Decreased Fibronectin Production Significantly Contributes to Dysregulated Repair of Asthmatic Epithelium

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Rationale: Damage to airway epithelium is followed by deposition of extracellular matrix (ECM) and migration of adjacent epithelial cells. We have shown that epithelial cells from children with asthma fail to heal a wound *in vitro*.

Objectives: To determine whether dysregulated ECM production by the epithelium plays a role in aberrant repair in asthma.

Methods: Airway epithelial cells (AEC) from children with asthma (n = 36), healthy atopic control subjects (n = 23), and healthy nonatopic control subjects (n = 53) were investigated by microarray, gene expression and silencing, transcript regulation analysis, and ability to close mechanical wounds.

Measurements and Main Results: Time to repair a mechanical wound *in vitro* by AEC from healthy and atopic children was not significantly different and both were faster than AEC from children with asthma. Microarray analysis revealed differential expression of multiple gene sets associated with repair and remodeling in asthmatic AEC. Fibronectin (FN) was the only ECM component whose expression was significantly lower in asthmatic AEC. Expression differences were verified by quantitative polymerase chain reaction and ELISA, and reduced FN expression persisted in asthmatic cells over passage. Silencing of FN expression in nonasthmatic AEC inhibited wound repair, whereas addition of FN to asthmatic AEC restored reparative capacity. Asthmatic AEC failed to synthesize FN in response to wounding or cytokine/growth factor stimulation. Exposure to 5', 2'-deoxyazacytidine had no effect on FN expression and subsequent analysis of the FN promoter did not show evidence of DNA methylation.

Conclusions: These data show that the reduced capacity of asthmatic epithelial cells to secrete FN is an important contributor to the dysregulated AEC repair observed in these cells.

Keywords: fibronectin; asthma; epithelium; wound repair; inflammation

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AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Injury of the airway epithelium is normally repaired by a process involving the deposition of extracellular matrix proteins by airway epithelial cells, promoting adhesion and migration to the site of injury. Dysregulated repair processes leading to epithelial remodeling may be important in asthma pathogenesis.

What This Study Adds to the Field

This study found that fibronectin (FN) is necessary for normal wound repair, and that asthmatic epithelial cells have persistently reduced FN production and deficient wound repair *in vitro* that can be restored with exogenous FN.

Asthma is the most common chronic respiratory disorder in children. Despite significant advances in our understanding of asthma, available therapies fail to alter the natural history and progression of the disease (1–6). Airway epithelial cells (AEC) are continuously exposed to and injured by environmental irritants, such as viruses and pollutants, and as such are ideally situated to orchestrate airway function in response to these stimuli. We and others have shown that the epithelium is intrinsically altered both structurally and biochemically in asthma, reflecting an increased susceptibility to injury, an inadequate repair response, or a combination of both (7–14). Importantly, many of the characteristic abnormalities can be detected early in disease progression and correlate with disease severity (15). For these reasons, it is essential to understand the complex regulatory mechanisms involved in epithelial repair and to identify novel mechanisms and therapeutic targets.

Under normal circumstances damage and loss of epithelial cells is followed by repair of the denuded luminal surface by the adjacent epithelium. The AEC surrounding the injury are triggered to synthesize and deposit extracellular matrix (ECM) on the exposed basement membrane to promote adhesion and migration of adjacent epithelial cells into the injury site (16). Fibronectin (FN) is one of the primary ECM proteins produced by AEC (17) and profoundly influences the survival, proliferation, and differentiation of these cells, suggesting it is an important contributor to epithelial wound repair (18–20).

TABLE 1. PATIENT DEMOGRAPHICS

Phenotype	Total (n)	Sex	Number	Average Age (yr)	Age Range (yr)	Average IgE (kU/L)	Hay Fever	Eczema	Hay Fever & Eczema
Healthy nonatopic	53	M	25	8.9 ± 4.6	2.5–12.7	49.7	3	2	1
		F	28	6.6 ± 3.0	2.9–16.1	63.7	4	5	0
Healthy atopic	23	M	14	7.3 ± 3.7	2.4–15.6	256	4	3	2
		F	9	10.1 ± 4.4	2.4–13.9	257	3	2	2
Atopic asthmatic	36	M	23	8.6 ± 3.5	2.4–16.9	729	4	2	12
		F	13	8.5 ± 3.0	3.6–13.2	414	4	3	4

Definition of abbreviations: F = female; M = male.

