Airway epithelial cells from asthmatic children differentially express proremodeling factors

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Background: The airway epithelium can express factors that drive subepithelial airway remodeling. TGF-β2, vascular epithelial growth factor (VEGF), a disintegrin and metalloprotease 33 (ADAM33), and periostin are hypothesized to be involved in subepithelial remodeling and are overexpressed in adult asthmatic airways. Epidemiologic data suggest that lung function deficits in asthmatic patients are acquired in childhood.

Objectives: We sought to determine whether airway epithelial cells (AECs) from asthmatic children differentially express TGF-β2, VEGF, ADAM33, or periostin compared with cells from atopic nonasthmatic and healthy children intrinsically or in response to IL-4/IL-13 stimulation.

Methods: Bronchial and nasal epithelial cells were obtained from brushings from well-characterized asthmatic (n = 16), atopic nonasthmatic (n = 9), and healthy (n = 15) children after achievement of anesthesia for elective procedures. After differentiation at an air-liquid interface (ALI) for 3 weeks, conditioned media were sampled and RNA was extracted from unstimulated and IL-4/IL-13–stimulated cultures. TGF-β2 and VEGF levels were measured with ELISA. ADAM33 and periostin expression was assessed by using real-time PCR.

Results: TGF-β2 and VEGF production was significantly greater in bronchial and nasal ALI cultures from asthmatic children than in cultures from atopic nonasthmatic and healthy children. TGF-β2 levels increased significantly in asthmatic cultures after IL-4/IL-13 stimulation. Within-subject correlation between nasal and bronchial ALI production of TGF-β2 ($r = 0.64$, $P = .001$) and VEGF ($r = 0.73$, $P < .001$) was good. Periostin expression was 3.7-fold higher in bronchial cells ($P < .001$) and 3.9-fold higher in nasal cells ($P < .004$) from asthmatic children than in cells from atopic nonasthmatic or healthy children. ADAM33 was not differentially expressed by AECs from asthmatic patients compared with that from cells from atopic nonasthmatic or healthy children.

Conclusion: AECs from asthmatic children differentially express TGF-β2, VEGF, and periostin compared with cells from atopic nonasthmatic and healthy children. Nasal epithelial cells might be a suitable surrogate for bronchial cells that could facilitate investigation of the airway epithelium in future longitudinal pediatric studies. (J Allergy Clin Immunol 2012;129:990-7.)

Key words: *Asthma, children, airway remodeling, epithelial cells vascular endothelial growth factor, a disintegrin and metalloprotease 33, periostin, TGF-β2*

Longitudinal studies following children with wheezing and asthma into the teenage years and adulthood have provided insight into the progression of asthma in children. Data from the Tucson Children's Respiratory Study suggest that pulmonary function in children who ultimately have persistent asthma is normal at birth, with deficits acquired by as early as 6 years of age.[1] Lung function data from a large cohort study in Melbourne, Australia, with follow-up from early childhood through age 35 years revealed that children with asthma had lower lung function during childhood and at age 35 years compared with that seen in cohort members without a history of childhood asthma.[2] Interestingly, lung function of cohort members, including those with severe asthma, did not worsen after age 10 years, suggesting that lifelong lung function deficits among asthmatic patients were acquired by age 10 years. Endobronchial biopsy specimens from asthmatic children show features of airway remodeling.[3,4] Together, these data suggest that structural changes in the asthmatic airway that might result in permanent lung function deficits occur early in the natural history of asthma.

In addition to serving as a passive barrier, the airway epithelium is the interface between the environment and the host and appears to play a critical role in orchestrating the host's inflammatory response.[5] There is an emerging consensus that the response of the asthmatic airway epithelium to environmental insults, infection, and inflammatory stimuli is dysfunctional, with resultant airway epithelial cell (AEC) expression of genes and factors that drive subepithelial airway remodeling.[6] *In vivo* animal models of airway remodeling[7-9] and descriptive data from human bronchial biopsy specimens[10-12] implicate several epithelium-derived cytokines in the promotion of airway fibrosis and angiogenesis, including TGF-β2 and vascular endothelial growth factor (VEGF). AECs can secrete TGF-β2, and this growth factor is overexpressed in airway epithelium obtained during bronchial biopsy specimens from adult asthmatic patients.[10] Vascular remodeling is another characteristic feature of the asthmatic airway.[13,14] Data from animal models suggest that VEGF is an important mediator of angiogenesis, vascular permeability, and structural changes in the asthmatic airway.[15,16] VEGF and VEGF receptor are reported to be overexpressed in

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Abbreviations used
ADAM33: A disintegrin and metalloprotease 33
AEC: Airway epithelial cell
ALI: Air-liquid interface
ATS: American Thoracic Society
BEGM: Bronchial epithelial growth medium
Ct: Cycle threshold
FEF_{25-75}: Forced expiratory flow between 25% and 75% of expiration
FENO: Fraction of exhaled nitric oxide
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
ICS: Inhaled corticosteroid
VEGF: Vascular epithelial growth factor

adult asthmatic airways,[17] and VEGF levels in sputum and lung tissue are increased in asthmatic patients and correlate with disease severity.[11,18,19]

A disintegrin and metalloprotease 33 (ADAM33) has been found in several diverse populations to be an asthma susceptibility gene.[20] This transmembrane protein is believed to play a role in cell proliferation, differentiation, adhesion, signaling, and apoptosis among other functions.[21] In a cohort of Dutch patients with asthma followed for 20 years, ADAM33 single nucleotide polymorphisms were found to be associated with an accelerated decrease in lung function.[22] In a clinical study of Korean adults with asthma, ADAM33 protein levels in bronchoalveolar lavage fluid were inversely associated with lung function.[23] In addition, ADAM33 expression was recently found to be higher in epithelial cells, submucosal cells, and smooth muscle from patients with moderate-to-severe asthma compared with that seen in patients with mild asthma and healthy control subjects.[24]

In a recent microarray study of gene expression in airway epithelial biopsy specimens, expression of the matricelluar protein periostin was found to be differentially upregulated in adults with asthma compared with that seen in healthy adults.[25] Furthermore, periostin expression has been shown to be upregulated in bronchial epithelial cells from adults with asthma grown *in vitro* and stimulated with IL-13 and appears to play a role in activating TGF-β signaling pathways and promoting type 1 collagen production by airway fibroblasts.[26]

Using bronchial epithelial cells obtained from well-characterized atopic asthmatic children, atopic nonasthmatic children, and healthy children, one of our objectives was to determine whether lower AECs from asthmatic children differentiated at an air-liquid interface (ALI) intrinsically express growth factors and genes that have been associated with airway remodeling in animal models and human studies of adults with asthma, including TGF-β2, vascular endothelial growth factor (VEGF), periostin, and ADAM33. In addition, we sought to characterize expression of these factors by AECs in response to T_H2 cytokine stimulation (IL-4 and IL-13). Our overall hypothesis was that AECs from children with asthma express a unique profile of factors that influence subepithelial mesenchymal remodeling. Finally, we compared the within-subject gene expression and cytokine production of bronchial and nasal epithelial cells to determine whether nasal cells can be used as a noninvasive proxy for bronchial cells in future longitudinal studies. Some of these results have been reported in abstract form.[27]

