Am J Physiol Lung Cell Mol Physiol 309: L1093–L1102, 2015.
First published September 4, 2015; doi:10.1152/ajplung.00019.2015.

CALL FOR PAPERS | *Bioengineering the Lung: Molecules, Materials, Matrix, Morphology, and Mechanics*

Differential deposition of fibronectin by asthmatic bronchial epithelial cells

Qi Ge,[1,2] Qingxiang Zeng,[1] Gavin Tjin,[1] Edmund Lau,[3] Judith L. Black,[1,2] Brian G. G. Oliver,[1,2]* and Janette K. Burgess[1,2]*

[1]*Respiratory Cellular and Molecular Biology Group, Woolcock Institute of Medical Research, Sydney, New South Wales, Australia;* [2]*Discipline of Pharmacology, Sydney Medical School, The University of Sydney, New South Wales, Australia; and* [3]*Royal Prince Alfred Hospital, Sydney, New South Wales, Australia*

Submitted 21 January 2015; accepted in final form 1 September 2015

Ge Q, Zeng Q, Tjin G, Lau E, Black JL, Oliver BGG, Burgess JK. Differential deposition of fibronectin by asthmatic bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 309: L1093–L1102, 2015. First published September 4, 2015; doi:10.1152/ajplung.00019.2015.— Altered 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 nonasthmatic 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 3 kinase, and activin-like kinase 5 (ALK5). Asthmatic HBECs deposited higher levels of Fn in the ECM than nonasthmatic cells under basal conditions, whereas 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 nonasthmatic cells. However, the excessive amounts of ECM Fn deposited by asthmatic cells after TGF-β1 stimulation persisted compared with nonasthmatic cells. Inhibition of ALK5 completely prevented TGF-β1-induced Fn deposition. Importantly, ECM Fn increased HBEC proliferation and IL-6 release, decreased PGE_2 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 represents a novel, therapeutic target.

fibronectin; asthma; airway epithelial cell; TGF-β1; ECM protein; IL-6

AIRWAY REMODELING IS A FEATURE of chronic severe asthmatic airways. One of the defining features of remodeling is altered 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 hyper-responsiveness (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 with 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, which contains one or two extra type III modules subjected to alternative splicing [extra-domain A and B (EDA and EDB, respectively)], 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 that polymerize both plasma Fn and/or cellular Fn (27).

Fn is known to elicit multiple functions. In vitro, plasma Fn, precoated 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 (15, 28). In addition, Fn inhibits epithelial cell apoptosis (11).

Fn can be produced by inflammatory cells and airway structural cells. Vignola and coworkers (46) found that the levels of Fn released by alveolar macrophages recovered from bronchoalveolar lavage fluid from asthmatic patients were higher than those from healthy controls. However, in human bronchial epithelial cells (HBECs), isolated and expanded in culture, Fn expression was lower in cells from children with asthma compared with those from otherwise healthy atopic children (23). In this same study, Kicic et al. (23) found that the addition of exogenous Fn to asthmatic HBECs restored the wound-repairing capacity that was deficient in the asthmatic cells.

The profibrotic growth factor, transforming growth factor (TGF)-β1, has been found to be a potent stimulus for Fn in vascular and ASM cells, lung fibroblasts, and the alveolar epithelial cell line, A549 (20–22, 25). The TGF-β1 signaling pathway is complicated and includes the Smad cascade, ERK, JNK, and p38 MAPK, as well as phosphatidylinositol 3 kinase (PI3K). The study in bronchial biopsy samples from asthmatic and healthy subjects found that activated TGF-β/Smad2 signaling is positively associated with the

* B. G. G. Oliver and J. K. Burgess contributed equally to this work.
Address for reprint requests and other correspondence: Q. Ge, Woolcock Institute of Medical Research, 431 Glebe Point Rd., Glebe, NSW 2037, Australia (e-mail: qi.ge@sydney.edu.au).

thickness of the basement membrane (39). In ASM cells, the ERK, p38 MAPK, and PI3K are all involved in TGF-β1-induced Fn mRNA expression in nonasthmatic cells, whereas only ERK and p38 MAPK were observed in asthmatic cells (20). However, the cellular signaling pathways for Fn production in HBECs under basal condition or with TGF-β1 stimulation are unclear. The aim of this study was to compare the expression of Fn by HBECs from individuals with and without asthma under basal conditions or in the presence of TGF-β1 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. HBECs 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 fiber-optic 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 HBSS, 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, Walkersville, MD), as described previously (9). The experiments were performed with cells between *passages 1* and *4*. The cells from each individual were regarded as one primary cell culture. In this study, the primary cultures were categorized into two groups: the asthmatic and nonasthmatic group. The data from the asthmatic group were averaged, as were those from the nonasthmatic group. The number of primary cell cultures used within a group in each experiment is represented by *n*. The patient and volunteer demographics are shown in Table 1.

Cell experiments. HBECs were seeded at 2×10^4 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) for 24 h. After quiescing, the medium was refreshed with BEGM in the absence or presence of TGF-β1 (0.1, 0.5, 1, and 5 ng/ml). The supernatants, ECM proteins, and total RNA lysates were collected from the HBECs at *day 0* (quiesced in BEBM for 24 h), following 1, 2, or 3 days growth in BEGM. Samples for *day 0* were collected at the time of stimulus addition after the cells had been quiesced for 24 h; 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 TGF-β1-modulated ECM Fn deposition. After quiescing in BEBM for 24 h, the cells were pretreated with the MEK (MAPK/ERK) inhibitor PD98059 (10 μM), the JNK inhibitor SP600125 (10 μM), the PI3K inhibitor LY294002 (3 μM; Calbiochem, San Diego, CA), the p38 MAPK inhibitor SB239063 (3 μM), and the TGF-β type I receptor activin-like kinase 5 (ALK5) inhibitor SB431542 (1, 3, and 10 μM; Tocris, Ellisville, MO) in BEGM for 30 min before stimulation with and without TGF-β1 (1 ng/ml). We used these specific protein kinase inhibitors at concentrations that 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 HBEC viability, proliferation, and the release of soluble cytokines and chemokines, human plasma Fn (BD Biosciences, Bedford, MA) 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 *days 1*, *2*, and *3*.

The role of soluble Fn was also examined. Confluent and quiesced HBECs 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 described previously 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 (0.016 M NH_4OH; Sigma, St. Louis, MO). At the time of analysis, human plasma Fn was used to generate a standard curve. A serial dilution of Fn at concentrations of 2,000, 1,000, 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 0.05% Tween 20 in PBS and blocked with 1% BSA (Sigma) in PBS. An anti-human Fn antibody (clone 868A11; Millipore, Billerica, MA) and a polyclonal rabbit anti-mouse Ig/horseradish peroxidase (Dako, Glostrup, Denmark) were used. After final washing, a liquid substrate system, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (Sigma), was used to detect the amount of ECM Fn deposited by cells. The absorbance was measured immediately at 405 nm using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). The readings from triplicate wells were averaged and the background absorbance subtracted. The data were expressed as nanograms per milliliter, 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 Fn ELISA kit (Millipore), according to the manufacturer's instructions. The detection limit for soluble Fn was 3 ng/ml. A standard curve was constructed, 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 described previously (9). The total RNA lysates were collected and extracted using a NucleoSpin RNA II kit, and Moloney murine leukemia virus RT was used for reverse transcription. A predeveloped, specific primer set for Fn, Hs00365058_m1, TaqMan Universal PCR Master Mix, and the StepOnePlus Real-Time PCR System was used for real-time PCR (Applied Biosystems, Branchburg, NJ). A predeveloped TaqMan reagent human 18S rRNA (Cat. #4319413; Applied Biosystems) was included in each real-time PCR reaction as an endogenous control. Data from the reactions were analyzed using StepOne Software, v 2.1 (Applied Biosystems).