Given our previous observations of incomplete epithelial repair by asthmatic epithelium (14), we hypothesized that intrinsic differences specifically relating to proliferation, differentiation, and migration contribute to the dysregulated epithelial response to injury. We investigated the global gene expression profile of pediatric asthmatic epithelium and identified FN as a target for further investigation. We show that knockdown of FN gene in normal cells impairs wound closure, whereas addition of FN to asthmatic cells largely restores reparative capacity. Fibronectin gene expression in asthmatic cells could not be induced through growth factor/cytokine treatment or mechanical wounding. Finally, we provide evidence to suggest that epigenetic regulation is not responsible for silencing FN expression in asthmatic epithelial cells. These observations suggest that functional alterations in epithelial wound repair are present early in the course of childhood asthma and that reduced production of FN is a key component of this process.

METHODS

Detailed descriptions of methodologies are provided in the online supplement.

Patients and Cell Types

Children (2.4–16.9 yr) with and without mild asthma were recruited before elective surgery. Asthma was defined by a physician diagnosis and positive responses on American Thoracic Society/European Respiratory Society respiratory questionnaires (21, 22). Children with asthma had not taken inhaled/oral glucocorticosteroids for at least 1 month before surgery. Atopy was determined by a positive radioallergen sorbent result to a panel of common allergens, including house dust mite, grass pollens, milk, mold, peanut, egg white, and animal dander. Epithelial cells were collected by bronchial brushing and cultured as previously described (8, 23, 24) and classified as healthy nonatopic (pAEC_{HNA}), healthy atopic (pAEC_{HA}), or atopic asthmatic (pAEC_{AA}).

Epithelial Wounding

Two wound models were used. To assess wound repair kinetics, circular wounds were created (25) and wound closure calculated as outlined in the online supplement. To assess the effects of small interfering RNA (siRNA), smaller wounds were made using a plastic pipette tip as described (14). Details are in the online supplement. In these experiments, supernatants, RNA, and cell pellets were collected at 24, 48, and 72 hours post wounding. For studies involving exogenous FN addition, confluent cultures were wounded. Purified human fibronectin (0–50 µg; Roche Diagnostics, Penzberg, Germany) was added and wound closure monitored.

Quantitative Polymerase Chain Reaction

Total RNA was extracted using an RNeasy mini kit (QIAGEN, Hilden, Germany). Fibronectin gene expression was determined via two-step reverse transcriptase polymerase chain reaction (RT-PCR) using the TaqMan Universal PCR Master mix and Taqman Gene Expression Assay (ID:Hs00365058_ml; Applied Biosystems, Melbourne, Victoria, Australia). Expression was normalized to 18S rRNA (Applied Biosystems).

Fibronectin Quantification

Soluble and cell lysate FN was measured by ELISA (Biomedical Technologies Inc, Stoughton, MA). Protein levels were normalized to cell number and expressed as pg/ml/10⁶ cells.

DNA Microarray, Hybridization, and Analysis

RNA from 16 hybridizations (pAEC_{HNA} = 9; pAEC_{AA} = 7) was quantified, assessed for quality using an Agilent Bioanalyser, and processed as previously described (23) for hybridization to Affymetrix Human Genome U133A Arrays. Data were normalized by GC Robust Multiarray Algorithm and differential gene expression between the groups was assessed.

Gene Silencing

siRNAs directed against human FN were commercially manufactured using previously published sequence information (<https://www1.qiagen.com/GeneGlobe>; QIAGEN). Gene silencing was performed as detailed in the online supplement.

