Differential deposition of fibronectin by asthmatic bronchial epithelial cells

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Abstract

Altered extracellular matrix (ECM) protein deposition is a feature in asthmatic airways. Fibronectin (Fn), an ECM protein produced by human bronchial epithelial cells (HBECs), is increased in asthmatic airways. This study investigated the regulation of Fn production in asthmatic or non-asthmatic HBECs, and whether Fn modulated HBEC proliferation and inflammatory mediator secretion. The signaling pathways underlying transforming growth factor (TGF)-β1 regulated Fn production were examined using specific inhibitors for ERK, JNK, p38 MAPK, phosphatidylinositol (PI)3 kinase and activin like kinase (ALK)5.

Asthmatic HBECs deposited higher levels of Fn in the ECM than non-asthmatic cells under basal conditions, whilst cells from the two groups had similar levels of Fn mRNA and soluble Fn. TGF-β1 increased mRNA levels, and ECM and soluble forms of Fn but decreased cell proliferation in both cells. The rate of increase in Fn mRNA was higher in non-asthmatic cells. However, the excessive amounts of ECM Fn deposited by asthmatic cells, after TGFβ1 stimulation, persisted compared to non-asthmatic cells. Inhibition of ALK5 completely prevented TGF-β1 induced Fn deposition. Importantly, ECM Fn increased HBECs proliferation and IL-6 release, decreased PGE2 secretion, but had no effect on VEGF release. Soluble Fn had no effect on cell proliferation and inflammatory mediator release.

Asthmatic HBECs are intrinsically primed to produce more ECM Fn, which when deposited into the ECM is capable of driving remodeling and inflammation. The increased airway Fn may be one of the key driving factors in the persistence of asthma and represent a novel therapeutic target.

Key Words: fibronectin, asthma, airway epithelial cell, TGFβ1, ECM protein, IL-6
**Introduction**

Airway remodeling is a feature of chronic severe asthmatic airways. One of the defining features of remodeling is altered extracellular matrix (ECM) in the airway wall. In addition to providing a structural framework the ECM plays an important role in regulating airway cell homeostasis by regulating processes such as cell adhesion, proliferation, migration, differentiation and the expression of inflammatory cytokines and contractile proteins. These changes in turn influence airway hyperresponsiveness (13-15).

Fibronectin (Fn) is one of the ECM proteins in the airway wall, which is increased in the basement membrane of the airways from patients with asthma compared to people without asthma (1, 37, 38). Fn is a 440 kDa dimeric glycoprotein, which exists in a soluble protomeric form in blood plasma and in an insoluble multimeric form when incorporated into the ECM. Plasma Fn is mainly synthesized in the liver by hepatocytes. The Fn synthesized locally in tissues by the surrounding cells is referred to as cellular Fn. Cellular Fn contains one or two extra type III modules subjected to alternative splicing (extradomain-A (EDA) and EDB), whereas plasma Fn contains neither ED. The ECM Fn can be formed by a cell-mediated process involving both integrins and specialized cell surface sites which polymerizes both plasma Fn and/or cellular Fn (27).

Fn is known to elicit multiple functions. *In vitro*, plasma Fn pre-coated in culture wells decreases the expression of contractile proteins and increases cell proliferation in airway smooth muscle (ASM) cells (13). Polymerized Fn induces cell spreading in collagen gels and cell contractility (17). Fn stimulates A549 lung epithelial cells and small airway epithelial cell migration and invasion, and the proliferation of BEAS-2B and 16-HBE (16, 28). In addition, Fn also inhibits epithelial cell apoptosis (11).
Fn can be produced by inflammatory cells and airway structural cells. Vignola and coworkers found that the levels of Fn released by alveolar macrophages recovered from bronchoalveolar lavage (BAL) fluid from asthmatic patients were higher than those from healthy controls (46). However, in human bronchial epithelial (HBE) cells isolated and expanded in culture Fn expression was lower in cells from children with asthma compared to those from otherwise healthy atopic children. In the same study, Kicic et al found that the addition of exogenous Fn to asthmatic HBE cells restored the wound repairing capacity which was deficient in the asthmatic cells (23).

The profibrotic growth factor, transforming growth factor (TGF)β1, has been found to be a potent stimulus for Fn in vascular and airway smooth muscle cells, lung fibroblasts and the alveolar epithelial cell line, A549 (20-22, 25). The TGFβ1 signaling pathway is complicated and includes the Smad cascade, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated kinase (MAPK), as well as phosphatidylinositol 3-kinase (PI3K). The study in bronchial biopsy samples from asthmatic and healthy subjects found that activated TGFβ/Smad2 signaling is positively associated with the thickness of the basement membrane (39). In ASM cells, the ERK, p38 MAPK and PI3K are all involved in TGFβ1 induced Fn mRNA expression in non-asthmatic cells while only ERK and p38 MAPK were observed in asthmatic cells (20). However, the cellular signaling pathways for Fn production in HBEs under basal condition or with TGFβ1 stimulation is unclear. The aim of this study was to compare the expression of Fn by HBE cells from individuals with and without asthma under basal conditions or in the presence of TGFβ1 and the regulating signaling pathways under these conditions. Furthermore, the role of exogenous Fn in
regulating cell proliferation and the release of the cytokines and inflammatory mediators was investigated.

