Insulin-Like Growth Factor-Binding Protein-5 Induces Pulmonary Fibrosis and Triggers Mononuclear Cellular Infiltration

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We have recently shown that insulin-like growth factor-binding protein (IGFBP)-5 is overexpressed in idiopathic pulmonary fibrosis lung tissues and increases collagen and fibronectin deposition. Here, we further examined the effect of IGFBP-5 *in vivo* by intratracheal administration of replication-deficient adenovirus expressing human IGFBP-5 (Ad5), IGFBP-3 (Ad3), or no cDNA (cAd) to wild-type mice. Increased cellular infiltration and extracellular matrix deposition were observed in mice after Ad5 administration compared with Ad3 and cAd. Mononuclear cell infiltration consisted predominantly of T lymphocytes at day 8. By day 14, the number of infiltrating T cells decreased, whereas that of B cells and monocytes/macrophages increased. IGFBP-5 also induced migration of peripheral blood mononuclear cells *in vitro*, suggesting that *in vivo* mononuclear cell infiltration may be the direct result of IGFBP-5 expression. α -Smooth muscle actin and Mucin-1 co-localized in cells of mice treated with Ad5, suggesting that IGFBP-5 induced epithelial-mesenchymal transition. In addition, exogenous IGFBP-5 induced α -smooth muscle actin expression in primary fibroblasts and epithelial-mesenchymal transition of pulmonary epithelial cells *in vitro*. In conclusion, our results suggest that overexpression of IGFBP-5 in mouse lung results in fibroblast activation, increased extracellular matrix deposition, and myofibroblastic changes. Thus, the IGFBP-5-induced fibrotic phenotype *in vivo* may represent a novel model to better understand the pathogen-

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Lung fibrosis in idiopathic pulmonary fibrosis (IPF) and systemic sclerosis (SSc) is characterized by excessive fibrosis attributable to fibroblast proliferation and activation resulting in excessive production of extracellular matrix (ECM).¹ However, the pathogenic mechanisms of fibrosis are still unknown. Fibrotic changes result in structural changes and lead to alveolar destruction and progressive restrictive lung disease.^{1,2} In IPF and SSc these changes result in significant morbidity and mortality.³ In SSc, pulmonary involvement is currently the leading cause of death.⁴ There is no effective treatment to halt the progression of pulmonary fibrosis in IPF and SSc, and the only current life-prolonging intervention is lung transplantation.⁵ The pathological features of IPF are injury and activation of alveolar epithelial cells in a heterogeneous subpleural distribution, the distinctive presence of fibroblastic foci, and excessive deposition of ECM proteins including collagen and fibronectin by resident fibroblasts.^{1,6} Thus, fibroblasts and epithelial cells play an important role in the pathogenesis of pulmonary fibrosis.

In addition, inflammation may also contribute to the pathogenesis of lung fibrosis.⁷ Increased numbers of lymphocytes have been detected in the bronchoalveolar lavage fluid of SSc patients with lung involvement.⁸ Inflammatory cells not only release cytokines, chemokines, and growth factors during the immunoinflammatory phase of the disease but also regulate the fibrogenic phase of the disease process through direct interaction with fibroblasts, thus resulting in the development of fibroproliferative lesions.^{9,10} Recent studies indicate that

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some chemokines recruit not only inflammatory cells but also fibrocytes, which are associated with ECM production and induction of fibrosis.^{11,12} To date, there are no reports of a single molecule that can simultaneously explain both resident fibroblast activation and recruitment of inflammatory cells.

We previously reported increased insulin-like growth factor-binding protein (IGFBP)-5 mRNA and protein levels in lung tissues of patients with lung fibrosis and in primary fibroblasts cultured from lung tissues of patients with IPF.¹³ In addition, we have shown that overexpression of IGFBP-5 in normal primary fibroblasts increases collagen and fibronectin deposition in the extracellular milieu.¹³

In this study, we report the *in vivo* effects of human IGFBP-5 expression in the mouse. Expression of IGFBP-5 induced prominent inflammatory changes and fibrosis resembling human fibrotic disease states. IGFBP-5 confers chemoattractant activity and can induce production and deposition of ECM through fibroblast activation and myofibroblast differentiation. IGFBP-5 also induced myofibroblastic changes of type II alveolar epithelial cells *in vivo*. These data suggest that IGFBP-5 is sufficient to initiate the histopathological changes characteristic of IPF and SSc, and in conjunction with our prior observations, strongly implicate IGFBP-5 in the pathogenesis of human IPF and SSc. In addition, the overexpression of IGFBP-5 provides a novel murine model to study the pathogenesis of human pulmonary fibrotic diseases such as IPF and SSc.