Transforming Growth Factor-β1, Dexamethasone, Phorbol-12-Myristate-13-Acetate, Cycloheximide, and Homocysteine Treatment

Quiesced cells were treated with either transforming growth factor-β1 (10 ng/ml), dexamethasone (10 µM), phorbol-12-myristate-13-acetate (10 ng/ml), or cycloheximide (10 µM) for 24 hours or homocysteine

Figure 1. Wound repair capacity of healthy and asthmatic airway epithelium. Healthy nonatopic airway epithelial cell (pAEC_{HNA}), healthy atopic airway epithelial cell (pAEC_{HA}), and atopic asthmatic airway epithelial cell (pAEC_{AA}) cultures were grown to confluence and mechanically wounded. Rates of repair were assessed using video time-lapse microscopy at 12-hour intervals over a 30-day period or until full wound closure. pAEC_{HNA} and pAEC_{HA} completely closed wounds between 7 and 9 days. Conversely pAEC_{AA} exhibited dramatic impairment of wound healing and were unable to achieve full wound closure even after 30 days. Data are represented as the calculated wound closure at each time point from three replicates. Experiments were performed in at least six separate individuals per phenotype and the bars represent the SD.

Figure 2. Differences in lower airway epithelial gene expression between healthy nonatopic children (HNA) and children with atopic asthma (AA). Differentially expressed genes based on a false discovery rate of less than 0.25 and an absolute fold change of greater than or equal to 1.5 were arranged using unsupervised two-dimensional hierarchical clustering. Each column represents a differentially expressed gene and each row represents an individual subject. Colors represent fold change in each individual, with red indicating up-regulated genes and green indicating down-regulated genes with respect to the average of the HNA subjects.

(0.1 mM) for 6 hours. Cells were collected 3, 6, and 24 hours post treatment and FN gene expression examined.

5'-Aza-2'-Deoxycytidine and Trichostatin A Treatment

Cultures of pAEC_{AA} at 50% confluence were placed in basal medium for 24 hours and then incubated with either the DNA methyltransferase inhibitor 5'-aza-2'-deoxycytidine (AZA) (5 μ M) for 96 hours or the histone deacetylase inhibitor (HDAC) trichostatin A (TSA) (15 nM). At 3, 6, and 24 hours post treatment, FN gene expression was analyzed by quantitative (qPCR).

Assessment of DNA Methylation

Cultures of pAEC_{AA} at 50% confluence were washed and incubated with media AZA (5 μ M) for 96 hours. DNA was extracted and bisulfite treatment of 0.5 to 1 μ g genomic DNA per sample performed using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA). Modified DNA was amplified by PCR using primer pairs specific for regions spanning from -770 to -640 and +380 to +583 bp from the FN transcription start site, purified using the DNA Clean and

Concentrator kit (Zymo Research), and directly sequenced using an ABI 3130xl 16-capillary genetic analyzer.

Statistics

Statistical significance was assessed using Mann-Whitney nonparametric analysis. Experiments were performed three to six times using matched samples and all values presented are means $\pm$ SD. All *P* values less than 0.05 were considered to be significant. We have previously shown little subject variation within each phenotype and validated this experimental approach (8).

RESULTS

Cohort Characteristics

The clinical characteristics and demographics for all subjects recruited to the study are provided in Table 1. Of the 112 subjects recruited for this study, 53 were nonasthmatic, showed no response to any of the allergens tested, and had a group

TABLE 2. FIFTEEN BIOLOGICAL PROCESS ONTOLOGIES WITH THE MOST SIGNIFICANTLY DECREASED EXPRESSION IN THE ASTHMATIC EPITHELIUM RELATIVE TO HEALTHY NONATOPIC CONTROL

GO ID	Gene Ontology Term	Genes in Ontology	No. Significant	<i>P</i> Value*
GO:0009607	Response to biotic stimulus	829	125	8.11E-17
GO:0006955	Immune response	718	113	1.38E-16
GO:0006952	Defense response	797	119	1.12E-15
GO:0009613	Response to pest, pathogen, or parasite	493	81	4.74E-13
GO:0006954	Inflammatory response	192	45	5.08E-13
GO:0051707	Response to other organism	498	81	8.26E-13
GO:0009605	Response to external stimulus	457	71	2.10E-10
GO:0009611	Response to wounding	356	60	2.25E-10
GO:0006950	Response to stress	942	112	2.01E-08
GO:0019884	Antigen presentation, exogenous antigen	14	9	6.63E-08
GO:0050896	Response to stimulus	1737	177	1.10E-07
GO:0007626	Locomotor behavior	116	26	1.19E-07
GO:0019886	Antigen processing, exogenous antigen via MHC class II	15	9	1.55E-07
GO:0006935	Chemotaxis	111	25	1.88E-07
GO:0042330	Taxis	111	25	1.88E-07

Definition of abbreviations: E = exponential; GO = gene ontology; MHC = major histocompatibility complex.

