Growth Factors, Cytokines, Cell Cycle Molecules

Insulin-Like Growth Factor Binding Proteins 3 and 5 Are Overexpressed in Idiopathic Pulmonary Fibrosis and Contribute to Extracellular Matrix Deposition

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Idiopathic pulmonary fibrosis (IPF) is a fibrotic disease of unknown etiology that results in significant morbidity and mortality. The pathogenesis of IPF is not completely understood. Because recent studies have implicated insulin-like growth factor-I (IGF-I) in the pathogenesis of fibrosis, we examined the expression and function of insulin-like growth factor binding proteins (IGFBP)-3 and -5 in IPF. IGFBP-3 and -5 levels were increased *in vivo* in IPF lung tissues and *in vitro* in fibroblasts cultured from IPF lung. The IGFBPs secreted by IPF fibroblasts are functionally active and can bind IGF-I, and IGFBPs secreted by primary fibroblasts bind extracellular matrix components. Our results also suggest that IGFBPs may be involved in the initiation and/or perpetuation of fibrosis by virtue of their ability to induce the production of extracellular matrix components such as collagen type I and fibronectin in normal primary adult lung fibroblasts. Although transforming growth factor- β increased IGFBP-3 production by primary fibroblasts in a time-dependent manner, IGFBP-5 levels were not increased by transforming growth factor- β . Taken together, our results suggest that IGFBPs play an important role in the development of fibrosis in IPF. (Am J Pathol 2005, 166:399–407)

Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic lung disease that often causes respiratory failure and death within 5 to 7 years of diagnosis.^{1,2} IPF typically afflicts individuals between the ages of 50 to 70 years with an estimated annual incidence of 7 and 10 cases per 100,000 for women and men, respectively.³ The pathological hallmarks of IPF are fibroblast proliferation, forma-

tion of fibroblastic foci, and production of excessive amounts of extracellular matrix (ECM) components such as collagen and fibronectin.⁴ These structural changes lead to alveolar destruction and progressive restrictive lung disease. The importance of fibroblast foci is supported by the observation that survival in IPF is greater in patients with fewer fibroblastic foci.² The pathogenesis of fibrosis in IPF remains unclear, however, several recent observations implicate insulin-like growth factor (IGF) and a family of proteins that bind IGF with high affinity, the insulin-like growth factor binding proteins (IGFBPs) 1 to 6.

IGF-I's biological activity on fibroblasts includes stimulation of collagen production and down-regulation of collagenase production,⁵ suggesting that IGF-I may be an important mediator in the development of pulmonary fibrosis. Indeed, several studies have reported changes in IGF-I in fibrotic conditions. Elevated IGF-I levels have been proposed as pathogenic in lung fibrosis in coal workers' pneumoconiosis⁶ and alveolitis in systemic sclerosis (SSc).⁷ IGF-I levels are elevated in alveolar and interstitial macrophages, interstitial mesenchymal cells, and epithelial cells in IPF.^{8,9} The biological activity of IGF-I is regulated at least in part by IGFBPs, which bind and regulate the access of IGF to its receptor.

The six IGFBPs vary in their tissue expression and in their response to, and regulation by, other growth factors. Two of these IGFBPs, IGFBP-3 and -5, can bind to ECM components such as heparan sulfate.^{10,11} IGFBP-3 is the major IGFBP in serum and the major IGFBP secreted into culture medium by fibroblasts. IGFBP-5 is the most conserved of the six IGFBPs. IGFBP-3 and -5 share features such as the presence of a nuclear localization sequence and the formation of ternary complexes with IGF and the

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acid-labile subunit in serum.^{11,12} IGFBPs bind IGF and thereby modulate the effects of IGF on target cells and protect IGF from degradation. Depending on context, IGFBPs can potentiate or inhibit the biological effects of IGF-I in a given tissue.

IGFBPs have been detected in adult rat lung¹³ and lung development in several species,^{14,15} but little is known about IGFBP expression in IPF. Spontaneous release of IGFBP-3 in bronchoalveolar lavage cells from patients with IPF was reported,⁸ and IGFBP-3 levels are also elevated in the bronchoalveolar lavage fluid and cells of patients with stage III sarcoidosis where it is believed to contribute to fibrogenesis.¹⁶ We have described a 20-fold increase in IGFBP-5 expression and an increase in IGFBP-3 levels in fibroblasts from the fibrotic skin of patients with SSc.^{17,18} To address the hypothesis that IGFBPs contribute to the development and/or perpetuation of ECM production and fibrosis in IPF, we determined whether aberrant expression of IGFBP-3 and -5 occurs in IPF lung tissue and in fibroblasts cultured from the lungs of patients with IPF, and whether IGFBP-5 alters matrix production by normal lung fibroblasts. Our results demonstrate that both IGFBP-3 and -5 are secreted in excess by fibroblasts from IPF patients compared to fibroblasts from normal lung, and that IGFBPs augment fibroblast matrix production.