Materials and Methods

Adenovirus Construct Preparation

Adenovirus constructs were obtained as previously reported.¹³ Briefly, the full-length cDNAs of human IGFBP-3 and -5 were obtained by reverse transcription-polymerase chain reaction (RT-PCR) using total RNA extracted from primary human lung fibroblasts. The cDNAs were subcloned into the shuttle vector pAdlox and used for the preparation of replication-deficient adenovirus serotype 5 expressing IGFBP-3 (Ad3), IGFBP-5 (Ad5), or no cDNA (cAd) in the Vector Core Facility at the University of Pittsburgh.

Mice and Adenoviral Administration

Eight-week-old wild-type C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All studies and procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Mice were intratracheally injected with phosphate-buffered saline (PBS), or 10^9 plaque-forming units (PFU) of cAd, Ad3, or Ad5 in a 55- μ l volume. Mice were sacrificed on days 8 and 14 after adenoviral administration. Harvested lung tissues were inflated and embedded in paraffin.

Culture of Primary Mouse Lung Fibroblasts

Primary mouse fibroblasts from lung tissues of C57BL/6J mice were cultured in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), penicillin, streptomycin, and anti-mycotic agent (Invitrogen Life Technologies), as previously described.¹⁴ Cells were used in early passage (passages 3 to 6).

Western Blot Analysis

Culture supernatants, ECM, and cellular lysates were obtained from cultured fibroblasts or the type II alveolar epithelial cell line A549, as previously described.¹⁵ In brief, 2.0×10^5 primary fibroblasts or A549 cells were cultured in 35-mm wells in the absence or presence of 500 ng/ml of recombinant human IGFBP-5. In parallel wells, fibroblasts were infected with cAd, Ad3, or Ad5 at a multiplicity of infection of 50. Culture supernatants, ECM, and cellular lysates were harvested at 48 hours. Culture supernatants were centrifuged for 10 minutes at 4°C to pellet cell debris. ECM was harvested as we have previously described.¹³ Samples were analyzed by Western blot analysis using one of the following antibodies: anti-human IGFBP-3 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-IGFBP-5 (Groppe Ltd., Thebarton, Australia), anti-fibronectin (Santa Cruz Biotechnology), or anti- α -smooth muscle actin (α -SMA) (Sigma-Aldrich). Signals were detected after incubation with horseradish peroxidase-conjugated secondary antibody and chemiluminescence (Perkin Elmer Life Sciences, Inc., Boston, MA).

Hydroxyproline Assay

Hydroxyproline incorporation was assayed as previously described.^{16,17} In brief, lung tissues were dried, acid-hydrolyzed, lyophilized, and assayed for hydroxyproline content using chloramine-T.

Histological Staining for Collagen

For the detection of collagen fibers, 6- μ m sections of paraffin-embedded skin tissues were deparaffinized and stained using the Chromaview Masson-trichrome stain kit (Richard-Allan Scientific, Kalamazoo, MI) following the manufacturer's recommendations.

Immunohistochemistry

Six- μ m sections of paraffin-embedded skin tissues were deparaffinized, and endogenous peroxidase and biotin were quenched using 10% H_2O_2 and a biotin blocking kit (Dakocytomation, Carpinteria, CA), respectively. The sections were blocked with 5% serum and incubated with one of the following primary antibodies: polyclonal anti-IGFBP-3, anti-IGFBP-5 (Groppe Ltd.), polyclonal anti-vimentin (LabVision Corp., Fremont, CA), polyclonal