* Fisher exact test.

Figure 3. Fibronectin (FN) gene and protein expression in healthy and asthmatic epithelium. (A) Total RNA was extracted from healthy nonatopic airway epithelial cells (pAEC_{HNA}) and atopic asthmatic airway epithelial cells (pAEC_{AA}) for assessment of FN gene expression using quantitative polymerase chain reaction. In pAEC_{AA} expression of FN was 5.1-fold lower than expression in pAEC_{HNA}. (B) pAEC_{HNA} and pAEC_{AA} cells were grown to confluence, supernatants and cell pellets collected, and intracellular and secreted FN levels determined by ELISA. Expression and secretion of FN was significantly lower in pAEC_{AA} compared with pAEC_{HNA} ($P < 0.001$). (C) Cultures of both pAEC_{HNA} and pAEC_{AA} were repetitively passaged, supernatants collected, and secreted FN levels measured by ELISA. The inability of pAEC_{AA} to synthesize FN was maintained over serial passage, but was greatest at initial passage ($P < 0.001$). Experiments were performed on cells from five to eight individuals per phenotype and the bars represent the SD.

mean total IgE of 57 IU/L. A further 23 subjects without asthma tested positive to one or more allergens and had a total group mean IgE of 256 IU/L. Thirty-six children were classified as atopic asthmatic with the majority also suffering from hay fever, eczema, or both. All subjects with asthma had mild disease and a total group mean IgE of 572 IU/L.

pAEC_{AA} Repair Wounds at a Significantly Slower Rate Than pAEC_{HNA}

We have recently shown that in the absence of exogenous stimuli, pAEC_{AA} cells proliferate faster than pAEC_{HNA} cells (8, 14). To examine whether this correlated with differences in wound closure capacity, pAEC_{HNA}, pAEC_{HA}, and pAEC_{AA} cells were grown to confluence and mechanically wounded. Although both pAEC_{HNA} and pAEC_{HA} cells were able to effectively close the wound within a similar time frame (7–9 d), pAEC_{AA} showed a dramatic impairment in wound healing ability, failing to completely close wounds even after 10 days (Figure 1). We followed repair in a subset of these cells over 30 days and found maximum wound closure to be less than 70% (data not shown).

Microarray Analysis Identified the Decreased Expression of Genes Related to Wound Repair in the Asthmatic Epithelium

To examine dysregulated repair of asthmatic epithelium, we undertook a global analysis of gene expression using Affymetrix microarrays using freshly harvested epithelial cells from children with and without asthma (Figure 2). Based on a log₂ ratio greater than or equal to 1.5 and false discovery rate (FDR) of 0.25, 764 genes were identified with increased gene expression (see Table E1 in the online supplement), including genes associated with apoptosis, metabolism, signal pathways and transduction, transcription, and transport. In addition, a further 848 genes showed decreased expression (Table E2), including those involved in cell adhesion, cell cycle, chemotaxis, immune response, protein transport, and cell motility. The complete data set is publicly available in the Gene Expression Omnibus public repository (<http://www.ncbi.nlm.nih.gov/geo/>) (accession no. GSE18965) in a format that complies with the Minimal Information About a Microarray Experiment guidelines. Gene ontology analysis of these differentially expressed genes identified a number of different processes, including biological processes (Table 2, Table E3), cellular components (Tables E4A and E4B), and molecular function (Tables E5A and E5B), that were significantly different. Each table illustrates the top 15 gene ontology terms with the most significantly increased (Tables E3, E4A, and E5A) or decreased (Table 2, Tables E4B and E5B) expression.

