure 11 illustrates the marked visible reduction of IL-13-induced mucin expression by the TGF-β2-neutralizing antibody. Because only two individual ALI cell cultures using the TGF-β2-neutralizing anti-



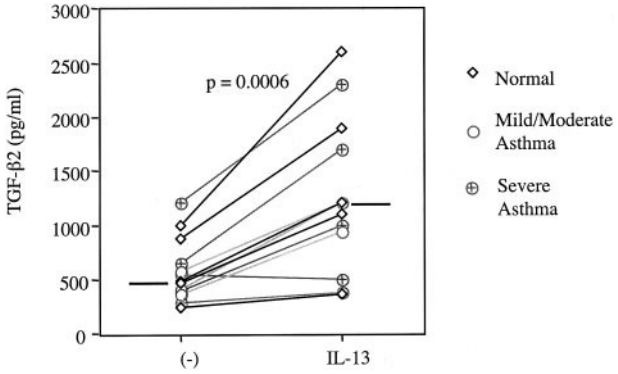


Figure 8. IL-13 significantly increased the release of total TGF-β2 protein from primary bronchial epithelial cells of both normal and asthmatic patients. Epithelial cells were cultured under the immersed culture conditions and incubated for 48 hours in the absence and presence of IL-13 (10 ng/ml).

body were available, no statistical analyses were attempted.

Discussion

This study demonstrated that TGF-β2 expression in asthmatic biopsy bronchial epithelium was significantly higher than in normal subjects and correlated with epithelial mucin expression. In contrast, expression of TGF-β1 (the most studied TGF-β isoform) in bronchial epithelial cells was low and not associated with airway mucin expression. *In vitro* data of cultured primary bronchial epithelial cells supported the *in vivo* data that epithelial cells express higher levels of TGF-β2 than TGF-β1. IL-13 induced the expression of TGF-β2, but not TGFβ-1. Furthermore, TGF-β2, but not TGF-β1, directly increased epithelial mucin expression at both mRNA and protein levels.

Previous studies have demonstrated that TGF-β1 and TGF-β2 similarly increased fibroblast collagen production.[19,20] However, comparison studies in epithelial cells have not been performed. Our current study is the first to compare the expression of both TGF-β1 and TGF-β2 in bronchial epithelia from biopsy specimens of asthmatic and normal subjects. TGF-β2 expression levels in the epithelia were higher than TGF-β1 in both asthmatic and normal subjects. Even without stimulation, a similar predominance of TGF-β2 was noted in cultured bronchial epithelial cells. Our findings of high levels of TGF-β2 and low levels of TGF-β1 in bronchial epithelia are consistent with a previous study showing absent TGF-β1 and high levels of TGF-β2 expression in human salivary glands and pleomorphic adenomas.[21] Moreover, TGF-β2 levels

Figure 7. Representative photomicrographs of a severe asthmatic bronchial epithelial cells grown under the ALI condition for 10 days and incubated in the absence (−) and presence of TGF-β1 (100 pg/ml) and TGF-β2 (100 pg/ml). **A:** H&E staining demonstrating mucociliary differentiation of epithelial cells, especially those treated with TGF-β2. **Blue** and **black arrows** indicate cilia and goblet cells, respectively. **B:** General mucin AB/PAS staining demonstrating intracellular mucin protein expression. **C:** Mucin MUC5AC immunofluorescent staining demonstrating intracellular MUC5AC protein expression. The green and blue colors represent MUC5AC and nuclear DAPI staining, respectively. The **white arrows** indicate MUC5AC-expressing cells. Original magnifications, ×400.

