

Role of Insulin-like Growth Factor Binding Protein-3 in Allergic Airway Remodeling

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Rationale: The hallmarks of allergic asthma are airway inflammation, obstruction, and remodeling. Airway remodeling may lead to irreversible airflow obstruction with increased morbidity and mortality. Despite advances in the treatment of asthma, the mechanisms underlying airway remodeling are still poorly understood. We reported that insulin-like growth factor (IGF) binding proteins (IGFBPs) contribute to extracellular matrix deposition in idiopathic pulmonary fibrosis; however, their contribution to airway remodeling in asthma has not been established.

Objectives: We hypothesized that IGFBP-3 is overexpressed in asthma and contributes to airway remodeling.

Methods: We evaluated levels of IGFBP-3 in tissues and bronchoalveolar lavage fluid from patients with asthma at baseline and 48 hours after allergen challenge, in reparative epithelium in an *in vitro* wounding assay, and in conditioned media from cytokine- and growth factor-stimulated primary epithelial cells.

Measurements and Main Results: IGFBP-3 levels and distribution were evaluated by Western blot, ELISA, and immunofluorescence. IGFBP-3 is increased *in vivo* in the airway epithelium of patients with asthma compared with normal control subjects. The concentration of IGFBP-3 is increased in the bronchoalveolar lavage fluid of patients with asthma after allergen challenge, its levels are increased in reparative epithelium in an *in vitro* wounding assay and in the conditioned medium of primary airway epithelial cell cultures stimulated with IGF-I.

Conclusions: Our results suggest that one mechanism of allergic airway remodeling is through the secretion of the profibrotic IGFBP-3 from IGF-I-stimulated airway epithelial cells during allergic inflammation.

Keywords: asthma; bronchoalveolar lavage; primary epithelial cells; insulin-like growth factor binding protein; fibrosis

Allergic asthma is a chronic inflammatory disease characterized by bronchoconstriction and airway remodeling. Classical teaching is that patients with asthma have preserved lung function between exacerbations, and during attacks respond well to bronchodilator and corticosteroid therapy (reversibility). Although this is true for a majority of patients with asthma, there is a subgroup of patients that will progress to irreversible airflow obstruction. It is generally believed that airway remodeling is a result of chronic inflammation, but the exact relationships

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Allergic airway remodeling causes significant morbidity and mortality among patients with asthma and currently there are no effective therapies available to prevent or reverse this process.

What This Study Adds to the Field

Our results suggest that one mechanism of allergic airway remodeling is through the secretion of profibrotic insulin-like growth factor binding protein-3 (IGFBP-3) from insulin-like growth factor-1 (IGF-1) stimulated airway epithelial cells during allergic inflammation.

between inflammatory mediators and the observed structural lung changes are unclear.

As a protective barrier, the epithelium is the first layer to be exposed to allergen, and can play a unique role in initiating the inflammatory process and regulating the injury-repair cycle. Epithelial cell sloughing and denudation, seen in asthmatic airway biopsies, suggest that, in chronic asthma, the epithelium is in a state of constant repair. Subepithelial fibrosis, a component of airway remodeling, could result from a chronic or abnormal injury-repair process. Multiple growth factors, such as transforming growth factor (TGF)- β , epidermal growth factor, insulin-like growth factor (IGF), and vascular endothelial growth factor, are up-regulated during airway inflammation (1), and induce the proliferation and differentiation of nearby mesenchymal cells toward increased collagen secretion, angiogenesis, and smooth muscle hyperplasia—hallmarks of remodeling.

The precise role of IGF-I and the associated IGF binding proteins (IGFBPs) in airway remodeling has not been elucidated. IGF-I is a potent mitogen and stimulant of collagen secretion in fibroblasts (2), and has been implicated in the pathogenesis of fibrosis in a number of lung disorders (3–5). IGF-I is up-regulated in the airways of mice after allergen challenge (6), and IGF-I levels in biopsies of patients with asthma correlate with subepithelial fibrosis (7). The regulation of IGF-I is mediated, in part, by a system of six IGFBPs (8).

We have previously demonstrated that IGFBP-3 plays a role in fibrosis and extracellular matrix (ECM) deposition in idiopathic pulmonary fibrosis (4). To investigate the hypothesis that IGFBP-3 is also overexpressed in asthma, and may contribute to airway remodeling, we examined IGFBP-3 levels in bronchoalveolar lavage (BAL) fluid of patients with mild asthma before and after allergic challenge, as well as by immunofluorescent staining of epithelial cells in patients with mild and severe asthma. Finally, we evaluated the influence of various cytokines and growth factors on IGFBP-3 secretion by primary airway epithelial cells.

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METHODS

Subjects

Group A, University of Pittsburgh. Patients with asthma of varying severity were recruited as part of ongoing studies of severe asthma, some of which were from the Severe Asthma Research Program. The patients with severe asthma all met the American Thoracic Society workshop definition of severe asthma (9). This definition includes the continuous use of high-dose inhaled and/or oral steroids for greater than 50% of the previous year, along with two of seven minor criteria. The patients with mild asthma were on low-dose or no inhaled corticosteroids (ICS), and had an FEV₁ of more than 75% predicted, with no urgent care visits or oral prednisone in the previous year. Normal subjects had no history of respiratory disease, and were not taking any medications. No subjects had more than a 5-pack-year history of cigarette smoking, and none had smoked in the previous year. Atopy was identified as the presence of one or more positive skin prick test (wheal $\geq$ histamine control) to local allergens. A history of mild but untreated seasonal allergic rhinitis was allowed in the normal control subjects. Informed consent was obtained from each subject before participation. The studies were approved by the National Jewish Medical and Research Center and the University of Pittsburgh (S.E.W.) institutional review boards (Table 1).