When examined more closely, 60 probes in the gene set involved in response to wounding showed significantly decreased expression in the asthmatic epithelium. These included genes involved in chemokine binding (CCL3,5,18), toll-like receptor (TLR) signaling (TLR2, 8, CD14), and interleukin production and signaling (IL1A, IL1B, ILR1, IL8RA) (Table E6). FN-1 was the only extra cellular matrix component to be significantly decreased out of this probe list. Gene Set Enrichment Analysis (GSEA) was then performed to identify coordinated changes in genes with common biological function. Using GSEA of gene sets that included FN, we found six gene sets demonstrating core enrichment in pAEC_{HNA} compared with pAEC_{AA} (Table E7), including the “response to wounding” gene set (Figure E1). Strikingly, of the 60 probes in this gene set, only one ECM component, FN-1, was significantly decreased in asthmatic cells (2.1-fold; Table E6), validating our initial observations. This finding was further confirmed by

Figure 4. Fibronectin (FN) gene expression and function in healthy nonatopic airway epithelial cell (pAEC_{HNA}) wound repair. (A) pAEC_{HNA} cultures were grown to confluence and mechanically wounded. Cell lysates were then collected at 24-hour intervals until full wound closure. Total RNA was extracted and FN gene expression measured over the course of repair. Expression of FN was significantly elevated in pAEC_{HNA} during the first 48 hours of repair and returned to baseline levels by 72 hours, coincident with full wound closure. (B) pAEC_{HNA} cultures were grown to confluence and treated with or without FN small interfering RNA (siRNA) for 24 hours, wounded, and repair assessed at 24-hour intervals until full repair was observed. Full wound closure was achieved in control cells by 72 hours. However, FN knockdown dramatically inhibited wound repair in pAEC_{HNA}. (C) pAEC_{HNA} cultures were grown to confluence, exposed to FN siRNA for 24 hours, and mechanically wounded. Purified human FN was then added at varying concentrations (0–50 μg) and rates of repair assessed at 24-hour intervals until wounds were closed. The blunted wound repair response evident with FN knockdown was significantly reversed by the addition of FN in a concentration-dependent and time-dependent manner ($P < 0.0001$ compared with the silenced control). Data are represented as the calculated wound closure at each time point from triplicate values from at least five separate individuals per phenotype and the bars represent the SD of the data points.

Figure 5. Fibronectin (FN) gene expression and role in atopic asthmatic airway epithelial cell (pAEC_{AA}) wound repair. (A) pAEC_{AA} cultures grown to confluence and mechanically wounded. Cell lysates were collected at 24-hour intervals until full wound closure was observed in healthy nonatopic airway epithelial cells (pAEC_{HNA}). Total RNA was extracted and FN gene expression measured over the course of repair. FN gene expression in pAEC_{AA} did not change. (B) pAEC_{AA} cultures were grown to confluence and treated with or without FN small interfering RNA (siRNA) for 24 hours, wounded, and repair assessed as described above. No evidence of wound repair was observed. (C) pAEC_{AA} cultures were grown to confluence, exposed to FN siRNA for 24 hours, and mechanically wounded. Purified human FN was then added at varying concentrations (0–50 μg) and rates of repair assessed at 24-hour intervals until wounds were closed. Wound repair was significantly enhanced in a concentration-dependent manner ($P < 0.001$ compared with the silenced control), to a maximum 70% of complete closure. Data are represented as the calculated wound closure (three replicates) at each time point from at least four individuals per phenotype and the bars represent the SD.

Figure 6. Regulation of fibronectin (FN) transcription in atopic asthmatic airway epithelial cells (pAEC_{AA}). (A–D). pAEC_{AA} cultures were grown to confluence, washed, and incubated in basal media for 24 hours before experimentation. Cells were then treated with (A) dexamethasone (10 μM), (B) phorbol-12-myristate-13-acetate (10 ng/ml), or (D) transforming growth factor (TGF)-β1 (10 ng/ml) for 24 hours. Cells were also treated with (C) homocysteine (0.1 mM) for 6 hours. Cells were lysed, RNA extracted, and FN gene expression analyzed at described time points. (A–C) Stimulation of glucocorticoid response element, cAMP response element, and iron response transcription activator-2 did not result in increased FN transcription. (D) Treatment with TGF-β1 induced a transient increase in FN expression at 3 hours ($P < 0.05$), which was not sustained over longer periods. Data are represented as mean expression of three replicates at each time point from at least four separate individuals per phenotype and the bars represent the SD of the data points.