anti-proliferating cell nuclear antigen (Santa Cruz Biotechnology), polyclonal anti-fibronectin (Santa Cruz Biotechnology), monoclonal anti- α -SMA (Sigma-Aldrich), monoclonal anti-CD3 (LabVision Corp.), monoclonal anti-B220 (BD Pharmingen, San Diego, CA), monoclonal anti-Mac-1 (Chemicon, Temecula, CA), and polyclonal anti-Mucin-1 (Fujirebio Diagnostics, Malvern, PA) antibodies. Sections were washed and incubated with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA). Bound secondary antibody was detected using the AEC Red kit (Zymed, San Francisco, CA) or fluorescence detection kit (Zymed). A light hematoxylin counterstain was used to identify nuclei using Hematoxylin QS (Vector Laboratories). Images were taken on a Nikon Eclipse 800 microscope (Nikon Instruments, Inc., Huntley, IL) or Olympus Fluoview 1000 (Olympus America Inc., Melville, NY) using identical camera settings.

Chemotaxis Assay

Chemoattractant activity was examined by *trans*-well migration assay using human peripheral blood mononuclear cells (PBMCs). Chemoattractant activity was assessed in a 24-well *trans*-well cell culture dish with 5- μ m-pore-size polycarbonate filters (Costar, Cambridge, MA). Human PBMCs were obtained from heparinized venous blood using Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) gradient centrifugation. PBMCs were resuspended in RPMI 1640 supplemented with 1% bovine serum albumin, and 2×10^5 of PBMCs in 0.1 ml of medium were added to the upper compartment of each chamber. Recombinant IGFBP-3, IGFBP-5, RANTES, or vehicle was diluted in RPMI 1640 supplemented with 1% bovine serum albumin and added to the lower compartment. After a 4-hour-incubation at 37°C, cells were harvested from both upper and lower chambers and manually counted under a phase contrast microscope. The percentage of migrated cells was calculated as a ratio of the cell count of the upper chamber to the total cell count of the upper and lower chambers.

Statistical Analysis

Statistical comparisons were performed using the Mann-Whitney *U*-test or Student's *t*-test as appropriate.

Results

In Vitro Expression of Human IGFBPs in Primary Mouse Fibroblasts

We had previously shown that replication-deficient adenovirus serotype 5 can be used for efficient overexpression of IGFBP-3 and -5 in primary human lung fibroblasts.¹³ To confirm the expression of adenovirally expressed human IGFBP in mouse fibroblasts, we infected primary mouse lung fibroblasts cultured from lung tissues of wild-type C57BL/6J mice with replication-defi-

Figure 1. A: IGFBP-3 and IGFBP-5 secretion by cultured primary mouse lung fibroblasts infected with control adenovirus (cAd, lanes 1 and 2), IGFBP-3-expressing (Ad3, lanes 3 and 4), and IGFBP-5-expressing adenovirus (Ad5, lanes 5 and 6). B: IGFBP-3 and IGFBP-5 expression in mouse lung. Control adenovirus (cAd) or adenovirus expressing human IGFBP-3 and IGFBP-5 (Ad3 and Ad5, respectively) was administered intratracheally. Mice were sacrificed and lung tissues harvested 14 days after injection. Expression of IGFBP-5 (a–d) and IGFBP-3 (e–g) was detected using immunofluorescence and that of IGFBP-5 was also detected by immunohistochemistry (h). Original magnifications, $\times 400$.

cient adenovirus expressing human IGFBP-3 or -5 (Ad3 and Ad5, respectively) or no cDNA (cAd). Human IGFBP-3 and -5 were secreted by mouse fibroblasts as detected by Western blot analysis of culture supernatants. No human IGFBP-3 or IGFBP-5 expression was detected in fibroblasts infected with a control adenovirus (cAd) (Figure 1A).

Detection of IGFBP-Expressing Cells In Vivo

Human IGFBP-expressing cells were detected in mouse lung tissues harvested 8 days after adenoviral administration. As shown in Figure 1B, immunohistochemical analysis showed increased expression of human IGFBP-5 in Ad5-treated mice (Figure 1B, c and d) compared with Ad3- and cAd-treated mice (Figure 1B, b and a, respectively). Weak expression of IGFBP-5 was detected in the Ad3-treated lung (Figure 1Bb). The anti-IGFBP-5 antibody, although directed against the human protein, may display some cross-reactivity with endogenous mouse IGFBP-5 because of the high degree of homology between human and mouse proteins. IGFBP-3 was detected in Ad3-treated lungs (Figure 1Bf) and to a lesser extent in Ad5-treated lungs (Figure 1Bg). In Ad5-treated lung, IGFBP-5 expression was primarily localized to scattered cells in the alveolar lumen and wall (Figure 1Bh).