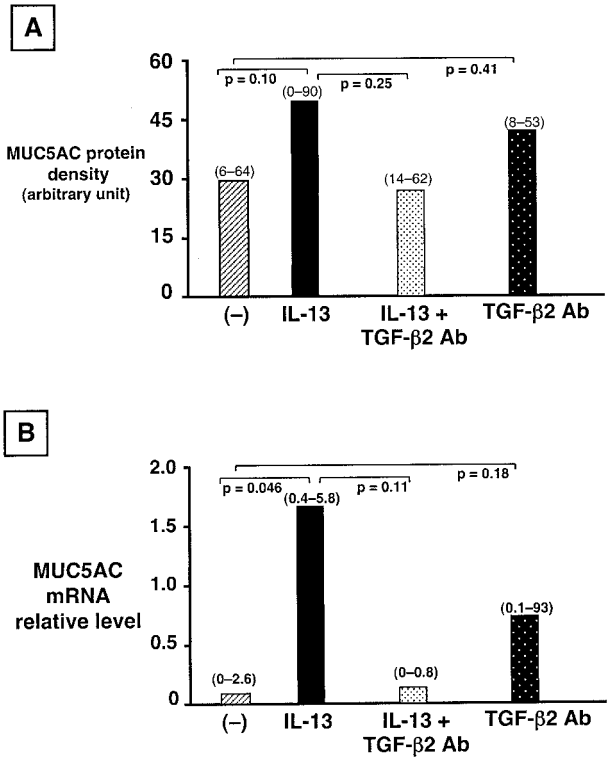


Figure 9. A TGF-β2-neutralizing antibody (TGF-β2 Ab) partially blocked IL-13-induced mucin MUC5AC intracellular protein (**A**) and mRNA (**B**) expression by primary bronchial epithelial cells of both normal and asthmatic patients. Epithelial cells were cultured under the immersed culture condition and incubated for 48 hours in the absence and presence of IL-13 (10 ng/ml), IL-13 (10 ng/ml) + a TGF-β2-neutralizing antibody (1 μg/ml), and a TGF-β2-neutralizing antibody (1 μg/ml) alone.

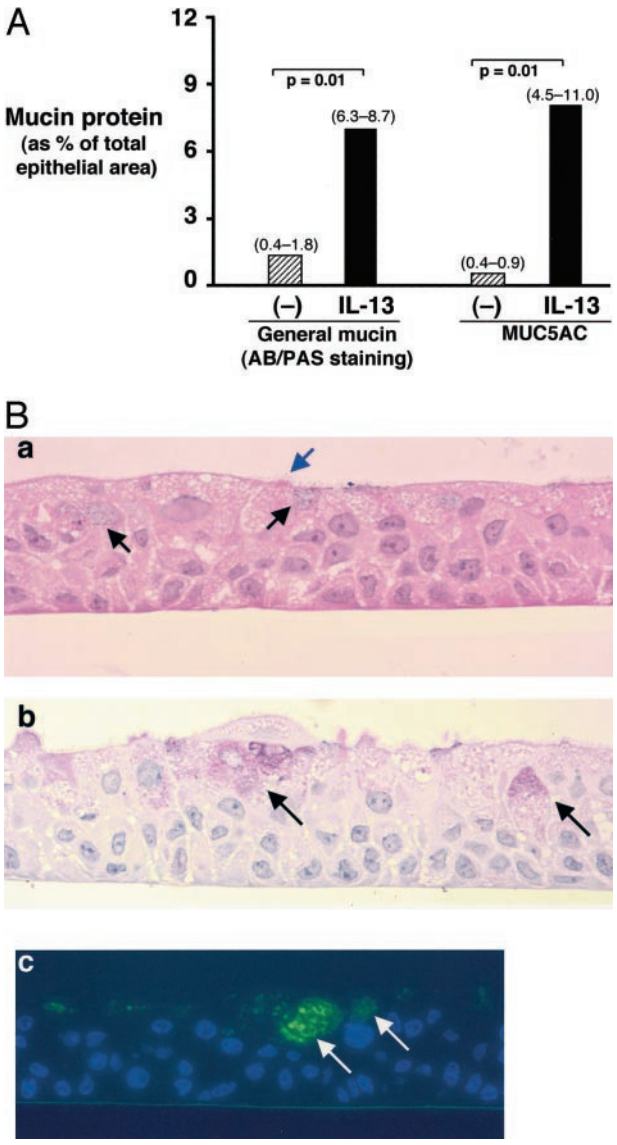


Figure 10. Effects of IL-13 on mucin expression in the ALI culture system. Epithelial cells were incubated for 10 days in the absence and presence of IL-13 (10 ng/ml). **A:** IL-13 significantly increased mucin protein expression. **B:** Representative photomicrographs of severe asthmatic bronchial epithelial cells grown under the ALI condition for 10 days with IL-13 (10 ng/ml). **a:** H&E staining demonstrating mucociliary differentiation of epithelial cells. **Blue** and **black arrows** indicate cilia and goblet cells, respectively. **b:** General mucin AB/PAS staining demonstrating intracellular mucin protein expression. **c:** Mucin MUC5AC immunofluorescent staining demonstrating intracellular MUC5AC protein expression. The green and blue colors represent MUC5AC and nuclear DAPI staining, respectively. The **white arrows** indicate MUC5AC-expressing cells. Data expressed as medians [interquartile (25 to 75%) range]. Original magnifications, ×400.