ELISA for IL-6, VEGF, and PGE_2. The IL-6, VEGF, and PGE_2 ELISAs were carried out following the manufacturers' instructions (IL-6, BD PharMingen, Franklin Lakes, NJ; VEGF, R&D Systems, Minneapolis, MN; and PGE_2, Cayman Chemical, Ann Arbor, MI).

Cell number, viability, and cytotoxicity assay. At the end point of experiments, the cell number, viability, and cytotoxicity were determined using manual 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 to the manufacturer's instructions (Sigma). The absorbance was measured using the SpectraMax M2 microplate reader. The data

Table 1. *Patient and volunteer demographics*

Donor #	Diagnosis	Age	Sex	Procedure	Smoking History	FEV1 % Predicted	FVC % Predicted	FEV1:FVC (%)	Experiments Where Used
1	Asthma	40	M	B	Nonsmoker	N/A	N/A	N/A	1, 2, 3
2	Asthma	27	M	B	Nonsmoker	82	105	64	1, 2
3	Asthma	20	F	B	Nonsmoker	85	110	N/A	1, 2, 3
4	Asthma	31	F	B	N/A	N/A	N/A	N/A	1, 2, 3, 6
5	Asthma	22	M	B	Nonsmoker	N/A	N/A	N/A	1, 2, 4, 5
6	Asthma	62	M	B	N/A	85	98	N/A	3, 4, 5, 6
7	Asthma	22	M	B	Nonsmoker	82	N/A	N/A	4, 5
8	Asthma	30	M	B	N/A	N/A	N/A	N/A	6, 9, 10, 13, 14
9	Asthma	27	F	B	N/A	81	99	70	5, 7, 9, 13, 14
10	Asthma	23	M	B	N/A	82	81	67	5, 7, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19
11	Healthy volunteer	43	M	B	Nonsmoker	N/A	N/A	N/A	1, 2
12	Emphysema	55	M	T	N/A	N/A	N/A	N/A	1, 2, 3, 9, 10, 13
13	Emphysema	64	F	T	Ex-smoker	N/A	N/A	N/A	1, 2, 3, 9, 10, 13, 14
14	Bronchiolitis	31	F	T	N/A	N/A	N/A	N/A	1, 2
15	Emphysema	56	F	T	N/A	N/A	N/A	N/A	1, 2, 9, 13, 14
16	Adeno Ca	69	M	T	N/A	N/A	N/A	N/A	3,
17	Bronchiectasis	39	M	T	Ex-smoker	36	57	54	1, 2, 3
18	Emphysema	64	M	T	N/A	N/A	N/A	N/A	4, 5
19	BOOP	45	M	T	N/A	N/A	N/A	N/A	4, 5
20	BOS	54	F	T	N/A	N/A	N/A	N/A	4, 5
21	α1 Antitrypsin BSSLTs	55	F	T	Ex-smoker, 30 pk/yr	26	63	33	4, 5, 6
22	Emphysema	64	M	T	N/A	N/A	N/A	N/A	6
23	Emphysema	52	M	T	N/A	N/A	N/A	N/A	6
24	Ca	60	M	R	Ex-smoker. 15/day, stopped 3 yr before	76	88	66	6
25	NSCCa, asthma, bronchiolitis	59	F	R	Ex-smoker, 10/day	94	99	81	6
26	Adeno Ca	77	M	R	Smoker, 11 pk/yr	48	46	75	15, 16, 18, 19
27	NSCCa	67	F	R	Ex-smoker	94	90	80	15, 16, 18, 19
28	Usual interstitial pneumonitis	59	M	T	N/A	N/A	N/A	N/A	1, 2
29	Kartagener syndrome	42	M	T	N/A	N/A	N/A	N/A	4, 5, 7
30	Cystic fibrosis	59	F	T	N/A	N/A	N/A	N/A	5, 7
31	NSCCa	70	M	*R*	Ex-smoker, stopped 15 yr before	67	67	72	7
32	Healthy volunteer	32	M	B	Smoker	N/A	N/A	N/A	9, 10, 14
33	Lesion	58	F	T	N/A	N/A	N/A	N/A	9, 10, 13, 14
34	Pulmonary fibrosis	68	M	T	Ex-smoker, 15 pk/yr	71	58	91	9, 10, 13, 14
35	Rejection (previous pneumonitis)	21	M	T	Nonsmoker	20	23	69	9, 10, 13, 14
36	NSCCa	54	F	*R*	Smoker	87	97	76	15, 16, 18, 19
37	Mononucleosis			T	N/A	N/A	N/A	N/A	15, 16, 18, 19
38	NSCCa	63	M	R	Ex-smoker, 75 pk/yr	69	69	76	15, 16, 18
39	NSCCa, mild COPD	58	M	R	Smoker, 60 pk/yr	70	N/A	N/A	15, 16, 18
40	Emphysema	64	M	T	N/A	17	44	28	15, 16, 18
41	Adeno Ca, mild COPD	70	M	R	Ex-smoker, 60 pk/yr	75	88	62	15, 16, 18
42	Ca	66	F	R	Ex-smoker, 25 pk/yr	N/A	N/A	N/A	8, 11
43	IPF	56	M	T	N/A	N/A	N/A	N/A	8, 11
44	NSCCa	71	M	R	Ex-smoker	79	81	71	8
45	Subarachnoid hemorrhage	52	M	Donor	N/A	N/A	N/A	N/A	9, 13
46	Asthma, Adeno Ca, COPD	64	M	R	Nonsmoker	38	68	44	9, 10
47	NSCCa, mild COPD	49	M	R	Ex-smoker	88	97	71	9, 10
48	Ca	82	F	R	NonSmoker	90	98	74	9, 10,

Continued

Table 1.—*Continued*

Donor #	Diagnosis	Age	Sex	Procedure	Smoking History	FEV1 % Predicted	FVC % Predicted	FEV1:FVC (%)	Experiments Where Used
49	Asthma, Ca, chronic cough, bronchiolitis	68	F	R	Nonsmoker	76	68	86	9, 10
50	NSCCa	60	F	R	Smoker	92	(2.9L)	83	9, 10, 13
51	Asthma, NSCCa	43	M	R	Nonsmoker	88	86	80	9, 10, 13
52	Asthma, NSCLC	75	M	R	Nonsmoker	(1.65L)	(2.61L)	63	11, 12, 17
53	Previous emphysema, BSLTx for BOS	58	F	T	N/A	N/A	N/A	N/A	11
54	ILD	34	F	T	Ex-smoker, 3 pk/yr	20	20	84	11, 12, 17
55	COPD	58	M	T	Ex-smoker 60 pk/yr	16	68	18	11
56	α1 Antitrypsin deficiency	57	M	T	N/A	N/A	N/A	N/A	11
57	Squamous cell Ca	65	M	R	Smoker 50 pk/yr	89	106	63	12, 17
58	Asthma, NSCCa	72	F	R	Ex-smoker, 35/day, stopped 23 yr before	56	64	67	12, 17
59	Adeno Ca	69	F	R	Nonsmoker	68	83	62	12, 17
60	Adeno Ca	57	F	R	N/A	N/A	N/A	N/A	12, 17
61	Viral myocarditis and PHT	49	F	T	Nonsmoker	52	61	69	12, 17
62	Adeno Ca COPD/emphysema	62	F	R	Smoker, 25 pk/yr	63	74	65	12, 17
63	Asthma, spindle cell melanoma	66	M	R	Ex-smoker, 10 pk/yr	N/A	N/A	N/A	15, 16, 18, 19
64	Asthma	45	F	B	Nonsmoker	N/A	N/A	N/A	9, 10, 12, 13, 14, 15, 16, 17, 18, 19
65	COPD	58	F	T	N/A	N/A	N/A	N/A	9, 10, 12, 13, 14, 15, 16, 17, 18, 19

FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; B, bronchoscopic biopsy + brushing; T, lung transplant; R, tissue resection; Ca, carcinoma; BOOP, Bronchiolitis obliterans organizing pneumonia; BSSLTs, bilateral sequential single-lung transplantations; NSCCa, nonsmall cell carcinoma; COPD, chronic obstructive pulmonary disease; IPF, idiopathic pulmonary fibrosis; NSCLC, nonsmall cell lung carcinoma; BSLT, bilateral sequential single-lung transplant; BOS, Bronchiolitis obliterans syndrome; ILD, interstitial lung disease; PHT, pulmonary hypertension; N/A, not available; pk/yr, pack years; experiments where used: 1, basal ECM fibronectin (Fn) protein expression; 2, ECM Fn expression and mitochondrial activity assay (MTT) assay with transforming growth factor receptor (TGF)-β1 stimulation; 3, soluble Fn release; 4, basal Fn mRN/A expression; 5, TGF-β1-stimulated Fn mRN/A expression; 6, ECM Fn deposition modulated by kinases' inhibitors; 7, Fn mRN/A expression regulated by activin-like kinase 5 inhibitor; 8, cell proliferation (CyQUANT assay) regulated by TGF-β1; 9, cell viability assay-MTT, treated with Fn precoated on the plates; 10, cell viability assay-lactate dehydrogenase (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, PGE_2 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, PGE_2 release for soluble Fn treatment. L, liters.

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, OR).

Statistical analysis. Data were expressed as means ± SE for the number of HBEC cultures (n) stated and analyzed 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. $P < 0.05$ was considered statistically significant.

RESULTS

The expression of Fn from HBECs under basal conditions. The levels of Fn mRNA expression were similar between asthmatic and nonasthmatic cells at *days 0* and *1* (Fig. 1*A*). There was an increase in cell viability/proliferation measured by MTT assay and soluble Fn release from asthmatic HBECs at *day 3*, but no differences in soluble Fn release and cell number were seen when comparing the asthmatic and nonasthmatic cells at *days 1*, *2*, and *3* (Fig. 1, *B* and *D*). However, asthmatic HBECs constitutively deposited greater amounts of ECM Fn than nonasthmatic cells (Fig. 1*C*).

TGF-β1 increased Fn production from HBECs. Following stimulation with TGF-β1, 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 TGF-β1, nonasthmatic HBECs expressed a greater amount of Fn mRNA compared with asthmatic cells (Fig. 2*A*). TGF-β1 increased both soluble and ECM Fn expression after 2 days of stimulation in nonasthmatic and asthmatic HBECs. Furthermore, the levels of ECM Fn produced by asthmatic cells were higher than those by nonasthmatic cells in the presence of TGF-β1 (Fig. 2, *B* and *C*, for *day 3*; *days 1* and *2* data not shown), although the percentage of increase in ECM Fn induced by TGF-β1 was

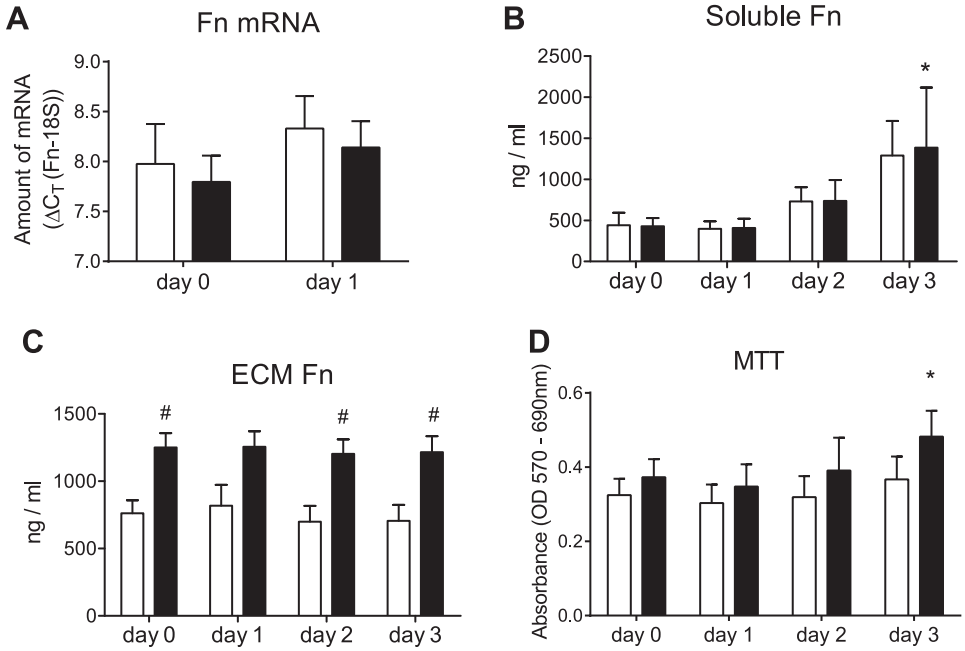

Fig. 1. The basal expression of fibronectin (Fn) in asthmatic (A) and nonasthmatic (NA) epithelial cells. Human bronchial epithelial cells (HBECs) were grown for 3 days in bronchial epithelial growth medium (BEGM) and quiesced for 24 h in bronchial epithelial basal medium (BEBM; *day 0*), followed by maintenance in BEGM for up to 3 days. Total RNA, supernatants, and ECM proteins were collected. Mitochondrial activity assay (MTT) assay was performed from *days 0* to *3*. Open bars, NA cells; closed bars, A cells. *A*: the levels of Fn mRNA expression at *days 0* and *1* (NA, $n = 7$; A, $n = 4$). ΔC_T, change in cycle threshold. *B*: soluble Fn release at *days 0–3* (NA, $n = 4$; A, $n = 4$). *C*: ECM Fn deposition at *days 0–3* (NA, $n = 7$; A, $n = 4$). *D*: MTT assay (NA, $n = 10$; A, $n = 5$). OD, optical density. Data are means ± SE. $^*P < 0.05$, significantly different from *day 0*; $\#P < 0.05$, significantly different between NA and A, 2-way ANOVA (repeated measures) with Bonferroni post-tests.

similar in nonasthmatic and asthmatic cells [at *day 3*, 5 ng/ml of TGF-β1; percentage increase over unstimulated 220.3 ± 54.6% for nonasthmatic, $n = 7$; 206.3 ± 44.0% for asthmatic, $n = 5$, respectively].

The HBEC viability and numbers modulated by TGF-β1 were monitored using MTT and CyQUANT direct cell proliferation assay. TGF-β1 reduced cell mitochondrial activity at *days 1*, *2*, and *3* in nonasthmatic and asthmatic cells (Fig. 3*A*). It also decreased DNA-bound fluorescence intensity in both cell groups (Fig. 3*B*), which confirmed that the HBEC number was not increased by TGF-β1.

