Asthmatic airway epithelial cells differentially regulate fibroblast expression of extracellular matrix components

Stephen R. Reeves, MD, PhD,[a,b] Tessa Kolstad, BS,[b] Tin-Yu Lien, BS,[b] Molly Elliott, BS,[a,b] Steven F. Ziegler, PhD,[c] Thomas N. Wight, PhD,[c] and Jason S. Debley, MD, MPH[a,b] *Seattle, Wash*

Background: Airway remodeling might explain lung function decline among asthmatic children. Extracellular matrix (ECM) deposition by human lung fibroblasts (HLFs) is implicated in airway remodeling. Airway epithelial cell (AEC) signaling might regulate HLF ECM expression.

Objectives: We sought to determine whether AECs from asthmatic children differentially regulate HLF expression of ECM constituents.

Methods: Primary AECs were obtained from well-characterized atopic asthmatic (n = 10) and healthy (n = 10) children intubated during anesthesia for an elective surgical procedure. AECs were differentiated at an air-liquid interface for 3 weeks and then cocultured with HLFs from a healthy child for 96 hours. Collagen I (COL1A1), collagen III (COL3A1), hyaluronan synthase (HAS) 2, and fibronectin expression by HLFs and prostaglandin E_2 synthase (PGE2S) expression by AECs were assessed by using RT-PCR. TGF-β1 and TGF-β2 concentrations in media were measured by using ELISA.

Results: COL1A1 and COL3A1 expression by HLFs cocultured with AECs from asthmatic patients was greater than that by HLFs cocultured with AECs from healthy subjects (2.2-fold, $P < .02$; 10.8-fold, $P < .02$). HAS2 expression by HLFs cocultured with AECs from asthmatic patients was 2.5-fold higher than that by HLFs cocultured with AECs from healthy subjects ($P < .002$). Fibronectin expression by HLFs cocultured with AECs from asthmatic patients was significantly greater than that by HLFs alone. TGF-β2 activity was increased in cocultures of HLFs with AECs from asthmatic patients ($P < .05$), whereas PGES2 was downregulated in AEC-HLF cocultures (2.2-fold, $P < .006$).

Conclusions: HLFs cocultured with AECs from asthmatic patients showed differential expression of the ECM constituents COL1A1 and COL3A1 and HAS2 compared with HLFs cocultured with AECs from healthy subjects. These findings support a role for altered ECM production in asthmatic airway remodeling, possibly regulated by unbalanced AEC signaling. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: *Asthma, children, airway remodeling, epithelial cells, human lung fibroblasts, extracellular matrix, collagen I, collagen III, hyaluronic acid, fibronectin, TGF-β2*

Childhood asthma is a significant global public health burden that affects the lives of millions of children worldwide, including an estimated 7.1 million American children.[1,2] In addition to the high cost of missed school attendance and health care use, poorly controlled asthma results in significant morbidity and even mortality.[3] Furthermore, evidence from population-based epidemiologic studies has shown that lung function in asthmatic children as a group declines between birth and school age. Indeed, the Tucson Children's Respiratory Study has established that children who subsequently have asthma demonstrate deficits in lung function by 6 years of age, despite being born with normal lung function.[4] Data from the Melbourne Asthma Study, the largest and longest-running asthma cohort study to monitor lung function, confirmed that children with persistent asthma had decreased lung function, which did not significantly worsen after age 10 years yet endured into adulthood and was not ultimately recovered.[5] This pattern is not observed in children who were early transient wheezers or children who never wheezed.[4] Unfortunately, significant phenotypic overlap among children who wheeze at an early age exists, making both treatment and early diagnosis of persistent asthma challenging.[6]

Airway remodeling, with changes to underlying airway structure, is a possible explanation for epidemiologic data demonstrating declines in lung function among children with asthma. Pathologic examination of the airways of asthmatic children has revealed evidence of airway remodeling, including goblet cell hyperplasia, matrix protein deposition in the basement membrane, angiogenesis, and both smooth muscle cell hypertrophy and hyperplasia.[7,8] Furthermore, thickening of the basement membrane is a common feature of both adult and pediatric asthmatic airway remodeling, suggesting that extracellular matrix (ECM) protein deposition in the epithelial basement membrane occurs in early childhood and persists into adulthood.[7-10] Evidence gathered from the histologic study of airways from asthmatic patients demonstrates increased amounts of collagen subtypes, fibronectin, hyaluronan, and several other ECM constituents.[11,12]

Although the components of the ECM have been thoroughly characterized, the active role of the ECM in airway remodeling and fibrosis is only beginning to be elucidated, as reviewed by Wight and Potter-Perigo.[13] One mechanism central to thickening of the basement membranes and promotion of airway fibrosis is

From [a]the Division of Pulmonary Medicine, Seattle Children's Hospital, University of Washington, Seattle; [b]the Center for Immunity and Immunotherapies, Seattle Children's Research Institute; and [c]the Benaroya Research Institute, Seattle.

Supported by the Firland Foundation 201208 (PI: S.R.R.); American Lung Association Senior Research Training Fellowship RT-268263-N (PI: S.R.R.); NIH R01AI068731 (PI: S.F.Z.); NIH PO1 HL098067 (PI: S.F.Z., Subproject T.N.W.).

Disclosure of potential conflict of interest: S. R. Reeves, T. Kolstad, T.-Y. Lien, M. Elliott, and J. S. Debley have received research support from the National Institutes of Health, the American Lung Association, and the Firland Foundation. S. F. Ziegler has received research support from the National Institutes of Health, the American Lung Association, and the Firland Foundation and has received payment for lectures from the American Academy of Allergy, Asthma & Immunology. T. Wight has received research support from the National Institutes of Health/National Heart, Lung, and Blood Institute.

Received for publication August 12, 2013; revised April 6, 2014; accepted for publication April 11, 2014.

Corresponding author: Jason S. Debley, MD, MPH, Pulmonary Division (MS OC.7.720), Seattle Children's Hospital, 4800 Sand Point Way NE, Seattle, WA 98105. E-mail: jason.debley@seattlechildrens.org.