in bronchial epithelia from endobronchial biopsies were higher in asthmatic as compared to normal subjects. In contrast to TGF-β2, epithelial TGF-β1 did not distinguish the groups, supporting a previous study showing no differences of TGF-β1 mRNA expression in bronchial epithelia between control and asthmatic patients.[2]

The potential roles of epithelial TGF-β2 in airway remodeling remain unclear, but increased TGF-β2 expression in asthmatic epithelia may contribute to subepithelial fibrosis, as well as modulating epithelial functions in an autocrine manner. Our initial observation of a significant correlation between epithelial TGF-β2 and mucin expression in bronchial biopsy tissues led us to hypothesize a potential role for TGF-β2 in induction of mucin expression. To test this hypothesis, we cultured first passage bronchial epithelial cells from asthmatic and normal subjects in both immersed and ALI culture systems, and evaluated mucin expression in the cells after TGF-β2 stimulation. TGF-β2 alone increased mucin expression at both transcriptional and translational levels with more profound induction in the ALI system than the immersed culture system. In contrast, TGF-β1 had no significant effects on mucin expression. Interestingly, after one passage *ex vivo*, epithelial cells from asthmatics and normal patients displayed similar mucin expression levels, both basally and with addition of TGF-β2. This suggests that TGF-β2-induced bronchial epithelial mucin expression is not a disease-specific phenomenon. A previous study of

Table 2. TGF-β Protein Levels (pg/ml) in Epithelial Supernatants (Air-Liquid Interface Culture)

TGF-β isoforms	Medium	IL-13	*P* value
Total TGF-β1	113 ± 15	78 ± 13	0.014
Active TGF-β1	<16	<16	NS
Total TGF-β2	1615 ± 384	4401 ± 1185	0.029
Active TGF-β2	22 ± 13	71 ± 22	0.015

NS = not significant.
N = 7 (3 normal subjects, 4 asthmatic subjects).

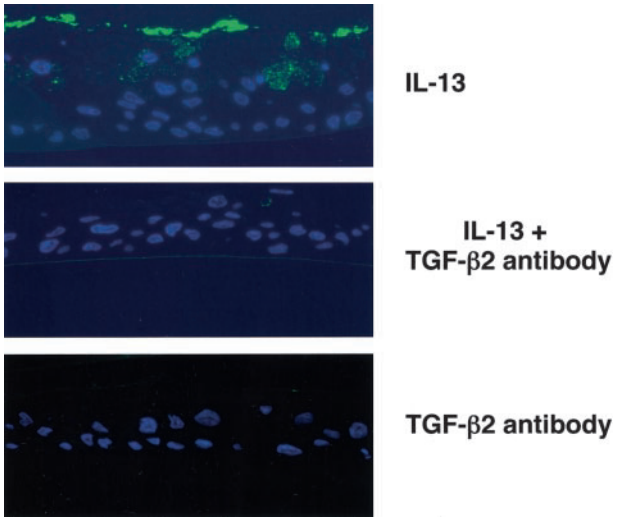


Figure 11. Representative photomicrographs of MUC5AC immunofluorescent staining of severe asthmatic bronchial epithelial cells grown under the ALI condition for 10 days with IL-13 (10 ng/ml), IL-13 + TGF-β2-neutralizing antibody (1 μg/ml), or TGF-β2-neutralizing antibody (1 μg/ml) alone.

cultured human airway fibroblasts showed similar patterns of responses between normal control and asthmatic patients.[15] However, our current study still suggests a role for disease-related TGF-β2 in airway mucin induction. Likely on the basis of environmental factors specific to asthma, TGF-β2 was increased in asthmatic bronchial epithelia *in vivo*, and *in vitro* significantly induced mucin expression by bronchial epithelial cells.