An ALK5 inhibitor SB431542 blocked TGF-β1-induced Fn expression. The inhibitors for ERK (PD98058), PI3K (LY294002), JNK (SP600125), p38 MAPK (SB239063), and ALK5 (SB431542) were used to block individual signaling pathways, which may be involved in the TGF-β1-induced ECM Fn deposition in HBECs. PD98059, LY294002, SP600125, and SB239063 had no effect on ECM Fn deposition in the absence and presence of TGF-β1 in either nonasthmatic or asthmatic HBECs (Fig. 4, *C–F*). However, SB431542 inhibited TGF-β1-induced Fn mRNA expression in nonasthmatic and asthmatic HBECs (Fig. 4*A*). Furthermore, SB431542 blocked TGF-β1-stimulated ECM Fn deposition but had no influence on constitutive ECM Fn deposition in either nonasthmatic or asthmatic cells (Fig. 4*B*).

Fn increased HBEC proliferation and regulated proinflammatory mediator release. To investigate the role of Fn in regulating cell proliferation and the release of proinflammatory mediators, the nonasthmatic HBECs 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, and *2*) the cells were grown to confluence and quiesced before plasma Fn was added as a stimulus.

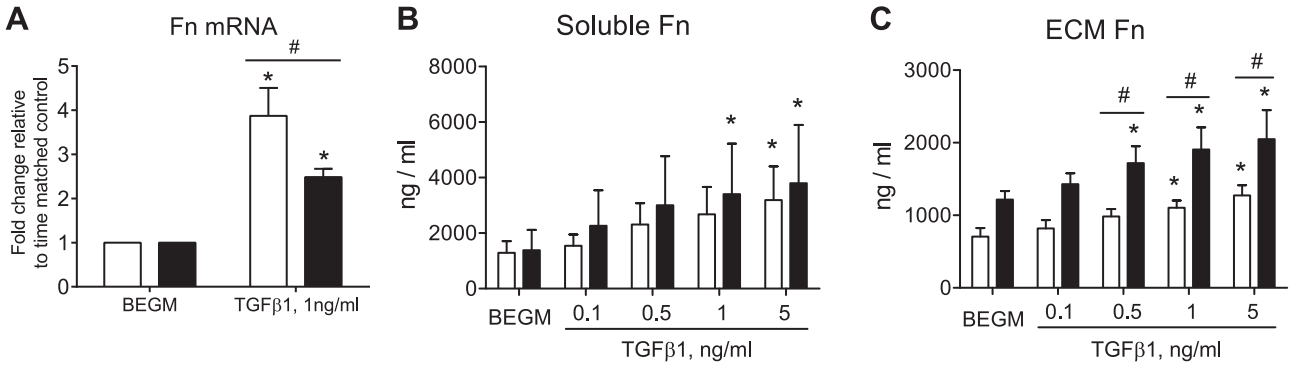

Fig. 2. Transforming growth factor (TGF)-β1 increases Fn production by HBECs. HBECs were grown in BEGM for 3 days and quiesced in BEBM for 24 h and then stimulated with or without TGF-β1 in BEGM. Total RNA was collected, and real-time PCR was used to detect Fn mRNA expression. Supernatants and ECM proteins were collected at *day 3* and the levels of Fn detected using ELISA. *A*: Fn mRNA expression at 24 h of TGF-β1 treatment in both NA and A cells. Data are expressed as means ± SE; fold change of time-matched control in the absence of TGF-β1 (BEGM). Open bars, NA ($n = 6$); closed 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 means ± SE. $^*P < 0.05$, significantly different from BEGM; $\#P < 0.05$, significantly different between NA and A, 2-way ANOVA (repeated measures) with Bonferroni post-tests.

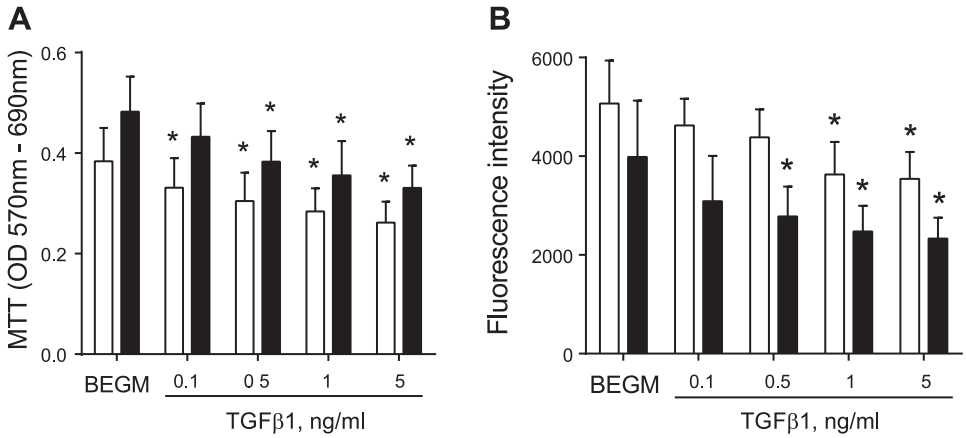

Fig. 3. TGF-β1 decreases cell viability and proliferation in HBECs. HBECs were grown in BEGM for 3 days and quiesced in BEBM for 24 h and then stimulated with or without TGF-β1 in BEGM. MTT assay and CyQUANT direct cell proliferation assay were performed after 3 days of stimulation. Open bars, NA cells; closed bars, 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 means ± SE. $^*P < 0.05$, significantly different from BEGM, 2-way ANOVA (repeated measures) with Bonferroni post-tests.