Materials and Methods

Tissues and Cells

Fibroblasts were cultured from the explanted lungs of patients with IPF who underwent lung transplantation at the University of Pittsburgh Medical Center, under a protocol approved by the University of Pittsburgh Institutional Review Board, and from normal lung tissue obtained from organ donors. Approximately 2-cm³ pieces of peripheral lung from which the pleural margin was removed were minced and fibroblasts 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.¹⁷ Cells were used in passages 3 to 7.

Immunohistochemistry

Seven- μ m sections of OCT-embedded lung tissues were prepared on a cryostat, fixed with ice-cold acetone, and then blocked with 5% horse serum. Polyclonal rabbit anti-human IGFBP-3 and IGFBP-5 antibodies (Groppe Ltd., Thebarton, SA, Australia) were used at a concentration of 10 μ g/ml, and rabbit serum was used as a negative control. Monoclonal mouse anti-human fibronectin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used to localize fibronectin. Sections were washed and incubated with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA). The sections were then washed and bound secondary antibody was

detected using the Zymed AEC Red kit (Zymed, San Francisco, CA). A light hematoxylin stain was used to identify nuclei. Representative images from three normal and three IPF lung specimens were taken on a Nikon Eclipse 800 microscope (Nikon Instruments, Inc., Huntley, IL) using identical camera settings for normal and IPF samples.

Ribonuclease Protection Assay (RPA)

RPAs were done as we have previously described.¹⁷ Briefly, the full-length cDNA for IGFBP-5 and IGFBP-3 was cloned into pGEMTe (Promega Corp., Madison, WI) and used for the preparation of a riboprobe using the SP6/T7 riboprobe system (Promega Corp.). RPA was done using RPA II (Ambion, Inc., Austin, TX) following the manufacturer's recommendations. Samples were electrophoresed on denaturing gels and signals were detected after autoradiography. A cDNA corresponding to GAPDH was used as an internal control.

Western Blot Analysis

Culture supernatant and ECM were obtained from confluent fibroblasts as previously described.¹⁹ Briefly, fibroblasts were cultured in 10-cm tissue culture dishes. Culture supernatants were collected and centrifuged for 10 minutes at 4°C to pellet cell debris. For the preparation of ECM, cells were rinsed with 1 $\times$ phosphate-buffered saline (PBS) and incubated with 1 $\times$ PBS/8 mol/L urea for 20 minutes at room temperature. Dishes were rinsed four times with 1 $\times$ PBS and the ECM was scraped directly in 2 $\times$ sodium dodecyl sulfate gel-loading buffer (100 mmol/L Tris-Cl, pH 6.8, 200 mmol/L mercaptoethanol, 4% sodium dodecyl sulfate, 0.2% bromophenol blue, 20% glycerol). Samples were analyzed by Western blot analysis using polyclonal goat anti-human IGFBP antibody, polyclonal anti-human collagen type I antibody, monoclonal anti-human fibronectin antibody (Santa Cruz Biotechnology, Inc.), and anti- β -actin antibodies (Sigma-Aldrich). Signals were detected after incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) and chemiluminescence (Perkin Elmer Life Sciences, Inc., Boston, MA).

Western Ligand Blot Assay

ECM fractions were electrophoresed under nonreducing conditions and transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in Tris-buffered saline/5% Tween-20 and incubated for 1 hour with biotinylated IGF-I (Groppe Ltd.). The membrane was washed and incubated for 1 hour with horseradish peroxidase-conjugated streptavidin (Amersham Biosciences, Piscataway, NJ) and the signal was detected using chemiluminescence (Perkin-Elmer Life Sciences, Inc.) and autoradiography.