Group B, University of Wisconsin. A total of 10 subjects with allergy with physician-diagnosed asthma were studied. All subjects were nonsmokers on short-acting β -agonists only; none were using ICS or systemic corticosteroids. All had a positive skin prick test to one or more aeroallergens, and did not have a respiratory infection or asthma exacerbation within 30 days of study, and had not received long-acting β -agonists within 2 days, antihistamines or leukotriene antagonists within 7 days, or corticosteroids within 30 days of study enrollment. Informed consent was obtained from each subject before participation. The study was approved by the University of Wisconsin, Madison (N.N.J.) Center for Health Sciences Human Subjects Committee (Tables 1 and 2).

Segmental Bronchoprovocation with Allergen and BAL

Bronchoscopy, BAL, and segmental bronchoprovocation with allergen (SBP-AG) were performed in two segments, as previously described (10). BAL (160 ml/segment) was performed before and 48 hours after SBP-AG. Allergen dose for the two segments was 10 and 20% of the dose of allergen causing a 20% fall in FEV₁ (Ag-PD₂₀). BAL fluid at baseline (0 h) and 48 hours after SBP-AG was analyzed for IGFBP-3 by Western blot and ELISA. Additional details are included in the online supplement. As a quality control measure to ensure that segmental allergen challenge does not cause a nonspecific increase in IGFBP-3 due to activation of airway epithelial cells by the allergen or the bronchoscopy procedure, a retrospective analysis was performed on BAL fluid obtained from nine normal subjects challenged with allergen (1,000 protein nitrogen units of ragweed). See the online supplement for details.

Endobronchial Biopsy and Immunofluorescence

Endobronchial biopsies were taken from the 3rd–4th level subcarinae of the lower lobes, as previously described (11). Sections (6 μ m) were

deparaffinized, antigen-retrieved, and incubated with a polyclonal rabbit anti-IGFBP-3 antibody (GroPep, Adelaide, SA, Australia) and fluorochrome-tagged secondary antibody (see the online supplement for a detailed protocol). Fluorescence intensity was analyzed using MetaMorph (Molecular Devices, Sunnyvale, CA). Subepithelial membrane (SBM) thickness was measured on formalin-fixed tissue sections stained with Movat's pentachrome stain, which differentially stains tissue structural elements, including collagen. Pictures of tissue sections were taken at 400 $\times$ magnification. SBM thickness was measured in areas of good epithelial orientation using Image-Pro software (Media Cybernetics, Inc., Bethesda, MD).

Cell Culture and Reagents

Primary human airway epithelial cells (HBEs) were cultured from excess pathological tissue after lung transplantation and organ donation under a protocol approved by the University of Pittsburgh Institutional Review Board. HBEs were cultured on human placental collagen-coated Costar Transwell filters (Catalog no. 3470, 0.33 cm², 0.4 μ m pore), as previously described (12), and used for experimentation after 4 to 6 weeks of culture in an air–liquid interface.

Polyclonal goat anti-human IGFBP-3 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used for Western blot analyses. Signals were detected after incubation with horseradish peroxidase-conjugated anti-goat secondary antibody (Santa Cruz Biotechnology, Inc.) and chemiluminescence (Perkin Elmer Life Sciences, Inc., Boston, MA). IGFBP-3 levels in undiluted BAL fluid were quantified using a commercially available ELISA (R&D Systems, Minneapolis, MN). The following cytokines were used for stimulation assays: IL-4, IL-13, and TNF- α (Leinco Technologies, St. Louis, MO); IGF-I (Sigma-Aldrich, St. Louis, MO); and TGF- β 1 (R&D Systems). Purified, low-endotoxin, natural *Felis domesticus* allergen 1 (nFel d 1) was purchased from Indoor Biotechnologies (Charlottesville, VA).

Epithelial Injury and Immunofluorescence

Mechanical scrape injury was induced by creating a line with a pipette tip across the surface of a confluent HBE cell layer, as previously described (13). At 18 hours after epithelial injury, IGFBP-3 expression was evaluated by immunofluorescence using polyclonal rabbit anti-IGFBP-3 (GroPep) and secondary antibody conjugated to Alexa-488 (Jackson Research, West Grove, PA). DRAQ5 (Alexis Corporation, Lausen, Switzerland) and fluorescently labeled phalloidin (Molecular Probes, Carlsbad, CA) were used as nuclear and cytoskeletal markers, respectively. Detailed methods appear in the online supplement.

Cell Stimulation Assays

Differentiated primary HBEs were transiently cultured in Dulbecco's modified Eagles medium in the presence of the indicated cytokines, growth factors, purified allergen, or dexamethasone for 24 or 48 hours. Equivalent surface area of differentiated HBEs (0.33 cm²) and media volumes were used per experimental condition, and equivalent apical and basolateral media volumes were used per well. The apical and basolateral media were collected and centrifuged for 10 minutes at 4°C to remove cell debris. IGFBP-3 levels in equivalent volumes of the sample supernatants were analyzed by Western blot.

TABLE 1. SUBJECT DEMOGRAPHICS

Group	Sex (F/M)	Race (C/AA/H)	Age* (yr)	Atopy (%)	FEV ₁ % Predicted†	ICS (Yes/No)	OCS (Yes/No)
Group A							
Normal control	3/4	7/0/0	29 $\pm$ 6	71	102 $\pm$ 2	0/6	0/6
Mild asthma	5/3	6/2/0	41 $\pm$ 11	75	84 $\pm$ 5	1/7	0/8
Severe asthma	3/4	6/1/0	47 $\pm$ 7	83	54 $\pm$ 2	7/0	4/3
Overall P value	NS	NS	P = 0.004	NS	P < 0.0001		
Group B							
Mild asthma	5/5	9/0/1	22 $\pm$ 3	100	94 $\pm$ 11	0/10	0/10

Definition of abbreviations: AA = African American; C = Caucasian; F = female; H = Hispanic; ICS = inhaled corticosteroids; M = male; NS = not significant; OCS = oral corticosteroids.

* Mean $\pm$ SD.

† mean $\pm$ SE.