METHODS

Subjects

Atopic asthmatic, atopic nonasthmatic, and healthy children aged 6 to 16 years who underwent an elective surgical procedure requiring endotracheal intubation and general anesthesia were recruited for this study. A detailed medical history was obtained at enrollment to ensure participants met the following inclusion and exclusion criteria. Asthmatic children had a 1-year or greater history of physician-diagnosed asthma, had physician-documented wheezing in the 12 months before enrollment, used albuterol twice or more a month or were taking a daily inhaled corticosteroids (ICSs) or leukotriene receptor antagonists, and were born at 36 weeks' gestation or later. Healthy and atopic nonasthmatic subjects were born at 36 weeks' gestation or later and had no history of asthma, reactive airway disease, chronic daily cough, or physician-diagnosed obstructive lung disease, and no history of prior treatment with a systemic corticosteroid or ICS, albuterol, or oxygen. Children with asthma, as well as atopic nonasthmatic children, had a history of 1 or more of the following atopic features: positive skin prick test or RAST results for a common aeroallergen, increased serum IgE levels (>100 IU/mL), physician-diagnosed allergic rhinitis, and physician-diagnosed atopic dermatitis. Healthy subjects lacked a history of any of these atopic features.

Written consent was obtained from subjects' parents, and assent was obtained for children 7 years or older. The study was approved by the Seattle Children's Hospital Institutional Review Board.

Epithelial cell isolation

Immediately after the endotracheal tube was secured, 3 bronchial epithelial cell samples were obtained from subjects after achievement of general anesthesia with 4-mm Harrell unsheathed bronchoscope cytology brushes (ConMed Corp, Utica, NY). As described by Lane et al,[28] the unprotected brush was inserted through an endotracheal tube, advanced until resistance was felt, and rubbed against the airway surface for 2 seconds. Simultaneous nasal brushings were also obtained in each subject. Cells were seeded onto T-25 cell culture flasks precoated with type I collagen. Cultures were maintained at 37°C in a 5% CO_2 atmosphere in a humidified incubator. Cells were cultured in bronchial epithelial growth medium (Clonetics BEGM; Lonza, Basel, Switzerland) containing gentamicin and amphotericin B and further supplemented with penicillin-streptomycin (100 μg/mL; Invitrogen, Carlsbad, Calif). Fluconazole (25 μg/mL) was added to primary cultures for the first 96 hours, after which medium was aspirated and replaced with BEGM without fluconazole. BEGM was thereafter changed every 48 hours until the culture reached approximately 70% to 90% confluence. When P_0 flasks became confluent, cells were passaged into 3 new $passage_1$ (P_1) flasks.

ALI epithelial cell cultures

For ALI cultures, Corning Costar 12-mm 0.4-μm Transwells (Corning Life Sciences, Corning, NY) were precoated with type I collagen and then seeded with P_2 epithelial cells at a concentration of 100,000 cells per transwell. All seeded cells screened negatively for *Mycoplasma* species infection (Mycoprobe Mycoplasma Kit; R&D Systems, Inc, Minneapolis, Minn). Cells were grown submerged in BEGM until 100% confluent, at which time apical medium was removed and basolateral medium was replaced with ALI medium consisting of a 1:1 mixture of BEGM and Dulbecco modified Eagle medium supplemented with all-trans retinoic acid (30 ng/mL), human recombinant epidermal growth factor (EGF; 0.5 ng/mL), $MgCl_2$ (0.6 mmol/L), $CaCl_2$ (1 mmol/L), and penicillin-streptomycin (100 μg/mL). ALI medium in the basolateral compartment was changed every other day, and cells were differentiated at an ALI for 21 days before initiation of experiments (see Fig E1 in this article's Online Repository at www.jacionline.org). Each experimental condition per cell line consisted of triplicate transwells. For transwells stimulated with IL-4 and IL-13, 50 ng/mL of each cytokine was added to basolateral medium. Sampling of the basolateral conditioned medium and RNA extraction was performed 48 hours after IL-4/IL-13 stimulation and 48 hours after a medium change for unstimulated transwells.

TABLE I. Subjects' characteristics

	Healthy control subjects (n = 15)	Atopic nonasthmatic patients (n = 9)	Asthmatic patients (n = 16)	*P* value
Age (y [SD])	12.2 (4.6)	10.8 (4.1)	11.4 (3.9)	.6
Female sex (%)	47	44	38	.8
Current use of ICSs (yes; %)	NA	NA	7 (44)	NA
IgE (IU/mL [SD])	40 (12)	252 (519)	341 (127)	**.02**
FVC (% predicted [SD])	105 (8.5)	110 (9.2)	106 (14.5)	.4
FEV_1/FVC ratio (SD)	0.84 (0.05)	0.85 (0.1)	0.77 (0.08)	**.03**
FEV_1 (% predicted [SD])	100 (10.5)	109 (11)	94 (14.7)	**.03**
FEF_{25-75} (% predicted [SD])	102 (27.7)	102 (23.3)	72 (25.6)	**.001**
BDR (yes; %)	NA	NA	9 (56)	NA
FENO (ppb [SD])	10.3 (5.4)	14.9 (15.5)	36.0 (22.1)	**.04**

BDR, Bronchodilator responsiveness (defined as an increase of ≥12% in FEV_1 or ≥25% in FEF_{25-75} after 2 puffs of albuterol through a metered-dose inhaler); *FVC*, forced vital capacity.

RNA extraction and real-time PCR

Three transwells from each experimental condition were harvested and pooled to isolate RNA by using the RNAqueous kit for total RNA purification from Ambion–Applied Biosystems (Austin, Tex). RNA concentration and integrity were determined by using the Agilent 2100 Bioanalyzer system and Agilent RNA 6000 Nano Chips (Agilent Technologies, Foster City, Calif). RNA samples (1 μg) with an RNA integrity number of 8 or greater were reverse transcribed with Moloney murine leukemia virus reverse transcriptase with a combination of random hexamers and oligo-dTs by using the SuperScript VILO cDNA Synthesis Kit from Invitrogen. Samples were diluted to a final volume of 100 μL (10 ng/μL). Semiquantitative real-time qPCR was performed by using the SensiMix II probe kit with SYBR Green (Bioline, London, United Kingdom) and in-house validated primers for periostin, ADAM33, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Additional details are provided in the Methods section in this article's Online Repository at www.jacionline.org.

ELISA analyses

For each condition, sampled basolateral medium from triplicate transwells was pooled. Measurement of protein levels of TGF-β2 and VEGF in sampled conditioned media was completed by using Duoset ELISA Development Kits (R&D Systems, Inc), according to the manufacturer's recommendations. The VEGF Duoset is specific for the VEGF-A isoform, without cross-reactivity to VEGF isoforms B, C, or D. All measurements were completed in duplicate.