0091-6749/$36.00

© 2014 American Academy of Allergy, Asthma & Immunology

http://dx.doi.org/10.1016/j.jaci.2014.04.007

Abbreviations used
AEC: Airway epithelial cells
ALI: Air-liquid interface
COL1A1: Collagen I
COL3A1: Collagen III
ECM: Extracellular matrix
FENO: Fraction of exhaled nitric oxide
FGM: Fibroblast growth media
FVC: Forced vital capacity
HA: Hyaluronan
HAS: Hyaluronan synthase
HLF: Human lung fibroblast
IHC: Immunohistochemistry
PGE_2: Prostaglandin E_2
PGE2S: Prostaglandin E_2 synthase
PTGS2: Prostaglandin-endoperoxide synthase 2

the accumulation of fibrillar collagens through either increased production or decreased degradation.[14] The primary source of the collagens found in the airways is thought to be derived from the resident fibroblasts, particularly those that have further differentiated into a myofibroblast phenotype (ie, those that express α smooth muscle actin).[15,16] Further evidence would suggest that collagen production is augmented by fibroblast to myofibroblast transition and that this process is highly dependent on TGF-β activity[17] and modulated by hyaluronan.[19,20] Separate studies have demonstrated that collagen production is increased in the airways of asthmatic patients and might also be related to inherent increases in TGF-β activity.[18,19]

In recent years, there has been a growing appreciation for the role of crosstalk between mesenchymal cells, such as fibroblasts, and airway epithelial cells (AECs) in the pathogenesis of asthma. Multiple lines of evidence now point to the AEC as playing an active role in the orchestration of inflammatory responses, wound repair, and host immunity.[20-22] Furthermore, data supporting the concept that dysfunctional regulation of these responses might play a role in the pathogenesis of asthma are accumulating.[22] Evidence of this dysfunctional regulation has been observed in both *in vivo* animal model systems and bronchial biopsy specimens from human subjects.[23-25]

To further investigate the role of cellular signaling from AECs on the production of ECM, we used a coculture model with primary AECs from both healthy and asthmatic children and human lung fibroblasts (HLFs) derived from a single healthy donor. We hypothesized that HLFs cocultured with AECs from asthmatic patients would exhibit greater expression of ECM components, specifically type I and type III collagen, hyaluronan, and fibronectin, compared with those cocultured with healthy AECs. Furthermore, we hypothesized that these changes are associated with increased TGF-β signaling or conversely decreased inhibitory signaling, such as prostaglandin E_2 (PGE2) activity. The latter could in turn be a mechanism to explain the predisposition for ECM deposition in the subepithelium, which is observed in the airways of asthmatic patients.

METHODS

Subjects

Atopic asthmatic and healthy nonatopic nonasthmatic children aged 6 to 18 years who were undergoing 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 that participants met the following inclusion and exclusion criteria. Children with asthma had at least a 1-year history of physician-diagnosed asthma, had physician-documented wheezing in the 12 months before study enrollment, used a short-acting β-agonist (ie, albuterol) twice a month or more or were taking a daily inhaled corticosteroid or leukotriene receptor antagonist, and were born at 36 weeks' gestation or later. Healthy subjects were born at 36 weeks' gestation or later; had no history of asthma, reactive airway disease, chronic daily cough, or physician-diagnosed obstructive lung disease; and had no history of prior treatment with a systemic or inhaled corticosteroids, short-acting β-agonists, or oxygen. Children with asthma had 1 or more of the following atopic features: history of positive skin prick test response, positive RAST result for a common aeroallergen (discussed below), increased serum IgE level (>100 IU/mL), history of physician-diagnosed allergic rhinitis, or history of physician-diagnosed atopic dermatitis. Healthy subjects lacked a history of any of the above atopic features and were excluded if they had any other atopic comorbidity.

A blood sample was drawn from each subject that was used to measure total serum IgE and RAST allergen-specific IgE levels to dust mites (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*), 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/European Respiratory Society guidelines[26] with a NIOX MINO nitric oxide analyzer (Aerocrine, Solna, Sweden). This device measures exhaled nitric oxide levels precisely only above a cutoff of 5 ppb and can thus only be used for clinical purposes. Forced vital capacity (FVC), FEV_1, and forced expiratory flow between 25% and 75% of FVC were measured according to American Thoracic Society guidelines by using a VMAX series 2130 spirometer (VIASYS Healthcare, Hong Kong, China). Spirometry was repeated 15 minutes after administration of 2 puffs of albuterol in children with asthma.

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

Epithelial cell isolation, proliferation, and differentiation

Immediately after the endotracheal tube was secured, 3 bronchial epithelial cell samples were obtained from subjects during general anesthesia with 4-mm Harrell unsheathed bronchoscope cytology brushes (CONMED, Utica, NY). As described by Lane et al,[27] the unprotected brush was inserted through an endotracheal tube, advanced until resistance was felt, and rubbed against the airway surface for 2 seconds. Cells were seeded onto T-25 cell-culture flasks precoated with type I collagen and proliferated under submerged culture conditions. By using passage 2 cells, epithelial cells were differentiated at an air-liquid interface (ALI), according to methods previously described by our laboratory. See the Methods section in this article's Online Repository at www.jacionline.org for further details.[28]

AEC-fibroblast cocultures

HLFs from a healthy child were obtained from a commercial vendor (Lonza, Walkersville, Md) and used for each experiment (≤passage 5). HLF cultures were established with a Fibroblast Cell Media Bulletkit (fibroblast growth media [FGM]) per the recommendations of Lonza. HLFs were seeded at a density of approximately 2500 cells/cm^2 in 12-well Collagen I BD BioCoat plates (Becton Dickinson, Bedford, Mass) and incubated for 7 days to achieve a confluent monolayer before initiation of AEC-HLF cocultures. Media changes of FGM occurred at 48-hour intervals. At 48 hours before experimental day 0, the HLF media was replaced with coculture media (1:1 FGM and PneumaCult ALI Maintenance Media). ALI transwells were placed in cocultures with the HLFs at experimental day 0. The coculture media in the basolateral chamber was changed every 24 hours and stored at −80°C for subsequent analysis. The media and cells were collected for studies 96 hours after initiation of coculture experiments.

TABLE I. Subjects' characteristics

	Healthy control subjects (n = 10)	Asthmatic patients (n = 10)	*P* value
Age (y), mean ± SD	10.3 ± 3.6	11.7 ± 3.4	.4
Female sex (%)	60	20	.07
Current use of inhaled steroids, yes, no. (%)		9 (90)	
History of eczema, yes, no. (%)		4 (40)	
History of allergic rhinitis, yes, no. (%)		9 (90)	
Positive RAST result, yes, no. (%)		9 (90)	
IgE (IU/mL), median (IQR)	20.5 (12-27)	242 (81-242)	.004
FVC (% predicted), mean ± SD	101 ± 13.2	100.7 ± 11.5	.9
FEV_1/FVC ratio, mean ± SD	0.89 ± 0.04	0.83 ± 0.05	.04
FEV_1 (% predicted), mean ± SD	99.8 ± 14.5	96.6 ± 12.1	.6
FEF_{25-75} (% predicted), mean ± SD	99 ± 20.4	88 ± 18.5	.2
F_{ENO} (ppb), mean ± SD	9.3 ± 4.6	14.9 ± 8.8	.2

FEF_{25-75}, Forced expiratory flow between 25% and 75% of expiration; *IQR*, interquartile range.

RNA extraction and real-time PCR

Total RNA was isolated from HLFs cocultured with AECs grown at an ALI. Three wells 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 MMLV reverse transcriptase with a combination of random hexamers and oligo-dTs by using the SuperScript VILO cDNA Synthesis Kit (Life Technologies, Grand Island, NY). Samples were diluted up to a final volume of 100 μL (10 ng/μL). Semiquantitative real-time qPCR was performed by using validated TaqMan probes (Life Technologies, Grand Island, NY) for human collagen I (COL1A1), collagen III (COL3A1), fibronectin (FNDC), prostaglandin E_2 synthase (PGE2S), prostaglandin-endoperoxide synthase 2 (PTGS2/COX-2), and hyaluronic acid synthase II. Although there are 3 hyaluronan synthase (HAS) enzyme isoforms (HAS1, HAS2, and HAS3), HAS2 is the major isoform expressed by HLFs and has previously been reported to be differentially expressed by fibroblasts from asthmatic patients.[29] Assays were performed with the TaqMan Fast Advanced Master Mix reagents and accompanying protocol and the Applied Biosystems StepOnePlus Real-Time PCR System with StepOne Software v2.2.2 (Life Technologies).