**Materials and Methods**

**Tissue collection and cell culture**

Approval for all experimental protocols with human lung was provided by the Human Research Ethics Committees of The University of Sydney and the Sydney South West Area Health Service. HBE cells were obtained from bronchial airways of volunteers with asthma or no lung disease and patients undergoing lung resection or transplantation. All donors provided written informed consent.

Bronchial brushing through the flexible fibreoptic bronchoscope was used to collect epithelial cells from volunteers. The lung tissue obtained at thoracotomy was dissected and the airways were isolated from macroscopically normal areas of lung. The epithelial layer was removed from the airways by macrodissection. After washing with Hank’s balanced salt solution, epithelial cells or tissue were placed in a tissue culture flask in Ham’s F-12 medium with growth supplements (9). The cells were maintained in Ham’s F-12 and tested negative for mycoplasmal contamination. The experiments were performed in bronchial epithelial growth medium (BEGM; Cambrex Bio Science, Walkersville, MD) as described previously (9). The experiments were performed with cells between passage 1 to 4. The cells from each individual were regarded as one primary cell culture. In this study, the primary cultures were categorised into two groups, the asthmatic and non-asthmatic group. The data from the asthmatic group were averaged as were those from the non-asthmatic group. n represents the number of primary cell cultures used within a group in each experiment. The patient and volunteer demographics are shown in online supplements table 1.
Cell experiments

HBE cells were seeded at 2x10^4 cell/cm^2 in 48-well plates in triplicate for ECM ELISA and in 12-well plates for collection of RNA lysates. The cells were grown for 3 days in BEGM, and then quiesced in bronchial epithelial basal medium (BEBM, Cambrex Bio Science, Walkersville, MD) for 24 hours. After quiescing, the medium was refreshed with BEGM in the absence or presence of TGFβ1 (0.1, 0.5, 1 and 5 ng/ml). The supernatants, ECM proteins and total RNA lysates were collected from the HBE cells at day 0 (quiesced in BEBM for 24 h), following 1, 2 or 3 days growth in BEGM. Samples for day 0 was collected at the time of stimulus addition after the cells had been quiesced for 24 hours, the measurements from samples collected at this time point were considered as basal levels.

Specific pharmacological protein kinase inhibitors were used to explore the signaling pathways involved in TGFβ1 modulated ECM Fn deposition. After quiescing in BEBM for 24 hours, the cells were pretreated with the MAPK kinase (MEK) inhibitor PD98059 (10 µM), the JNK inhibitor SP600125 (10 µM), the PI3K inhibitor LY294002 (3 µM) (Calbiochem, San Diego, California), the p38 MAP kinase inhibitor SB 239063 (3 µM) and the TGFβ type I receptor ALK5 inhibitor SB431542 (1 µM, 3 µM and 10 µM) (Tocris, Ellisville, Missouri) in BEGM for 30 minutes before stimulation with and without TGFβ1 (1 ng/ml). We used these specific protein kinase inhibitors at concentrations which have been previously proven to be effective in human airway cells (9, 10, 18, 20, 41). ECM samples were collected after 3 days.

To test the effect of Fn on HBE cell viability, proliferation and the release of soluble cytokines and chemokines, human plasma Fn (BD Biosciences, Bedford, Massachusetts) was precoated on plates at 0, 1.58, 5 and 15.8 µg/ml in PBS overnight at 37°C. The plates were
washed with sterile PBS and then the cells were seeded in BEGM. Samples were collected at day 1, 2 and 3.

The role of soluble Fn was also examined. Confluent and quiesced HBE cells were treated with Fn at 0, 1.58, 5 and 15.8 µg/ml in BEGM. After 1, 2 and 3 days of incubation, cell viability and proliferation were determined and supernatants were collected.

**ECM Fn ELISA**

The ECM Fn ELISA was performed as previously described with some modifications (19). At the end of time points, the medium was removed from the plates. The plates were washed with PBS and the cells were lysed with hypotonic ammonium hydroxide (0.016M NH₄OH, Sigma, Saint Louis, Missouri). At the time of analysis human plasma Fn was used to generate a standard curve. A serial dilution of Fn at concentrations of 2000, 1000, 500, 250, 125 and 62.5 ng/ml was added to the empty wells of the plates followed by incubation at 4°C overnight. The following day, the plates were washed with T-PBS (0.05% Tween-20 in PBS) and blocked with 1% BSA (bovine serum albumin) (Sigma, Saint Louis, Missouri) in PBS.