qPCR, which showed a fivefold reduction in FN gene expression in pAEC_{AA} (Figure 3A; $P = 0.003$). The decrease in FN gene expression was mirrored by a substantially reduced production of FN protein by pAEC_{AA} (Figure 3B; $P < 0.001$) measured in both culture supernatants and cell lysates. The diminished capacity to synthesize FN was maintained over repeated passage (Figure 3C; $P < 0.05$). To address whether this may be an effect of atopy, we also assessed FN expression in pAEC_{HNA} and found gene expression to be similar to pAEC_{HNA} ($P = 0.675$; Figure 3A). Although the amount of FN protein released by pAEC_{HNA} was significantly lower than pAEC_{HNA} ($P = 0.004$ and $P = 0.01$ for lysate and soluble FN production, respectively), the amounts were still significantly higher than that from pAEC_{AA} ($P = 0.003$ and $P = 0.001$ for lysate and soluble FN production, respectively; Figure E2A). Because atopy may precede the development of asthma, we assessed the effect of age on the rate of wound closure and FN gene and protein production and found that these parameters were not related to age (data not shown).

FN Profile during pAEC_{HNA} and pAEC_{AA} Wound Repair

We then used siRNA to determine the contribution of FN to epithelial wound repair. Using this approach FN gene expression was reduced by 89% in pAEC_{HNA} and 85% in pAEC_{AA} (Figure E2B). In pAEC_{HNA}, FN gene expression increased more than fivefold during the first 48 hours of wound repair (Figure 4A), returning to baseline levels by 72 hours, coinciding with complete wound closure (Figure 4B). As expected, FN knockdown dramatically inhibited wound repair (Figure 4B). To determine whether FN was necessary for wound repair we added FN back to cultures in which FN gene expression was silenced. As shown in Figure 4C, the addition of FN promoted complete wound closure by 72 hours, suggesting that FN is necessary and sufficient for epithelial restoration under normal conditions.

In contrast to pAEC_{HNA}, FN gene expression in pAEC_{AA} was not altered by wounding (Figure 5A) and coincident with

this, wound closure over the same period was minimal. Furthermore, when FN was silenced in pAEC_{AA}, wound repair was virtually abolished (Figure 5B). Addition of FN to pAEC_{AA} enhanced wound repair in a concentration-dependent manner. Although full wound closure was not achieved by 72 hours, approximately 70% of the wound had closed in the presence of FN compared with approximately 20% in the untreated cultures (Figure 5B; $P < 0.001$).

FN Gene Expression in pAEC_{AA} Is Not Regulated by Binding of Regulatory Factors to Promoter Transcription Factor Binding Sites

Having confirmed a functional role for FN in epithelial wound repair, we next sought to identify possible mechanisms that could be responsible for suppressing its expression in pAEC_{AA}. *In silico* analysis of the FN promoter revealed several putative transcription factor consensus sequences, including glucocorticoid response element (GRE), cAMP response element (CRE), iron response transcription activator (ATF-2), and Smad binding sites. We then exposed pAEC_{HNA} and pAEC_{AA} to selective agonists known to activate these transcription factor consensus sequences. However, as shown in Figures 6A–6C, stimulation of GRE, CRE, ATF-2, or Smad (Figures 6A–6D) had little effect on inducing FN expression.

The FN Promoter Is Not Epigenetically Silenced in pAEC_{AA}

The down-regulation of FN gene expression through the hypermethylation of 5' CpG islands in its promoter region has been suggested to play a functional role in the development and progression of certain malignancies (26). We evaluated the extent of CpG methylation in the FN promoter as well as 100 bp upstream of the transcriptional start site in pAEC_{AA} using direct sequencing of bisulfite-converted genomic DNA. However, we found no evidence of DNA methylation in the regions tested (Figure 7A). Similarly, treatment with the DNA methyltransferase inhibitor AZA had little effect in activating FN gene expression (Figure 7B). We also investigated the potential