Immunohistochemistry

Sterilized 12-mm round glass cover slips were coated with type I collagen and placed in the bottom of one of the replicate chambers of the 12-well plates before seeding the HLFs. After 96 hours of coculture, the cover slips were carefully removed and placed in a separate 12-well plate for immunohistochemistry (IHC). Cover slips containing cells were then washed 3 times in room temperature PBS to remove residual media and were then fixed with 50:50 methanol and acetone at 20°C for 10 minutes. Cover slips were then washed with PBS and blocked with 10% FBS for 30 minutes. After this, cover slips were washed again with PBS and then incubated with primary antibodies to COL1A1 (1:1000 ab6310; Abcam, Cambridge, Mass) and hyaluronan (1:500 ab53842, Abcam) for 1 hour at room temperature. Additional PBS washes were performed before incubation with appropriate secondary antibodies for 1 hour at room temperature (1:1000 A11016 and 1:1000 A21202, Life Technologies). Three final PBS washes preceded mounting the cover slips with ProLong Gold antifade reagent with DAPI (Life Technologies). Images were acquired with an automated Leica DM6000B fluorescent microscope (Leica Microsystems, Wetzlar, Germany) in 7 × 7 grids at ×400 magnification and stitched together with LASAF software to provide higher-resolution images of larger areas of tissue. Given that cover slips required coating with COL1A1 to support HLF cultures, we did not perform IHC studies for COL1A1 because of concern for background staining.

ELISA analyses

For each condition, sampled basolateral medium from triplicate transwells was pooled. Measurement of protein levels of activated TGF-β1 and TGF-β2 in sampled basolateral conditioned media was completed with Duoset ELISA Development Kits (R&D Systems, Minneapolis, Minn), according to the manufacturer's recommendations. All measurements were completed in duplicate. Samples in which concentrations were less than the assay detection level were assigned a value of one half the lower limit of detection for analysis.

Statistical analysis

Protein levels are presented as means ± SDs when all groups (eg, healthy, asthmatic, and HLF alone) were normally distributed and as medians with interquartile ranges if 1 or more groups were not normally distributed. The Kolmogorov-Smirnov test was used to determine whether data were normally distributed. One-way ANOVA or the Kruskal-Wallis test if data in 1 or more groups was nonnormally distributed was used to compare the distributions of protein levels in coculture media across the 3 groups. *Post hoc* comparisons between pairs of groups (eg, between HLFs and asthma cocultures) were made by using the Dunn multiple comparisons test, with the significance level set at a *P* value of less than .05. For age, sex, lung function parameters, F_{ENO} values, and IgE levels, the paired *t* test or Wilcoxon signed-rank test for nonnormally distributed data was used for comparisons between asthmatic and healthy subjects. Statistical analyses of clinical data and protein levels in ALI cultures were performed with Prism 6.0 software (GraphPad Software, San Diego, Calif). The relative expression of COL1A1, COL3A1, HAS2, FNDC, PGE2S, and PTGS2/COX-2 was standardized by using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a nonregulated reference gene. Analyses of real-time qPCR results were performed with GenEx version 5.0.1 (MultiD Analyses AB, Göteborg, Sweden) based on methods described by Pfaffl.[30] Statistical significance was set at a *P* value of less than .05.

RESULTS

Bronchial epithelial brushings were obtained from 10 healthy subjects and 10 atopic asthmatic patients and used for the coculture studies. Characteristic data from the subjects, summaries of laboratory findings, and lung function test results obtained during the follow-up visit are summarized in Table I. Subjects were of comparable age (healthy subjects, 10.3 ± 3.6 years; asthmatic patients, 11.7 ± 3.4 years; $P = .4$); however, there were more female subjects in our healthy group compared with the asthmatic group. The majority of our asthmatic patients were using daily inhaled corticosteroids. Most of the asthmatic patients had positive RAST results to a specific aeroallergen (90%). Compared with healthy subjects, the asthmatic patients

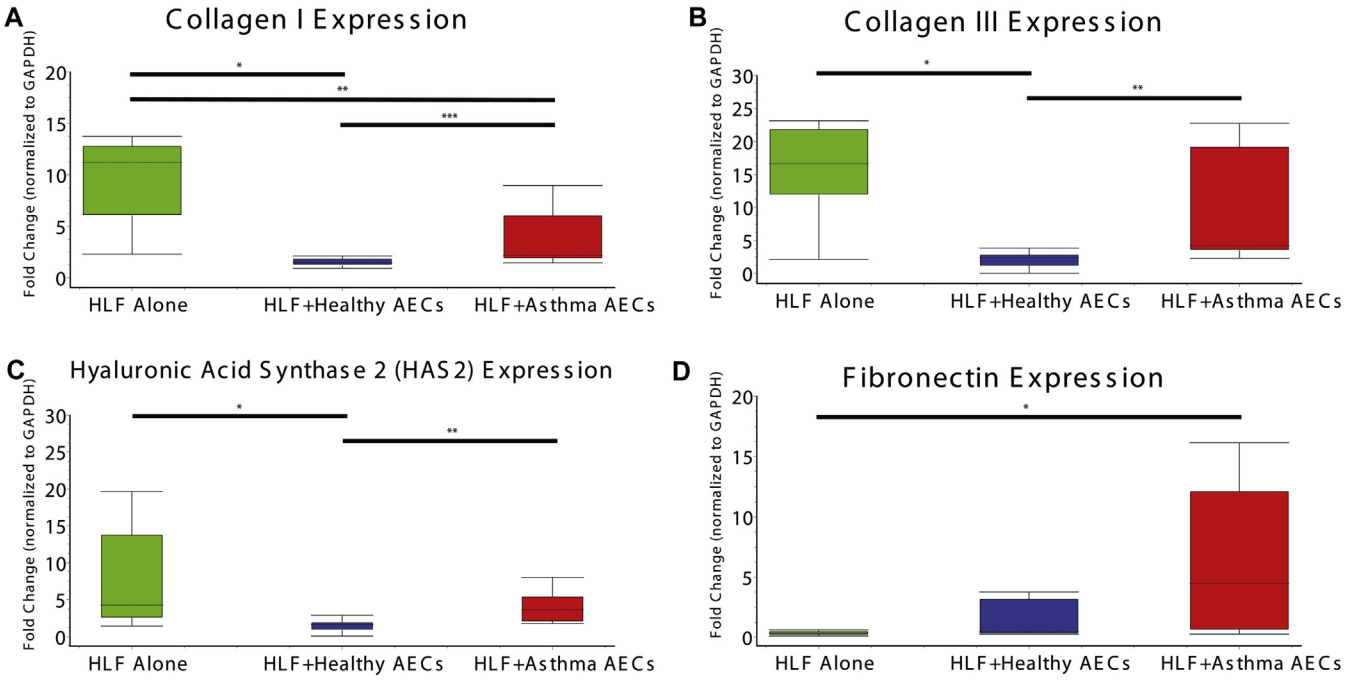


FIG 1. **A,** Expression of COL1A1 mRNA by HLFs alone compared with coculture with AECs from healthy subjects or AECs from asthmatic patients. **B,** Expression of COL3A1 mRNA by HLFs alone compared with coculture with AECs from healthy subjects or AECs from asthmatic patients. **C,** Expression of HAS2 mRNA by HLFs alone compared with coculture with AECs from healthy subjects or AECs from asthmatic patients. **D,** Expression of fibronectin (FNDC) by HLFs alone compared with coculture with AECs from healthy subjects or AECs from asthmatic patients. *GAPDH,* Glyceraldehyde-3-phosphate dehydrogenase.