TABLE 2. SUBJECT BASELINE CHARACTERISTICS AND BRONCHOALVEOLAR LAVAGE FLUID PROFILE BEFORE AND 48 HOURS AFTER SEGMENTAL BRONCHOPROVOCATION ALLERGEN

Subject No.	Screen					Baseline BAL (D0)			48-h Post-AG BAL (D2)		
	Sex	Age (yr)	Meth PC ₂₀ (mg/ml)	FEV ₁ % Predicted	Ag	Return Volume (%)	BAL Cells × 10 ⁶	BAL EOS (%)	Return Volume (%)	BAL Cells × 10 ⁶	BAL EOS (%)
1	M	19	2.5	76	RW	68	30	0.1	63	110	53.8
2	F	24	0.4	92	CAT	79	30	0.3	73	43	23.6
3	F	27	0.5	95	CAT	76	21	0.3	73	47	28.9
4	M	21	1.3	99	CAT	70	31	3.3	68	42	22.7
5	M	26	9.9	106	HDM	74	34	0.9	71	197	52.5
6	F	20	0.2	97	RW	63	34	0.2	65	51	30.6
7	M	19	2.9	94	HDM	77	39	1.0	82	82	24.6
8	F	18	1.4	93	RW	70	37	0.6	70	176	63.1
9	F	26	2.9	111	CAT	73	36	2.2	72	141	62.5
10	M	24	2.5	75	HDM	76	28	8.0	76	806	72.3
5 F; 5 M 22 ± 3 2.5 ± 2.8 94 ± 11 3 RW; 4 CAT; 3 HDM						73 ± 5	32 ± 5	1 ± 1	71 ± 5	169 ± 231	44 ± 19

Definition of abbreviations: AG = allergen; BAL = bronchoalveolar lavage; CAT = cat allergen; D0 = before SBP-AG; D2 = 48 hours after SBP-AG; EOS = eosinophils; F = female; HDM = house dust mite; M = male; Meth = methacholine; PC₂₀ = concentration of methacholine causing a 20% fall in FEV₁; RW = ragweed; SBP = segmental bronchoprovocation.

Statistical Analysis

Data were assessed for normalcy of distribution. All data were normally distributed and therefore analyzed by analysis of variance or Student's *t* test. When an overall difference among the groups was observed, intergroup differences were analyzed by Tukey-Kramer testing. A *P* value less than 0.05 was considered significant for all results.

RESULTS

IGFBP-3 Protein Levels Are Increased in BAL Fluid of Patients with Asthma after Allergen Challenge

IGFBP-3 levels were examined by Western blot of equal volumes of BAL fluid from 10 patients with mild asthma before (0 h) and 48 hours after segmental allergen bronchoprovocation (Figure 1A). The qualitative increase in IGFBP-3 was quantified by ELISA (Figure 1B). IGFBP-3 levels increased variably

between a minimum of 2.5-fold (Subject 2) to greater than 50-fold (Subject 10), with a mean baseline concentration of 0.27 (±0.63) ng/ml, and a mean post-challenge concentration of 14.85 (±13.64) ng/ml. Increased levels of IGFBP-3 were induced by challenge with three different allergens (house dust mite, ragweed, and cat). After allergen challenge, total numbers of BAL cells and percentage of BAL eosinophils were significantly increased (169 ± 231 × 10⁶ vs. 32 ± 5 × 10⁶ total cells, *P* < 0.001; 44 ± 19% eosinophils vs. 1.7 ± 2.5% eosinophils, *P* < 0.001) (Table 2), and correlated with levels of IGFBP-3 (Spearman's correlation coefficient > 0.7; *P* ≤ 0.004). BAL fluid recovery was not significantly different after challenge (73 ± 5% at baseline vs. 71 ± 5% after challenge) (Table 2). Allergen challenge of normal, nonallergic subjects with no airway disease did not induce airway eosinophilia or marked changes in BAL fluid concentrations of IGFBP-3. BAL eosino-

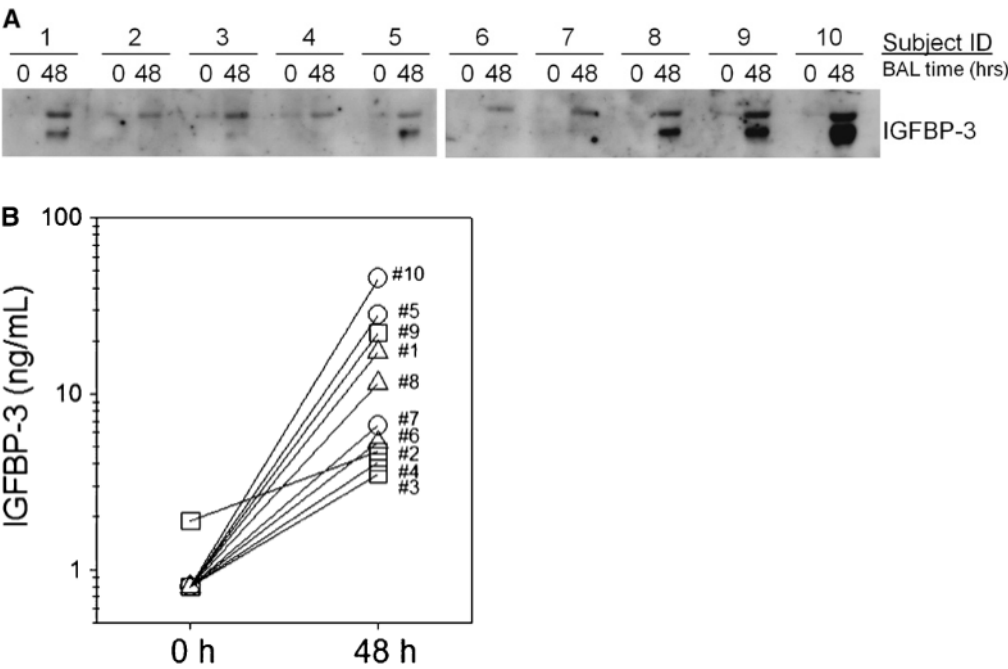


Figure 1. Insulin-like growth factor binding protein (IGFBP)-3 levels are increased in bronchoalveolar lavage (BAL) fluid of patients with asthma 48 hours after allergen challenge. (A) Western blot analysis demonstrating increased IGFBP-3 levels in BAL fluid from 10 patients with mild asthma after allergen challenge. Standard reducing sodium dodecyl sulfate sample buffer was added to a final concentration of 1× to undiluted BAL fluid, and equal volumes of BAL fluid were loaded per lane. BAL fluid samples designated "0" are baseline (before SBP-AG challenge) and those designated "48" are 48 hours after challenge. (B) ELISA quantification of IGFBP-3 levels in aliquots of the BAL fluid samples depicted in (A). Analysis was performed on undiluted samples obtained before and 48 hours after challenge with house dust mite (squares), ragweed (triangles), or cat (circles) allergen, and data are expressed as nanograms per milliliter of BAL fluid.