Subjects' clinical characterization

A blood sample was drawn and used to measure total serum IgE and RAST allergen-specific IgE levels to dust mite, cat epithelium, dog epithelium, *Alternaria tenuis*, *Aspergillus fumigatus*, and timothy grass. The fraction of exhaled nitric oxide (FENO) was measured according to American Thoracic Society guidelines with a NIOX chemiluminescent nitric oxide analyzer (Aerocrine, Solna, Sweden). Forced expiratory volumes and flows (forced vital capacity, FEV_1, and forced expiratory flow between 25% and 75% of expiration [FEF_{25-75}]) were measured according to American Thoracic Society guidelines with a VMAX series 2130 spirometer (VIASYS Healthcare, Hong Kong, China). Spirometry was repeated 15 minutes after administration of 2 puffs of albuterol in asthmatic children.

Statistical analysis

Protein levels are presented as means ± SDs. The Kolmogorov-Smirnov test was used to determine whether data were normally distributed. One-way ANOVA or the Kruskal-Wallis test for nonnormally distributed data was used to compare lung function parameters and FENO values among asthmatic, atopic nonasthmatic, and healthy children and to compare protein levels in ALI culture basolateral conditioned medium among cells from the 3 groups of subjects. The unpaired *t* test or the Mann-Whitney test for nonnormally distributed data was used to compare ALI culture protein levels between the asthmatic and healthy groups and between the asthmatic and atopic nonasthmatic groups. The paired *t* test or the Wilcoxon signed-rank test for nonnormally distributed data was used to compare protein levels in ALI cultures before and after IL-4/IL-13 stimulation. The Pearson coefficient was calculated to determine the intrasubject correlation of protein levels between ALI cultures from bronchial and nasal epithelial cells. Statistical analyses of clinical data and protein levels in ALI cultures were performed with Prism 5.0 software (GraphPad Software, Inc, San Diego, Calif). The relative expression of periostin and ADAM33 was standardized by using GAPDH as a nonregulated reference gene. We used the REST (Relative Expression Software Tool)–MCS software to compare gene expression.[29,30] Final results were expressed as the fold change on a $\log_2$ scale relative to median expression of genes in healthy control samples. Additional details are provided in the Methods section in this article's Online Repository.

RESULTS

Airway epithelial brushings were obtained from 21 asthmatic children, 11 atopic children without asthma, and 19 healthy children. ALI cultures were successfully established, and experiments were completed with cells from 16 asthmatic children, 9 atopic children without asthma, and 15 healthy children. Airway brushings were well tolerated by all subjects, with no adverse events. Baseline characteristics of subjects completing the study are presented in Table I. The mean age of children from the 3 groups was similar. Consistent with inclusion criteria, serum IgE levels were significantly higher in asthmatic and atopic nonasthmatic children than in healthy children. The FEV_1/forced vital capacity ratio, FEV_1, and FEF_{25-75} percent predicted values were significantly lower and the FENO value was significantly higher among children with asthma compared with atopic nonasthmatic and healthy children.

The concentration of TGF-β2 in ALI conditioned culture media of bronchial epithelial cells from children with asthma (Asthma BE group) was significantly higher than that in conditioned media of cells from atopic nonasthmatic (Atopic BE group) and healthy (Healthy BE group) children (Asthma BE group, 1018 ± 709 pg/mL; Atopic BE group, 456 ± 327 pg/mL; and Healthy BE group, 554 ± 372 pg/mL; $P = .02$; Fig 1). TGF-β2 production was also significantly higher in nasal epithelial cell ALI cultures from asthmatic (Asthma NE group) compared with atopic nonasthmatic (Atopic NE group) and healthy (Healthy NE group) children (Asthma NE group, 1067 ± 851 pg/mL; Atopic NE group, 449 ± 244 pg/mL; and Healthy NE group, 516 ± 293 pg/mL; $P = .05$; Fig 1). Stimulation of bronchial and nasal epithelial ALI cultures with IL-4 and IL-13 led to significant increases in TGF-β2 production in cells from asthmatic but not

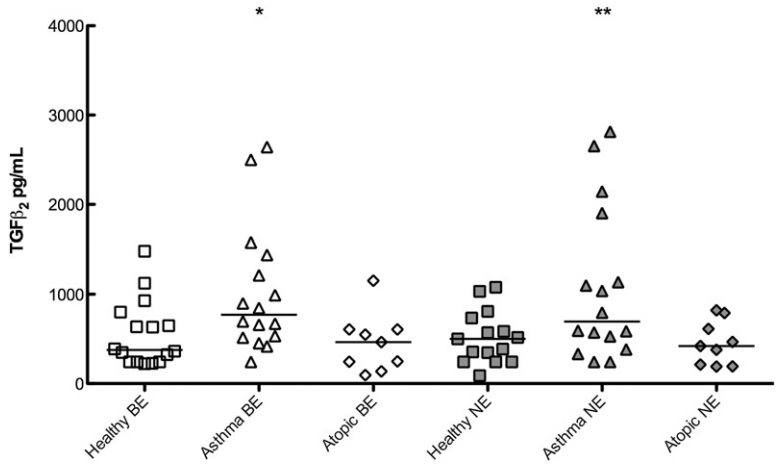


FIG 1. TGF-β2 concentrations in ALI cultures from bronchial epithelial cells from healthy subjects *(Healthy BE),* bronchial epithelial cells from asthmatic patients *(Asthma BE),* bronchial epithelial cells from atopic nonasthmatic patients *(Atopic BE),* nasal epithelial cells from healthy subjects *(Healthy NE),* nasal epithelial cells from asthmatic patients *(Asthma NE),* and nasal epithelial cells from atopic nonasthmatic patients *(Atopic NE).* *Three-way group comparison of Asthma BE versus Healthy BE and Atopic BE: $P = .02$ (Kruskal-Wallis test). Comparison of Asthmatic BE with Healthy BE: $P = .01$ (Mann-Whitney test). Comparison of Asthmatic BE with Atopic BE: $P = .02$ (Mann-Whitney test). **Three-way group comparison of Asthma NE versus Healthy NE and Atopic NE: $P = .05$ (Kruskal-Wallis test). Comparison of Asthmatic NE with Healthy NE: $P = .04$ (Mann-Whitney test). Comparison of Asthmatic NE to Atopic NE: $P = .04$ (Mann-Whitney test).

atopic nonasthmatic or healthy children (see Fig E2 in this article's Online Repository at www.jacionline.org).