had a significantly greater serum IgE level (916 IU/mL in asthmatic patients vs 21.3 IU/mL in healthy subjects, $P < .0005$). The FEV_1/FVC ratio was significantly lower in asthmatic patients compared with that seen in healthy subjects (89% ± 4% vs 83% ± 5%, respectively; $P = .04$). Asthmatic patients and healthy subjects had comparable F_{ENO} levels (14.9 ± 8.8 vs 9.3 ± 4.6 ppb, $P = .2$).

Expression of COL1A1 mRNA production, as assessed by using RT-PCR, from HLFs alone (noncocultured time control) was 8.3-fold greater compared with that seen in HLFs cocultured with AECs from healthy subjects (95% CI, 5.7-10.8; $P = .002$; Fig 1, *A*). COL1A1 expression by HLFs alone was 6.6-fold greater than that by HLFs cocultured with AECs from asthmatic patients (95% CI, 3.1-10.1; $P = .002$). Direct comparison of COL1A1 expression by HLFs cocultured with AECs from healthy subjects and HLFs cocultured with AECs from asthmatic patients revealed 2.2-fold greater gene expression in the asthma cocultures (95% CI, 0.4-4.3; $P = .02$), which is consistent with a lesser degree of attenuation than that seen in healthy cocultures. We observed similar findings for COL3A1, with expression by HLFs alone 15.6-fold higher than that by HLFs cocultured with AECs from healthy subjects (95% CI, 7.5-23.8; $P = .002$; Fig 1, *B*). Comparison of COL3A1 expression by HLFs cocultured with AECs from asthmatic patients with that by HLFs cocultured with AECs from healthy subjects demonstrated 10.8-fold greater gene expression than seen in the asthma cocultures (95% CI, 3.2-24.9; $P = .02$). No significant difference in expression between HLFs cultured alone and HLFs cocultured with AECs from asthmatic patients was observed. Staining of HLFs for COL3A1 protein accumulation in each group was performed in parallel to RNA expression studies. Representative images are shown in Fig 2. Additionally, we examined HAS2 expression from HLF time controls, as well as HLFs cocultured with both AECs from healthy subjects and AECs from asthmatic patients. Expression of HAS2 by HLFs alone was 6.3-fold greater than that by HLFs cocultured with healthy AECs (95% CI, 1.8-10.8; $P = .005$; Fig 1, *C*). When compared with HLFs cocultured with AECs from asthmatic patients, a trend toward greater expression by HLFs alone was observed but did not reach statistical significance (3.8-fold; 95% CI, −0.6 to 8.2; $P = .07$). Compared with HLFs cocultured with AECs from healthy subjects, HLFs cocultured with AECs from asthmatic patients expressed 2.5-fold greater HAS2 mRNA (95% CI, 1.2-3.8; $P = .002$). Hyaluronan accumulation was assessed by using IHC staining of HLFs treated in parallel with those that were used for RNA extraction. Representative images are compared in Fig 3. Expression of fibronectin by HLFs cocultured with AECs from asthmatic patients was 5-fold greater than that for HLFs alone (95% CI, 2-16; $P = .05$); however, no significant difference between HLFs cocultured with AECs from asthmatic patients and HLFs cocultured with AECs from healthy subjects was detected.

To examine potential mediators of the altered gene expression, we performed ELISAs for TGF-β1 and TGF-β2 activity in media samples obtained at the 96-hour coculture time point. Mean TGF-β1 concentrations for HLFs alone (n = 4), HLFs cocultured with AECs from healthy subjects (n = 8), and HLFs cocultured with AECs from asthmatic patients (n = 8) are depicted in Fig 4, *A*. No significant differences were observed among the groups. Similar values were also present at the 48-hour time point (data not shown). In contrast, a separate ELISA performed for TGF-β2

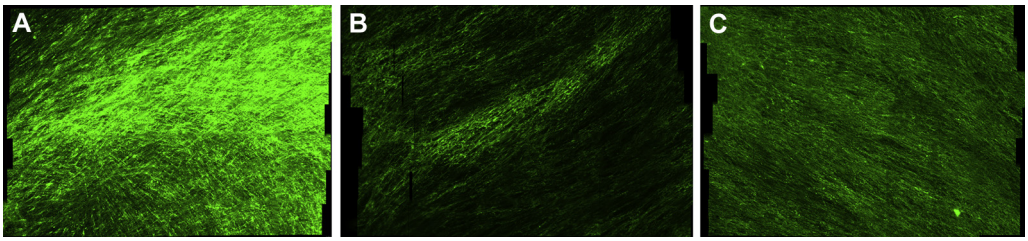


FIG 2. Collagen III expression assessed by using IHC among HLFs alone **(A)**, HLFs plus AECs from healthy subjects **(B)**, and HLFs plus AECs from asthmatic patients **(C)**.

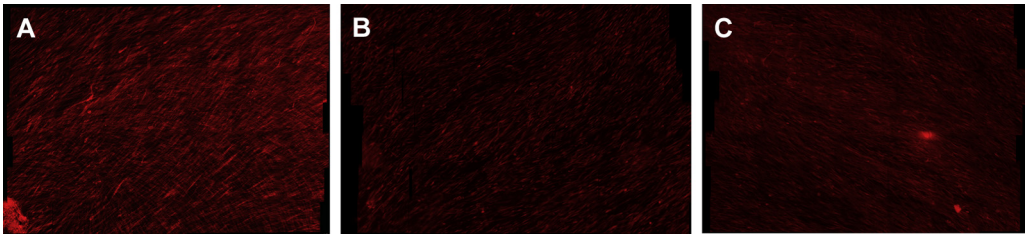


FIG 3. Hyaluronic Acid expression assessed by using IHC among HLFs alone **(A)**, HLFs plus AECs from healthy subjects **(B)**, and HLFs plus AECs from asthmatic patients **(C)**.

activity by using a second aliquot of the same samples revealed significantly greater detectable levels of TGF-β2 present in the culture media at the 96-hour coculture time point in cocultures with AECs from asthmatic patients compared with those seen in HLFs alone and HLFs cocultured with AECs from healthy subjects ($P = .01$; Fig 4, *B*).