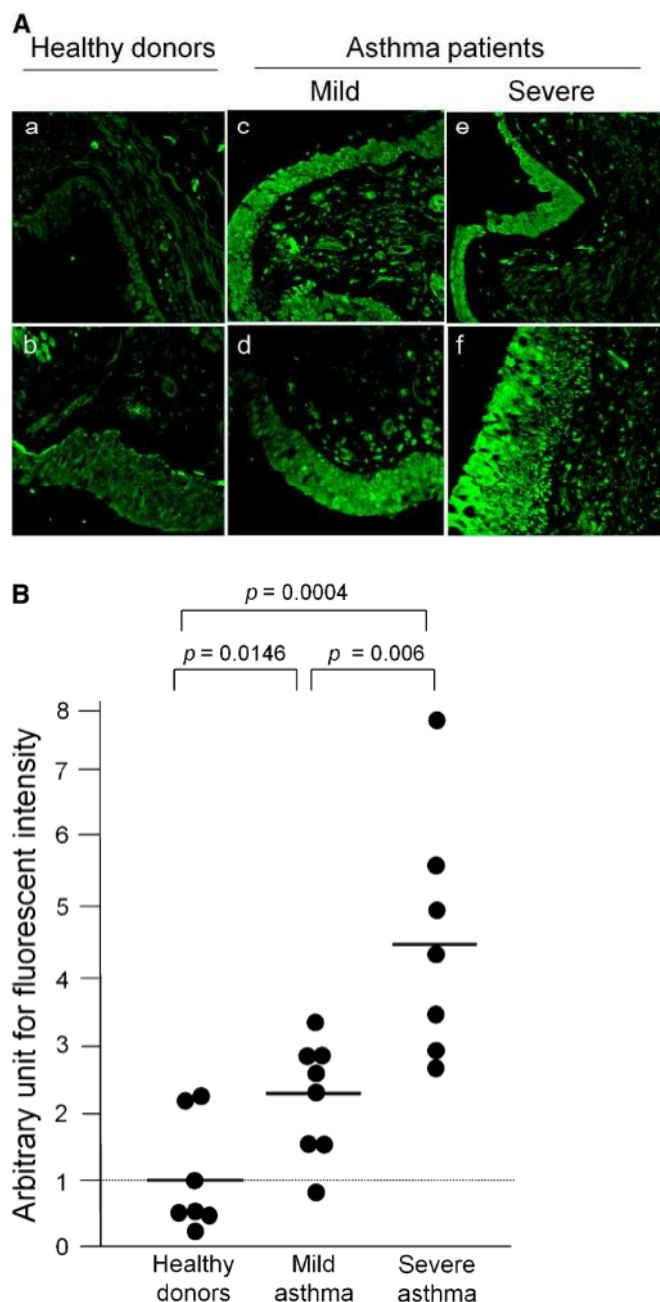


Figure 2. Insulin-like growth factor binding protein (IGFBP)-3 protein is increased *in vivo* in airway epithelial cells of patients with asthma versus subjects without asthma. (A) IGFBP-3 expression in airway tissues from healthy donors (a and b), patients with mild asthma (c and d), and those with severe asthma (e and f). IGFBP-3 levels were detected by immunofluorescence (green). Images were taken at 600 $\times$ magnification. (B) Analysis of the signal intensity in airway epithelial cell layers in airway tissues. Airway tissues obtained from eight patients with mild asthma, seven patients with severe asthma, and seven healthy control subjects. Five individual fields were randomly selected in each tissue specimen and fluorescence intensity was quantified using MetaMorph. Mean immunofluorescence intensity of healthy donors was defined as one, and the ratio of the sample-to-mean immunofluorescence intensity of healthy donors was calculated as an arbitrary unit.

phils were 0.3 (± 0.7)% at baseline, 0.7 (± 0.3)% 48 hours after saline challenge, and 0.6 (± 0.7)% 48 hours after allergen challenge. Concentrations of IGFBP-3 in BAL fluid were 0.9 (± 0.1) ng/ml at baseline, 2.0 (± 1.4) ng/ml 48 hours after saline challenge, and 1.9 (± 0.9) ng/ml 48 hours after allergen challenge (see Figure E1 in the online supplement). The lack of a marked increase in IGFBP-3 after allergen or saline challenge of normal subjects suggests that neither the allergen nor the bronchoscopic procedure provide sufficient signals to induce IGFBP-3 secretion by airway epithelium.

IGFBP-3 Is Highly Expressed *In Vivo* in Airway Epithelial Cells of Patients with Asthma

To further examine IGFBP-3 expression levels in the airways of patients with asthma, and to determine the source of IGFBP-3 *in vivo*, airway tissue sections were analyzed using immunofluorescence to evaluate the amount and distribution of cell-associated IGFBP-3 protein. As shown in Figure 2A, IGFBP-3 was highly expressed in the epithelial cell layer in patients with mild and severe asthma compared with healthy donors. To quantify the expression of IGFBP-3 *in vivo*, we analyzed the mean fluorescence intensity in the airway epithelial layer in tissues from eight patients with mild asthma, seven patients with severe asthma, and seven healthy control subjects. As shown in Figure 2B, the intensity was 2.2 times (2.2 ± 0.8) higher in mild and 4.4 times (4.4 ± 1.7) higher in severe asthmatic airways compared with healthy control airways ($P < 0.0146$ and $P < 0.0004$, respectively). Epithelial cell expression of IGFBP-3 correlated negatively with FEV₁ % predicted ($r = -0.54$; $P = 0.01$), but did not correlate with SBM thickness (data not shown).