Fibrotic and Proinflammatory Effects of IGFBP-5

To analyze the *in vivo* effects of human IGFBP-5 expression in mouse lung, histopathological changes were analyzed using hematoxylin and eosin (H&E) staining and Masson's trichrome staining. As shown in Figure 2A, cellular infiltration and deposition of collagen were prominent, especially around airways, in Ad5-treated mice (Figure 2Ad) compared with PBS- (Figure 2Aa), Ad3- (Figure 2Ab), and cAd-treated (Figure 2Ac) mice at 14 days after adenoviral administration. In Figure 2B, ECM deposition (homogenous-acidic deposits) was observed in Ad5-treated mice at high magnification (arrows in Figure 2Bc) compared with cAd (Figure 2Ba) and Ad3 (Figure 2Bb) mouse lung tissues. Masson's Trichrome stain demonstrated increased deposition of collagen in Ad5 lung tissues (Figure 2Bf) compared with cAd (Figure 2Bd) and Ad3 (Figure 2Be) lungs.

Characterization of Infiltrating Cells in IGFBP-5-Expressing Lungs

Immunohistochemistry was performed to characterize the infiltrating mononuclear cells in Ad5-treated lungs. As shown in Figure 3A, on day 8 T cells were the predominant cell type in the alveolar area (Figure 3Aa). Some scattered B cells (Figure 3Ab) and several monocytes/macrophages (Figure 3Ac) were also observed around the vessels at day 8 after adenoviral administration. At day 14, the number of T cells decreased (Figure 3, Ad and B) ($104.80 \pm 39.95/\text{HPF}$ versus $22.65 \pm 22.07/\text{HPF}$, $P = 5.5 \times 10^{-9}$) and the number of B cells (Figure 3Ae) and monocytes/macrophages (Figure 3Af) increased ($50.30 \pm 30.64/\text{HPF}$ versus $107.60 \pm 83.02/\text{HPF}$ and $17.4 \pm 10.81/\text{HPF}$ versus $57.55 \pm 30.36/\text{HPF}$, $P = 0.008$, and $P = 9.8 \times 10^{-6}$, respectively) (Figure 3B). Moreover, B cells appeared to form germinal center-like clusters (not shown). Infiltrating mononuclear cells were rare in cAd-treated lungs (day 8: T cells, $5.50 \pm 2.43/\text{HPF}$; B cells, $2.67 \pm 1.15/\text{HPF}$; monocytes/macrophages, $2.57 \pm$

Figure 2. *In vivo* effects of IGFBP-3 and IGFBP-5 overexpression in mouse lung. **A:** Increased collagen deposition in IGFBP-5-expressing lungs. Phosphate-buffered saline (PBS) (a), 10^9 PFU control adenovirus (cAd) (c), or IGFBP-3- and IGFBP-5-expressing adenovirus [Ad3 (b) and Ad5 (d), respectively] were administered intratracheally to mice. Mice were sacrificed 2 weeks later, and lung tissue sections were stained with Masson's Trichrome. **B:** Mononuclear cell infiltration and ECM deposition in IGFBP-5-expressing lungs. Lung sections from cAd-, Ad3-, and Ad5-treated mice were stained with H&E (a–c) or Masson Trichrome staining (d–f). Original magnifications: $\times 20$ (A); $\times 200$ (Ba–Bc); $\times 100$ (Bd–Bf).

2.44/HPF; day 14: T cells, $5.11 \pm 2.57/\text{HPF}$; B cells, $4.17 \pm 3.13/\text{HPF}$; monocytes/macrophages, $1.75 \pm 1.48/\text{HPF}$).