Given the interesting findings of suppressed ECM production in the cocultures of HLFs and AECs from healthy subjects, we sought to examine the possibility that factors inhibiting fibroblast expression of ECM might be differentially expressed by asthmatic AECs. To study this, we examined mRNA expression of PGE2S, as well as expression of PTGS2/COX-2, in AECs from asthmatic patients and AECs from healthy subjects that were cocultured with HLFs (Fig 5). We found that PGE2S mRNA expression by AECs from healthy subjects was 2.2-fold greater than that by AECs from asthmatic patients (95% CI, 1.3- to 3.8-fold; $P = .006$). Conversely, PTGS2/COX-2 expression by AECs from healthy subjects was not significantly different than that by AECs from asthmatic patients (95% CI, 1.8- to 11-fold; $P = .18$).

DISCUSSION

This is the first study to examine the regulation of ECM component expression by HLFs imposed by coculture with differentiated primary human AECs from well-characterized healthy and asthmatic children. Herein, we report that coculture of HLFs with both AECs from healthy subjects and AECs from asthmatic patients regulates the expression of ECM components, specifically COL1A1, COL3A1, and hyaluronan. However, HLFs cocultured with AECs from asthmatic patients displayed decreased regulation compared with cocultures with AECs from healthy subjects. In addition, we found that TGF-β2, but not TGF-β1, is more highly expressed in cocultures with AECs from asthmatic patients, whereas expression of PGE2S was downregulated in these culture systems. Furthermore, HLFs cocultured with AECs from asthmatic patients had greater expression of fibronectin than HLFs alone, suggesting perhaps a positive AEC-derived stimulus as opposed to inhibitory regulation. Taken together, these data point to a dynamic system that is influenced by the interaction of the AECs and HLFs and suggests that an altered balance of proremodeling and antiremodeling signals from the AECs from asthmatic patients might regulate HLF expression of ECM constituents.

One of the more striking findings of this study was that coculture with AECs from healthy subjects markedly attenuated expression of ECM components compared with HLF time controls, suggesting that tonic regulation of HLF phenotype through crosstalk with AECs must be occurring. In a previous study, Lama et al[31] demonstrated that murine fibroblasts grown alone in cell culture exhibited increased proliferation compared with those grown in coculture with murine AECs. That study further linked the downregulation of cells grown in coculture to the COX-2 pathway and the production of the antiproliferative signaling of PGE_2 using cells derived from transgenic mice. In line with this, our study demonstrates greater expression of PGE2S in cocultures of HLFs with AECs from healthy subjects, which correlated with attenuated production of ECM components. In our study PTGS2/COX2 was not significantly different between the groups; however, it is important to note that production of PGE_2 is a complex pathway with multiple areas of potential regulation. In a separate study Hostettler et al[32] reported that incubation of cultured HLFs with conditioned media obtained from AEC cell lines resulted in an approximately 50% reduction in fibroblast proliferation. This effect was negated by means of preincubation with indomethacin, a PGE_2 inhibitor. Interestingly, in the same series of experiments, the authors demonstrated that adding a TGF-β neutralizing antibody to the conditioned media also blocked the inhibitory effect and concluded that TGF-β was likely involved in the induction of the PGE_2 axis. Similar findings were also reported with conditioned media from bovine AECs in another study.[33] The findings of the latter 2 studies highlight the interdependence of the PGE_2 and TGF-β pathways; however, accumulating evidence would suggest that the activity of

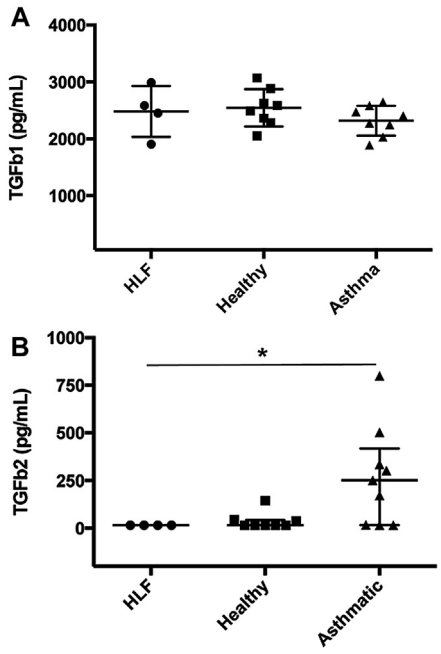


FIG 4. A, Concentrations of TGF-β1 measured in culture media collected at 96 hours from HLFs alone compared with cocultures of AECs from healthy subjects and HLFs and cocultures of AECs from asthmatic patients and HLFs (*lines* at means with SDs). **B,** Concentrations of TGF-β2 measured in culture media collected at the 96-hour time point from HLFs alone, cocultures of HLFs plus AECs from healthy subjects, and cocultures of HLFs plus AECs from asthmatic patients (*lines* at medians with interquartile ranges). $*P = .01$.

TGF-β is likely far more complex than these studies might suggest. One important concept that these studies support is that there is crosstalk between the AECs and fibroblasts. Other studies have highlighted the effects of fibroblasts on AEC proliferation[34,35]; however, these effects were not directly examined in the present study.

The concept that the TGF-β family of signaling molecules exhibits proremodeling effects on fibroblasts is becoming widely accepted.[36-38] Correlation of increased TGF-β expression and increased subepithelial fibrosis has been reported in adults with severe eosinophilic asthma.[39,40] A more recent study by Brown et al[41] demonstrated increased TGF-β1 levels in brochoalveolar lavage fluid obtained from asthmatic children, which was associated with markers of increased oxidative stress and evidence of airway obstruction documented by pulmonary function tests. In addition to its other profibrotic effects, TGF-β has been shown to enhance the production of ECM constituents, including collagens.[42,43] TGF-β might also exert remodeling effects by augmenting the expression of tissue inhibitors of metalloproteinases, which in turn disrupt the ability of matrix metalloproteinases to turn over secreted ECM components, such as hyaluronan.[44,45] The latter is essential for appropriate wound repair after epithelial damage.

It is important to note that multiple isoforms of TGF-β exist, including TGF-β1 (the most abundant isoform, characteristically associated with endothelial, hematopoietic, and connective tissue cells), TGF-β2 (primarily synthesized by AECs and neuronal cells), and TGF-β3 (principally secreted by mesenchymal cells).[46] Both TGF-β1 and TGF-β2 have been shown to enhance fibroblast activity in the context of inflammatory remodeling in respiratory epithelium.[47] In the latter study both isoforms were capable of activating fibroblasts in a concentration-dependent fashion. In reality, it is likely that both TGF-β1 and TGF-β2 are simultaneously active in orchestrating fibrosis *in vivo* with contributions from the epithelium (TGF-β2), eosinophils (TGF-β1), and macrophages (TGF-β1).[48,49] Bronchial fibroblasts have been shown to respond to both isoforms, and intriguingly, fibroblasts derived from asthmatic donors displayed enhanced activity to both TGF-β1 and TGF-β2, suggesting the possibility that greater responses to TGF-β could be accounted for by augmentation of both ends of the signaling axis.[36] Additionally, our laboratory has previously reported increased secretion of TGF-β2 from AECs obtained from asthmatic children.[28] Data from the present study are in line with previously published findings; however, it is important to note that the relative amount of TGF-β2 present in this system is less than the overall levels of TGF-β1 detected. Radaev et al[50] have recently reported that TGF-β2 has significantly greater affinity for the TGF-β receptor subunit I and additionally might simultaneously engage TGF-β receptor subunit I and TGF-β receptor subunit II, whereas TGF-β1 sequentially engages these receptor complexes and thus has augmented activation kinetics. These variations in receptor subtype engagement by TGF-β1 and TGF-β2 might contribute to variable downstream signaling and have significant functional consequences.