VEGF production in ALI cultures of bronchial epithelial cells from children with asthma was significantly higher than that in cells from atopic nonasthmatic and healthy children (Asthma BE group, 1416 ± 864 pg/mL; Atopic BE group, 449 ± 290 pg/mL; and Healthy BE group, 636 ± 449 pg/mL; $P < .001$; Fig 2). VEGF production was also significantly higher in nasal epithelial cell ALI cultures from asthmatic compared with atopic nonasthmatic and healthy children (Asthma NE group, 1331 ± 1076 pg/mL; Atopic NE group, 358 ± 267 pg/mL; and Healthy NE group, 443 ± 289 pg/mL; $P < .001$; Fig 2). There was no change in VEGF production in bronchial or nasal epithelial cells from asthmatic, atopic nonasthmatic, or healthy children after stimulation with IL-4/IL-13 (see Fig E2). Production of TGF-β2 and VEGF in bronchial epithelial cells from asthmatic children who reported ICS use at the time of airway brushings was similar to production in cells from asthmatic children not using ICSs (see Fig E3 in this article's Online Repository at www.jacionline.org).

Production of TGF-β2 in nasal epithelial cells was significantly correlated with bronchial epithelial cell TGF-β2 production within subjects ($r = 0.64$, $P = .001$, Fig 3). Similarly, there was good within-subject correlation of nasal and bronchial epithelial cell production of VEGF ($r = 0.73$, $P < .001$, Fig 3).

In unstimulated bronchial epithelial cell ALI cultures, expression of periostin mRNA was 3.7-fold higher in cells from children with asthma than in cells from healthy children ($P = .0001$, Fig 4), whereas periostin expression in atopic nonasthmatic cultures was similar to expression seen in healthy cells. Periostin expression was 3.9-fold higher in nasal epithelial cells from asthmatic children than in cells from healthy children ($P = .003$), whereas atopic and healthy nasal cell periostin expression was similar. Periostin expression was 2.4-fold higher in asthmatic bronchial epithelial cells stimulated with IL-4 and IL-13 compared with that in unstimulated asthmatic cells ($P = .046$, see Fig E4 in this article's Online Repository at www.jacionline.org). There was no significant difference in periostin expression after IL-4/IL-13 stimulation in bronchial epithelial cells from healthy and atopic subjects. Periostin expression in asthmatic bronchial epithelial cells from children reporting use of ICSs at the time of airway brushings and from asthmatic children who were not using ICSs were both greater than that seen in cells from healthy children (see Fig E5 in this article's Online Repository at www.jacionline.org).

ADAM33 was not differentially expressed in asthmatic AECs compared with cells from atopic nonasthmatic or healthy children (Fig 4). Furthermore, there was no change in ADAM33 expression in AECs from asthmatic, atopic nonasthmatic, or healthy children in response to IL-4 and IL-13 stimulation (see Fig E4).

DISCUSSION

In this *in vitro* study of differentiated AECs from well-characterized asthmatic, atopic nonasthmatic, and healthy children, we found that several factors believed to influence subepithelial airway remodeling were differentially expressed in AECs from asthmatic children. Specifically, production of TGF-β2 and VEGF was significantly higher in AECs from asthmatic children than in cells from atopic or healthy children. In AECs from asthmatic children, stimulation with IL-4 and IL-13 led to an increase in TGF-β2 production. We demonstrated good intrasubject correlation of *in vitro* bronchial and nasal epithelial production of both TGF-β2 and VEGF. Finally, we showed that expression of periostin was significantly higher in both bronchial and nasal epithelial cells from children with asthma than in cells from atopic or healthy children.

TGF-β2 expression in bronchial epithelial cells from adult asthmatic patients has been shown to be greater than expression in cells from healthy subjects.[28] We have now demonstrated that TGF-β2 expression in the airway epithelium is also increased in children with asthma compared with that seen in atopic or

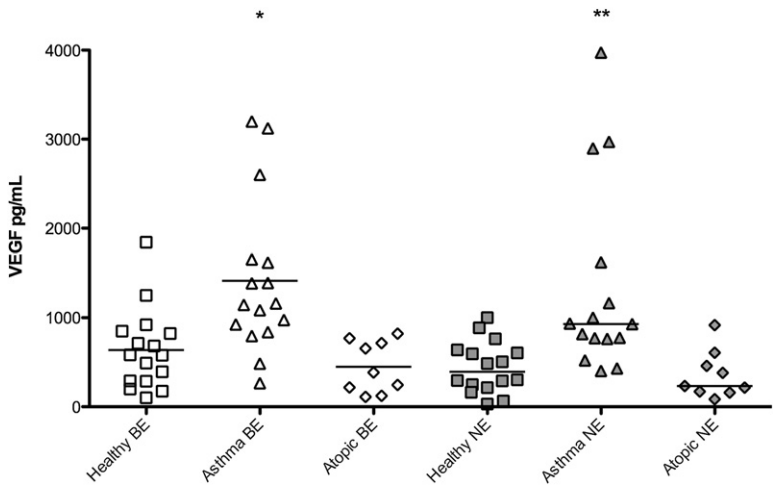


FIG 2. VEGF concentration in ALI cultures from bronchial epithelial cells from healthy subjects *(Healthy BE)*, bronchial epithelial cells from asthmatic patients *(Asthma BE)*, bronchial epithelial cells from atopic nonasthmatic patients *(Atopic BE)*, nasal epithelial cells from healthy subjects *(Healthy NE)*, nasal epithelial cells from asthmatic patients *(Asthma NE)*, and nasal epithelial cells from atopic nonasthmatic patients *(Atopic NE)*. *Three-way group comparison of Asthma BE versus Healthy BE and Atopic BE: $P < .001$ (1-way ANOVA). Comparison of Asthmatic BE with Healthy BE: $P = .003$ (unpaired *t* test). Comparison of Asthmatic BE with Atopic BE: $P = .004$ (unpaired *t* test). **Three-way group comparison of Asthma NE versus Healthy NE and Atopic NE: $P < .05$ (Kruskal-Wallis test). Comparison of Asthmatic NE with Healthy NE: $P < .001$ (Mann-Whitney test). Comparison of Asthmatic NE with Atopic NE: $P = .001$ (Mann-Whitney test).

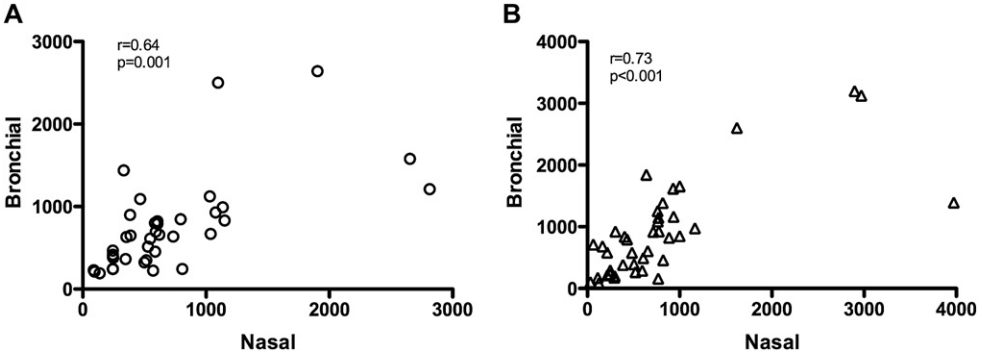


FIG 3. Correlation between bronchial and nasal epithelial cell ALI cultures for production of TGF-β2 **(A)** and VEGF **(B)**.