IGFBP-3 Is Up-Regulated at the Leading Edge of Wound Repair in an *In Vitro* Epithelial Cell Wound Healing Assay

Based on the high expression of IGFBP-3 in the epithelial cell layer of patients with asthma, we hypothesized that up-regulation of IGFBP-3 may result from epithelial cell injury and repair. To test this hypothesis, we evaluated IGFBP-3 levels by immunofluorescence in an *in vitro* model of epithelial cell wound repair. As demonstrated in Figure 3, IGFBP-3 levels are significantly increased at the leading edge of wound repair, and subsequently return to baseline levels in regions where the epithelial monolayer has been successfully restored to a normal phenotype.

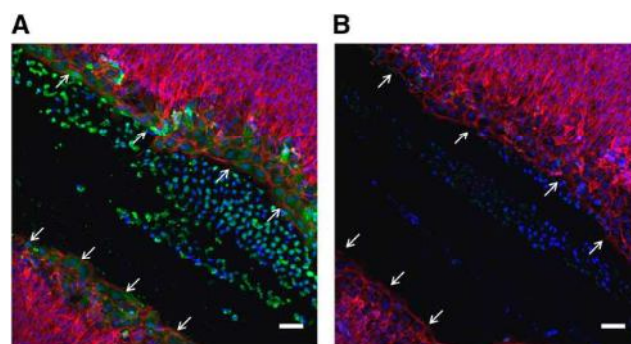


Figure 3. Insulin-like growth factor binding protein (IGFBP)-3 is up-regulated in reparative airway epithelium. Well differentiated human airway epithelial cell cultures were mechanically denuded, cultured for 18 hours, and subsequently immunostained (green) with (A) anti-IGFBP-3 polyclonal antibody or (B) rabbit IgG. The cytoskeleton (red) and nuclei (blue) demonstrate cellular morphology. Note the increased expression of IGFBP-3 along the reparative wound edge (arrows). Bar = 50 μ m. Images shown are representative of three independent experiments performed using tissues cultured from three donors.

IGF-I Up-Regulates IGFBP-3 Production in Airway Epithelial Cells

Because IGFBP-3 was markedly up-regulated in BAL fluid of patients with asthma after challenge, as well as in airway epithelial cells of patients with asthma, we examined potential inducers of IGFBP-3. Primary airway epithelial cells were stimulated for 24 and 48 hours with cytokines and growth factors, as listed in Figure 4. At 24 hours, IGF-I induced a 300-fold increase in IGFBP-3 secretion at the apical surface, and a 10-fold increase from the basolateral surface. In comparison, TGF- β 1 alone induced a more modest increase in IGFBP-3 (83-fold and 5-fold, respectively). When combined with IGF-I, TGF- β 1 inhibited IGF-I stimulation of IGFBP-3 at the apical and basolateral surfaces (60 and 10%, respectively). Of note, IGFBP-3 exists as two predominant glycosylation variants, with approximate molecular masses of 41 and 45 kD, that can be reduced to a single approximately 36-kD band by deglycosylation; all three of which have the capacity to bind IGF ligands (14).

To ensure that exposure to allergen does not result in a non-specific increase in IGFBP-3 production by airway epithelial cells, we examined the effect of nFel d 1 on IGFBP-3 secretion and degradation. *In vitro*, nFel d 1 does not increase IGFBP-3 secretion or degradation in primary airway epithelial cells in either the presence or absence of IGF-I (Figure E2).

Because a subset of patients with asthma received corticosteroids, we evaluated whether exposure to corticosteroids alone results in increased IGFBP-3. Primary airway epithelial cells were incubated for 48 hours with dexamethasone in either the presence or absence of IGF-I. Dexamethasone alone does not result in increased IGFBP-3 secretion or detection of degraded IGFBP-3, and, in fact, demonstrates a trend toward diminished IGFBP-3 secretion in the presence of IGF-I (Figure E3).

DISCUSSION

We previously demonstrated that IGFBP-3 is associated with fibrosis and ECM deposition in IPF (4). We therefore tested the hypothesis that IGF and its binding proteins are also involved in the remodeling process of asthma. In this study, we demonstrate an increase in levels of IGFBP-3 protein in BAL fluid of patients with asthma after allergen challenge that correlates with percentages of BAL eosinophils—a hallmark of the inflammatory response in asthma. We show that baseline cell-associated IGFBP-3 levels in airway epithelial cells are higher in patients with asthma versus healthy donors, and that levels increase significantly with disease severity and worsening lung function, although they do not correlate directly with SBM thickness. Taken together, these observations demonstrate that IGFBP-3 is increased in the airway epithelium of patients with asthma, and suggest that it is released into the lumen (where it is detectable by BAL) in significant quantities only in response to irritant stimuli, such as inhaled allergen.

We have demonstrated that intracellular IGFBP-3 expression is increased at the leading edge of epithelial wound repair in an *in vitro* system, and that IGF-I is sufficient to dramatically up-regulate secreted IGFBP-3 levels in nondisrupted, differentiated primary airway epithelial cell culture. These observations support our hypothesis that increased epithelial cell-associated IGFBP-3 in asthma is associated with the cyclic damage and repair of the epithelium that characterizes allergic asthma. Injured airway epithelial cells are therefore one source of IGFBP-3 in post-challenge BAL, and, based upon our previous observations (4), we hypothesize that secreted IGFBP-3 is involved in the development and/or perpetuation of subepithelial fibrosis and airway remodeling in asthma.