Chakir et al[51] have previously reported a link to the expression of TGF-β and the deposition of both collagen I and collagen III in bronchial biopsy specimens. Notably, expression of both collagen I and collagen III in that study was significantly greater in the subjects with more severe asthma. This effect was not modified after treatment with corticosteroids, suggesting that remodeling that had already occurred was not reversible. Earlier work by Minshall et al[52] also confirmed the presence of greater amounts of collagen I and III in asthmatic airways, which correlated with asthma severity. Similar studies have demonstrated that proteoglycan deposition in the ECM is increased in patients with moderate-to-severe asthma and correlates with asthma severity.[53] In addition, hyaluronan has also been implicated in playing a significant role in the increased deposition of ECM in asthmatic patients.[54] Beyond its role as a scaffold for collagen deposition after airway inflammation,[55] hyaluronan has also been implicated in the persistence of eosinophils and enhanced production of TGF-β in asthmatic patients.[56] The latter finding suggests not only a significant role for hyaluronan in airway remolding but also modulation of a variety of inflammatory and signaling mediators. Our findings of enhanced production of collagens and hyaluronan by HLFs cocultured with AECs from asthmatic patients compared with HLFs cocultured with AECs from healthy subjects are in line with these previous reports and provide a novel model in which relevant signaling pathways can be further investigated.

There are some inherent limitations to this study design. Our population of asthmatic patients exhibited mild airflow obstruction, which is consistent with milder phenotypes of asthma independent of treatment. Alternatively, this could be related to good adherence to controller medications because our subjects reported that 90% of the cohort was presently taking daily inhaled corticosteroids. Given that the cells used in the cocultures were multiple passages beyond the initial sample collection, it is unlikely that any medications being taken at the time of

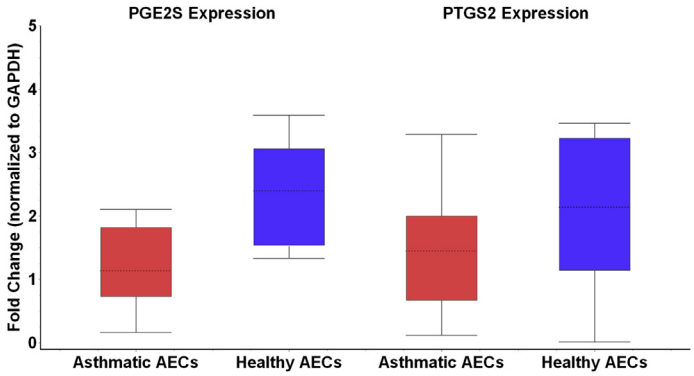


FIG 5. PGE2S mRNA expression is depicted on the *left side of the figure* in either AECs from asthmatic patients or AECs from healthy subjects. The *right side of the figure* demonstrates mRNA expression of PTGS2/COX-2 in AECs from asthmatic patients or AECs from healthy subjects, respectively.

recruitment would still have an effect on the cells; however, we cannot completely exclude that possibility. If present, this effect would bias toward the null hypothesis and likely make differences between the groups less apparent. Despite a relatively mild asthma phenotype, our asthmatic patients had significantly lower FEV_1/FVC ratios than the healthy subjects, indicating the presence of airflow obstruction. Another limitation of this study is that gene expression was only assessed at a single time point. Given the findings of this study, future investigation into additional signaling pathways and the balance between profibrotic and antifibrotic signaling pathways and assessment of gene expression at multiple time points are warranted. Once a more clear understanding of the likely multiple pathways that contribute to this crosstalk are achieved, future studies specifically targeting either or both profibrotic and antifibrotic signaling might lead to a better understanding of how to ameliorate the increased deposition of ECM components observed in the airways of asthmatic patients.

Although this is an *in vitro* model of cellular function, similar *in vivo* studies in healthy and asthmatic children would not be ethical or feasible. In addition, there are many inherent strengths to this approach. We have used a common healthy donor HLF cell line across the experiments, which not only limits the biologic variability of the HLFs but also helps to isolate differences seen between the healthy and asthmatic groups to AEC activity. Furthermore, our model allows for multifaceted characterization of several outcome measures, including gene expression, bound and excreted protein production, and histologic examination. Additionally, we are able to clinically characterize our population based on history, laboratory testing, and lung function.

In conclusion, we have demonstrated that HLFs from a common healthy donor behave differently when cocultured with either AECs obtained from a healthy donor or AECs obtained from an asthmatic donor. Furthermore, we have shown differential expression of important ECM components, including type I and type III collagens, as well as hyaluronan and fibronectin, depending on whether the HLFs were cocultured with AECs from healthy subjects or AECs from asthmatic patients. These findings are clinically relevant given the role that ECM deposition plays in airway remodeling and might have further-reaching implications given that we are just beginning to understand the active role that the ECM plays in airway remodeling and fibrosis.[13] In addition, we have shown that TGF-β2 levels are increased in cocultures of HLFs with ACEs from asthmatic patients, despite finding no differences in TGF-β1 levels compared with those seen in cocultures of HLFs with AECs from healthy subjects. The latter finding is consistent with previous work published by our group[28] and might be an important signaling mechanism driving the augmented ECM deposition observed in the airways of asthmatic patients. In addition, we have shown in our model that PGE2S is downregulated in cocultures of HLFs with AECs from asthmatic patients, which is in line with previous reports from other groups.[31,32] Taken together, these findings are consistent with the paradigm that signaling from the AEC is an important regulatory mechanism in the functional coordination of airway cellular subsets[22] and provide further evidence that intrinsic dysregulation of the epithelial-mesenchymal trophic unit might underlie asthma pathogenesis, airway remodeling, or both.[57]

Clinical implications: ECM components expressed by lung fibroblasts are less regulated by AECs from asthmatic children compared with AECs from healthy children, which might explain the airway remodeling observed in asthmatic patients.