healthy children. Chu et al[31] found that epithelial TGF-β2 expression in bronchial mucosa from adult asthmatic patients was correlated with mucin expression, and *in vitro* they showed that IL-13 stimulation of bronchial epithelial cells significantly increased TGF-β2 expression. Furthermore, they found that TGF-β2 stimulation of bronchial epithelial cells grown *in vitro* directly increased mucin expression. Our data are consistent with the findings of Chu et al[31]; however, in our experiments cells were stimulated with a combination of IL-4 and IL-13. Using normal bronchial epithelial cells differentiated at an ALI and cocultured with normal human fibroblasts, Thompson et al[32] showed that an epithelial scrape injury led to a 2- to 3-fold increase in TGF-β2 production, which subsequently enhanced α-smooth muscle actin expression in the underlying collagen-embedded fibroblasts and secretion of tenascin-C into the matrix. By using a similar model system, Malavia et al[33] demonstrated that IL-13 stimulation also resulted in an increase in TGF-β2 release by bronchial epithelial cells and that TGF-β2 stimulation of AECs led to collagen secretion in the subepithelial matrix. These findings, together with our data, suggest that differential expression of TGF-β2 by the asthmatic airway epithelium might play a role in the development of subepithelial airway remodeling in asthmatic children.

Vascular remodeling is another characteristic feature of the asthmatic airway.[13,14] Data from animal models suggest that VEGF is an important mediator of angiogenesis, vascular permeability, and structural changes in the asthmatic airway.[15] VEGF and VEGF receptor are reported to be overexpressed in adult human asthmatic airways,[18] VEGF levels are increased in sputum from asthmatic children,[34] and VEGF expression is increased in lung tissue from adult asthmatic patients and correlated with disease severity.[11,18,19] Recently, Sharma et al[35] found that VEGF polymorphisms were associated with childhood asthma, lung function, and airway responsiveness. To our knowledge, we now demonstrate, for the first time, using bronchial epithelial cells grown and differentiated *in vitro* from well-characterized children that VEGF expression is significantly greater in AECs from asthmatic children than in cells from atopic or healthy children. Our finding supports the hypothesis that epithelium-derived

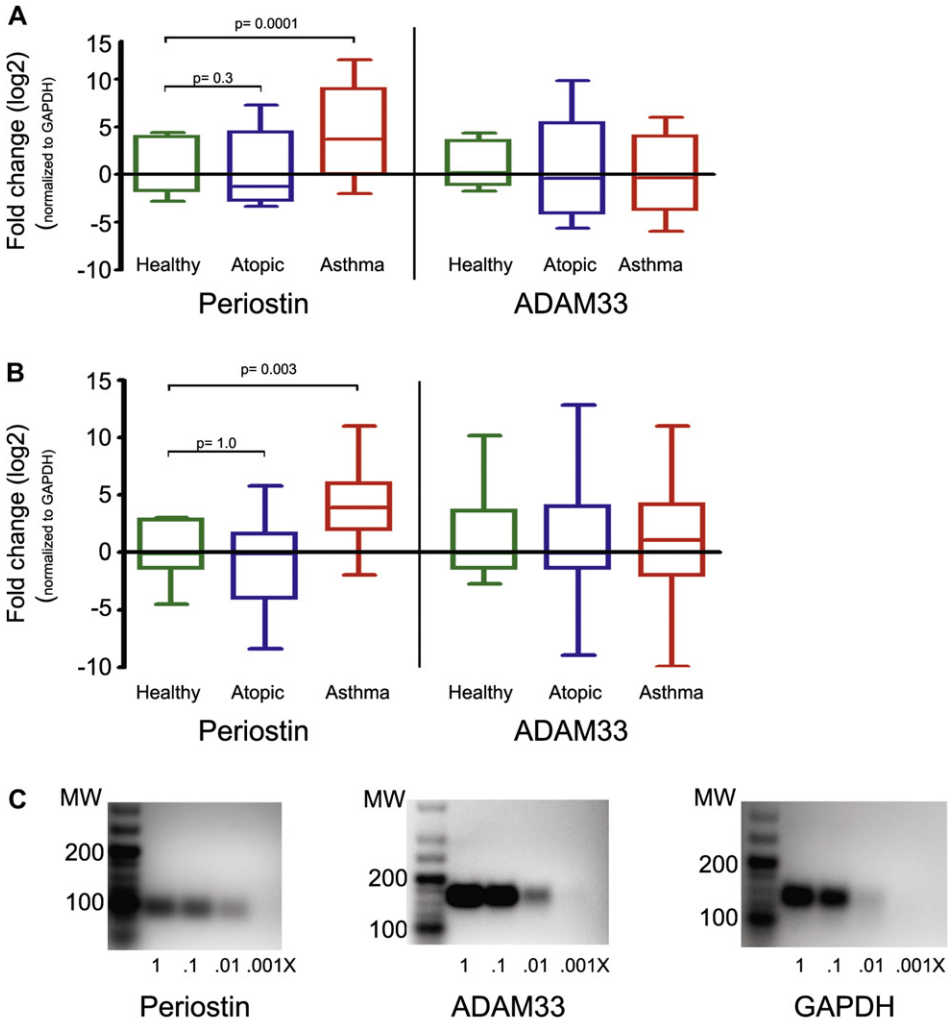


FIG 4. Periostin and ADAM33 mRNA expression in asthmatic bronchial **(A)** and nasal **(B)** epithelial cell ALI cultures compared with expression in cultures from atopic nonasthmatic and healthy children normalized to the housekeeping gene GAPDH. Fig 4, *A*: Periostin overall group differences, $P = .001$; asthmatic patients versus healthy subjects, $P = .0001$. ADAM33 showed no significant between-group differences. Fig 4, *B*: Periostin overall group differences, $P = .05$; asthmatic patients versus healthy subjects, $P = .003$. ADAM33 showed no significant differences between groups. **C,** Periostin, ADAM33, and GAPDH amplicons determined by means of gel electrophoresis.

VEGF might contribute to airway remodeling early in the natural history of asthma.

In this study we have demonstrated significantly greater expression of periostin in bronchial and nasal epithelial cells from children with asthma than in cells from atopic or healthy children, with increased expression in cells from asthmatic patients that are stimulated with IL-4 and IL-13. Periostin is a 90-kd disulfide-linked matricellular protein expressed by connective tissues, bone, myocardium, heart valves, and skin[36,37] that is believed to interact with multiple integrins and be involved in cell proliferation and regeneration, epithelial-mesenchymal transformation, fibrosis, and angiogenesis.[38-40] In 2006, Takayama et al reported that periostin expression was higher in asthmatic than normal airways.[41] Genome-wide profiling of airway epithelium from adult asthmatic patients and healthy control subjects was performed by Woodruff et al,[25] who reported that periostin was differentially upregulated in epithelial tissue from adult asthmatic patients compared with that from healthy subjects and smokers. Sidhu et al[26] recently demonstrated in epithelial ALI cultures that periostin is basally secreted and that its production is enhanced by IL-13 stimulation. Furthermore, they showed that AEC stimulation by periostin led to increased expression of type I collagen and evidence of epithelial to mesenchymal transition. Our finding of differential expression of periostin in AECs from pediatric asthmatic patients is consistent with the findings of Woodruff et al[25] and Sidhu et al[26] and provide additional evidence that periostin might be associated with subepithelial changes in asthma, even at a young age.