Although this is the first study to demonstrate mucin induction by TGF-β2 in primary human bronchial epithelial cells, the mechanisms by which TGF-β2, but not TGF-β1, induces mucin expression remain unknown. Theoretically, differences in cell proliferation and signaling involved in mucociliary differentiation are likely responsible for the different effects of TGF-β2 and TGF-β1 on mucin expression. The ALI culture system appeared to show an increase in cell layers after TGF-β1, TGF-β2, as well as IL-13, suggesting a role of these mediators in epithelial proliferation. Future studies to address the potential proliferative effects of TGF-β on epithelial cells in parallel with effects on epithelial mucociliary differentiation should help to clarify this relationship. Previous studies have suggested that the family of TGF-β (1, 2, and 3) stimulates cells through the binding and activation of TGF-β receptors. Ligand binding of type II TGF-β receptor recruits and phosphorylates the type I TGF-β receptor, which eventually leads to regulation of TGF-β-responsive genes through activation and nuclear translocation of Smad transcription factors.[22] The differential signaling by TGF-β1 and TGF-β2 has not been well studied, but the considerable phenotypic differences in TGF-β1 versus TGF-β2 gene knockout mice suggest the differential signaling of TGF-β1 and TGF-β2 in certain cell types.[23–25] Whether this difference occurs at the receptor level or downstream requires further study. A recent study suggested that activated Smad3/4 may induce mucin MUC2 expression in a human colon epithelial cell line via the activation of nuclear factor-κB.[26] Similar to MUC2, the MUC5AC promoter also has the binding site for nuclear factor-κB, suggesting a similar activation process for MUC5AC gene transcription. Further studies are needed to address the potential differences of TGF-β1 and TGF-β2 signaling pathways and their contribution to mucin expression in cultured human primary bronchial epithelial cells.

In the current study, we attempted to elucidate the mechanisms by which bronchial epithelial TGF-β2, but not TGF-β1, was up-regulated *in vivo*. IL-13 consistently induced the expression of TGF-β2, but not TGF-β1, in primary bronchial epithelial cells from both asthmatic and normal subjects. These results are similar to two previous studies that showed increased release of TGF-β2 from bronchial epithelial cells after IL-13 stimulation under immersed culture conditions.[10,11] In the study by Wen and co-workers,[10] the bronchial epithelial cells were obtained from bronchial tissue of a nonasthmatic patient at autopsy. Although Richter and co-workers[11] evaluated the effects of IL-13 on TGF-β2 release from cultured bronchial epithelial cells, only asthmatics were recruited and TGF-β1 release was not simultaneously evaluated with TGF-β2. Therefore, our current study has extended previous studies by including both asthmatic and normal subjects, and by comparing the effects of IL-13 on both TGF-β1 and TGF-β2 in both immersed and ALI culture systems. In the ALI culture system, IL-13 slightly decreased TGF-β1 release, which has not been reported in human primary airway epithelial cells. Thus, these data, in addition to data from others all indicate a pivotal role of the Th2 cytokine IL-13 in the induction of TGF-β2 expression by bronchial epithelial cells. Besides IL-13, other factors could also regulate TGF-β2 expression by bronchial epithelial cells. Mechanical stress, direct mechanical damage to the cells, and IL-4 have also been shown to increase TGF-β2 expression.[27,28] On the other hand, the Th1 cytokine interferon-gamma inhibits IL-13-induced TGF-β2 production.[29]

Although elucidating the mechanisms by which IL-13 induces airway epithelial mucin expression was not the major focus of this study, the role of TGF-β2 in IL-13-induced mucin expression was explored. We found that IL-13-induced mucin expression was partially blocked by a TGF-β2-neutralizing antibody. Those cells with higher TGF-β2 protein expression after IL-13 stimulation showed the most significant reduction in intracellular mucin expression with TGF-β2-neutralizing antibody treatment. Although considerable work remains to determine the mechanisms by which IL-13 up-regulates TGF-β2 and consequently induces mucin expression, it is conceivable that TGF-β2 may be an important mediator in IL-13-induced mucin expression in asthma when the following data are considered: 1) IL-13, a cytokine known to be increased in asthmatic lungs,[30,31] increased TGF-β2 expression; 2) TGF-β2 directly induced mucin expression; and 3) IL-13-induced mucin expression was partially blocked by a TGF-β2-neutralizing antibody.

In summary, our study suggests that Th2 cytokines such as IL-13 can induce TGF-β2 expression *in vitro*, which may in part explain the increased expression of

TGF-β2 by asthmatic bronchial epithelial cells *in vivo*. Although the significance of TGF-β2 in airway epithelial biology remains to be determined, this study suggests that TGF-β2 may be involved in host defense and wound repair by promoting mucin expression. Future studies are warranted to elucidate the molecular mechanisms by which IL-13 augments airway epithelial TGF-β2 production, and TGF-β2 induces mucin expression. It is anticipated that these future studies will determine whether TGF-β2 signaling may be one of the therapeutic targets for the treatment of mucin overexpression in asthma and perhaps other pulmonary diseases.

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