IGFBP-5 Has Chemoattractant Activity for PBMCs

To determine whether IGFBP-5 can directly recruit immune cells, we examined the chemoattractant activity of IGFBP-5 using transwell migration assays. As shown in Figure 3C, 500 ng/ml of recombinant IGFBP-5 induced significant migration of human PBMCs compared with vehicle control or recombinant IGFBP-3 at the same concentration ($19.40 \pm 4.87\%$ versus $3.22 \pm 1.46\%$ or

Figure 3. Characterization of infiltrating immune cells. **A:** Immunohistochemistry for the detection of T lymphocytes, B lymphocytes, and monocytes/macrophages. Mice were infected with 10^9 PFU control-, IGFBP-3-, or IGFBP-5-expressing adenovirus (cAd, Ad3, and Ad5, respectively), and lung samples were analyzed at 8 days and 14 days after injection. **Solid arrows** point to representative positive cells. **B:** Number of T cells, B cells, and monocytes/macrophages in mouse lungs. Two representative samples were randomly selected from each group of mice and the number of each cell subset was counted in 10 random fields/sample at $\times 400$ magnification. Statistical analysis was done using Student's *t*-test. Each bar denotes the mean of each group. **C:** PBMC migration assay. Purified human PBMCs were used in a migration assay with vehicle control, recombinant IGFBP-3 (BP3), recombinant human IGFBP-5 (BP5), or RANTES as a positive control. Statistical analysis was done using the Mann-Whitney *U*-test. **P* < 0.007. Original magnifications, $\times 400$.

Figure 4. **A:** Collagen production and deposition in lung tissues. The amount of collagen was measured in lungs injected with 10^9 PFU control adenovirus-, IGFBP-3-, or IGFBP-5-expressing adenoviruses (cAd, Ad3, and Ad5, respectively) using a hydroxyproline assay. The bar in each group denotes mean collagen amount. Statistical analysis was done using the Mann-Whitney *U*-test. $*P < 0.03$. **B:** Expression of fibronectin and α -SMA in mouse lungs 14 days after injection. Expression of fibronectin and α -SMA was examined in mouse lung samples infected with 10^9 PFU control adenovirus (cAd), IGFBP-3- or IGFBP-5-expressing adenovirus (Ad3 and Ad5, respectively). Fibronectin (**a–c**) and α -SMA (**d–f**) were detected by immunohistochemistry. **Solid arrows** point to representative positive cells. **C:** Detection of fibronectin and α -SMA by Western blot analysis. Primary mouse lung fibroblasts were incubated with vehicle control or recombinant human IGFBP-5 (rIGFBP-5). ECM and lysates were harvested after 48 hours and analyzed by Western blot for fibronectin and α -SMA. β -Actin was detected as a loading control. Each experiment was done in duplicate. Original magnifications: $\times 200$ (**a–f**); $\times 1000$ (**inset**).

$4.90 \pm 2.13\%$; $P < 0.007$ and $P < 0.007$, respectively). IGFBP-5 concentrations used are within physiological range 200 to 800 ng/ml.^{18–20}

Quantitation of Collagen Deposition in IGFBP-5-Expressing Lungs

To quantify the collagen levels deposited in mouse lung tissues, the amount of hydroxyproline incorporation in lungs harvested at day 14 after adenoviral administration was measured. As shown in Figure 4A, deposition of collagen was significantly increased in lung tissues of Ad5-treated mice compared with those of Ad3- or cAd-treated mice ($597.11 \pm 60.64 \mu\text{g}$ versus $518.58 \pm 100.73 \mu\text{g}$ or $516.38 \pm 88.10 \mu\text{g}$; $P < 0.03$ and $P < 0.03$, respectively).

Comparison of Fibronectin Levels and Fibroblast Phenotype

The deposition of fibronectin was examined by immunohistochemistry. As shown in Figure 4B, fibronectin staining was increased around airways and blood vessels in Ad5 lung tissues (Figure 4Bc) compared with cAd (Figure 4Ba) and Ad3 (Figure 4Bb) lung tissues. To characterize the fibroblast phenotype, α -SMA expression was examined. As shown in Figure 4B, α -SMA-positive fibroblasts were more abundant in Ad5 lung tissues (Figure 4Bf) compared with cAd (Figure 4Bd) and Ad3 lungs (Figure 4Be). To determine whether IGFBP-5 can also induce fibronectin and α -SMA expression *in vitro*, primary mouse lung fibroblasts were incubated with a physiological concentration of recombinant IGFBP-5. As shown in Figure