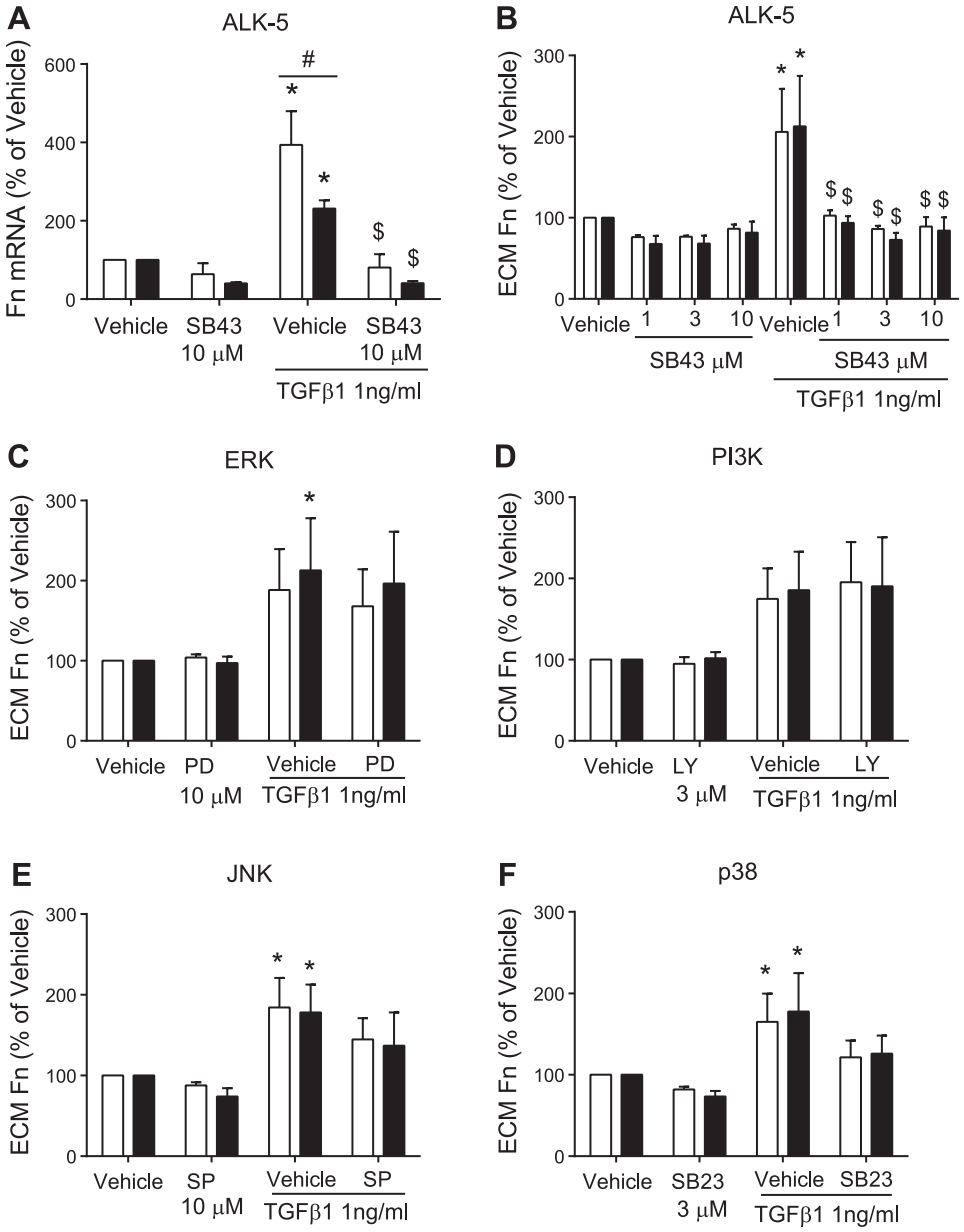

Fig. 4. SB431542 inhibited Fn expression in HBECs. Confluent HBECs were quiesced in BEBM for 24 h, and then the cells were pretreated with inhibitors or vehicle control for 1 h in BEGM, followed by stimulation with or without TGF-β1 in the presence of inhibitors or vehicle controls. Total RNA lysates 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, NA cells; closed bars, A cells. *A*: the expression of Fn mRNA from NA ($n = 4$) and A HBECs ($n = 4$). *B–F*: ECM Fn deposition in the absence or presence of activin-like kinase 5 (ALK5) inhibitor SB431542 (SB43; *B*), ERK inhibitor PD98059 (PD; *C*), phosphatidylinositol 3 kinase (PI3K) inhibitor LY294002 (LY; *D*), JNK inhibitor SP600125 (SP; *E*), or p38 MAPK inhibitor SB239063 (SB23; *F*; NA, $n = 4$; A, $n = 4$). Data are expressed as percentage of vehicle, means ± SE. $^*P < 0.05$, significantly different from vehicle; $\#P < 0.05$, significantly different between NA and A; $\$P < 0.05$, significantly different from vehicle with TGF-β1, 2-way ANOVA (repeated measures) with Bonferroni post-tests.

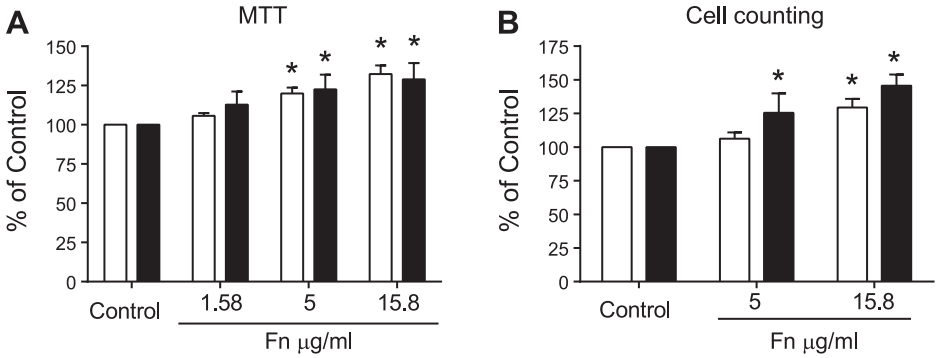

Fig. 5. Precoated Fn increased cell viability and proliferation in HBECs. HBECs were seeded in BEGM, in plates precoated with Fn or PBS (Control). The MTT assay and manual cell counting were performed at *day 2*. Open bars, NA cells; closed bars, A cells. *A*: MTT assay (NA, $n = 12$; A, $n = 7$). *B*: manual cell counting (NA, $n = 8$; A, $n = 4$). Data are expressed as percentage of control, means ± SE. $*P < 0.05$, significantly different from control (PBS), 2-way ANOVA (repeated measures) with Sidak's (*A*) or Dunnett's (*B*) multiple comparisons test.

When HBECs were seeded on the plate precoated with Fn, Fn increased cell viability at *days 1*, *2*, and *3*, as measured by MTT (Fig. 5*A* for *day 2* in asthmatic and nonasthmatic cells; *days 1* and *3* data not shown) and LDH (Table 2) assay. The precoated Fn induced HBEC proliferation (Fig. 5*B*), 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 HBECs, as measured by released LDH ($n = 12$; data not shown).

Growth of the HBECs in tissue-culture wells precoated with Fn also altered the release of soluble factors important in asthma. Figure 6 illustrates IL-6 (*A*), PGE_2 (*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 HBECs, had no effect on cell viability and proliferation, nor did it affect cytotoxicity (nonasthmatic $n = 8$ and asthmatic $n = 3$ for MTT and LDH assays; nonasthmatic $n = 7$ and asthmatic $n = 4$ for cell counting; data not shown). Similarly, soluble Fn did not alter the regulation of IL-6, PGE_2, and VEGF release in HBECs (nonasthmatic $n = 9$ and asthmatic $n = 3$ for IL-6 and VEGF release; nonasthmatic $n = 5$ and asthmatic $n = 3$ for PGE_2 release; data not shown).

DISCUSSION

This study compared the expression of Fn in HBECs from the airways of asthmatic and nonasthmatic adults constitutively or following stimulation with TGF-β1 and discovered that HBECs produce both soluble and the ECM-bound form of Fn. Interestingly, asthmatic HBECs deposited greater amounts of ECM Fn than nonasthmatic cells. TGF-β1 increased ECM Fn and soluble Fn in both nonasthmatic and asthmatic HBECs, and this effect was regulated by the ALK5/Smad signaling pathway. In addition, exogenous deposited Fn increased primary HBEC proliferation and regulated the release of proinflammatory mediators.

The present study showed that the levels of ECM Fn, under both basal conditions and after stimulation with TGF-β1, were higher in asthmatic epithelial cells compared with nonasthmatic cells, although the percentages of increase induced by TGF-β1 were similar in both groups of cells. However, nonasthmatic and asthmatic epithelial cells expressed similar amounts of Fn mRNA and release of soluble Fn under basal conditions. When stimulated with TGF-β1, nonasthmatic 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 TGF-β1 in nonasthmatic HBECs, as we reported previously (9). The reason why asthmatic cells deposit greater amounts of Fn, even as 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 HBECs and the elevated levels of TGF-β1 in asthmatic airways (1, 37).