An anti-human Fn antibody (clone 868A11, Millipore, Billerica, MA) and a polyclonal rabbit anti-mouse immunoglobulin/horse radish peroxidase (HRP) (Dako, Glostrup, Denmark) were used. After final washing, a liquid substrate system, 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS, Sigma, Saint Louis, Missouri) was used to detect the amount of ECM Fn deposited by cells. The absorbance was immediately measured at 405 nm using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA). The readings from triplicate wells were averaged and the background absorbance subtracted. The data were expressed as ng/ml, which was calculated using the equation generated from the standard curve.
Soluble Fn ELISA

The levels of Fn released by the cells into the supernatant were determined using a QuantiMatrix human fibronectin ELISA kit (Millipore, Billerica, MA) according to the manufacturer’s instructions. The detection limit for soluble Fn was 3 ng/ml. A standard curve was constructed and the Fn concentration was interpolated from the standard curve and GraphPad Prism (Version 5.0) was used for further analysis.

Real time PCR

Real time PCR was performed as previously described (9). The total RNA lysates were collected and extracted using a NucleoSpin RNA II kit and M-MLV reverse transcriptase was used for reverse transcription. A pre-developed specific primer set for Fn, Hs00365058_m1, TaqMan Universal PCR MasterMix and the StepOne Plus Real-Time PCR System were used for real time PCR (Applied Biosystems, Branchburg, New Jersey USA). A pre-developed TaqMan reagent human 18S rRNA (Cat# 4319413, Applied Biosystems, Branchburg, New Jersey) was included in each real time PCR reaction as an endogenous control. Data from the reactions were analyzed using StepOne Software v2.1 (Applied Biosystems, Branchburg, New Jersey USA).

ELISA for IL-6, VEGF and PGE₂

The IL-6, VEGF and PGE₂ ELISAs were carried out following the manufacturers’ instructions (IL-6, BD Pharmingen, BD, Franklin Lakes, NJ; VEGF, R&D Systems, Minneapolis, MN; PGE₂, Cayman Chemical Company, Ann Arbor, MI).
Cell number, viability and cytotoxicity assay

At the end point of experiments, the cell number, viability and cytotoxicity were determined using manually cell counting, a lactate dehydrogenase (LDH) assay and/or a mitochondrial activity assay (MTT) respectively. The LDH assay was a means of measuring either the number of cells via total cytoplasmic LDH or membrane integrity (cytotoxicity) as a function of the amount of cytoplasmic LDH released into the medium. The MTT assay was a means of measuring the activity of living cells via mitochondrial dehydrogenases. Both assays were carried out according the manufacturer’s instructions (Sigma, Saint Louis, Missouri). The absorbance was measured using the SpectraMax M2 microplate reader. The data from each treatment were averaged and background absorbance subtracted and GraphPad Prism (Version 5.0) was used for further analysis.

The cell proliferation status and membrane integrity were also confirmed using CyQUANT Direct Cell Proliferation Assay according to the manufacturer’s instructions (Molecular Probes, Eugene, Oregon).

Statistical analysis

Data were expressed as mean ± the standard error of the mean (SEM) for the number of HBE cell cultures (n) stated and analysed using GraphPad Prism (Version 6.0). After testing for normal distribution and equal variance, the differences were assessed by unpaired Student’s t test, one-way or two-way ANOVA using Dunnett’s or Sidak’s multiple comparisons test or Bonferroni post tests with repeated measures as appropriate. A p-value of less than 0.05 was considered statistically significant.
Results

The expression of Fn from HBE cells under basal conditions

The levels of Fn mRNA expression were similar between asthmatic and non-asthmatic cells at day 0 and 1 (Fig. 1a). There was an increase in cell viability/proliferation measured by MTT assay and soluble Fn release from asthmatic HBE cells at day 3, but no differences in soluble Fn release and cell number were seen when comparing the asthmatic and non-asthmatic cells at days 1, 2 and 3 (Fig. 1b and Fig. 1d). However, asthmatic HBE cells constitutively deposited greater amounts of ECM Fn than NA cells (Fig. 1c).

TGFβ1 increased Fn production from HBE cells

Following stimulation with TGFβ1 the Fn mRNA expression was increased after 8 h, maximal at 24 h and while beginning to decline from maximum the levels were still above baseline at 48 and 72 h (n = 4, data not shown). Interestingly, after 24 h stimulation with TGFβ1, non-asthmatic HBE cells expressed a greater amount of Fn mRNA compared to asthmatic cells (Fig 2a). TGFβ1 increased both soluble and ECM Fn expression after 2 days of stimulation in non-asthmatic and asthmatic HBE cells. Furthermore, the levels of ECM Fn produced by asthmatic cells were higher than those by non-asthmatic cells in the presence of TGFβ1 (Fig. 2b and c for day 3, data for day 1 and 2 not shown), although the percentage of increase in ECM Fn induced by TGFβ1 was similar in non-asthmatic and asthmatic cells (at day 3, 5 ng/ml of TGFβ1, percentage increase over unstimulated 220.3 ± 54.6 % for NA n = 7, 206.3 ± 44.0 % for A n = 5, respectively).