Figure 5. Characterization of alveolar epithelial cells. **A:** Immunohistochemistry for α -SMA and Mucin-1. Mice were infected with 10^9 PFU control adenovirus (cAd) or IGFBP-5-expressing adenovirus (Ad5). Lung tissues were harvested 14 days after injection. Cells expressing α -SMA (green, **a** and **d**), Mucin-1 (red, **b** and **e**), and both α -SMA and Mucin-1 (yellow merge, **c** and **f**) are detected in IGFBP-5-expressing lung (**white arrows**). **Solid arrows** point to representative positive cells. Red blood cells had high levels of autofluorescence even in the absence of antibody. The **broken arrow** with **asterisk** (**c**) shows representative autofluorescence of red blood cells (shown in yellow) in the vessels (shown in green). **B:** High-magnification immunofluorescence detection of α -SMA and Mucin-1. Merged images of α -SMA and Mucin-1 staining are shown in cAd-treated lung (**a**) and Ad5-treated lung (**b**). Mucin-1 (**c**) and α -SMA (**d**) staining in Ad5-treated mice are also shown. **Solid arrows** indicate positive cells. **C:** Western blot analysis of α -SMA expression in A549 cells induced by IGFBP-5. **a:** Western blot of α -SMA in cellular lysates of A549 cells incubated with increasing volume of supernatant from IGFBP-5-expressing primary lung fibroblasts (Ad5 sup). **b:** Western blot of α -SMA in cellular lysates of A549 cells incubated with 500 μ l (25% of final volume) of culture supernatant from cAd- or Ad5-infected primary fibroblasts. GAPDH was detected as a loading control. Original magnifications: $\times 1600$ (**A**); $\times 2000$ (**B**).

4C, expression of fibronectin and α -SMA increased in the presence of exogenous recombinant IGFBP-5 compared with vehicle control. Thus, IGFBP-5 induces increased deposition of fibronectin and transformation of lung fibroblasts into a myofibroblastic phenotype.

Epithelial-Mesenchymal Transition of Type II Alveolar Epithelial Cells Is Induced by IGFBP-5

We further examined the effect of IGFBP-5 on type II alveolar epithelial cells. As shown in Figure 5A, type II epithelial cells expressing Mucin-1 (Figure 5A, b and e) and α -SMA (Figure 5A, a and d) were increased in Ad5-treated lungs (Figure 5A, d-f) compared with cAd-lungs (Figure 5A, a-c). Increased numbers of cells expressing both Mucin-1 and α -SMA were detected in Ad5-treated mice (Figure 5, Af and Bb), suggesting that IGFBP-5

results in α -SMA expression *in vivo*. To examine the effect of IGFBP-5 on α -SMA expression by type II alveolar epithelial cells *in vitro*, A549 cells were cultured with various dilutions of cAd- or Ad5-infected primary fibroblast culture supernatants. Culture supernatants of fibroblasts infected with Ad5 secrete abundant levels of human IGFBP-5 as shown in Figure 1. As shown in Figure 5C, culture supernatants of Ad5-infected fibroblasts induce expression of α -SMA in A549 cells in a dose-dependent manner (Figure 5Ca). When 500 μ l of culture supernatant was added to A549 cells (25% of the final volume), α -SMA expression was up-regulated in A549 cells incubated with supernatants from Ad5-infected fibroblasts compared with cAd-infected fibroblasts (Figure 5Cb). Thus, IGFBP-5 overexpression can induce epithelial-mesenchymal transition of type II alveolar epithelial cells.

Discussion

We previously reported increased IGFBP-5 mRNA and protein levels in lung tissues of patients with lung fibrosis and in primary fibroblasts cultured from lung tissues of patients with IPF.¹³ In addition, we have shown that overexpression of IGFBP-3 and -5 in normal primary fibroblasts increases the levels of collagen and fibronectin deposited in the extracellular milieu.¹³ We now report the *in vivo* effect of human IGFBP expression in mouse lung by intratracheal injection of IGFBP-expressing recombinant adenovirus.