Immunocytochemistry

Primary fibroblasts from healthy donor lung and the lungs of IPF patients were cultured in four-chamber tissue culture slides (Becton Dickinson, Franklin Lakes, NJ). Cells were fixed with 2% paraformaldehyde and permeabilized with 0.1% Triton X-100. Slides were blocked with 10% serum at 4°C for 16 hours. Anti-IGFBP-3 and anti-IGFBP-5 antibodies (Groppe Ltd.) were used at a dilution of 1:150 in 5% serum. Slides were washed, incubated with biotinylated secondary antibody (Amersham Biosciences) at a dilution of 1:400 and signal detected using the Vectastain ABC Kit (Vector Laboratories, Inc.). Images were taken using a Nikon Eclipse 800 microscope (Nikon).

Co-Immunoprecipitations

Polyclonal anti-IGFBP-3 and anti-IGFBP-5 antibodies (Santa Cruz Biotechnology, Inc.) were bound to protein A-agarose (Invitrogen Life Technologies). Supernatant and ECM fractions of normal and IPF lung fibroblasts corresponding to equal cell numbers were incubated with the agarose-bound antibodies at 4°C. Bound complexes were washed and analyzed by Western blot analysis using polyclonal anti-collagen type I antibody (Santa Cruz Biotechnology, Inc.).

Culture of Fibroblasts on Collagen and Fibronectin

Tissue culture dishes (10-cm) were coated with 5 $\mu\text{g}/\text{cm}^2$ of rat tail collagen I or 1 $\mu\text{g}/\text{cm}^2$ of human plasma fibronectin (Becton Dickinson Labware, Bedford, MA) for 1 hour at room temperature. Dishes were rinsed and 1×10^6 fibroblasts were plated per dish. Cells were cultured for 4 days until the fibroblasts reached confluence. ECM was harvested as described above, and samples representing 1×10^5 cells were analyzed by Western blot analysis using anti-collagen type I antibody (Santa Cruz Biotechnology, Inc.)

Recombinant IGFBP-3 and -5 Expression

The full-length cDNAs for IGFBP-3 and IGFBP-5 were obtained by reverse transcriptase-polymerase chain reaction using total RNA from human primary lung fibroblasts. The cDNAs were expressed in baculovirus in Sf9 insect cells following the manufacturer's recommendation (Invitrogen Life Technologies). Recombinant proteins were purified as previously described.²⁰

Adenovirus Construct Preparation

The full-length cDNA of human IGFBP-5 was cloned into the shuttle vector pAdlox. The construct was then used for the preparation of replication-deficient adenovirus type 5 expressing IGFBP-5 in the Vector Core facility at the University of Pittsburgh. Primary adult lung fibroblasts were infected with adenovirus type 5 expressing IGFBP-5,

Figure 1. IGFBP-3 and -5 levels in IPF lung tissues. Serial sections of lung tissues from a healthy donor (**A–D**) and an IPF patient undergoing lung transplantation (**E–H**) were analyzed by immunohistochemistry using anti-fibronectin (**B, F**), anti-IGFBP-3 (**C, G**), or anti-IGFBP-5 (**D, H**) antibodies. **A** and **E** are representative images from tissue sections in which primary antibody was omitted. Fibronectin antibody labeled alveolar septae (**arrowhead** in **B**) and fibroblasts (**arrows** in **F**). IGFBP-3 and -5 antibodies labeled cells having the appearance of alveolar type II cells (**arrows** in **C** and **D**) but not normal alveolar septae (**arrowheads** in **C** and **D**). In IPF samples, IGFBP-3 and -5 strongly labeled fibroblasts in thickened alveolar septae (**arrows** in **G** and **H**). Data are representative of three NL and three IPF lung tissues. Original magnifications, $\times 400$.

or a control empty recombinant adenovirus at a multiplicity of infection of 50.

Statistical Analysis

Data were analyzed using the unpaired *t*-test (Microsoft Excel).

Results

IGFBP-3 and -5 Expression Are Increased in IPF Lungs

IGFBP-3 and -5 protein expression in lung sections from healthy donors and patients with IPF who underwent lung transplantation were analyzed by immunohistochemistry. As shown in Figures 1 and 2, IGFBP-3 and -5 expression

Figure 2. IGFBP-5 expression in IPF. IGFBP-5 antibody labeled interstitial fibroblasts (arrows) and epithelial cells in residual alveolar spaces (arrowheads in A) and in bronchiolar epithelial cells (arrowheads in B). Original magnifications, $\times 400$.

was higher in IPF lung sections compared to lung sections obtained from a normal donor lung. Levels of IGFBP-5 were more intense than those of IGFBP-3 and parallel levels of fibronectin. IGFBP-5, and to a lesser extent, IGFBP-3, localized to fibroblasts surrounding airspaces, and to epithelial cells in bronchioles and alveoli.