The HBE cell viability and numbers modulated by TGFβ1 were monitored using MTT and CyQUANT direct cell proliferation assay. TGFβ1 reduced cell mitochondrial activity at day 1, 2 and 3 in non-asthmatic and asthmatic cells (Fig 3a). It also decreased DNA-bound
fluorescence intensity in both cell groups (Fig 3b) which confirmed that the HBE cell number was not increased by TGFβ1.

An ALK5 inhibitor, SB431542, blocked TGFβ1 induced Fn expression

The inhibitors for ERK (PD98058), PI3 kinase (LY294002), JNK (SP600125), p38 MAP kinase (SB239063) and ALK5 (SB431542) were used to block individual signaling pathways which may be involved in the TGFβ1 induced ECM Fn deposition in HBE cells. PD98059, LY294002, SP600125 and SB239063 had no effect on ECM Fn deposition in the absence and presence of TGFβ1 in either non-asthmatic or asthmatic HBE cells (Fig 4c, d, e and f).

However, SB431542 inhibited TGFβ1 induced Fn mRNA expression in non-asthmatic and asthmatic HBE cells (Fig 4a). Furthermore, SB431542 blocked TGFβ1 stimulated ECM Fn deposition but had no influence on constitutive ECM Fn deposition in either non-asthmatic or asthmatic cells (Fig 4b).

Fn increased HBE cell proliferation and regulated proinflammatory mediator release

To investigate the role of Fn in regulating cell proliferation and the release of proinflammatory mediators, the non-asthmatic HBE cells were treated with Fn in two ways: (1) plasma Fn was precoated on the plates and the cells were seeded on top of the Fn; (2) the cells were grown to confluence and quiesced before plasma Fn was added as a stimulus.

When HBE cells were seeded on the plate precoated with Fn, Fn increased cell viability at day 1, 2 and 3 as measured by MTT (Fig 5a for day 2 in asthmatic and non-asthmatic cells, day 1 and 3 data not shown) and LDH (table 2) assay. The precoated Fn induced HBE cell proliferation (Fig 5b) and this was also confirmed using a CyQUANT direct cell proliferation assay (table 2). Furthermore, the rates of cell proliferation measured by MTT, LDH, cell
counting and CyQUANT assay were similar (table 2). Precoated Fn had no effect on cytotoxicity of HBE cells as measured by released LDH (n = 12, data not shown).

Growth of the HBE cells in tissue culture wells precoated with Fn also altered the release of soluble factors important in asthma. Figure 6 illustrates IL-6 (a), PGE2 (b) and VEGF (c) release in the presence or absence of precoated Fn after correction for cell number as measured by MTT.

In contrast, soluble Fn when added to confluent HBE cells had no effect on cell viability and proliferation, nor did it affect cytotoxicity (non-asthmatic n = 8 and asthmatic n = 3 for MTT and LDH assays, non-asthmatic n = 7 and asthmatic n = 4 for cell counting, data not shown). Similarly, soluble Fn did not alter the regulation of IL-6, PGE2 and VEGF release in HBE cells (non-asthmatic n = 9 and asthmatic n = 3 for IL-6 and VEGF release, non-asthmatic n = 5 and asthmatic n = 3 for PGE2 release, data not shown).

4.4 Discussion

This study compared the expression of Fn in HBE cells from the airways of asthmatic and non-asthmatic adults constitutively or following stimulation with TGFβ1, and discovered that HBE cells produce both soluble and the ECM bound form of Fn. Interestingly, asthmatic HBE cells deposited greater amounts of ECM Fn than non-asthmatic cells. TGFβ1 increased ECM Fn and soluble Fn in both non-asthmatic and asthmatic HBE cells, and this effect was regulated by the ALK5/Smad signaling pathway. In addition, exogenous deposited Fn increased primary HBE cell proliferation and regulated the release of pro-inflammatory mediators.
The present study showed that the levels of ECM Fn, under both basal conditions and after stimulation with TGFβ1, were higher in asthmatic epithelial cells when compared to non-asthmatic cells although the percentages of increase induced by TGFβ1 were similar in both groups of cells. However, non-asthmatic and asthmatic epithelial cells expressed similar amounts of Fn mRNA and release of soluble Fn under basal conditions. When stimulated with TGFβ1, non-asthmatic cells expressed a greater amount of Fn mRNA than asthmatic cells which may be the result of the increased Smad2 activities (phospho-Smad2) induced by TGFβ1 in NA HBE cells as we previously reported (9). The reason why asthmatic cells deposit greater amounts of Fn, even while those cells express the same or lower levels of mRNA, is not clear and requires further investigation. The increased Fn in the subepithelial basement membrane in asthmatic airways may be the compound result of both enhanced ECM Fn deposition by asthmatic HBE cells and the elevated levels of TGFβ1 in asthmatic airways (1, 37).