Adenovirally expressed IGFBP-3 and -5 were detected in airway epithelial cells, alveolar epithelial cells, and alveolar macrophages. Masson's trichrome and H&E staining revealed deposition of ECM and prominent infiltration of mononuclear cells in Ad5 lungs compared with Ad3 and cAd lungs. IGFBP-5, but not IGFBP-3, induced fibrosis in a peribronchial, perivascular, and interstitial pattern. IGFBP-5, but not IGFBP-3, also induced infiltration of mononuclear cells in mouse lung tissues. Lung-infiltrating cells in IGFBP-5-expressing lungs were predominantly T lymphocytes at day 8 after administration, whereas B lymphocytes and monocytes/macrophages were more abundant at day 14, suggesting a temporal migration of different immune cell types. Migration assays demonstrated that IGFBP-5 itself has a chemoattractant activity for PBMCs. Mononuclear cell infiltration occurs in chronic inflammation and can result in *de novo* formation of organized lymphoid tissue.²¹ Marchal-Somme and colleagues²² recently described lymphoid neogenesis in lung tissues of patients with IPF. In our model, in Ad5-treated lung tissues, B lymphocytes formed clusters that resemble the recently reported lymphoid neogenesis.²² Whether these clusters are involved in the development and/or perpetuation of fibrosis remains to be determined.

The association between fibrosis and inflammation in IPF is still unclear. In SSc-associated lung fibrosis, alveolitis usually precedes chronic fibrosis, suggesting that inflammation contributes to the development of lung fibrosis.²³ Our *in vivo* and *in vitro* findings support a role for IGFBP-5 in the recruitment of inflammatory cells and the activation of fibroblasts. It has been proposed that infiltrating inflammatory cells play an important role in the progression of fibrosis⁷; however, the significance of the interaction between inflammatory cells and fibroblasts has not been clarified. Fibroblasts respond to altered microenvironmental signals by facilitating the recruitment and retention of immune effector cells, and by cell-matrix or cell-cell interactions, to regulate the synthesis and degradation of matrix components.^{9,10} Several studies have suggested that a shift to a T-helper 2 (Th2) CD4⁺ T-cell response is associated with the development of fibroproliferative diseases.²⁴ Th2 cytokines such as interleukin (IL)-4 and IL-13 can induce macrophage-derived insulin-like growth factor I (IGF-I) expression.²⁵ Furthermore, macrophage-derived IGF-I protects myofibroblasts from apoptosis.²⁶ IGFBP-5 may be involved in this axis through binding IGF-I to increase its bioavailability²⁷ and induce myofibroblastic changes in resident fibroblasts. Taken together, these findings suggest that the interac-

tion between fibroblasts and inflammatory cells is important for the progression of fibrosis.

Increased collagen and fibronectin deposition were detected in Ad5-lung tissues, demonstrating that IGFBP-5 also induces ECM production and fibrosis *in vivo*. Moreover, α -SMA-expressing cells were increased in Ad5 lung tissues, and expression of α -SMA and fibronectin was also increased in cultured mouse lung fibroblasts by IGFBP-5, suggesting that resident lung fibroblasts are a source of increased ECM and that these fibroblasts are activated and have acquired a myofibroblastic phenotype. Furthermore, type II alveolar epithelial cells, detected using anti-Mucin-1 antibody, were increased in Ad5 lungs compared with Ad3 and cAd lungs and also expressed α -SMA. These results suggest that type II alveolar epithelial cells are acquiring a myofibroblastic phenotype in IGFBP-5-expressing lungs. Taken together, our findings suggest that overexpression of IGFBP-5 induces an inflammatory response and fibrosis through the transformation of fibroblasts and epithelial cells into myofibroblastic cells that then contribute to the resulting fibrosis.

We have shown that myofibroblastic changes are induced by IGFBP-5 in primary lung fibroblasts. IPF patient lung tissues have evidence of active fibrosis with increased numbers of activated fibroblasts, many of which have the phenotypic characteristics of myofibroblasts.²⁸ At these sites, increased ECM deposition is evident with effacement of the normal alveolar architecture.²⁸ A myofibroblast is strictly defined by electron microscopic findings as a cell that is vimentin and/or α -SMA-positive, with prominent rough endoplasmic reticulum, modestly developed peripheral myofilaments with focal densities, fibronexus junctions, some Golgi apparatus producing collagen secretion granules, and gap and actin-filament-based junctions.²⁹ However, most studies use an immunohistochemical definition without electron microscopy—a spindle-shaped cell expressing α -SMA.³⁰ Thus fibroblasts expressing α -SMA in our model are undergoing a myofibroblastic change, and this *in vivo* phenomenon can be triggered by exogenous IGFBP-5.