IPF Fibroblasts Have Increased Expression of IGFBP-3 and -5 mRNA

Having demonstrated that IGFBP-3 and -5 expression is higher in IPF lung compared to normal, we used primary fibroblasts cultured from these tissue sources to determine whether increased expression was because of increased fibroblast number in the IPF lungs, or to increased expression per fibroblast. Steady-state mRNA levels for IGFBP-5 were analyzed by RPA using total RNA from equal numbers of primary adult normal and IPF lung fibroblasts in passage 3. As shown in Figure 3, IGFBP-5 and IGFBP-3 mRNA levels are significantly increased in IPF fibroblasts compared to normal lung fibroblasts ($P = 0.001$ and $P = 0.048$, respectively). Steady-state IGFBP-5 mRNA levels are increased in all IPF fibroblasts with a mean twofold increase compared to normal control fibroblasts. Steady-state mRNA levels of IGFBP-3 are increased in three of five IPF fibroblasts, with a mean fourfold increase when all normal and IPF samples are compared (Figure 3C).

Increased Secretion and Deposition of IGFBP-3 and -5 by IPF Fibroblasts

To determine whether fibroblasts from IPF secrete more IGFBP-3 and -5 than normal fibroblasts, Western blot analysis was performed on the ECM secreted by primary normal lung and IPF fibroblasts. All fibroblasts were used before passage 7. Culture supernatant prepared from 1×10^5 fibroblasts was used in Western blot analysis. As shown in Figure 4, IGFBP-3 and -5 protein levels were increased in the media conditioned by IPF fibroblasts. Levels of IGFBP-5 secreted by IPF fibroblasts were on average 100 times higher than those secreted by normal lung fibroblasts. Differences in secreted IGFBP-5 levels were statistically significant ($P = 0.0007$). Levels of IGFBP-3 in culture supernatant were on average 3.8 times higher in IPF fibroblasts compared to normal lung fibroblasts ($P = 0.04$). IGFBP-3 and -5 levels were also

Figure 3. Increased IGFBP-5 and -3 in IPF fibroblasts. Increased IGFBP-5 and -3 mRNA in fibroblasts from IPF lung tissue. **A:** IGFBP-5 mRNA levels: IGFBP-5 mRNA levels were analyzed by RPA in passage 3 lung fibroblasts from two healthy donors (NL) and five IPF patients (IPF). Yeast RNA was used as control (lane C). GAPDH mRNA levels were used to normalize the signal. **B:** IGFBP-3 mRNA levels: IGFBP-3 mRNA levels were analyzed by RPA as described in A. **C:** Graphical presentation of the data analyzed by scanning densitometry. Values represent mean $\pm$ SE.

increased in the ECM of IPF lung fibroblasts (Figure 5). The increase in IGFBP-3 and -5 production in the ECM fraction of IPF fibroblasts paralleled that of collagen and fibronectin (Figure 5). To determine whether increased

Figure 4. **A:** Increased secretion of IGFBP-3 and -5 by IPF fibroblasts. Fibroblasts from control (NL) and IPF lung tissues were cultured and used in early passage. Cells were plated at equal numbers and conditioned media collected. Supernatant from 1×10^5 fibroblasts was analyzed by Western blot using anti-IGFBP-5 and anti-IGFBP-3 antibodies. **B:** Graphical presentation of the data analyzed by scanning densitometry. Values represent mean $\pm$ SE.

Figure 5. Increased levels of collagen I, fibronectin, and IGFBP-3 and -5 in ECM from IPF fibroblasts: Western blot analysis of IGFBP-3 and -5 in ECM from control and IPF fibroblasts. ECM was prepared from NL and IPF fibroblasts and analyzed by Western blot using anti-IGFBP-3, IGFBP-5, collagen, and fibronectin antibodies. ECM fractions from 1×10^5 fibroblasts were loaded in each lane. Comparisons are representative of fibroblasts from seven different IPF patients and four healthy controls.