The differences in Fn expression between nonasthmatic and asthmatic HBECs detected in this study were not consistent with the findings reported by Kicic and colleagues (23). In their study, healthy, nonatopic bronchial epithelial cells produced higher levels of Fn mRNA and soluble and cell lysate Fn than atopic asthmatic cells (23). The differences between our study and theirs may be due to the different tissue sources. In the current study, HBECs were obtained from adults with or without asthma; in the Kicic et al. study (23), the epithelial cells were collected from children with or without asthma. It is of interest that there was a differential expression of Fn by HBECs 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 nonasthmatic

Table 2. *The rates of cell proliferation stimulated by precoated Fn*

Fn Concentration, μg/ml	PBS	1.58	5	15.8
MTT assay, $n = 14$	100	105.3 ± 1.90	119.3 ± 3.68*	131.7 ± 5.27*
LDH assay, $n = 12$	100	110.3 ± 2.41	119.2 ± 3.73*	136.9 ± 10.85*
CyQUANT assay, $n = 7$	100		121.5 ± 11.03	127.4 ± 11.21*
Cell counting, $n = 8$	100		118.9 ± 9.47	124.8 ± 9.19*

Human bronchial epithelial cells were seeded in bronchial epithelial growth medium 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), means ± SE. $*P < 0.05$, significantly different from control (PBS), 1-way ANOVA (repeated measures) with Dunnett's multiple comparison test.

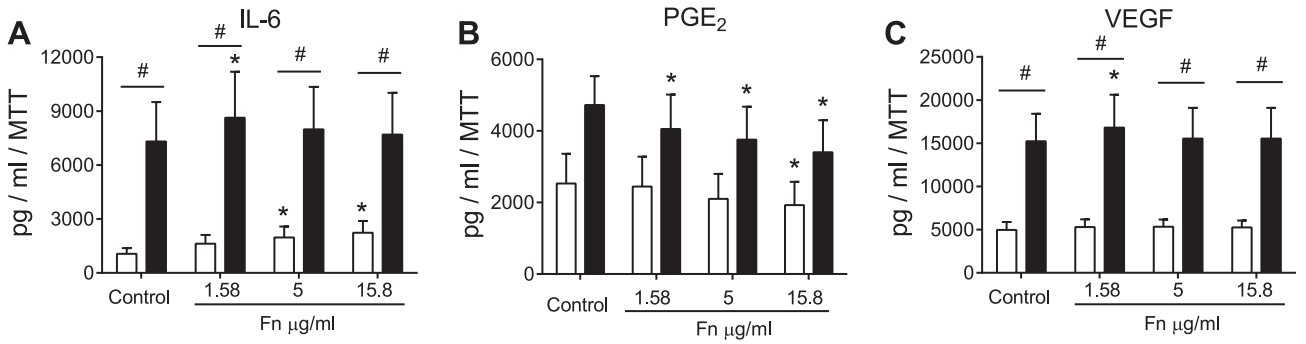

Fig. 6. Precoated Fn increased IL-6 release but decreased PGE_2 release in HBECs. HBECs were seeded in BEGM in plates precoated with increasing concentrations of Fn or PBS (Control). The supernatants were collected after 2 days of incubation. Release of IL-6 (*A*), PGE_2 (*B*), and VEGF (*C*) was measured using an ELISA, and data were normalized against MTT measurements in the same wells. Open bars, NA HBECs ($n = 9$); closed bars, A cells ($n = 4$). Data are means ± SE. $*P < 0.05$, significantly different from control; $\#P < 0.05$, significantly different between NA and A, 2-way ANOVA (repeated measures) with Sidak's multiple comparisons test.

airways (5, 8, 12, 24, 44, 45). The findings from these studies indicate that the characteristics of asthmatic HBECs 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 TGF-β1 augmented both soluble and ECM Fn production by asthmatic and nonasthmatic HBECs, and the increase was, at least partly, regulated at the level of transcription, since TGF-β1 also induced Fn mRNA expression. These results were consistent with the study conducted by Doerner and Zuraw (6).

In this study, specific inhibitors for ERK, JNK, PI3K, p38 MAPK, and TGF-β type I receptor ALK5 were used to test which signaling pathways were involved in basal and TGF-β1-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 MAPK also had no effect on Fn deposition in the presence of TGF-β1, indicating that these signaling pathways were not involved in the TGF-β1-induced Fn deposition. This finding was different from our previous observations in ASM cells, in which both ERK and p38 MAPK were involved in TGF-β1-induced Fn expression in nonasthmatic and asthmatic ASM cells, whereas 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 TGF-β1-induced Fn ECM protein and mRNA expression in the HBECs implied that TGF-β1 increases Fn production, mainly via the ALK5/Smad2/3 pathway in nonasthmatic and asthmatic HBECs. Given that the levels of active Smad2 are higher in airways from asthmatic patients compared with those from healthy volunteers (39), one could speculate that HBECs 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 TGF-β1-treated cells. The MTT assay is based on intracellular proteinase activities that can represent cell numbers in most conditions (4, 26, 31, 35, 42). The limitation of the MTT assay is that some treatments may

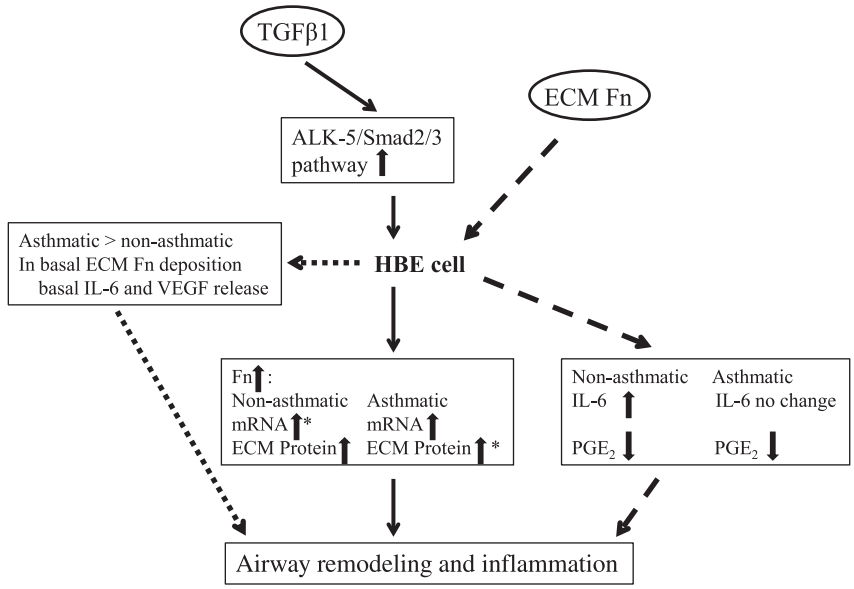

Fig. 7. A schematic summary of the proposed role of TGF-β1 and Fn in airway remodeling and inflammation in asthma. A HBECs produce greater amounts of constitutively and TGF-β1-induced Fn than NA cells. TGF-β1 increases Fn via ALK5/Smad signaling in A and NA HBECs. Fn increases the release of IL-6 in NA cells but decreases PGE_2 in both A and NA HBECs. ↑, increased expression compared with unstimulated cells; *, increased expression in A cells compared with NA cells under TGF-β1 stimulation; ↓, decreased expression compared with unstimulated cells.

influence certain enzyme activities that 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, TGF-β1 reduced MTT measurements in both asthmatic and nonasthmatic HBECs. The CyQUANT assay further confirmed that TGF-β1 decreased DNA content in nonasthmatic HBECs. These data indicated that TGF-β1 was not a proliferative stimulus for HBECs in the current treatment period. These results are consistent with the report by Semlali et al. (40) that TGF-β1 does not affect baseline proliferation in asthmatic and nonasthmatic HBECs.