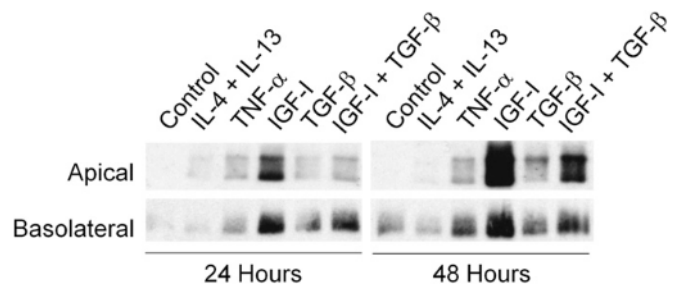


Figure 4. Insulin-like growth factor (IGF)-I induces secretion of IGF binding protein (IGFBP)-3 by primary airway epithelial cells *in vitro*. IGF-I induces a 300-fold increase in IGFBP-3 apical secretion and a 10-fold increase in basolateral secretion in well differentiated human airway epithelial cell cultures. Transforming growth factor (TGF)- β alone induced a more modest increase in apical and basolateral IGFBP-3 (83-fold and 5-fold, respectively), and, when combined with IGF-I, inhibited IGF-I stimulation of IGFBP-3 secretion (60% apically and 10% basolaterally). Equivalent volumes of apical or basolateral culture media (corresponding to 5% of the 0.33 cm² surface area of the differentiated epithelial cell monolayer) were loaded per lane. Concentrations used were as follows: IL-4 (10 ng/ml); IL-13 (10 ng/ml); TNF- α (40 ng/ml); IGF-I (250 ng/ml), TGF- β (10 ng/ml).

Damage to the epithelium can range from loss of important components, such as cilia, to shedding of the epithelium and denudation of the basement membrane. Epithelial cells have been shown to release multiple profibrogenic factors in response to exogenous chemical or mechanical stimuli in an *in vitro* coculture model with myofibroblasts, including TGF- β , fibroblast growth factor-2, endothelin-1, platelet-derived growth factor-BB, and IGF-I (15, 16). In this model, collagen gene expression is increased and myofibroblast proliferation is enhanced (16). Our data identify IGFBP-3 as a product of airway epithelial cells induced in response to injury and to IGF-I elaboration. Taken together with our previous findings that IGFBP-3 induces a profibrotic phenotype *in vitro* (4), our results suggest that IGFBP-3 is another mediator of subepithelial fibrosis and allergic airway remodeling. The activity of IGF-I is modulated by IGFBPs in a cell- and tissue-specific manner (2), and IGF-I can also modulate IGFBP expression (8). Our data suggest that the latter may be the case in allergic airway inflammation. This is supported by two recent studies implicating IGF-I in airway subepithelial fibrosis in human and murine asthma models (6, 7). Hoshino and colleagues (7) reported a significant correlation between IGF-I levels in patients with asthma and collagen thickening of the lamina reticularis of the basement membrane. Furthermore, Yamashita and colleagues (6) found that IGF-I was up-regulated in the BAL of mice after allergen challenge, with a concomitant increase in IGFBP-3 mRNA in lung tissue. Treatment with neutralizing antibody against IGF-I attenuated airway hyperresponsiveness and subepithelial fibrosis, and resulted in a significant decrease in lung tissue levels of IGFBP-3 mRNA (6).

We recognize that there are certain limitations to studies of this nature. First, our sample size may be too small to detect a significant correlation between IGFBP-3 expression and SBM thickness; alternatively, our observations may be reflective of a necessary, but not sufficient, role for IGFBP-3 in the complex mechanism of chronic airway remodeling and subepithelial fibrosis. Second, all subjects in the severe asthma cohort were on ICSs, which may affect IGFBP-3 expression independent of disease severity. However, *in vitro* exposure of cultured primary airway epithelial cells to corticosteroids does not result in

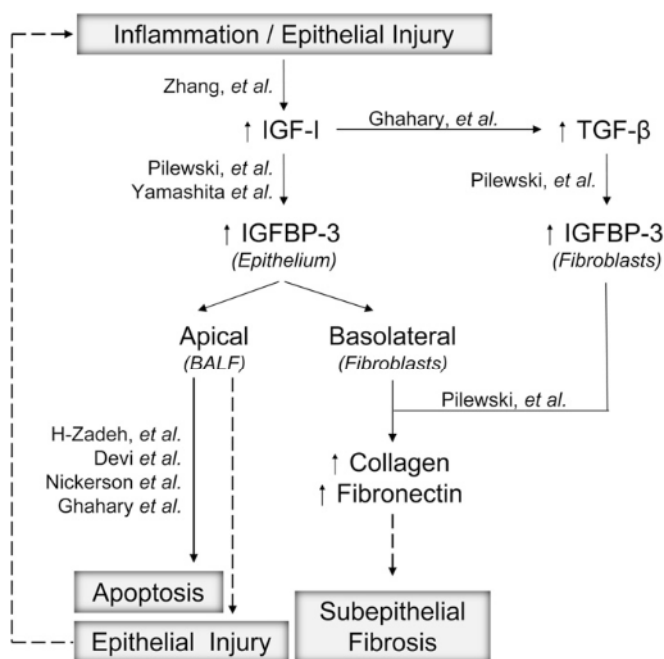


Figure 5. Proposed mechanism for the contribution of insulin-like growth factor (IGF)-1 and IGF binding protein (IGFBP)-3 to subepithelial fibrosis in asthma. BALF = bronchoalveolar lavage fluid.

increased IGFBP-3 levels (Figure E3), and IGFBP-3 expression was also significantly increased in non-steroid-treated subjects with mild asthma compared with normal control subjects (Figure 2); thus, increased IGFBP-3 in asthmatic airways is at least partially independent of steroid exposure. Lastly, it is possible that allergen directly activates airway epithelial cell release of IGFBP-3; however, this is not likely, based on the observation that SBP-AG did not induce IGFBP-3 in normal subjects without allergy (Figure E1). Furthermore, addition of allergen to *ex vivo* cultures of airway epithelial cells did not increase IGFBP-3 levels (Figure E2). Thus, allergen appears to have an indirect effect on epithelial cell production of IGFBP-3.