Foley et al[24] reported that ADAM33 expression was higher in endobronchial biopsy samples from adults with moderate-to-severe asthma compared with that seen in patients with mild asthma and healthy subjects and that ADAM33 immunostaining

was increased in the epithelium, submucosal glands, and smooth muscle in biopsy specimens from patients with severe asthma. In contrast, Yang et al[42] found that ADAM33 was silenced due to DNA methylation in primary bronchial epithelial cells from asthmatic and healthy adults. In this study we were able to detect AEC ADAM33 expression by using PCR, which we confirmed with gel electrophoresis. However, we found no difference in ADAM33 expression among AECs from asthmatic patients, atopic patients, and healthy subjects. Possible explanations for the apparent discrepancy between our findings and those of Foley et al[24] include that our asthmatic patients had mild disease, whereas Foley et al reported increased ADAM33 expression in patients with moderate and severe asthma, and that our investigation consisted of *in vitro* experiments using P_2 AECs as opposed to an analysis of bronchial biopsy specimens, which likely reflects interactions between multiple cell types, including immune cells.

An important finding of this study was good correlation between bronchial and nasal epithelial expression of proremodeling factors. This suggests that nasal epithelial cells might serve as an acceptable and less invasive proxy for lower AEC numbers in future longitudinal studies and ultimately as a potential adjunctive diagnostic strategy for asthma. Specific noninvasive methods to identify infants and young children with persistent asthma or children with asthma at risk for progressive airway remodeling would be useful because clinicians are generally unable to distinguish young children with recurrent transient wheezing from those with early persistent asthma or to predict which patients are at risk for progressive decreases in lung function.

Some data exist to suggest that higher doses of ICSs can reduce reticular basement membrane thickening in adult asthmatic patients.[43,44] In our study differential expression of TGF-β2, VEGF, and periostin was not affected by a history of ICS use at the time of bronchial epithelial sampling, although the small numbers of subjects included in these subset analyses preclude detection of subtle differences. However, given that our *in vitro* experiments were performed with cells several passages removed from exposure, we did not expect to see effects on expression of proremodeling factors by a history of ICS treatment.

There are several limitations to this study. First, cells used in our experiments were several passages removed from the host and interactions with other cell types that occur *in vivo*. However, differential expression of factors by P_2 AECs that are associated with asthma or airway remodeling support an inherent epithelial cell role in the pathogenesis of asthma.

Second, in our T_H2 cytokine stimulation experiments, cells were stimulated with a combination of IL-4 and IL-13. Therefore we were unable to determine which of these cytokines was directly responsible for the observed increase in TGF-β2 expression. However, *in vivo* AECs are likely exposed to both cytokines simultaneously in asthmatic patients.

Another limitation of our study was that our asthmatic patients had relatively mild disease. Our finding of differential expression of TGF-β2, VEGF, and periostin by AECs from children with mild asthma suggest that these factors play important roles in the disease during childhood. Our observed lack of differential expression of these factors by AECs from atopic nonasthmatic children contrasts with the findings of several groups demonstrating changes in the basement membrane of atopic nonasthmatic adults.[45,46] However, we did not directly evaluate the *in vivo* airway structure of children in this study, and therefore we cannot conclude that lower airway basement membrane changes do not occur in atopic nonasthmatic children.

In conclusion, we have demonstrated that AECs from well-characterized children with asthma grown *in vitro* differentially express several sentinel factors believed to influence subepithelial airway remodeling, namely TGF-β2, VEGF, and periostin. Stimulation of AECs with IL-4 and IL-13 led to enhanced TGF-β2 production by cells from asthmatic patients. We observed good correlation between bronchial and nasal epithelial expression of these proremodeling factors, suggesting that nasal epithelial cells might be useful as surrogates for lower AEC numbers and potentially allowing for serial sampling in future longitudinal pediatric studies. Our findings of differential expression of TGF-β2, VEGF, and periostin in epithelial cells from pediatric asthmatic patients are consistent with the global hypothesis by Holgate et al[6] and others, suggesting an intrinsic dysregulation of the epithelial-mesenchymal trophic unit in patients with asthma.[47]

Clinical implications: AECs from children with mild asthma exhibit greater expression of factors associated with subepithelial airway remodeling than cells from atopic and healthy children.

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METHODS

RNA extraction and real-time PCR

Total RNA was isolated from cells grown at an ALI. Three transwells from each experimental condition were harvested and pooled to isolate RNA by using the RNAqueous kit for total RNA purification from Ambion–Applied Biosystems. RNA concentration and integrity were determined by using the Agilent 2100 Bioanalyzer system and Agilent RNA 6000 Nano Chips (Agilent Technologies). RNA samples (1 μg) with an RNA integrity number of 8 or greater were reverse transcribed with Moloney murine leukemia virus reverse transcriptase with a combination of random hexamers and oligo-dTs by using the SuperScript VILO cDNA Synthesis Kit from Invitrogen. Samples were diluted up to a final volume of 100 μL (10 ng/μL). Semiquantitative real-time qPCR was performed by using the SensiMix II probe kit wit SYBR Green (Bioline). We designed specific primers and validated the optimal conditions for amplification of periostin (NM_006475.2; forward, 5′-ACAGCTCAGAG TCTTCGTATATCG-3′; reverse, 5′-CCGTTTCTCCCTTGCTTACTCC-3′) and GAPDH (NM_002046.3; forward, 5′-CATCCCTGCCTCTACTGGCG-3′; reverse, 5′-TAGACGGCAGGTCAGGTCCAC-3′). ADAM33 (NM_025 220) primers were purchased from SA Biosciences (Qiagen). A library of representative samples containing cDNAs were made, and serial dilutions of 1×, 0.1×, 0.01×, 0.001×, and 0.0001× were prepared for the validation of primers and to determine amplification efficiency. These dilutions were run in parallel with samples to adjust the efficiency in each set of samples analyzed. Samples of 15 μL were prepared by using 0.1 μmol/L of each of the forward and reverse primers and 1 μL of 1× cDNA.

Real-time qPCR reactions containing 1.5 μL of cDNA, 0.2 μmol/L primers, and the 2× SYBR Green Supermix were run in the Bio-Rad iQ thermal cycler (Bio-Rad Laboratories, Hercules, Calif) with programs specific for the respective gene. Reactions were run in triplicate, and genes of interest were normalized to the GAPDH housekeeping gene. All primers were designed in-house and optimized for efficiency, and the absence of primers-dimers was confirmed for primer specificity. Single amplicons of the appropriate base pair were confirmed by means of gel electrophoresis.