REFERENCES

1. Papadopoulos NG, Arakawa H, Carlsen KH, Custovic A, Gern J, Lemanske R, et al. International consensus on (ICON) pediatric asthma. Allergy 2012;67:976-97.
2. American Lung Association, Epidemiology and Statistic Unit, Research and Program Services. Estimated prevalence and incidence of lung disease by Lung Association territory [electronic resource]. New York: American Lung Association; 2006.
3. Bloom B, Cohen RA, Freeman G. Summary health statistics for U.S. children: national health interview survey, 2007. Vital Health Stat 10 2009;239:1-80.
4. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. The Group Health Medical Associates. N Engl J Med 1995;332:133-8.
5. Phelan PD, Robertson CF, Olinsky A. The Melbourne Asthma Study: 1964-1999. J Allergy Clin Immunol 2002;109:189-94.
6. Brand PL, Baraldi E, Bisgaard H, Boner AL, Castro-Rodriguez JA, Custovic A, et al. Definition, assessment and treatment of wheezing disorders in preschool children: an evidence-based approach. Eur Respir J 2008;32:1096-110.
7. Payne DN, Rogers AV, Adelroth E, Bandi V, Guntupalli KK, Bush A, et al. Early thickening of the reticular basement membrane in children with difficult asthma. Am J Respir Crit Care Med 2003;167:78-82.
8. Barbato A, Turato G, Baraldo S, Bazzan E, Calabrese F, Panizzolo C, et al. Epithelial damage and angiogenesis in the airways of children with asthma. Am J Respir Crit Care Med 2006;174:975-81.

9. Malmstrom K, Pelkonen AS, Malmberg LP, Sarna S, Lindahl H, Kajosaari M, et al. Lung function, airway remodelling and inflammation in symptomatic infants: outcome at 3 years. Thorax 2011;66:157-62.
10. Saglani S, Payne DN, Zhu J, Wang Z, Nicholson AG, Bush A, et al. Early detection of airway wall remodeling and eosinophilic inflammation in preschool wheezers. Am J Respir Crit Care Med 2007;176:858-64.
11. Laitinen A, Altraja A, Kampe M, Linden M, Virtanen I, Laitinen LA. Tenascin is increased in airway basement membrane of asthmatics and decreased by an inhaled steroid. Am J Respir Crit Care Med 1997;156:951-8.
12. Roberts CR, Burke AK. Remodelling of the extracellular matrix in asthma: proteoglycan synthesis and degradation. Can Respir J 1998;5:48-50.
13. Wight TN, Potter-Perigo S. The extracellular matrix: an active or passive player in fibrosis? Am J Physiol Gastrointest Liver Physiol 2011;301:G950-5.
14. Wynn TA. Cellular and molecular mechanisms of fibrosis. J Pathol 2008;214:199-210.
15. Zhang K, Rekhter MD, Gordon D, Phan SH. Myofibroblasts and their role in lung collagen gene expression during pulmonary fibrosis. A combined immunohistochemical and in situ hybridization study. Am J Pathol 1994;145:114-25.
16. Phan SH. The myofibroblast in pulmonary fibrosis. Chest 2002;122(suppl):286S-9S.
17. Petrov VV, Fagard RH, Lijnen PJ. Stimulation of collagen production by transforming growth factor-beta1 during differentiation of cardiac fibroblasts to myofibroblasts. Hypertension 2002;39:258-63.
18. Johnson PR, Burgess JK, Ge Q, Poniris M, Boustany S, Twigg SM, et al. Connective tissue growth factor induces extracellular matrix in asthmatic airway smooth muscle. Am J Respir Crit Care Med 2006;173:32-41.
19. Goulet S, Bihl MP, Gambazzi F, Tamm M, Roth M. Opposite effect of corticosteroids and long-acting beta(2)-agonists on serum- and TGF-beta(1)-induced extracellular matrix deposition by primary human lung fibroblasts. J Cell Physiol 2007;210:167-76.
20. Knight DA, Holgate ST. The airway epithelium: structural and functional properties in health and disease. Respirology 2003;8:432-46.
21. Crosby LM, Waters CM. Epithelial repair mechanisms in the lung. Am J Physiol Lung Cell Mol Physiol 2010;298:L715-31.
22. Holgate ST, Roberts G, Arshad HS, Howarth PH, Davies DE. The role of the airway epithelium and its interaction with environmental factors in asthma pathogenesis. Proc Am Thorac Soc 2009;6:655-9.
23. Kelly MM, Leigh R, Bonniaud P, Ellis R, Wattie J, Smith MJ, et al. Epithelial expression of profibrotic mediators in a model of allergen-induced airway remodeling. Am J Respir Cell Mol Biol 2005;32:99-107.
24. Torrego A, Hew M, Oates T, Sukkar M, Fan Chung K. Expression and activation of TGF-beta isoforms in acute allergen-induced remodelling in asthma. Thorax 2007;62:307-13.
25. Kumar RK, Herbert C, Foster PS. Expression of growth factors by airway epithelial cells in a model of chronic asthma: regulation and relationship to subepithelial fibrosis. Clin Exp Allergy 2004;34:567-75.
26. American Thoracic Society/European Respiratory Society. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. Am J Respir Crit Care Med 2005;171:912-30.
27. Lane C, Burgess S, Kicic A, Knight D, Stick S. The use of non-bronchoscopic brushings to study the paediatric airway. Respir Res 2005;6:53.
28. Lopez-Guisa JM, Powers C, File D, Cochrane E, Jimenez N, Debley JS. Airway epithelial cells from asthmatic children differentially express proremodeling factors. J Allergy Clin Immunol 2012;129:990-7.e6.
29. Liang J, Jiang D, Jung Y, Xie T, Ingram J, Church T, et al. Role of hyaluronan and hyaluronan-binding proteins in human asthma. J Allergy Clin Immunol 2011;128:403-11.e3.
30. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.
31. Lama V, Moore BB, Christensen P, Toews GB, Peters-Golden M. Prostaglandin E2 synthesis and suppression of fibroblast proliferation by alveolar epithelial cells is cyclooxygenase-2-dependent. Am J Respir Cell Mol Biol 2002;27:752-8.
32. Hostettler KE, Roth M, Burgess JK, Gencay MM, Gambazzi F, Black JL, et al. Airway epithelium-derived transforming growth factor-beta is a regulator of fibroblast proliferation in both fibrotic and normal subjects. Clin Exp Allergy 2008;38:1309-17.
33. Nakamura Y, Tate L, Ertl RF, Kawamoto M, Mio T, Adachi Y, et al. Bronchial epithelial cells regulate fibroblast proliferation. Am J Physiol Lung Cell Mol Physiol 1995;269:L377-87.
34. Semlali A, Jacques E, Rouabhia M, Milot J, Laviolette M, Chakir J. Regulation of epithelial cell proliferation by bronchial fibroblasts obtained from mild asthmatic subjects. Allergy 2010;65:1438-45.
35. Skibinski G, Elborn JS, Ennis M. Bronchial epithelial cell growth regulation in fibroblast cocultures: the role of hepatocyte growth factor. Am J Physiol Lung Cell Mol Physiol 2007;293:L69-76.
36. Michalik M, Pierzchalska M, Legutko A, Ura M, Ostaszewska A, Soja J, et al. Asthmatic bronchial fibroblasts demonstrate enhanced potential to differentiate into myofibroblasts in culture. Med Sci Monit 2009;15:BR194-201.
37. Halwani R, Al-Muhsen S, Al-Jahdali H, Hamid Q. Role of transforming growth factor-beta in airway remodeling in asthma. Am J Respir Cell Mol Biol 2011;44:127-33.
38. Broide DH. Immunologic and inflammatory mechanisms that drive asthma progression to remodeling. J Allergy Clin Immunol 2008;121:560-72.
39. Redington AE, Madden J, Frew AJ, Djukanovic R, Roche WR, Holgate ST, et al. Transforming growth factor-beta 1 in asthma. Measurement in bronchoalveolar lavage fluid. Am J Respir Crit Care Med 1997;156:642-7.
40. Ohno I, Nitta Y, Yamauchi K, Hoshi H, Honma M, Woolley K, et al. Transforming growth factor beta 1 (TGF beta 1) gene expression by eosinophils in asthmatic airway inflammation. Am J Respir Cell Mol Biol 1996;15:404-9.
41. Brown SD, Baxter KM, Stephenson ST, Esper AM, Brown LA, Fitzpatrick AM. Airway TGF-beta1 and oxidant stress in children with severe asthma: association with airflow limitation. J Allergy Clin Immunol 2012;129:388-96, e1-8.
42. Raghu G, Masta S, Meyers D, Narayanan AS. Collagen synthesis by normal and fibrotic human lung fibroblasts and the effect of transforming growth factor-beta. Am Rev Respir Dis 1989;140:95-100.
43. Reed MJ, Vernon RB, Abrass IB, Sage EH. TGF-beta 1 induces the expression of type I collagen and SPARC, and enhances contraction of collagen gels, by fibroblasts from young and aged donors. J Cell Physiol 1994;158:169-79.
44. Qing J, Zhang Y, Derynck R. Structural and functional characterization of the transforming growth factor-beta-induced Smad3/c-Jun transcriptional cooperativity. J Biol Chem 2000;275:38802-12.
45. Piek E, Ju WJ, Heyer J, Escalante-Alcalde D, Stewart CL, Weinstein M, et al. Functional characterization of transforming growth factor beta signaling in Smad2- and Smad3-deficient fibroblasts. J Biol Chem 2001;276:19945-53.
46. Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. N Engl J Med 2000;342:1350-8.
47. Serpero L, Petecchia L, Sabatini F, Giuliani M, Silvestri M, Di Blasi P, et al. The effect of transforming growth factor (TGF)-beta1 and (TGF)-beta2 on nasal polyp fibroblast activities involved upper airway remodeling: modulation by fluticasone propionate. Immunol Lett 2006;105:61-7.
48. Eisma RJ, Allen JS, Lafreniere D, Leonard G, Kreutzer DL. Eosinophil expression of transforming growth factor-beta and its receptors in nasal polyposis: role of the cytokines in this disease process. Am J Otolaryngol 1997;18:405-11.
49. Levi-Schaffer F, Garbuzenko E, Rubin A, Reich R, Pickholz D, Gillery P, et al. Human eosinophils regulate human lung- and skin-derived fibroblast properties in vitro: a role for transforming growth factor beta (TGF-beta). Proc Natl Acad Sci U S A 1999;96:9660-5.
50. Radaev S, Zou Z, Huang T, Lafer EM, Hinck AP, Sun PD. Ternary complex of transforming growth factor-beta1 reveals isoform-specific ligand recognition and receptor recruitment in the superfamily. J Biol Chem 2010;285:14806-14.
51. Chakir J, Shannon J, Molet S, Fukakusa M, Elias J, Laviolette M, et al. Airway remodeling-associated mediators in moderate to severe asthma: Effect of steroids on TGF-β, IL-11, IL-17, and type I and type III collagen expression. J Allergy Clin Immunol 2003;111:1293-8.
52. Minshall E, Chakir J, Laviolette M, Molet S, Zhu Z, Olivenstein R, et al. IL-11 expression is increased in severe asthma: association with epithelial cells and eosinophils. J Allergy Clin Immunol 2000;105:232-8.
53. Huang J, Olivenstein R, Taha R, Hamid Q, Ludwig M. Enhanced proteoglycan deposition in the airway wall of atopic asthmatics. Am J Respir Crit Care Med 1999;160:725-9.
54. Roberts CR. Is asthma a fibrotic disease? Chest 1995;107(suppl):111S-7S.
55. Cheng G, Swaidani S, Sharma M, Lauer ME, Hascall VC, Aronica MA. Hyaluronan deposition and correlation with inflammation in a murine ovalbumin model of asthma. Matrix Biol 2011;30:126-34.
56. Ohkawara Y, Tamura G, Iwasaki T, Tanaka A, Kikuchi T, Shirato K. Activation and transforming growth factor-beta production in eosinophils by hyaluronan. Am J Respir Cell Mol Biol 2000;23:444-51.
57. Hackett TL, Knight DA. The role of epithelial injury and repair in the origins of asthma. Curr Opin Allergy Clin Immunol 2007;7:63-8.