Based on our findings, we hypothesize the mechanism diagrammed in Figure 5 to incorporate the IGF-I/IGFBP-3 profibrotic pathway into the complex cascade of events leading to subepithelial fibrosis and chronic airway remodeling in allergic asthma. In this model, IGF-I is up-regulated in airway epithelial cells in patients with asthma during allergic inflammatory injury, and is released by injured airway epithelial cells and inflammatory cells (15). IGF-I then stimulates the epithelium to produce IGFBP-3, a potent inducer of fibroblast ECM production (4). This is supported by previous findings that neutralization of IGF-I in mice caused a significant decrease in IGFBP-3 mRNA (6), and our current findings that IGF-I induces production and secretion of IGFBP-3, both apically and basolaterally, by cultured HBES. IGFBP-3 secreted basolaterally can trigger ECM production by resident fibroblasts, with resulting subepithelial fibrosis, and IGFBP-3 secreted apically into the lumen may cause further injury to airway epithelium and perpetuate the injury-repair cycle, as IGFBP-3 can induce epithelial cell apoptosis (17–19). The injury-repair cascade may be additionally perpetuated by IGF-I through induction of TGF- β secretion by fibroblasts (20), which is a potent stimulus for both ECM production and IGFBP-3 secretion by fibroblasts (4). Additionally, Minshall and colleagues (21) have shown that eosinophils are a source of TGF- β in asthma. Thus, our observed association of IGFBP-3 levels with BAL eosinophil

percentages raises the intriguing hypothesis that eosinophils could affect airway remodeling via TGF- β , either directly or indirectly through the induction of IGFBP-3 expression by subepithelial fibroblasts; however, our data do not demonstrate causality. A vicious loop would thereby be established, which serves to augment the response to epithelial injury and perpetuate fibrosis.

Irreversible airway remodeling and resultant loss of lung function is a profound complication of asthma that occurs despite current available therapy, and results in significant morbidity and mortality. This is the first report demonstrating IGF-I induction of IGFBP-3 in human airways and its relationship to airflow limitation. Future studies are warranted to investigate the roles that IGF-I and IGFBP-3 may play in human airway hyper-responsiveness, the induction of proinflammatory mediators, and allergic airway remodeling. Our findings have important clinical implications, as this pathway represents a novel target for the rational design of new pharmacologic therapies.

Conflict of Interest Statement: K.L.V. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; B.T.G. has served as a speaker from GlaxoSmithKline from 2007 to the present, receiving \$4,000 in 2007 and \$2,000 in 2008; H.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.M.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; E.A.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; S.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; N.N.J. received \$2,000 in 2007–2008 for serving on an advisory board for Genentech and \$2,000 in 2007–2008 for serving on an advisory board for GlaxoSmithKline, \$4,000 in 2008 for speaking at a conference sponsored by Merck, and works as a consultant for Asthmatic (\$8,000 in 2005) and Genentech (\$2,000 in 2007–2008). In 2007–2008, N.N.J. received research grants for participating in multicenter clinical trials from GlaxoSmithKline (\$150,000), Merck (\$494,000), Genentech (\$164,000), and MedImmune (\$365,000); J.M.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; S.E.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; C.A.F-B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Role of Insulin-like Growth Factor Binding Protein-3 in Allergic Airway Remodeling

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Online Data Supplement

Methods

Segmental bronchoprovocation (SBP) and bronchoalveolar lavage (BAL) of atopic subjects with asthma. To determine allergen dose for SBP, a graded inhaled allergen (AG) challenge was performed. The AG dose leading to a 20% FEV₁ fall (AG PD₂₀) was calculated from the dose-response curve. Allergens used in SBP included ragweed (GS Ragweed mix, Greer Labs, Lenoir, NC), cat AG (Fel D1, Bayer Allergy Products, Spokane, WA), and house dust mite (*Dermatophagoides farinae*, Miles Allergy Products, Spokane, WA). Bronchoscopy and SBP were performed as previously described (1). Two different bronchopulmonary segments were identified. For each segment, a wedge position was achieved by the fiberoptic bronchoscope and a baseline BAL was performed with four aliquots of 40 ml of pre-warmed 0.9% NaCl (160 ml per segment). Ten percent of the AG PD₂₀ (diluted in 10 ml of sterile saline) was instilled into the first segment. If this dose was well-tolerated by the subject (no change in vital signs, pulse oximetry or symptoms), a second segment was challenged with a dose of 20%. After 48 hours, bronchoscopy was repeated and BAL was performed on each of the two previously challenged segments. BAL fluid from the two segments was pooled for analysis.

Segmental bronchoprovocation (SBP) and bronchoalveolar lavage (BAL) of nonasthmatic subjects. As a quality control measure to ensure that segmental allergen challenge does not cause a nonspecific increase in IGFBP-3 due to activation of airway epithelial cells by the allergen or the bronchoscopy procedure, a retrospective analysis was performed on BAL fluid obtained from normal (n=9) or allergic (n=3) subjects immediately prior to challenge and 48 hours after challenge of two different segments with saline or ragweed allergen. Normal subjects had normal spirometry, were skin-prick test negative, and had no history of allergy or asthma.

Two different bronchopulmonary segments were identified. One segment received saline and a second segment was challenged with ragweed allergen. BAL was performed at 5 minutes and 48 hours after challenge. Normal subjects received 1000 protein nitrogen units (PNU) of ragweed and allergic subjects received 15% of the amount of allergen that induced a 20% fall in FEV₁ following an inhalation challenge (AG PD₂₀) for doses of 6, 400, and 1000 PNU.

Endobronchial Biopsy Immunofluorescence. Six µm sections of paraffin-embedded lung tissues were de-paraffinized and endogenous biotin was blocked using the Biotin blocking kit (Dakocytomation, Carpinteria, CA). The sections were blocked with 5% serum and incubated with polyclonal rabbit anti-human IGFBP-3 (GroPep, Adelaide, Australia). Sections were washed and incubated with fluorochrome-tagged secondary antibody. Signal was detected using the Fluorescence avidin kit (Vector laboratories, Burlingame, CA), and images were taken on a Fluoview 1000 confocal microscope (Olympus America Inc., Melville, NY). Fluorescent intensity was analyzed using MetaMorph (Molecular Devices, Sunnyvale, CA) in the Center for Biological Imaging at the University of Pittsburgh School of Medicine. Mean immunofluorescence intensity of healthy donors was defined as one, and the ratio of the sample-to-mean immunofluorescence intensity of healthy donors was calculated as an arbitrary unit.