IGFBP levels were because of decreased IGFBP proteolytic activity, IPF lung fibroblast supernatants were mixed with medium conditioned by primary adult normal lung fibroblasts for 1 to 16 hours at 37°C, in the presence and absence of protease inhibitors, and analyzed by Western blot analysis. IGFBP-3 and -5 levels were not decreased in the presence of normal lung fibroblast conditioned medium (data not shown) suggesting that increased IGFBP accumulation in the media conditioned by IPF fibroblasts is not because of decreased degradation of IGFBPs. To date, increased IGFBP-3 and -5 secretion has been confirmed in a total of 12 IPF samples. All 12 samples overexpressed IGFBP-5 and 10 of 12 samples overexpressed IGFBP-3 relative to matched normal lung fibroblasts.

Increased levels of IGFBP-3 and IGFBP-5 were also examined in cultured fibroblasts by immunocytochemistry. Figure 6 shows increased IGFBP-3 and IGFBP-5 cytoplasmic and nuclear staining in IPF fibroblasts compared to normal lung fibroblasts. Taken together, these results demonstrate that the increased expression of IGFBP-3 and -5 detected *in vivo* in IPF lung tissues is paralleled by increased levels in cultured IPF fibroblasts *in vitro*.

IGFBP-3 and -5 Are Detected in ECM as Functional IGF Binding Proteins

Having demonstrated increased mRNA and protein levels of IGFBP-3 and -5 in IPF fibroblasts, we next determined whether functional IGFBPs could be detected in ECM fractions. To do so, a Western ligand blot assay

Figure 6. Cell-associated IGFBP-3 and -5 expression is increased in cultured IPF fibroblasts compared to normal fibroblasts. Primary fibroblasts from normal and IPF lung tissues were cultured in four-chamber slides. Cells were fixed and stained with anti-IGFBP-3 and -5 antibodies as described in the Materials and Methods section. As a control (C), primary antibody was omitted from the incubation steps. Original magnifications, $\times 200$.

Figure 7. Western ligand blot analysis of IGFBP. Functional IGFBPs in ECM were analyzed by Western ligand blot using biotinylated IGF-I. ECM extracted from 1×10^5 cells was loaded in each lane. IGFBP-3 and -5 monomers correspond to the 30- to 35-kD and 40- to 48-kD bands indicated by asterisked arrows. Higher molecular weight bands represent dimers and multimers of IGFBP-3 and -5 based on molecular weight estimates. Comparisons are representative of two normal and two IPF lung fibroblast lines. Experiments with each pair were repeated three times.

using biotinylated IGF-I was used. As shown in Figure 7, increased IGFBP levels were also detected using Western ligand blot analysis. Arrows indicate all IGF binding proteins detected using this method. IGFBP-3 and -5 monomers most likely correspond to the 40- to 48-kD and 30- to 35-kD bands indicated by arrows and asterisks, respectively, based on comparisons of molecular weights of IGFBPs detected in ECM by Western blot analysis. IGFBP-3 is detected as a doublet. Because samples analyzed by Western ligand blot are electrophoresed under nonreducing conditions, IGFBPs are detected as monomers, dimers, and multimers (indicated by arrows). These results demonstrate that the IGFBP-3 and -5 secreted by IPF fibroblasts are functionally active by virtue of their ability to bind IGF-I.

IGFBP-3 and -5 Bind ECM Components in Normal and IPF Lung Fibroblasts

IGFBP-3 and -5 secreted by normal and IPF lung fibroblasts and deposited in the ECM were immunoprecipitated using protein A-agarose-bound anti-IGFBP-3 and -5. To determine whether IGFBP-3 and -5 secreted by normal and IPF lung fibroblasts bound ECM components, immunoprecipitated IGFBPs were analyzed by Western blot analysis for collagen-bound IGFBPs. Figure 8 shows that both IGFBP-3 and -5 bind collagen I in the supernatant and ECM of both normal and IPF fibroblasts. Supernatant and ECM aliquots from an equal number of cells (1×10^5 cells) were used for both samples. These data demonstrate increased levels of IGFBP-bound collagen I

Figure 8. Binding of IGFBP-3 and -5 to collagen type I. Supernatant (SUP) and ECM fractions of fibroblasts from control and IPF lung were incubated with protein A agarose-bound IGFBP-3 or IGFBP-5. Samples were analyzed by Western blot using a polyclonal anti-collagen I antibody. Note increased collagen I-bound IGFBP-3 and -5 in IPF relative to control.

in both the supernatant and ECM fractions of IPF fibroblasts compared to normal lung fibroblasts.