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 HBEC proliferation in both asthmatic and nonasthmatic 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 TGF-β1 (>3 days) treatment promotes ASM cell proliferation via interaction between TGF-β1-induced ECM proteins, including Fn, and the integrin receptor α5β1 (29, 32, 48). In this study, despite the fact that precoated Fn induced HBEC proliferation, and Fn production was enhanced by TGF-β1, TGF-β1 did not increase HBEC proliferation. This might be due to insufficient time for the cells to react to TGF-β1-induced Fn. However, studies have shown that TGF-β1 may inhibit epithelial cell proliferation via suppression of proto-oncogene c-myc (30, 43).

To extend our knowledge of the role of Fn on HBECs, we examined the release of proinflammatory mediators and growth factors modulated by Fn in HBECs. The unique finding in this study is that precoating with Fn increased IL-6 release but decreased PGE_2 production by HBECs. IL-6 is able to stimulate the differentiation of T cells (T helper 2 and 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. PGE_2 is a pleiotropic inflammatory mediator that has a protective role in the respiratory system (33). It inhibits allergen-induced bronchial hyperresponsiveness (34) and reduces ASM cell proliferation (3) and TGF-β1-induced ECM protein mRNA expression in ASM cells (2). Since HBECs are one of the primary sources for endogenous PGE_2, the decrease of PGE_2 release by Fn in HBECs may attenuate its protective role in asthmatic airways. Thus the increased deposition of Fn by asthmatic HBECs may contribute to the thickened basal epithelial cell layer, airway inflammation, and bronchial hyper-responsiveness in asthmatic airways. The lack of response in IL-6 release when asthmatic HBECs were treated with precoated Fn could be explained by observations from this study that asthmatic HBECs 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 HBECs 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, HBECs constitutively release soluble Fn, as reported in this study. The exogenous soluble Fn added to the HBECs 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 nonasthmatic donors were not always derived from patients who had only asthma or from healthy volunteers, due to the difficulties in collecting sufficient HBECs. 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 HBECs deposited greater amounts of ECM Fn than nonasthmatic cells under basal conditions and in the presence of TGF-β1. TGF-β1-induced Fn production was dependent on the TGF-β1-ALK5-Smad2/3 signaling pathway. Deposited Fn modulated cell proliferation and proinflammatory cytokine and mediator release in HBECs (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.

ACKNOWLEDGMENTS

The authors thank the cardiopulmonary transplant team; the pathologists at St. Vincent's Hospital; and the thoracic physicians and pathologists at Royal Prince Alfred Hospital, Concord Repatriation Hospital, Strathfield Private Hospital, and Davies Campbell de Lambert Pathology, Sydney, for their ongoing contributions to this research.

GRANTS

Support for this work was provided by the National Health and Medical Research Council (NHMRC) Australia (Grants NHMRC 571098 to J. L. Black, NHMRC 1032695 to B. G. G. Oliver, NHMRC 1026880 to J. K. Burgess, and NHMRC 1003263).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: Q.G., J.L.B., B.G.G.O., and J.K.B. conception and design of research; Q.G., Q.Z., and G.T. performed experiments; Q.G. and Q.Z. analyzed data; Q.G., J.L.B., B.G.G.O., and J.K.B. interpreted results of experiments; Q.G. prepared figures; Q.G. drafted manuscript; Q.G., Q.Z., G.T., E.L., J.L.B., B.G.G.O., and J.K.B. edited and revised manuscript; Q.G., Q.Z., G.T., E.L., J.L.B., B.G.G.O., and J.K.B. approved final version of manuscript.

REFERENCES

1. **Bousquet J, Vignola AM, Chanez P, Campbell AM, Bonsignore G, Michel FB.** Airways remodelling in asthma: no doubt, no more? *Int Arch Allergy Immunol* 107: 211–214, 1995.
2. **Burgess JK, Ge Q, Poniris MH, Boustany S, Twigg SM, Black JL, Johnson PR.** Connective tissue growth factor and vascular endothelial growth factor from airway smooth muscle interact with the extracellular matrix. *Am J Physiol Lung Cell Mol Physiol* 290: L153–L161, 2006.
3. **Chambers LS, Black JL, Ge Q, Carlin SM, Au WW, Poniris M, Thompson J, Johnson PR, Burgess JK.** PAR-2 activation, PGE2, and COX-2 in human asthmatic and nonasthmatic airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 285: L619–L627, 2003.