The differences in Fn expression between non-asthmatic and asthmatic HBE cells detected in this study were not consistent with the findings reported by Kicic and colleagues. In their study, healthy nonatopic bronchial epithelial cells produced higher levels of Fn mRNA, soluble and cell lysate Fn than atopic asthmatic cells (23). The differences between these two studies may be due to the different tissue sources. In the current study, HBE cells were obtained from adults with or without asthma; in Kicic’s study, the epithelial cells were collected from children with or without asthma. It is of interest that there was a differential expression of Fn by HBE cells derived from adults and children. Other studies also found the discrepant results when comparing cellular responses from cells from children and adults in growth factor release and ECM deposition when comparing asthmatic and non-asthmatic airways (12, 24, 45)(8)(5, 44). The findings from these studies indicate that the
characteristics of asthmatic HBE cells and the ECM proteins deposited in asthmatic airways are altered. However, the alterations in asthmatic airways may be different between adults and children but this requires further investigation to understand the mechanisms underlying these changes.

The current study found that TGFβ1 augmented both soluble and ECM Fn production by asthmatic and non-asthmatic HBE cells; and the increase was at least partly regulated at the level of transcription since TGFβ1 also induced Fn mRNA expression. These results were consistent with the study conducted by Doerner and coworkers (6).

In this study, specific inhibitors for ERK, JNK, PI3K, p38 MAP kinase and TGFβ type I receptor ALK-5 were used to test which signaling pathways were involved in basal and TGFβ1 induced Fn deposition. The results showed that none of the inhibitors affected ECM Fn deposition under basal conditions, indicating that these signaling pathways were not involved in the basal ECM Fn deposition. Blockage of ERK, JNK, PI3K and p38 MAP kinase also had no effect on Fn deposition in the presence of TGFβ1 indicating that these signaling pathways were not involved in the TGFβ1 induced Fn deposition. This finding was different from our previous observations in ASM cells in which both ERK and p38 MAP kinase were involved in TGFβ1 induced Fn expression in non-asthmatic and asthmatic ASM cells, while PI3K was implicated only in asthmatic ASM cells (20). These results suggest that differential signaling pathways regulate Fn expression in different types of airway cells. The fact that inhibition of ALK5/Smad2 reversed TGFβ1 induced Fn ECM protein and mRNA expression in the HBE cells implied that TGFβ1 increases Fn production mainly via the ALK5/Smad2/3 pathway in non-asthmatic and asthmatic HBE cells. Given that the levels of active Smad2 are higher in airways from asthmatic patients compared to those from healthy
volunteers (39), one could speculate that HBE cells are an important contributor to asthmatic airway remodeling through the deposition of ECM Fn into the basement membrane.

The MTT and CyQUANT direct cell proliferation assays were used in this study to measure the cell number, viability and membrane integrity in TGFβ1 treated cells. The MTT assay is based on intracellular proteinase activities which can represent cell numbers in most conditions (4, 26, 31, 35, 42). The limitation of the MTT assay is that some treatments may influence certain enzyme activities which could affect the MTT measurement but have no effect on the live cell number or the mitochondrial activity per cell (49). In the current study, TGFβ1 reduced MTT measurements in both asthmatic and non-asthmatic HBE cells. The CyQUANT assay further confirmed that TGFβ1 decreased DNA content in non-asthmatic HBE cells. These data indicated that TGFβ1 was not a proliferative stimulus for HBE cells in the current treatment period. These results are consistent with the report by Semlali that TGFβ1 does not affect baseline proliferation in asthmatic and non-asthmatic HBE cells (40).

The role of coated Fn in epithelial cell proliferation has been reported previously in the bronchial cell lines BEAS-2B and 16-HBE (11). This current study found that precoating the culture vessel with Fn induced primary HBE cell proliferation in both asthmatic and non-asthmatic groups. This was determined using both the metabolic enzyme activity assays and DNA quantification assay, and confirmed by direct cell counting. The rates of increased proliferation in the presence of precoated Fn were similar in the four different measurements which demonstrated that quantification of DNA is a simple and reliable method to measure epithelial cell proliferation. In ASM cells, prolonged TGFβ1 (longer than 3 days) treatment promotes ASM cell proliferation via interaction between TGFβ1 induced ECM proteins, including Fn, and the integrin receptor α5β1 (29, 32, 48). In this study, despite the fact that
precoated Fn induced HBE cell proliferation and Fn production was enhanced by TGFβ1, TGFβ1 did not increase HBE cell proliferation. This might be due to insufficient time for the cells to react to TGFβ1-induced fibronectin. However, studies have shown that TGFβ1 may inhibit epithelial cell proliferation via suppression of proto-oncogene c-myc (30, 43).