METHODS

Epithelial cell isolation

Immediately after the endotracheal tube was secured, 3 bronchial epithelial cell samples were obtained from subjects during general anesthesia by using 4-mm Harrell unsheathed bronchoscope cytology brushes (CONMED). As described by Lane et al,[E1] the unprotected brush was inserted through an endotracheal tube, advanced until resistance was felt, and rubbed against the airway surface for 2 seconds. Nasal brushings were also obtained in each subject and banked for future studies. 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) containing gentamicin and amphotericin B and further supplemented with penicillin-streptomycin (100 μg/mL, Invitrogen). 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 70% to 90% confluent, cells were passaged into 3 new P_1 T-25 flasks.

ALI epithelial cell cultures

For ALI cultures, we used the PneumaCult ALI media system (StemCell Technologies, Vancouver, British Columbia, Canada).[E2] Before seeding, the cells were incubated with PneumaCult ALI Induction Media for 24 hours in the T-25 flasks, according to the manufacturer's recommendations. After induction, cells were then trypsinized with 2 mL of 0.025% Trypsin-EDTA and then seeded onto collagen I–precoated Corning Costar 12-mm 0.4-μm Transwells (Corning Life Sciences, Tewksbury, Mass) at a concentration of 100,000 cells per transwell. Cells were then kept in submerged culture by using PneumaCult ALI Expansion Media in both the apical and well chambers for 7 days or until confluent, according to the manufacturer's guidelines. Once confluent, cells were then changed to PneumaCult ALI Maintenance Media in the lower well chamber only, and the remaining apical media was aspirated. ALI Maintenance Media in the basolateral compartment were changed every other day, and cells were differentiated at an ALI for 21 days before initiation of cell-culture experiments. Each experimental condition per cell line consisted of triplicate transwells. Sampling of basolateral conditioned media and extraction of RNA were performed 96 hours after initiation of cocultures or at the corresponding time point for HLF-only time controls.

REFERENCES

E1. Lane C, Burgess S, Kicic A, Knight D, Stick S. The use of non-bronchoscopic brushings to study the paediatric airway. Respir Res 2005;6:53.

E2. Wadsworth SJ, Riedel M, Eskandar Afshari A, Louis S, Dorscheid D. PneumaCultTM-ALI: an improved media for mucociliary differentiation of primary human bronchial epithelial cells. Am J Respir Crit Care Med 2012;185:A6345.