4. **Chao ES, Dunbar D, Kaminsky LS.** Intracellular lactate dehydrogenase concentration as an index of cytotoxicity in rat hepatocyte primary culture. *Cell Biol Toxicol* 4: 1–11, 1988.
5. **Chu Hong W, Halliday Janet L, Martin Richard J, Leung Donald YM, Szefler Stanley J, Wenzel SE.** Collagen deposition in large airways may not differentiate severe asthma from milder forms of the disease. *Am J Respir Crit Care Med* 158: 1936–1944, 1998.
6. **Doerner A, Zuraw B.** TGF-beta1 induced epithelial to mesenchymal transition (EMT) in human bronchial epithelial cells is enhanced by IL-1beta but not abrogated by corticosteroids. *Respir Res* 10: 100–115, 2009.
7. **Elias JA.** Airway remodeling in asthma. Unanswered questions. *Am J Respir Crit Care Med* 161: S168–S171, 2000.
8. **Fedorov IA, Wilson SJ, Davies DE, Holgate ST.** Epithelial stress and structural remodelling in childhood asthma. *Thorax* 60: 389–394, 2005.
9. **Ge Q, Moir LM, Black JL, Oliver BG, Burgess JK.** TGFbeta1 induces IL-6 and inhibits IL-8 release in human bronchial epithelial cells: the role of Smad2/3. *J Cell Physiol* 225: 846–854, 2010.
10. **Griego SD, Weston CB, Adams JL, Tal-Singer R, Dillon SB.** Role of p38 mitogen-activated protein kinase in rhinovirus-induced cytokine production by bronchial epithelial cells. *J Immunol* 165: 5211–5220, 2000.
11. **Han SW, Roman J.** Fibronectin induces cell proliferation and inhibits apoptosis in human bronchial epithelial cells: pro-oncogenic effects mediated by PI3-kinase and NF-kappa B. *Oncogene* 25: 4341–4349, 2006.
12. **Hastie AT, Kraft WK, Nyce KB, Zangrilli JG, Musani AI, Fish JE, Peters SP.** Asthmatic epithelial cell proliferation and stimulation of collagen production: human asthmatic epithelial cells stimulate collagen type III production by human lung myofibroblasts after segmental allergen challenge. *Am J Respir Crit Care Med* 165: 266–272, 2002.
13. **Hirst SJ, Twort CH, Lee TH.** Differential effects of extracellular matrix proteins on human airway smooth muscle cell proliferation and phenotype. *Am J Respir Cell Mol Biol* 23: 335–344, 2000.
14. **Hocking DC.** Fibronectin matrix deposition and cell contractility: implications for airway remodeling in asthma. *Chest* 122: 275S–278S, 2002.
15. **Hocking DC, Chang CH.** Fibronectin matrix polymerization regulates small airway epithelial cell migration. *Am J Physiol Lung Cell Mol Physiol* 285: L169–L179, 2003.
17. **Hocking DC, Sottile J, Langenbach KJ.** Stimulation of integrin-mediated cell contractility by fibronectin polymerization. *J Biol Chem* 275: 10673–10682, 2000.
18. **Ip WK, Wong CK, Lam CW.** Interleukin (IL)-4 and IL-13 up-regulate monocyte chemoattractant protein-1 expression in human bronchial epithelial cells: involvement of p38 mitogen-activated protein kinase, extracellular signal-regulated kinase 1/2 and Janus kinase-2 but not c-Jun NH2-terminal kinase 1/2 signalling pathways. *Clin Exp Immunol* 145: 162–172, 2006.
19. **Johnson PR, Black JL, Carlin S, Ge Q, Underwood PA.** The production of extracellular matrix proteins by human passively sensitized airway smooth-muscle cells in culture: the effect of beclomethasone. *Am J Respir Crit Care Med* 162: 2145–2151, 2000.
20. **Johnson PR, Burgess JK, Ge Q, Poniris M, Boustany S, Twigg SM, Black JL.** Connective tissue growth factor induces extracellular matrix in asthmatic airway smooth muscle. *Am J Respir Crit Care Med* 173: 32–41, 2006.
21. **Kaiura TL, Itoh H, Kubaska SM, 3rd McCaffrey TA, Liu B, Kent KC.** The effect of growth factors, cytokines, and extracellular matrix proteins on fibronectin production in human vascular smooth muscle cells. *J Vasc Surg* 31: 577–584, 2000.
22. **Kasai H, Allen JT, Mason RM, Kamimura T, Zhang Z.** TGF-beta1 induces human alveolar epithelial to mesenchymal cell transition (EMT). *Respir Res* 6: 56, 2005.
23. **Kicic A, Hallstrand TS, Sutanto EN, Stevens PT, Kobor MS, Taplin C, Pare PD, Beyer RP, Stick SM, Knight DA.** Decreased fibronectin production significantly contributes to dysregulated repair of asthmatic epithelium. *Am J Respir Crit Care Med* 181: 889–898, 2010.
24. **Kicic A, Sutanto EN, Stevens PT, Knight DA, Stick SM.** Intrinsic biochemical and functional differences in bronchial epithelial cells of children with asthma. *Am J Respir Crit Care Med* 174: 1110–1118, 2006.
25. **Krimmer DI, Burgess JK, Wooi TK, Black JL, Oliver BG.** Matrix proteins from smoke exposed fibroblasts are pro-proliferative. *Am J Respir Cell Mol Biol* 46: 34–39, 2012.
26. **Legrand C, Bour JM, Jacob C, Capiaumont J, Martial A, Marc A, Wudtke M, Kretzmer G, Demangel C, Duval D, Hache J.** Lactate dehydrogenase (LDH) activity of the number of dead cells in the medium of cultured eukaryotic cells as marker. *J Biotechnol* 25: 231–243, 1992.
27. **Magnusson MK, Mosher DF.** Fibronectin: structure, assembly, and cardiovascular implications. *Arterioscler Thromb Vasc Biol* 18: 1363–1370, 1998.
28. **Meng XN, Jin Y, Yu Y, Bai J, Liu GY, Zhu J, Zhao YZ, Wang Z, Chen F, Lee KY, Fu SB.** Characterisation of fibronectin-mediated FAK signalling pathways in lung cancer cell migration and invasion. *Br J Cancer* 101: 327–334, 2009.
29. **Moir LM, Burgess JK, Black JL.** Transforming growth factor beta 1 increases fibronectin deposition through integrin receptor alpha 5 beta 1 on human airway smooth muscle. *J Allergy Clin Immunol* 121: 1034–1039.e4, 2008.
30. **Moses HL.** TGF-beta regulation of epithelial cell proliferation. *Mol Reprod Devel* 32: 179–184, 1992.
31. **Mosmann T.** Rapid colorimetric assay for cellular growth and survival—application to proliferation and cyto-toxicity assays. *J Immunol Methods* 65: 55–63, 1983.
32. **Oenema TA, Mensink G, Smedinga L, Halayko AJ, Zaagsma J, Meurs H, Gosens R, Dekkers BG.** Cross-talk between transforming growth factor-beta(1) and muscarinic M(2) receptors augments airway smooth muscle proliferation. *Am J Respir Cell Mol Biol* 49: 18–27, 2013.
33. **Pavord ID, Tattersfield AE.** Bronchoprotective role for endogenous prostaglandin E2. *Lancet* 345: 436–438, 1995.
34. **Pavord ID, Wong CS, Williams J, Tattersfield AE.** Effect of inhaled prostaglandin E2 on allergen-induced asthma. *Am Rev Respir Dis* 148: 87–90, 1993.
35. **Perri M, Pingitore A, Cione E, Vilardi E, Perrone V, Genchi G.** Proliferative and anti-proliferative effects of retinoic acid at doses similar to endogenous levels in Leydig MLTC-1/R2C/TM-3 cells. *Biochim Biophys Acta* 1800: 993–1001, 2010.
36. **Rincon M, Anguita J, Nakamura T, Fikrig E, Flavell RA.** Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4+ T cells. *J Exp Med* 185: 461–469, 1997.
37. **Roberts CR.** Is asthma a fibrotic disease? *Chest* 107: 111S–117S, 1995.
38. **Roche WR, Beasley R, Williams JH, Holgate ST.** Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* 1: 520–524, 1989.
39. **Sagara H, Okada T, Okumura K, Ogawa H, Ra C, Fukuda T, Nakao A.** Activation of TGF-β/Smad2 signaling is associated with airway remodeling in asthma. *J Allergy Clin Immunol* 110: 249–254, 2002.
40. **Semlali A, Jacques E, Plante S, Biardel S, Milot J, Laviolette M, Boulet LP, Chakir J.** TGF-beta suppresses EGF-induced MAPK signaling and proliferation in asthmatic epithelial cells. *Am J Respir Cell Mol Biol* 38: 202–208, 2008.
41. **Shinkai M, Foster GH, Rubin BK.** Macrolide antibiotics modulate ERK phosphorylation and IL-8 and GM-CSF production by human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 290: L75–L85, 2006.
42. **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* 293: L69–L76, 2007.
43. **Takizawa H.** [Studies on the regulation of normal bronchial epithelial cell proliferation and proto-oncogene expression]. *Nihon Kyobu Shikkan Gakkai Zasshi* 30, *Suppl*: 27–32, 1992.
44. **Tomkowicz A, Kraus-Filarska M, Bar J, Rabczynski J, Jelen M, Piesiak P, Fal A, Panaszek B.** Bronchial hyper-responsiveness, subepithelial fibrosis, and transforming growth factor-beta(1) expression in patients with long-standing and recently diagnosed asthma. *Arch Immunol Ther Exp (Warsz)* 56: 401–408, 2008.
45. **Vignola AM, Chanez P, Chiappara G, Merendino A, Pace E, Rizzo A, la Rocca AM, Bellia V, Bonsignore G, Bousquet J.** Transforming growth factor-beta expression in mucosal biopsies in asthma and chronic bronchitis. *Am J Respir Crit Care Med* 156: 591–599, 1997.
46. **Vignola AM, Chanez P, Chiappara G, Merendino A, Zinnanti E, Bousquet J, Bellia V, Bonsignore G.** Release of transforming growth factor-beta (TGF-beta) and fibronectin by alveolar macrophages in airway diseases. *Clin Exp Immunol* 106: 114–119, 1996.
47. **Wang YH, Wills-Karp M.** The potential role of interleukin-17 in severe asthma. *Curr Allergy Asthma Rep* 11: 388–394, 2011.
48. **Xie S, Sukkar MB, Issa R, Khorasani NM, Chung KF.** Mechanisms of induction of airway smooth muscle hyperplasia by transforming growth factor-beta. *Am J Physiol Lung Cell Mol Physiol* 293: L245–L253, 2007.
49. **Zhang ZD, Cox G.** MTT assay overestimates human airway smooth muscle cell number in culture. *Biochem Mol Biol Int* 38: 431–436, 1996.