Figure 7. Assessment of fibronectin (FN) promoter methylation or histone acetylation in atopic asthmatic airway epithelial cells (pAEC_{AA}). (A) Methylation analysis of two CpG islands located -770 to -640 and $+380$ to $+583$ from the FN1 transcription start site for (a) 5'-aza-2'-deoxycytidine (AZA)-treated samples and (b) control samples using genomic bisulfite sequencing. Each circle shows a CpG site in the primary DNA sequence (open circles, nonmethylated; solid circles, methylated). (B) Cultures of pAEC_{AA} were grown until 50% confluent, washed, and incubated in basal medium for 24 hours before experimentation. Cultures were then treated with either AZA (5 μ M), the histone deacetylase inhibitor trichostatin A (TSA, 15 nM), or a combination of both over a designated period. Cells were then collected 3, 6, and 24 hours post treatment and FN gene expression examined. Treatment of pAEC_{AA} with TSA or AZA failed to stimulate FN gene transcription. Furthermore, pretreatment of pAEC_{AA} with AZA followed by TSA stimulation also failed alter FN gene expression. Data are represented as determined FN gene expression of three replicates at each time point from at least four separate individuals per phenotype and the bars represent the SD of the data points.

regulatory roles of HDAC on FN gene expression. Exposure to the HDAC inhibitor TSA, which would normally result in increased levels of histone acetylation, also failed to stimulate transcription and resulting FN gene expression even over extended exposure periods (Figure 7B). Combined exposure to TSA and AZA had no effect (Figure 7B).

DISCUSSION

We have previously shown biochemical and functional differences between the epithelium of children with and without asthma (8), and that asthmatic epithelial cells exhibit dysregulated responses to injury (14). In the current study, we extend these earlier findings by undertaking a global gene expression analysis on freshly isolated epithelial cells obtained from children with and without asthma focusing on expression of ECM genes and others associated with wound repair. Gene ontology analysis revealed that the expression of the gene set "response to wounding" was reduced in pAEC_{AA}. Within this gene set, FN was the only ECM protein significantly down-regulated in pAEC_{AA}. This finding was confirmed by qPCR and analysis of protein expression. Furthermore, knocking down FN

expression in pAEC_{HNA} resulted in a significant impairment in wound healing and addition of FN to these cells fully rescued wound healing capacity. In pAEC_{AA} addition of FN significantly improved, but did not completely restore, wound healing ability. Addition of growth factors and cytokines known to induce FN expression did not impact the reduced FN expression in pAEC_{AA}. Similarly, wounding itself had no effect on FN gene expression in pAEC_{AA}. Finally, we found no evidence of this in pAEC_{AA}. Collectively our data demonstrate that the response to wounding is dysregulated in AEC from children with asthma, and that a reduced ability to synthesize FN is a key factor in this response.

The epithelium is constantly faced with inflammatory and potentially injurious stimuli. To maintain the integrity of the barrier, rapid repair mechanisms are necessary (27, 28). Under normal conditions, repair is believed to follow a coordinated series of processes involving inflammation, proliferation, and reepithelialization, which is accompanied by remodeling of the underlying ECM (18, 20). We show that pAEC_{HNA} were able to fully repair wounds 1 mm wide within 7 days. This time is similar to that seen in animal studies *in vivo* (18, 29). In contrast, pAEC_{AA} exhibit a significantly impaired ability to complete

wound repair, failing to fully close wounds even after 30 days. Although dysregulated wound repair in asthmatic epithelium has been previously suggested (30, 31), to our knowledge these data are the first to functionally assess the wound-healing capacity of pediatric asthmatic airway epithelium. Importantly, our findings provide further mechanistic support for the concept that compromised repair processes are an innate feature of asthmatic epithelium.