To extend our knowledge of the role of Fn on HBE cells, we examined the release of pro-inflammatory mediators and growth factors modulated by Fn in HBE cells. The unique finding in this study is that pre-coating with Fn increased IL-6 release but decreased PGE2 production by HBE cells. IL-6 is able to to stimulate the differentiation of T cells (T helper 2 and T helper 17), B cells and macrophages, and contribute to the initiation and maintenance of inflammation in the asthmatic airway (7, 36, 47). The enhanced release of IL-6 by deposited Fn indicates that altered ECM proteins may indirectly influence airway inflammation. The mechanism of the Fn induced IL-6 release is still unclear and requires further investigation. PGE2 is a pleiotropic inflammatory mediator which has a protective role in the respiratory system (33). It inhibits allergen induced bronchial hyperresponsiveness (34), reduces ASM cell proliferation (3) and TGFβ1 induced ECM protein mRNA expression in ASM cells (2). Since HBE cells are one of the primary sources for endogenous PGE2, the decrease of PGE2 release by Fn in HBE cells may attenuate its protective role in asthmatic airways. Thus, the increased deposition of Fn by asthmatic HBE cells may contribute to the thickened basal epithelial cell layer, airway inflammation and bronchial hyperresponsiveness in asthmatic airways. The lack of response in IL-6 release when asthmatic HBE cells were treated with precoated Fn could be explained by observations from this study that asthmatic HBE cells produce high levels of cytokines constitutively.
It is interesting that the soluble Fn which was added to confluent epithelial cells had no
effects on cell proliferation or cytokine release. The cell numbers in the wells with or without
soluble Fn treatment had reached similar levels to the numbers observed in the highest
concentration of Fn in the Fn precoating experiments. It is possible that the HBE cells could
not proliferate in the presence of the soluble Fn as they had already formed a confluent
monolayer. The concept of this study was to test the same concentration of Fn in both the
soluble and the precoated forms. However, HBE cells constitutively release soluble Fn, as
reported in this study. The exogenous soluble Fn added to the HBE cells may not have been
able to cause further stimulation above the levels of endogenous Fn.

One of the limitations of this study was the source of the cells. Cells from asthmatic or non-
asthmatic donors were not always derived from patients who had only asthma or from healthy
volunteers due to the difficulties in collecting sufficient HBE cells. We attempted to limit any
confounding effects by deriving all epithelial cells from macroscopically normal areas of
lungs. However we cannot exclude the possibility that the presence of another disease may
confound these results.

In conclusion, this study showed that asthmatic HBE cells deposited greater amounts of ECM
Fn than non-asthmatic cells under basal conditions and in the presence of TGFβ1. TGFβ1
induced Fn production was dependent on the TGFβ1-ALK5-Smad 2/3 signaling pathway.
Deposited Fn modulated cell proliferation and proinflammatory cytokine and mediator
release in HBE cells (Fig 7). The altered expression of Fn in asthmatic bronchial epithelial
cells could be a potential therapeutic target for reversing asthmatic airway remodeling and
inflammation.
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Disclosures:
No conflicts of interest, financial or otherwise are declared by the authors.


Figure Captions:

Figure 1. The basal expression of Fn in asthmatic (A) and non-asthmatic (NA) epithelial cells. Human bronchial epithelial (HBE) cells were grown for 3 days in BEGM and quiesced for 24 h in BEBM (day 0) followed by maintenance in BEGM for up to 3 days. Total RNA, supernatants and ECM proteins were collected. MTT assay was performed from day 0 to day 3. Open bars, NA cells; black bars, A cells. a) The levels of Fn mRNA expression at day 0 and 1, NA n=7 and A n=4. b) Soluble Fn release at day 0, 1, 2 and 3, NA n=4 and A n=4. c) ECM Fn deposition at day 0, 1, 2 and 3, NA n=7 and A n=4. d) MTT assay, NA n=10, A n=5. Data are mean ± SEM. * P < 0.05, significantly different from day 0; # P < 0.05, significant difference between NA and A; two-way ANOVA (repeated measures) with Bonferroni post tests.

Figure 2. TGFβ1 increases Fn production by human bronchial epithelial (HBE) cells. HBE cells were grown in BEGM for 3 days and quiesced in BEBM for 24 h, and then stimulated with or without TGFβ1 in BEGM. Total RNA were collected and real time PCR was used to detect Fn mRNA expression. Supernatants and ECM proteins were collected at day 3 and the levels of Fn detected using ELISA. a) Fn mRNA expression at 24 h of TGFβ1 treatment in both non-asthmatic (NA) and asthmatic (A) cells. Data are expressed as mean ± SEM, fold change of time matched control in the absence of TGFβ1 (BEGM). Open bars, NA n = 6; black bars, A n = 5. b) Soluble Fn release at day 3, NA n = 4, A n = 4. c) ECM Fn deposition at day 3, NA n = 7, A n = 5. Data are expressed as mean ± SEM. * p < 0.05, significantly different from BEGM; # p < 0.05, significantly different between NA and A; two-way ANOVA (repeated measures) with Bonferroni post tests.

Figure 3. TGFβ1 decreases cell viability and proliferation in human bronchial epithelial (HBE) cells. HBE cells were grown in BEGM for 3 days and quiesced in BEBM for 24 h,
and then stimulated with or without TGFβ1 in BEGM. MTT assay and CyQUANT Direct Cell Proliferation Assay were performed after 3 days of stimulation. Open bars, non-asthmatic (NA) cells; black bars, asthmatic (A) cells. a) MTT assay, NA n = 9, A n = 5. b) CyQUANT Direct Cell Proliferation Assay, NA n = 4, A n = 3. Data are expressed as mean ± SEM. * p < 0.05, significantly different from BEGM; two-way ANOVA (repeated measures) with Bonferroni post tests.