The relative expression of periostin and ADAM33 was standardized by using GAPDH as a nonregulated reference gene. The expression ratio was calculated by using the real-time PCR efficiency and the Δ cycle threshold (Ct; intersection between a threshold point and the amplification curve). We used the REST (Relative Expression Software Tool)–MCS software to compare gene expression.[E1] The software uses PCR efficiencies and the average crossing point deviations among subject groups (asthmatic, atopic nonasthmatic, and healthy groups). The expression ratios are tested for statistical significance by using a Pair Wise Fixed Re-allocation Randomisation Test and plotted by using SE estimation through the Taylor algorithm.[E2] All PCR analysis algorithms used to estimate relative gene expression use changes in Ct values. The relative expression ratio of a target gene is computed based on its real-time PCR efficiencies *(E)* and the difference *(Δ)* of 1 unknown sample (treatment) versus 1 control (ΔCt control − treatment). The Pfaffl algorithm is based on the following equation:

$$\text{Ratio} = \text{E(target)}^{\Delta\text{Ct target (Mean control}-\text{Mean sample)}} / \text{E(reference)}^{\Delta\text{Ct reference (Mean control}-\text{Mean sample)}}.$$

In addition to using this general algorithm, the REST software incorporates a Pair Wise Fixed Re-allocation Randomization Test to test for statistical significance and the Taylor algorithm to plot by SE estimation.

Our final PCR results were expressed as the fold increase on a $\log_2$ scale relative to median expression of genes in healthy control samples.

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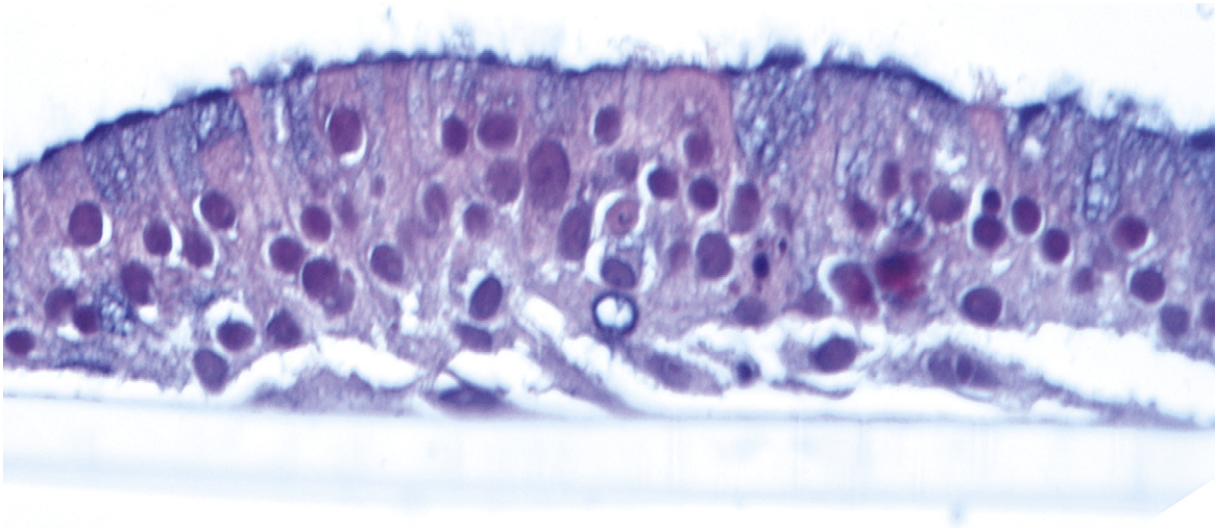


FIG E1. Hematoxylin and eosin–stained bronchial epithelial cells from an asthmatic patient differentiated at an ALI.

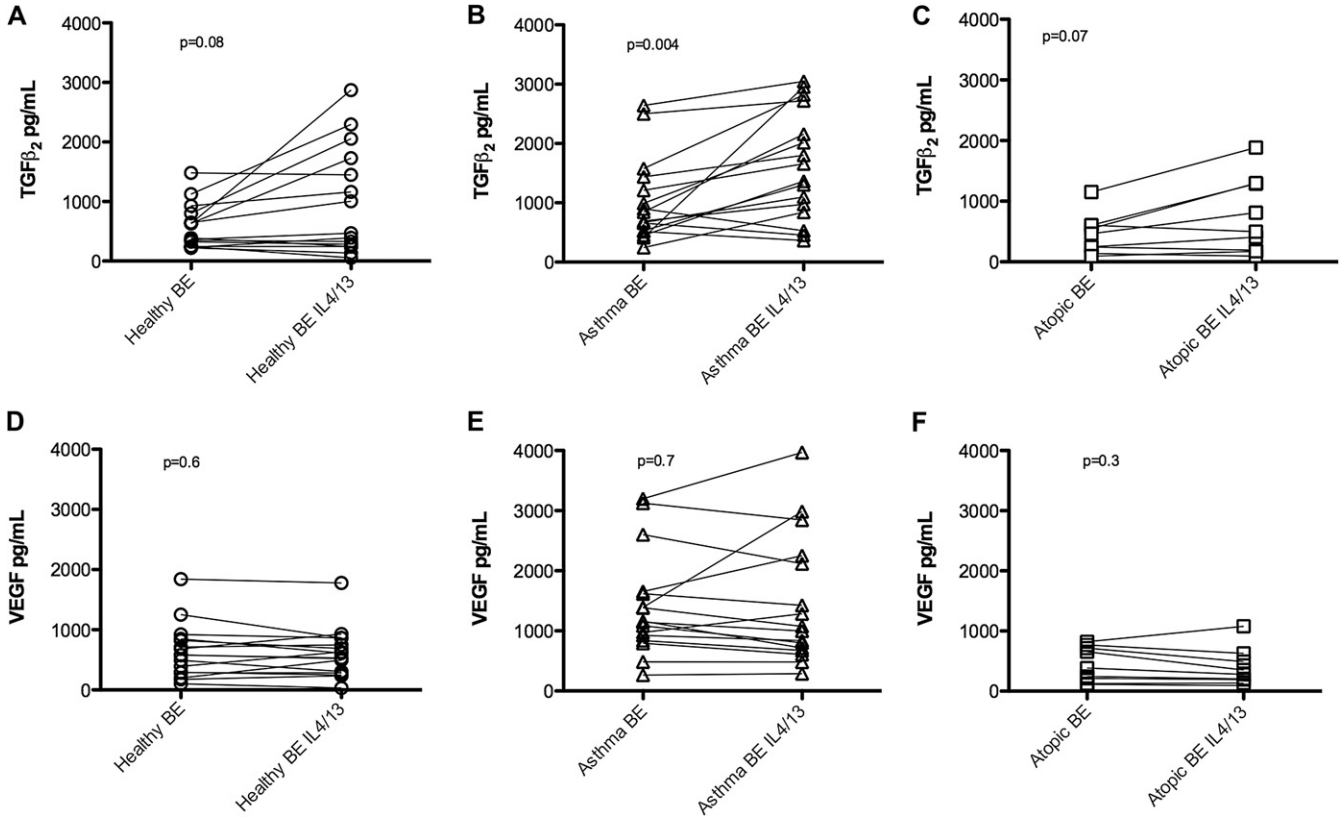


FIG E2. Change in TGF-β2 concentrations in ALI conditioned culture media from healthy bronchial **(A)**, asthmatic bronchial **(B)**, and atopic nonasthmatic bronchial **(C)** epithelial cells after stimulation with IL-4 and IL-13. Change in VEGF concentrations in ALI conditioned culture media from healthy bronchial **(D)**, asthmatic bronchial **(E)**, and atopic nonasthmatic bronchial **(F)** epithelial cells after stimulation with IL-4 and IL-13. Analyses were conducted with paired *t* tests or Wilcoxon matched-pairs test, as appropriate.