Culture of Lung Fibroblasts on Collagen or Fibronectin Increases IGFBP-3 and -5 in the ECM

Because IGFBP-3 and -5 can bind ECM components and thus be protected from protease degradation,¹⁰ we determined the effect of ECM on the accumulation of IGFBP-3 and -5 in the ECM of lung fibroblasts. As shown in Figure 9, normal lung fibroblasts cultured on collagen type I or fibronectin exhibit increased IGFBP-3, IGFBP-5, and fibronectin accumulation in the ECM compared to fibroblasts cultured on uncoated tissue culture dishes. This suggests that ECM components can stimulate production of IGFBPs, thereby creating a feedback mechanism for modulation of IGF-I and IGFBP activity.

IGFBP-3 and -5 Can Induce ECM Production by Normal Lung Fibroblasts

To determine whether the converse phenomenon occurs, specifically whether IGFBP-3 and -5 can induce production of ECM components, normal lung fibroblasts were incubated with recombinant IGFBP-3 and -5 at a concentration of 250 ng/ml for 48 hours. ECM was prepared and fibronectin levels analyzed by Western blot. IGFBP-3 induced a 14-fold and IGFBP-5 induced a 16-fold increase in matrix fibronectin in normal lung fibroblasts (Figure 10A). This effect was time-dependent and increased

Figure 9. Culture of normal lung fibroblasts on collagen or fibronectin increases IGFBP-3 and -5 levels. Tissue culture dishes were coated with collagen I or fibronectin. Primary normal lung fibroblasts were cultured on uncoated, collagen-coated, or fibronectin-coated dishes for 4 days. ECM from 1×10^5 fibroblasts was collected and analyzed by Western blot analysis for IGFBP-3, IGFBP-5, and fibronectin.

Figure 10. IGFBP-3 and -5 induce ECM production. Primary adult lung fibroblasts were incubated with recombinant IGFBP-3 or -5 for 48 hours (A) or with IGFBP-5 for the indicated time points (B). ECM was extracted and analyzed by Western blot using anti-fibronectin antibody. Note induction of fibronectin by both IGFBP-3 and -5. C: Primary lung fibroblasts were infected with adenovirus expressing IGFBP-5. Increased expression of IGFBP-5 in adenovirus-IGFBP-5-infected fibroblasts was confirmed by Western blot analysis. D: ECM and conditioned media were collected from uninfected (lane C) and adenovirus-IGFBP-5-infected fibroblasts 4 or 5 days after infection, and analyzed by Western blot analysis using anti-fibronectin and anti-collagen antibodies, respectively. Lysates from cells used for the extraction of ECM were analyzed using anti- β actin antibody. E: As a control, fibroblasts were uninfected (No Ad) or infected with a control adenovirus (C-Ad). ECM was extracted and analyzed as described above.

gradually between 3 and 48 hours from 4- to 12-fold, respectively (Figure 10B). To determine the effect of overexpressing IGFBP on ECM production, primary adult lung fibroblasts were infected with adenovirus type 5 expressing human IGFBP-5. Increased IGFBP-5 secretion by adenovirus-IGFBP-5-infected fibroblasts was confirmed by Western blot analysis (Figure 10C). Overexpression of IGFBP-5 resulted in increased secretion and deposition of fibronectin and collagen type I in the ECM of the fibroblasts (Figure 10D). Overexpression of IGFBP-5 for 4 days induced a threefold and twofold increase in fibronectin and collagen expression, respectively. Overexpression of IGFBP-5 for 5 days resulted in a sixfold and fourfold increase in fibronectin and collagen I levels, respectively. Increases in ECM components were not observed when cells were infected at the same multiplicity of infection with control adenovirus (Figure 10E).

Increased IGFBP-5 Is Not Due to Transforming Growth Factor (TGF)- β Stimulation

Because TGF - β has been implicated in the development of IPF, we determined whether TGF - β alters IGFBP production by fibroblasts. Normal lung fibroblasts were treated with 1 ng/ml of recombinant human TGF - β for 1 to 72 hours. IGFBP-3 and -5 were analyzed in culture supernatants of untreated and TGF - β -treated fibroblasts using Western blot analysis. Supernatant fractions equivalent to 4.5×10^3 fibroblasts were analyzed in each lane. IGFBP-3 increased in a time-dependent manner between 24 and 72 hours in TGF - β -treated lung fibroblasts, whereas

Figure 11. TGF- β stimulates IGFBP-3, but not IGFBP-5 production by normal fibroblasts. Primary normal lung fibroblasts were treated with 1 ng/ml of TGF- β for the indicated times. Conditioned media from 4.5×10^5 fibroblasts were analyzed by Western blot for IGFBP-3 and IGFBP-5. C denotes absence of TGF- β .

IGFBP-5 levels did not change, and even decreased somewhat at 72 hours after stimulation (Figure 11).

Discussion

IPF is a disease of unknown etiology with significant morbidity and mortality. To date, no effective therapeutic approaches are available. IGF-I has been detected by immunohistochemistry in IPF lung tissues in alveolar and interstitial macrophages, interstitial mesenchymal cells, and epithelial cells.^{8,9} The levels and function of IGF-I can be modulated by IGFBPs via both IGF-dependent and -independent mechanisms.²¹ We have therefore begun to elucidate the role of IGFBPs in IPF. We determined that IGFBP-3 and -5 expression is increased in IPF lung tissue. To determine whether fibroblasts are a source of these IGFBPs in IPF lung, and whether increased tissue IGFBP reflects increased fibroblast production, primary fibroblasts were cultured from healthy donor and IPF lung tissues. Our studies reveal that the increase in IGFBP-3 and -5 levels in IPF lung tissues are due to, at least partly, increased production of these IGFBPs by cultured fibroblasts.

We have previously reported increased IGFBP-5 production by primary dermal fibroblasts from patients with SSc, demonstrating increased IGFBP-5 in fibrotic skin fibroblasts.^{17,18} We report here that IGFBP-5, as well as IGFBP-3, are also increased in the extracellular milieu of primary fibroblasts cultured from fibrotic lung tissues of patients with IPF. Our data further show that the increase in IGFBP protein levels is most significant in the ECM of the fibroblasts. This suggests that IPF fibroblasts overexpress IGFBPs that are secreted and deposited in the ECM. IGFBPs secreted by IPF fibroblasts are functionally active by virtue of their ability to bind IGF-I in a Western ligand blot assay.

Increased IGFBP could be an initiating event in ECM production, or reflect a feedback mechanism from ECM. Our results indicate that the increase in IGFBP levels is a primary event in pulmonary fibrosis because adenoviral-mediated expression of IGFBP-5, or addition of recombinant IGFBP-3 or -5, leads to increased ECM production by primary normal adult lung fibroblasts. Previous reports have suggested that exogenous and endogenous IGFBPs may exert different effects on cells.²² In normal primary lung fibroblasts, both exogenously added recombinant IGFBP-5, which may mimic epithelial production of IGFBP-5, and endogenously overexpressed IGFBP-5, increased the secretion and deposition of ECM

proteins. This is consistent with IGFBPs being sufficient to promote lung fibrosis and raises the question whether epithelial injury could lead to IGFBP-5 production by epithelial cells, and subsequent activation of fibroblasts to produce ECM proteins and endogenous IGFBP-5. In addition, secretion of IGF-I by interstitial macrophages and epithelial cells^{8,9} could induce IGFBP-5 production by fibroblasts *in vivo*. However, it does not explain the sustained overexpression of IGFBPs maintained by IPF fibroblasts *in vitro* several months and passages after their removal from their *in vivo* milieu.

IGFBPs may also promote fibrosis via interactions with ECM, thereby promoting establishment of a vicious cycle of matrix deposition that if not accompanied by matrix degradation, eventuates in fibrosis. We and others have observed binding of IGFBP-3 and -5 to collagen type I and fibronectin in the ECM.^{21,23,24} This interaction may form the basis for the initiation of signal and its perpetuation since stimulation of collagen and fibronectin production provides additional IGFBP-binding partners in the extracellular milieu, thus generating an uncontrolled loop of increased ECM components and increased binding sites for IGFBPs.

The increased IGFBP levels by IPF fibroblasts may be the result of increased IGFBP secretion and/or decreased IGFBP degradation. Secreted IGFBPs can be proteolytically cleaved and degraded by several proteases identified to date.²¹ The increased amounts of IGFBP-3 and -5 in IPF fibroblast supernatants do not appear to result from reduced proteolytic degradation since media conditioned by normal lung fibroblasts that contain the secreted proteases with known IGFBP proteolytic activity²⁵⁻²⁷ do not affect IGFBP levels secreted by IPF fibroblasts. In contrast, it is possible that the increased IGFBP deposited in the ECM fractions is due to decreased proteolytic cleavage since IGFBPs that are bound to ECM are protected from proteolytic degradation¹⁰ and also since we have been able to demonstrate binding of IGFBP-3 and -5 to extracellular collagen type I (Figure 8) and fibronectin (data not shown) in the extracellular milieu of the fibroblasts. In turn, the excessive production of collagen and fibronectin by IPF fibroblasts creates additional binding sites for IGFBPs, thus promoting their accumulation in the ECM. Collagen and fibronectin may in turn protect IGFBPs from proteolytic degradation. This is supported by findings in Figure 9 that culture of fibroblasts on collagen or fibronectin results in increased accumulation of IGFBP-3 and -5 in the ECM, as well as additional accumulation of ECM components such as fibronectin.

One of the limitations of our analysis is that IPF fibroblasts were derived from patients with late-stage disease. This suggests that IGFBPs contribute to the propagation of the fibrotic phenotype, but our observation of increased collagen and fibronectin production upon incubation of normal primary lung fibroblasts with recombinant IGFBP-3 or -5 and overexpression of IGFBP-5 implicates IGFBPs in the initiation phase of fibrosis as well. This is further supported by our findings that IGFBP-5 mRNA levels are increased not only in clinically involved and thus fibrotic skin of patients with SSc, but

also in their yet clinically uninvolved skin.^{17,18} Further support comes from microarray analysis that revealed increased IGFBP-3 and -5 mRNA levels in human IPF lung tissues and increased IGFBP-3 in lung tissues of bleomycin-treated mice.^{28,29} Taken together, these observations suggest that IGFBPs may be involved early in the cascade of events leading to increased ECM production and fibrosis. Studies are currently underway to determine the mechanism by which IGFBPs exert their effects on primary lung fibroblasts.

IGFBPs are involved in maintaining equilibrium between the synthesis and the degradation of ECM components. This is accomplished via IGF-dependent and IGF-independent pathways. Several cells including fibroblasts can synthesize and respond to IGF-I. In primary fibroblasts, IGF-I can induce latent TGF- β 1 thus leading to matrix modulation.³⁰ IGF-I can induce a twofold increase in the rate of transcription of TGF- β in fibroblasts and this effect can be partially blocked by the addition of neutralizing anti-TGF- β antibodies, suggesting that the induction of TGF- β by IGF-I can trigger an autoinduction of TGF- β .³¹ In IPF, IGF-I levels are increased in alveolar and interstitial macrophages, interstitial mesenchymal cells, and epithelial cells.^{8,9} TGF- β has been shown to increase IGFBP-3 in foreskin fibroblasts,³² thus indirectly modulating IGF-I's levels by increasing those of its binding protein. As such, TGF- β can alter the sensitivity of fibroblasts to IGF-I. We demonstrate that TGF- β can also increase IGFBP-3 secretion by primary adult lung fibroblasts. The induction of IGFBP-3 by TGF- β was delayed, starting at 24 hours and continuing through 72 hours. However, TGF- β did not stimulate the secretion of IGFBP-5 by primary lung fibroblasts. Thus, increased production of IGFBP-5 by IPF fibroblasts is not due to TGF- β . At 72 hours after stimulation, a slight decrease in IGFBP-5 levels was observed, as has been previously reported in muscle cells.^{33,34} These data suggest that although TGF- β may account for increased IGFBP-3 in IPF, other mechanisms are responsible for induction of IGFBP-5.

Recently, ubiquitous overexpression of IGFBP-5 in transgenic mice was reported. Increased IGFBP-5 production resulted in neonatal mortality, reduced fertility, growth inhibition, and delayed muscle development,³⁵ reflecting the important role of IGFBP-5 in growth and development. Mice transgenic for IGFBP-3 exhibit impaired bone formation, reduced birth weight, and selective organomegaly involving the spleen, liver, and heart.³⁶ These models do not provide insight into lung disease because of the excessive neonatal mortality rate reflecting the important role of IGFBPs in development.

In conclusion, the current study demonstrates that 1) IGFBP expression is increased in IPF lung tissues, 2) cultured IPF fibroblasts secrete and deposit increased amounts of IGFBP-3 and -5 into their ECM, 3) IGFBPs deposited in the ECM of IPF fibroblasts are functionally active and bind IGF-I, and 4) IGFBP-3 and -5 induce production and deposition of collagen and fibronectin in primary adult lung fibroblasts. These data identify an important pathway for initiation and propagation of a fibrotic reaction in the lung, and reveal a novel potential target for therapeutic intervention.

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