Figure 4. SB431542 inhibited Fn expression in human bronchial epithelial (HBE) cells. Confluent HBE cells were quiesced in BEBM for 24 h, and then the cells were pretreated with inhibitors or vehicle control for 1 h in BEGM, followed by stimulation with or without TGFβ1 in the presence of inhibitors or vehicle controls. Total RNA lysates were collected after 24 h stimulation and the expression of Fn mRNA was determined using real time PCR. ECM proteins were collected after 3 days of stimulation and determined using ELISA. Open bars, non-asthmatic (NA) cells; black bars, asthmatic (A) cells. a) The expression of Fn mRNA from NA, n = 4 and A HBE cells, n = 4. b) to f), ECM Fn deposition in the absence or presence of ALK-5 inhibitor SB431542 (SB43) (b), ERK inhibitor PD98059 (PD) (c), PI3K inhibitor LY294002 (LY) (d), JNK inhibitor SP600125 (SP) (e) or p38 MAP kinase inhibitor SB239063 (SB23) (f); NA n = 4; A n = 4. Data are expressed as percentage of vehicle, mean ± SEM. * p < 0.05, significantly different from vehicle; # p < 0.05, significantly different between NA and A; $ p < 0.05, significantly different from vehicle with TGF-β1; two-way ANOVA (repeated measures) with Bonferroni post tests.

Figure 5. Precoated Fn increased cell viability and proliferation in HBE cells. HBE cells were seeded in BEGM in plates precoated with Fn or PBS (control). The MTT assay and manual cell counting were performed at day 2. Open bars, non-asthmatic (NA) cells; black bars, asthmatic (A) cells. a) MTT assay, NA n = 12, A n = 7. b) Manual cell counting, NA n = 8, A
Figure 6. Precoated Fn increased IL-6 release but decreased PGE2 release in HBE cells. HBE cells were seeded in BEGM in plates precoated with increasing concentration of Fn or PBS (control). The supernatants were collected after 2 days of incubation. Release of IL-6 a), PGE2 b) and VEGF c) was measured using an ELISA and data were normalised against MTT measurements in the same wells. Open bars, non-asthmatic (NA) HBE cells, n = 9 and black bars, asthmatic (A) cells n = 4. Data are mean ± SEM. * p < 0.05, significantly different from control; # p < 0.05, significantly different between NA and A; two-way ANOVA (repeated measures) with Sidak’s multiple comparisons test.

Figure 7. A schematic summary of the proposed role of TGF β1 and fibronectin in airway remodelling and inflammation in asthma. Asthmatic HBE cells produce greater amounts of constitutively and TGF β1 –induced Fn than non-asthmatic cells. TGFβ1 increases Fn via ALK-5/Smad signaling in asthmatic and non-asthmatic HBE cells. Fn increases the release of IL-6 in non-asthmatic cells but decreases PGE2 in both asthmatic and non-asthmatic HBE cells. ↑ increased expression when compared to unstimulated cells, * increased expression in asthmatic cells when compared to non-asthmatic cells under TGFβ1 stimulation, ↓ decreased expression when compared to unstimulated cells.
a) Fn mRNA
Amount of mRNA
\(\Delta C_T (\text{Fn-18S})\)

b) Soluble Fn
ng / ml

c) ECM Fn
ng / ml

d) MTT
Absorbance (OD 570-690nm)
Figure 1: Effects of TGFβ1 on FN expression and secretion.

(a) FN mRNA expression levels were measured by qRT-PCR and normalized to time-matched controls. Bars represent mean ± SEM of fold change relative to controls.

(b) Soluble FN concentrations were determined by ELISA. Bars represent mean ± SEM of ng/ml in culture supernatant.

(c) ECM FN levels were assessed by qELISA. Bars represent mean ± SEM of ng/ml ECM.

Significance was determined using one-way ANOVA with Tukey’s post-hoc test. *p < 0.05 vs. BEGM, #p < 0.05 vs. 0.1 ng/ml TGFβ1.
a

MTT (OD 570nm - 690nm)

- BEGM
- TGFβ1, ng/ml

b

Fluorescence intensity

- BEGM
- TGFβ1, ng/ml
a  MTT  

% of Control  

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b  Cell counting  

% of Control  

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IL-6 pg / ml / MTT

Control 1.58 5 15.8

Fn g/ml

IL-6

PGE2 pg / ml / MTT

Control 1.58 5 15.8

Fn µg/ml

PGE2

VEGF pg / ml / MTT

Control 1.58 5 15.8

Fn µg/ml

VEGF
Asthmatic > non-asthmatic
In basal ECM Fn deposition
basal IL-6 and VEGF release

**HBE cell**

- ECM Fn
- ALK-5/Smad2/3 pathway ↑

**TGFβ1**

- Fn ↑:
  - Non-asthmatic mRNA ↑*
  - ECM Protein ↑*
  - Asthmatic mRNA ↑
  - ECM Protein ↑*