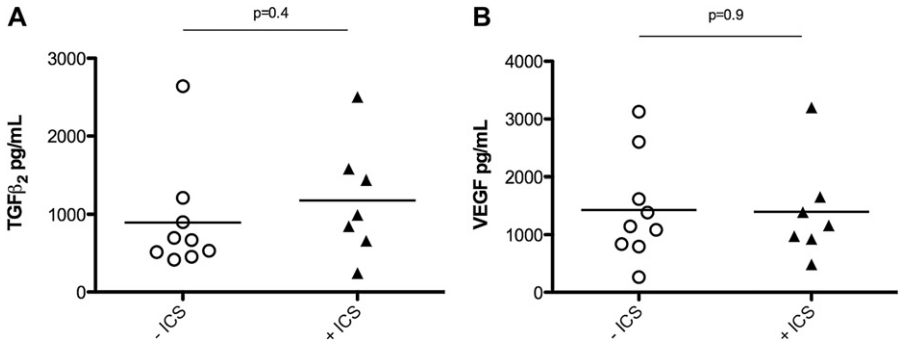


FIG E3. Production of TGF-β2 **(A)** and VEGF **(B)** in bronchial epithelial cell ALI cultures from asthmatic children who reported use of ICSs at the time of airway brushings *(+ICS)* was similar to production in cells from asthmatic children who were not using inhaled steroids *(−ICS)*.

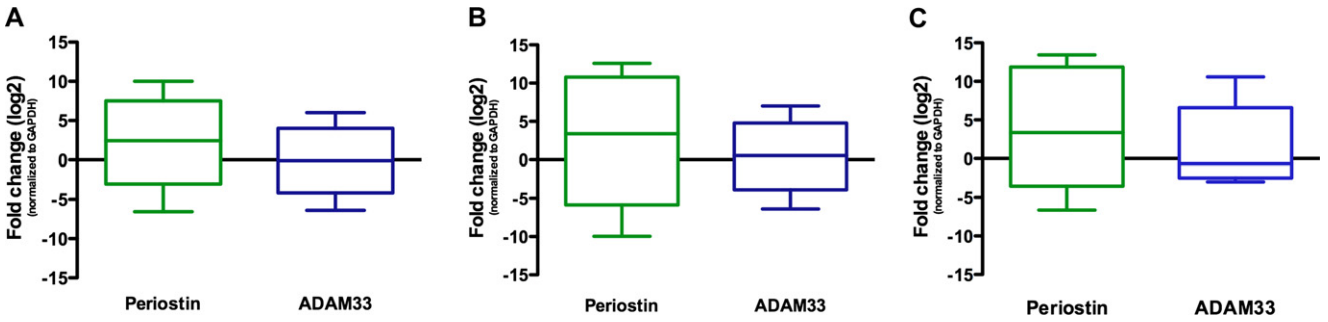


FIG E4. Periostin expression *(green box)* was 2.4-fold higher in bronchial epithelial cells from asthmatic patients stimulated with IL-4 and IL-13 compared with that seen in unstimulated cells from asthmatic patients (**A**; $P = .046$). There was no significant change in periostin expression after IL-4 and IL-3 stimulation by bronchial epithelial cells from healthy subjects (**B**; $P = .5$) or atopic nonasthmatic patients (**C**; $P = .4$). ADAM33 expression *(blue box)* by AECs from asthmatic (Fig E4, *A*), healthy (Fig E4, *B*), or atopic nonasthmatic (Fig E4, *C*) children did not change in response to IL-4 and IL-13 stimulation.

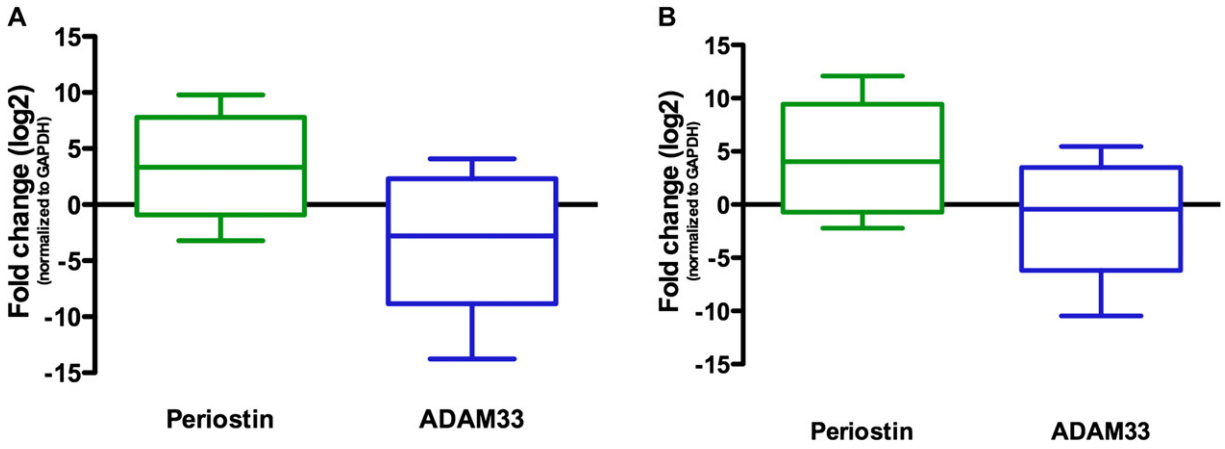


FIG E5. Periostin expression *(green box)* was 3.3-fold higher in asthmatic bronchial epithelial cells from children not using inhaled steroids *(−ICS)* than expression in healthy children (**A**; $P = .003$). Periostin expression in asthmatic children who were using inhaled steroids *(+ICS)* was 4-fold higher than expression in cells from healthy children (**B**; $P = .001$). ADAM33 *(blue box)* was not differentially expressed by either −ICS (Fig E5, *A*; $P = .2$) or +ICS (Fig E5, *B*; $P = .9$) asthmatic children in comparison with healthy children.