The source of myofibroblasts is controversial. Kinetic studies suggest that one of the origins of myofibroblasts in pulmonary fibrosis is resident peribronchial and perivascular adventitial fibroblasts.³¹ This observation is supported by *in vitro* biochemical and morphological evidence of the fibroblastic origin of myofibroblasts.^{32,33} Furthermore, cultured fibroblasts *in vitro* can be induced to differentiate into myofibroblasts by treatment with cytokines such as transforming growth factor- β .³⁴ Epithelial-mesenchymal transition is proposed as yet another source of myofibroblasts and can also be induced by transforming growth factor- β .^{30,35,36} It was recently reported that transitioned cells, which co-express epithelial cell markers and α -SMA, are abundant in lung tissues from IPF patients.³⁰ Because transmembrane Mucin-1 is a sialylated glycoprotein that is expressed in human airway and type II alveolar epithelial cells,^{37,38} we used Mucin-1 as an epithelial cell marker and α -SMA as a mesenchymal cell marker. We have shown that type II epithelial cells are increased and are in a transition phase

in IGFBP-5-expressing lungs compared with those that express IGFBP-3. Taken together, our findings suggest that human IGFBP-5 is a novel inducer of epithelial-mesenchymal transition in type II epithelial cells.

We have previously shown that both IGFBP-3 and IGFBP-5 induce ECM production by primary normal lung fibroblasts.¹³ However, our current findings indicate that IGFBP-5, and not IGFBP-3, is profibrotic *in vivo*. This may be attributable to 1) species-specific posttranslational modifications of IGFBP-3 because both IGFBP-3 and IGFBP-5 cDNAs are human; 2) the fact that adenovirally mediated expression of IGFBP-3 and -5 *in vivo* results in expression of these proteins in alveolar macrophages and epithelial cells whereas our *in vitro* findings were from primary lung fibroblasts; and 3) IGFBP-5 induces migration of PBMCs, whereas IGFBP-3 does not. The induction of fibrosis *in vivo* is a complex process that is likely to require cell-cell interactions of inflammatory cells and other cell types. Thus, the infiltration of mononuclear cells triggered by IGFBP-5 may be mediating its profibrotic effects.

The mechanism by which IGFBP-5 induces its profibrotic effects is currently under investigation. Our preliminary findings suggest that IGFBP-5's effects on ECM production by cultured primary lung fibroblasts is IGF-I independent because preliminary findings suggest that neutralizing anti-IGF-I antibodies do not hamper the induction of collagen and fibronectin deposition in the ECM (C.A.F.-B. and H.Y., unpublished observations). This is further supported by the fact that targeted overexpression of IGF-I to the lung does not trigger fibrosis.³⁹

Recently, ubiquitous overexpression of IGFBP-5 in transgenic mice was reported.⁴⁰ Increased IGFBP-5 production resulted in neonatal mortality, reduced fertility, growth inhibition, and delayed muscle development, reflecting the important role of IGFBP-5 in growth and development.⁴⁰ This model does not provide useful insights into fibrotic diseases because of excessive neonatal mortality. IGFBP-5 has been shown to play an important role in the maintenance of bone mineral density,⁴¹ maintenance of hair shaft medulla structure,⁴² and myoblast differentiation.^{43,44} Based on these reports and our current findings, we propose that IGFBP-5 plays an important role in the development and/or differentiation of mesenchymal cells.

In summary, we have shown that IGFBP-5 has profibrotic and chemoattractant activity *in vivo* and *in vitro*. IGFBP-5 induces the production of ECM via activation of fibroblasts and type II alveolar epithelial cells and the development of a myofibroblastic phenotype. IGFBP-5 overexpression provides a novel mouse model to study the pathogenesis of lung fibrosis.

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