- Non-asthmatic IL-6 ↑
- PGE₂ ↓
- Asthmatic IL-6 no change
- PGE₂ ↓

Airway remodeling and inflammation
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<td>55</td>
<td>COPD</td>
<td>M</td>
<td>58</td>
<td>T</td>
<td>Ex-smoker, 60 pk/yrs</td>
<td>16</td>
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<tr>
<td>56</td>
<td>α1antitrypsin deficiency</td>
<td>M</td>
<td>57</td>
<td>T</td>
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<tr>
<td>57</td>
<td>squamous cell Ca</td>
<td>M</td>
<td>65</td>
<td>R</td>
<td>Smoker, 50 pk/yrs</td>
<td>89</td>
<td>106</td>
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<td>12, 17</td>
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<td>58</td>
<td>Asthma, NSSCa</td>
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<td>72</td>
<td>R</td>
<td>Ex-smoker, 35/d, stopped 23y prior</td>
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<td>64</td>
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<tr>
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<td>R</td>
<td>Non-smoker</td>
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<td>83</td>
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<td>NA</td>
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<tr>
<td>61</td>
<td>Viral myocarditis &amp; PHT</td>
<td>F</td>
<td>49</td>
<td>T</td>
<td>Non-smoker</td>
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<td>61</td>
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<tr>
<td>62</td>
<td>Adeno Ca COPD/Emphysema</td>
<td>F</td>
<td>62</td>
<td>R</td>
<td>Smoker, 25 pk/yrs</td>
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<td>74</td>
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<td>Asthma, spindle cell melanoma</td>
<td>M</td>
<td>66</td>
<td>R</td>
<td>Ex-smoker, 10 pk/yrs</td>
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<td>64</td>
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<td>B</td>
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<td>T</td>
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<td>13, 14, 15, 16, 17, 18, 19</td>
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</table>

Abbreviations used: B: bronchoscopic biopsy + brushing; T: lung transplant; R: tissue resection; NA: not available; Ca, carcinoma; NSCCa: non-small cell carcinoma; NSCLC: non-small cell lung carcinoma; BOOP: bronchiolitis obliterans organizing pneumonia; BSSLT’s: bilateral sequential single lung transplantations; BSLT: bilateral sequential single lung transplant; BOS: Bronchiolitis obliterans syndrome; IPF: idiopathic pulmonary fibrosis; COPD: chronic obstructive pulmonary disease; PHT: pulmonary hypertension; ILD: interstitial lung disease; pk/yrs: pack years.

1: basal ECM Fn protein expression; 2: ECM Fn expression and MTT assay with TGFβ1 stimulation; 3: soluble Fn release; 4: basal Fn mRNA expression; 5: TGFβ1 stimulated Fn mRNA expression; 6: ECM Fn deposition modulated by kinase’s inhibitors; 7: Fn mRNA expression regulated by ALK-5 inhibitor; 8, cell proliferation (CyQuant assay) regulated by TGFβ1; 9: cell viability assay-MTT, treated with Fn precoated on the plates; 10: cell viability assay-LDH, treated with Fn precoated on the plates; 11: cell proliferation-CyQuant assay, treated with Fn precoated on the plates; 12: cell proliferation-manual cell counting, treated with Fn precoated on the plates; 13: IL-6 and VEGF release with Fn precoated on the plates; 14: PGE2 release with Fn precoated on the plates; 15: cell viability assay-MTT for soluble Fn treatment; 16: cell viability assay-LDH for soluble Fn treatment; 17: cell proliferation-manual cell counting for soluble Fn treatment; 18: IL-6 and VEGF release for soluble Fn treatment; 19: PGE2 release for soluble Fn treatment.
Table 2 The rates of cell proliferation stimulated by precoated Fn

<table>
<thead>
<tr>
<th>Fn concentration (µg/ml)</th>
<th>PBS</th>
<th>1.58</th>
<th>5</th>
<th>15.8</th>
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<tbody>
<tr>
<td>MTT Assay (n = 14)</td>
<td>100</td>
<td>105.3±1.90</td>
<td>119.3±3.68*</td>
<td>131.7±5.27*</td>
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<tr>
<td>LDH Assay (n = 12)</td>
<td>100</td>
<td>110.3±2.41</td>
<td>119.2±3.73*</td>
<td>136.9±10.85*</td>
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<tr>
<td>CyQUANT Assay (n = 7)</td>
<td>100</td>
<td>121.5±11.03</td>
<td>127.4±11.21*</td>
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<td>Cell counting (n= 8)</td>
<td>100</td>
<td>118.9±9.47</td>
<td>124.8±9.19*</td>
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</tr>
</tbody>
</table>

HBE cells were seeded in BEGM in plates precoated with Fn or PBS (control). The MTT, LDH and CyQUANT assays and manual cell counting were performed at day 2. Data are expressed as percentage of control (PBS), mean ± SEM. * P < 0.05, significantly different from control (PBS); one-way ANOVA (repeated measures) with Dunnett’s multiple comparison test.