Microarray technology has been used to identify gene profiles associated with a number of lung diseases, including asthma, pulmonary fibrosis, and chronic obstructive pulmonary disease in the context of understanding diseases in adults (32–39). We used microarray to identify gene expression differences in children with asthma, focusing on the epithelium. Globally, we identified 1,612 genes as differentially expressed (764 increased, 848 decreased) in pAEC_{AA} and that expression of a large numbers of genes involved in the “response to wounding” gene ontology were reduced in these cells. This included a number of genes previously implicated in the pathophysiology of asthma, as well as a number of genes not previously known to be involved in this condition. These findings strongly support a fundamental alteration in the epithelium that contributes to onset and progression of asthma. In addition to the deficient wound repair response, other signals in the asthmatic epithelium point to deficient immune responses, consistent with two recent investigations demonstrating that asthmatic AEC have reduced capacity to control rhinovirus infection related to deficient innate immune response (40, 41).

Fibronectin is a large multifunctional glycoprotein that promotes cell migration, attachment, and spreading and has been shown to play an important role in epithelial wound repair (14, 42–46). In the “response to wounding” ontology, FN expression was significantly decreased in asthmatic cells. This was confirmed by qPCR analysis and at the protein level. These results differ from those of others (47) who have demonstrated that epithelial cells from adult patients with asthma release spontaneously or after stimulation more FN than their healthy counterparts. We think it is unlikely that the differences in FN production are the result of the sampling technique *per se*, but that they are likely to be related to cohort differences, namely, pediatric mild asthma and adult moderate to severe asthma. Similarly, the relative contribution of epithelial cell-specific FN as measured in this study versus the contribution of plasma leakage, epithelium, and other tissue sources measured in BAL *in vivo* studies (48) is unknown. We show that silencing FN in pAEC_{HNA} profoundly disrupted wound repair to a rate comparable to pAEC_{AA}. Moreover, addition of FN dose-dependently restored the reparative ability of these cells, confirming that FN is necessary and sufficient for full epithelial wound closure. In contrast, although addition of FN significantly improved the rate and extent of wound repair in pAEC_{AA}, full wound closure was not achieved, suggesting the involvement of other as-yet unknown interacting factors. These may include specific FN integrins and/or growth factor receptors, both of which are under investigation.

Because FN synthesis in pAEC_{AA} cells is constitutively low compared with pAEC_{HNA}, we next examined whether pAEC_{AA} could be stimulated to synthesize and secrete FN. Mechanical wounding, a nonspecific but powerful stimulus, did not induce FN synthesis in pAEC_{AA} cells. Analysis of the FN promoter revealed a number of transcription factor consensus sequences, such as Sp1, GRE, CRE, and ATF-2 sequences (27, 49–61). However, activation of these binding sites after treatment with transforming growth factor- β 1, dexamethasone, phorbol-12-myristate-13-acetate, or homocysteine also failed to increase FN expression.

The reduction in FN expression seen in pAEC_{AA} persisted over serial passage suggesting that it is not dependent on an *in vivo* environment. Epigenetic control of gene expression by either methylation of CpG motifs in the promoter region or histone modifications can inhibit gene transcription by restricting the binding of specific transcription factors or recruiting repressive chromatin-modifying complexes such as HDACs. Methylation of CpG islands within the promoter region of several ECM genes, including FN, have been described in several cancers and are highly correlated with reduced FN expression (25, 61). *In silico* analysis of the FN gene revealed three CpG islands in the promoter and a total of 24 throughout the entire gene. We used two approaches to investigate whether the FN promoter was methylated. First, we treated proliferating cells with the DNA methyl transferase inhibitor, AZA. However, this had no effect on FN expression. To confirm this finding, we used bisulfite sequencing and again found no evidence that the FN promoter was methylated. Furthermore, treatment with the potent HDAC inhibitor TSA also had little effect on FN gene expression in pAEC_{AA}, suggesting that histone deacetylation was not involved in FN suppression. Current studies are focusing on microRNAs and in particular miR-17, which has been shown to regulate FN gene expression in other disease settings (62–64).

In summary, we reveal that the global gene expression profile of epithelium from children with mild asthma is intrinsically different from that of nonatopic children. Using this approach, qPCR, and ELISA, we show that expression of the ECM protein is reduced in asthmatic cells. Our data suggest that this defect was not related to control of FN expression by DNA methylation or histone acetylation. Given that FN is an essential component of the provisional ECM, providing a surface for epithelial migration and proliferation, our data highlight a potential mechanism for the abnormal epithelial repair seen in asthmatic airways.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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