Epithelial Injury and Immunofluorescence. Mechanical scrape injury was induced by creating a line with a pipette tip across the surface of a confluent HBE cell layer as previously described (2). Following injury, the apical surface was washed with DMEM to remove denuded epithelial cells, and the area of injury was examined to assure uniformity. Eighteen hours following epithelial injury, the transwell inserts were fixed in 4% paraformaldehyde, permeabilized with

Triton X-100, and blocked with 5% goat serum. The HBE cultures were then incubated with 0.01 mg/ml rabbit polyclonal antibody against IGFBP-3 (GroPep, Thebarton SA, Australia) or control rabbit IgG (Lab Vision, Fremont, CA) for 1 hour. Species-appropriate secondary antibody conjugated to Alexa-488 (Jackson Research, West Grove, PA) was used to visualize IGFBP-3. Additionally, DRAQ5 (Alexis Corporation, Lausen, Switzerland) and fluorescently labeled phalloidin (Molecular Probes, Carlsbad, CA) were used as nuclear and cytoskeletal markers, respectively. Images were taken using an Olympus Fluoview 500 confocal microscope at 200X magnification and analyzed using ImageJ (3). Auto-leveling was applied to the red (cytoskeleton) and blue (nuclear) images and the brightness was increased by 20% in order to improve clarity. Images shown are representative of three independent experiments performed using tissues cultured from three donors.

Figure Legends

Figure E1. *Segmental allergen challenge does not cause an increase in IGFBP-3 in normal, nonallergic subjects.* ELISA quantification of IGFBP-3 levels in undiluted BAL fluid samples obtained from nine normal control subjects at baseline (0 hours) or 48 hours after segmental instillation of either saline or ragweed allergen (1000 protein nitrogen units (PNU)). As a control, IGFBP-3 was also measured in three allergic subjects challenged with 15% AgPD₂₀ (for doses of 6, 400, and 1000 PNU). IGFBP-3 does not significantly increase following allergen challenge of normal subjects or following saline challenge in allergic subjects. Data are presented as medians with interquartile ranges.

Figure E2. *Allergen does not increase secretion of IGFBP-3 by primary airway epithelial cells in vitro.* Exposure to purified cat allergen (Fel d 1) does not increase secretion of IGFBP-3 or its degradation products into either the apical or basolateral compartment. Molecular mass markers indicate known glycosylation variants of IGFBP-3 (4). Samples were harvested after 48 hours and equivalent volumes of apical or basolateral culture media (corresponding to 5% of the 0.33 cm² surface area of the differentiated epithelial cell monolayer) were loaded per lane. Concentrations used are as follows: Purified nFel d 1 (10 mcg/mL in the apical media), IGF-I (250 ng/mL in both the apical and basolateral media). Data presented are replicates from single donor primary cells and are representative of results obtained from four separate donors.

Figure E3. *Steroids do not increase secretion of IGFBP-3 by primary airway epithelial cells in vitro.* In the absence of IGF-I, dexamethasone (Dex) does not alter levels of secreted IGFBP-3 or its degradation products in either the apical or basolateral compartment. In the presence of IGF-I, there is a trend towards diminished secretion of IGFBP-3 in response to dexamethasone. Molecular mass markers indicate known glycosylation variants of IGFBP-3 (4). Samples were harvested after 48 hours and equivalent volumes of apical or basolateral culture media (corresponding to 5% of the 0.33 cm² surface area of the differentiated epithelial cell monolayer) were loaded per lane. Concentrations used are as follows: Dexamethasone (50 nM in the apical media), IGF-I (250 ng/mL in both the apical and basolateral media). Data presented are replicates from single donor primary cells and are representative of results obtained from four separate donors.

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Figure E1:

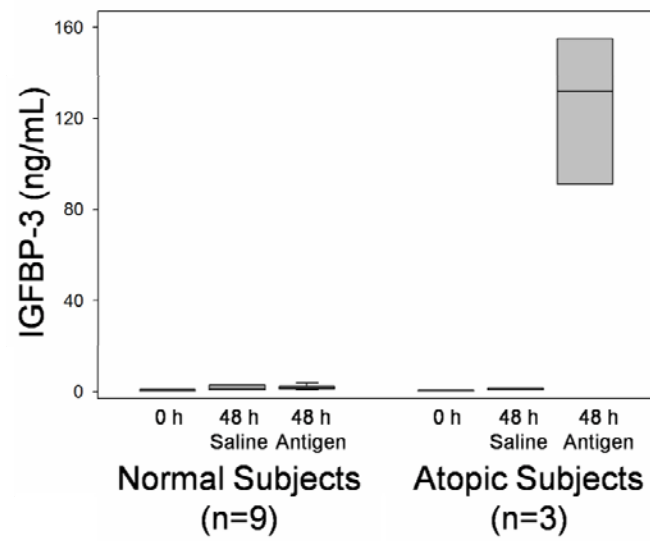


Figure E2:

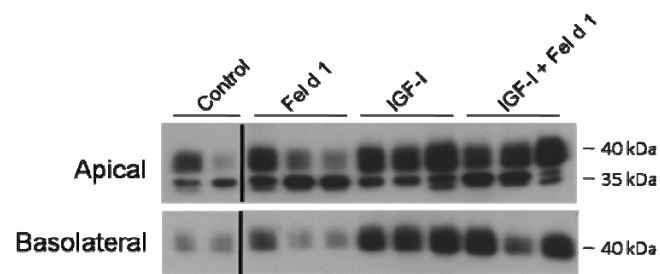


Figure E3:

