The Pro-Fibrotic Factor IGFBP-5 Induces Lung Fibroblast and Mononuclear Cell Migration

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We have previously shown that insulin-like growth factor-binding protein-5 (IGFBP-5) is overexpressed in fibrotic lung tissues and that it induces production of extracellular matrix components such as collagen and fibronectin both *in vitro* and *in vivo*. We recently observed mononuclear cell infiltration in lung tissues of mice expressing IGFBP-5. We therefore examined the role of IGFBP-5 on the migration of immune cells. Migration assays demonstrated that IGFBP-5 induced migration of peripheral blood mononuclear cells (PBMCs) in a dose-dependent manner. Preferential migration of monocytes/macrophages, natural killer cells, and T cells was observed. Moreover, the CD4/CD8 ratio of migrating cells was significantly higher *in vitro* and *in vivo* in response to IGFBP-5. IGFBP-5 resulted in preferential migration of activated CD4⁺ T cells and monocytes. Interestingly, IGFBP-5 also induced migration of primary human lung fibroblasts. Exogenous administration of IGFBP-5 induced activation of mitogen-activated protein kinase (MAPK) signaling cascade but not PI3K in PBMCs. IGFBP-5-induced migration was blocked by the MEK1/2 inhibitor U0126, suggesting that IGFBP-5-induced migration occurs via MAPK activation. Furthermore, monocytes treated with recombinant IGFBP-5 expressed the mesenchymal markers α -smooth muscle actin and fibronectin *in vitro* and *in vivo*, suggesting that IGFBP-5 can induce the transformation of monocytes into mesenchymal cells. Collectively, our results suggest that IGFBP-5 induces cell migration via MAPK-dependent and IGF-I-independent mechanisms.

Keywords: IGFBP-5; mononuclear cells; migration; MAPK; fibrosis

Pulmonary fibrosis is histologically characterized by the deposition of extracellular matrix (ECM) components in the lung (1). The hallmarks of pulmonary fibrosis include cell proliferation, production of ECM by resident fibroblasts, and the presence of immune cells (2, 3). Pulmonary fibrosis results in significant morbidity and mortality (4, 5). There is currently no good treatment to halt the progression of the fibrosis, and organ transplantation remains the only therapeutic option.

Several reports have emphasized the role of chronic inflammation triggered by unknown stimuli that subsequently leads to lung injury and fibrosis (6). The role of inflammation is supported by the presence of interstitial and alveolar inflammatory cytokines, especially IL-1 β , IL-8, TGF- β , and TNF- α , in the lungs of

CLINICAL RELEVANCE

Insulin-like growth factor-binding protein-5 (IGFBP-5) is a novel fibrotic factor. Very little is known about the mechanism by which IGFBP-5 exerts its effect. This research identifies a chemoattractant activity of IGFBP-5 for immune cells and describes the mechanism mediating it. Thus, these findings provide insights into the pathogenic mechanisms mediating pulmonary fibrosis.

patients with fibrosis (7, 8), and the existence of an autoimmune response in fibrotic diseases such as systemic sclerosis (SSc) (9) and idiopathic pulmonary fibrosis (IPF) (10). Moreover, increased numbers of lymphocytes have been detected in the bronchoalveolar lavage fluid (BALF) of patients with SSc with lung involvement (11), and patchy lymphocytes and plasma cell infiltration of the alveolar walls were observed in the early and end stages of SSc-associated pulmonary fibrosis (3, 12). Multiple surgical lung biopsies of patients with pulmonary fibrosis revealed that the disease process may begin with a predominantly cellular pattern and progresses to end-stage fibrosis or usual interstitial pneumonia, with an intermediate stage that is characterized by a mixed pattern comprising both cellular and fibrotic components (13). These findings suggest that inflammation is an important feature of pulmonary fibrosis, especially in the early phase of the disease.

Insulin-like growth factor-binding protein (IGFBP)-5 is one of six known IGFBPs that were characterized by their ability to bind insulin-like growth factor I (IGF-I). Our group previously reported increased mRNA levels of IGFBP-5 in primary early-passage dermal fibroblasts from patients with SSc (14). Increased IGFBP-3 and IGFBP-5 mRNA and protein levels were also detected *in vivo* in lung tissues of patients with IPF and in primary fibroblasts cultured from these tissues (15). We have also demonstrated increased IGFBP-3 and -5 *in vitro* in primary fibroblasts cultured from skin and lung tissues of patients with SSc (15, 16). *In vitro*, IGFBP-5 induced production of ECM components, thus recapitulating the fibrotic phenotype. *In vivo*, IGFBP-5 triggered the development of dermal and pulmonary fibrosis (16, 17). IGFBP-5 expression *in vivo* resulted in infiltration of mononuclear cells into lung tissues with T cell infiltration preceding that of other mononuclear cells (17). IGFBP-5 also induced the development of a myofibroblastic phenotype (17).

To determine whether IGFBP-5 can mediate mononuclear cell migration and identify the subpopulations of cells responsive to IGFBP-5, we examined the effect of IGFBP-5 on peripheral blood mononuclear cells (PBMCs). Our results demonstrate that IGFBP-5 induces migration of PBMCs and fibroblasts. Cells migrating in response to IGFBP-5 had an activated phenotype. IGFBP-5 also induced expression of mesenchymal markers on monocytes *in vitro* and *in vivo*. Moreover, the effect of IGFBP-5 was IGF-I independent, and required activation of the mitogen-activated protein kinase (MAPK) pathway. Our results suggest

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that IGFBP-5 has a chemoattractant activity and is a novel target for the modulation of inflammation and the early phase of fibrosis.

MATERIALS AND METHODS

Reagents

Eight-week-old wild-type C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Ficoll-Paque PLUS was from Amersham Biosciences (Uppsala, Sweden). RPMI 1640 medium was from Cambrex (Walkersville, MD). Dulbecco's modified Eagle's medium was from Mediatech (Herndon, VA). Transwell culture dishes with filters were from Corning Incorporated (Corning, NY). Culture dishes (35-mm well) were from Costar (Cambridge, MA). Anti-IGF-I antibody and recombinant human RANTES were from R&D Systems (Minneapolis, MN). MEK1/2 inhibitor U0126, anti-phospho Raf, anti-phospho MEK1/2, anti-phospho p44/p42 MAPK, anti-phospho Akt, and anti-total Akt antibodies were from Cell Signaling (Beverly, MA). Anti-fibronectin, anti-total MEK1/2, anti-total p44/p42 MAPK, and anti-glyceraldehyde phosphate dehydrogenase (anti-GAPDH) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for flow cytometry were from BD Biosciences (San Jose, CA). Anti-CD4 and CD8 antibodies for immunohistochemistry were from Dako cytometations (Theburton, MA), and anti-Mac-1 antibody was from Chemicon (Temecula, CA). Anti- α -smooth muscle actin (α -SMA) antibody was from Sigma-Aldrich (St. Louis, MO). Recombinant human IGFBP-3 and IGFBP-5 were from Gropep Limited (Adelaide, Australia). Chemiluminescence reagents were from Perkin Elmer Life Sciences, Inc (Boston, MA). OCT compound was from Sakura Fine-technical Co., Ltd. (Tokyo, Japan). The AEC Red kit was from Zymed (San Francisco, CA). Fluorescence-conjugated or biotinylated secondary antibodies and Hematoxylin QS were from Vector laboratories (Burlingame, CA). Anti-CD3 antibody was from Lab Vision Corporation (Fremont, CA).

Human Subjects

Whole blood samples and lung tissues were obtained from healthy donors with informed consent under a protocol approved by the Institutional Review Board. Human PBMCs were obtained from heparinized venous whole blood using Ficoll-Paque PLUS gradient centrifugation as previously described (17, 18). Primary human lung fibroblasts were cultured from explanted lungs of healthy donors as we have previously described (15).

Migration Assay

The chemoattractant activity of IGFBP-5 was assessed in trans-well migration assays using human PBMCs and fibroblasts as we have previously described (17) with some modifications. Chemoattractant activity was assessed in a 24-well Trans-well cell culture dish with 5- μ m pore-size polycarbonate filters for PBMCs and 8- μ m pore size filters for fibroblasts. PBMCs or fibroblasts were resuspended in RPMI 1640 or DMEM, respectively, supplemented with 1% bovine serum albumin (BSA), and 2×10^5 of PBMCs or 5×10^4 fibroblasts were applied to the upper compartment of each chamber. Recombinant IGFBP-3, IGFBP-5, RANTES, or vehicle (10 mM HCl) were diluted in medium supplemented with 1% BSA and added to the lower compartment. Concentrations of recombinant IGFBP used are within physiologic levels as we have previously reported (17). These concentrations are below the estimated levels of IGFBP-5 in fibrotic lung tissues as assessed by Western blot analysis (see Figure E1 in the online supplement). In some experiments, PBMCs were preincubated with antibodies or MEK1/2 inhibitor (U0126) for 1 hour at 37°C at the indicated concentrations and directly applied to the upper chamber without replacement of medium. For PBMCs, after a 4-hour incubation with recombinant proteins 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 lower chamber to the total cell count of the upper and lower chambers. Harvested cells were also analyzed by flow cytometry. For fibroblasts, after a 6-hour incubation, cells penetrating through the filter pores and adhering to the bottom side of the membrane were defined as migrated cells, stained with hematoxylin, and counted.

Western Blot Analysis

Cellular lysates were obtained from cultured monocytes and macrophages as previously described (16, 17) with some modification. Briefly, 2.0×10^6 PBMCs were cultured in 35-mm wells for 1 hour. Nonadherent cells were removed, and adherent cells (which consisted of > 95% CD14⁺ cells) were used for the following experiments. PBMCs or adherent monocytes were stimulated with 50 to 500 ng/ml recombinant human IGFBP-5. Greater than 98% of adherent cells expressed Mac-1 (Figure E2). Culture supernatants and cellular lysates were harvested at the indicated time points. Samples were analyzed by Western blot using one of the following antibodies: anti-phospho Raf, anti-phospho MEK1/2, anti-phospho p44/p42 MAPK, anti-phospho Akt, anti-total Akt, anti-total MEK1/2, anti-total p44/p42 MAPK, fibronectin, and α -SMA antibodies. Anti-GAPDH antibody was used as a loading control. Signals were detected after incubation with horseradish peroxidase-conjugated secondary antibody and chemiluminescence.

Flow Cytometric Analysis

The phenotype of the cells was analyzed by flow cytometry by means of a FACScalibur cytometer using CellQuest software (BD Biosciences) as previously described (18). Cells were stained with FITC-conjugated, PE-conjugated, Cy5-PE-conjugated and/or APC-conjugated monoclonal antibodies against the following surface markers: CD3, CD4, CD8, CD14, CD16, CD45RA, CD45RO, CD56, CD69, HLA-DR. Appropriate isotype controls were used for each analysis. The number of migrated cells was calculated from the percentage obtained by flow cytometry.

Adenovirus Constructs

The adenovirus constructs were previously described (15). Briefly, the full-length cDNA of human IGFBP-5 was obtained by RT-PCR using total RNA extracted from primary human fibroblasts. The cDNA was subcloned into the shuttle vector pAdlox and used for the preparation of replication-deficient adenovirus serotype 5 expressing IGFBP-5 (Ad5) in the Vector Core Facility at the University of Pittsburgh. Adenovirus lacking a specific cDNA insert (cAd) was used as a control.

Mice and Adenoviral Administration

Administration of adenovirus to mice was done as previously described (17). All studies and procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Eight-week-old wild-type C57BL/6J mice were intratracheally injected with 10^9 plaque-forming units (PFU) of Ad5 or cAd in a 55- μ l volume. Mice were killed 8 or 14 days after adenoviral administration. Harvested lung tissues were inflated, embedded in paraffin or frozen in OCT compound, and kept at -80°C .

Immunohistochemistry and Immunofluorescence

Immunohistochemistry was done as previously described (17). In brief, 6- μ m sections of OCT-compound-embedded (for immunohistochemistry) or paraffin-embedded lung tissues (for immunofluorescence) were used. In other experiments, adherent monocytes from human PBMCs were used for immunofluorescence. The sections were blocked with 5% serum and incubated with one of the following primary antibodies: anti-CD3, anti-CD4, anti-CD8, anti-Mac-1, anti-fibronectin, or anti- α -SMA antibodies, after antigen retrieval and endogenous peroxidase quenching for paraffin-embedded sections. Primary antibodies were used at 1:100 dilution, except for anti- α -SMA, Mac-1, and fibronectin antibodies, which were diluted 1:50. Sections were washed and incubated with fluorescence-conjugated or biotinylated secondary antibody. Bound biotinylated secondary antibody was detected using the AEC Red kit, and a light hematoxylin counterstain was used to identify nuclei using hematoxylin QS. Images were captured on a Nikon Eclipse 800 microscope (Nikon Instruments, Inc., Melville, NY) or Olympus Fluoview 1,000 (Olympus America, Inc., Center Valley, PA) using identical camera settings.

Statistical Analysis

Statistical comparisons were performed using the Mann-Whitney *U*-test.

RESULTS

IGFBP-5 Induces Migration of PBMCs *In Vitro*

We recently demonstrated infiltration of mononuclear cells in IGFBP-5-expressing mouse lung tissues *in vivo* (17). To determine if IGFBP-5 elicits influx of mononuclear cells, migration assays were performed using PBMCs from three healthy donors. As shown in Figure 1A, recombinant IGFBP-5 induced 5.3 times more cell migration than vehicle ($P < 0.02$) ($24.4 \pm 1.3\%$ versus $4.6 \pm 0.5\%$, respectively). We had previously compared the migratory activity of IGFBP-5 to that of recombinant IGFBP-3. IGFBP-3 did not induce cell migration in this system (17). Induction of PBMC migration by IGFBP-5 was dose-dependent (Figure 1B). Recombinant IGFBP-3 and IGFBP-5 used in these migration assays were produced in mammalian cells. Endotoxin levels were measured at 0.02 EU/ μg , which is lower than the acceptable concentrations of endotoxin in commercially available chemokines such as RANTES. Thus the induction of cell migration by IGFBP-5 is not due to endotoxin. In contrast to its effect on

PBMC migration, IGFBP-5 did not induce migration of neutrophils (data not shown), further confirming our *in vivo* observations of mononuclear, but not polymorphonuclear, cell infiltration in response to IGFBP-5 expression (17). Since IGFBP-5 can exert its effects in IGF-I-dependent (19) and -independent manners (20, 21), we determined whether the effect of IGFBP-5 on cell migration was IGF-I independent. The efficacy of anti-IGF-I antibody in neutralizing IGF-I activity was assessed. Anti-IGF-I blocked Akt phosphorylation (Figure 1C) and IGF-I-induced PBMC migration (Figure 1D) at a concentration consistent with the neutralizing dose (ND)₅₀ reported by the manufacturer. In the presence of anti-IGF-I antibody, Akt phosphorylation levels were comparable to those seen at baseline in unstimulated cells and IGF-I-induced migration was blocked. Using the optimal neutralizing dose of anti-IGF-I antibody, autocrine/paracrine IGF-I secreted by PBMCs was neutralized. As shown in Figure 1E, blocking IGF-I had no effect on IGFBP-5-induced migration. Our findings suggest that IGFBP-5 has chemoattractant activity for PBMCs that is likely IGF-I independent.

Figure 1. Insulin-like growth factor (IGF)-binding protein-5 (IGFBP-5) induces cell migration in a dose-dependent, IGF-I-independent manner. (A) Summary of migration assays. The chemoattractant activity of IGFBP-5 was assessed by migration assay using 500 ng/ml recombinant IGFBP-5 or 10 mM HCl as a vehicle control. The number of cells in the upper and lower chambers of Transwells was counted, and the percentage of migrated cells was calculated. Peripheral blood mononuclear cells (PBMCs) were obtained from three donors; migration of PBMCs was assessed in one sample from Donor 1, two serial blood samples from Donor 2, and three serial samples from Donor 3. Each dot represents the average of three independent experiments. $*P < 0.02$ by Mann-Whitney U-test. (B) Results of PBMC migration assays using different concentrations of IGFBP-5. Results are representative of two independent experiments and shown as a mean $\pm$ SD. (C) Inhibition of IGF-I signaling by neutralizing anti-IGF-I antibody. Primary human lung fibroblasts were treated with recombinant IGF-I for 10 minutes in the presence of a control IgG or anti-IGF-I antibody. Akt phosphorylation was assessed by Western blot. (D) Migration of PBMC in response to 50 ng/ml recombinant IGF-I was examined in the presence of 5 or 10 $\mu\text{g/ml}$ control antibody or anti-IGF-I antibody. (E) Results of migration assays with anti-IGF-I antibody using PBMCs from a healthy donor. PBMC migration was examined in the presence of 5 $\mu\text{g/ml}$ of IGF-I-neutralizing antibody or a control antibody. Assays were done in the presence of 500 ng/ml IGFBP-5 (+) or vehicle (-). Results represent three independent experiments. Data are shown as a mean $\pm$ SD.

Figure 2. IGFBP-5 preferentially induces migration of monocytes, natural killer (NK) cells, and T cells. Migrated PBMCs used in migration assays were analyzed by flow cytometry. Migration assays were performed with vehicle (10 mM HCl), 500 ng/ml recombinant IGFBP-3 (rBP3), 500 ng/ml recombinant IGFBP-5 (rBP5), or RANTES (50 ng/ml) in the lower chamber. Migrated cells were harvested from the lower chamber after a 4-hour incubation and analyzed by flow cytometry. Lymphocytes (for B, T, and NK cells) and monocytes were gated using forward and side scatter. Arrows indicate peaks of positive cells detected by flow cytometry (black). Gray lines indicate PBMCs stained with isotype control.

Phenotype of Cells Migrating in Response to IGFBP-5

To identify the migrating immune cells, the phenotype of cells migrating in response to IGFBP-5 was examined using flow cytometry (Figure 2). PBMCs consisted of 65% CD3⁺ cells,

TABLE 1. PERCENTAGE OF MIGRATED CELLS BY CELL SUBSET (n = 3)

	Marker	Vehicle	rBP3	rBP5	RANTES
T cells	CD3	0.6 ± 0.2	1.0 ± 0.3	3.1 ± 0.3*	18.6 ± 5.0*
B cells	CD19	0.6 ± 0.2	0.9 ± 0.3	1.5 ± 0.3	7.1 ± 0.2*
NK cells	CD56	2.3 ± 1.3	6.7 ± 2.0	16.0 ± 1.2*	37.1 ± 9.0*
Monocytes	CD14	3.0 ± 1.3	5.8 ± 3.4	25.7 ± 9.7*	19.0 ± 0.1*

Definition of abbreviations: NK cells, natural killer cells; RANTES, regulated on activation, normal T cells expressed and secreted; rBP3, recombinant insulin-like growth factor-binding protein-3; rBP5, recombinant insulin-like growth factor-binding protein-5.

* $P < 0.05$ by Mann-Whitney *U*-test, compared with vehicle.

10% CD56⁺ cells, 20% CD14⁺ cells, and 5% CD19⁺ cells. As shown in Table 1, the percentage of migrated cells in each subset was examined. IGFBP-5 induced migration of monocytes (25.7%; 6,189 ± 5,663 cells), NK cells (16.0%; 3,665 ± 1,271 cells), and T cells (3.1%; 3,055 ± 777 cells), but did not significantly affect B cell migration. However, the number of migrating T cells, although reflecting 3% of migrating T cells, represents 25.3% of total PBMCs migrating in response to IGFBP-5. As a comparison, monocytes and NK cells migrating in response to IGFBP-5 represent 41.8 ± 19.9% and 31.8 ± 14.3% of total migrating PBMC, respectively. Of the T cells migrating in response to IGFBP-5 *in vitro*, CD4⁺ T cells were predominant (Figure 3A, Table 2). The CD4/CD8 ratio was significantly higher in IGFBP-5-treated cells compared with vehicle, IGFBP-3, or RANTES ($P < 0.05$). *In vivo*, in IGFBP-5-expressing mouse lungs, infiltration of CD3⁺ cells was prominent (Figure 3B, panels a and b) (17). To determine whether the preferential migration of CD4⁺ T cells *in vitro* also occurs *in vivo* in response to IGFBP-5, we examined infiltrating T cells

Figure 3. IGFBP-5 induces preferential migration of CD4⁺ T cells. (A) Detection of CD4⁺ and CD8⁺ cells in migrated cells. The expression of CD4 and CD8 molecules on migrated cells was analyzed by flow cytometry. Migrated cells were gated to lymphocytes using forward and side scatter. The numbers in upper left and lower right quadrants denote percentage of CD4⁺ and CD8⁺ cells in total lymphocytes, respectively. (B) Representative staining of lung tissues from C57BL/6J mice treated with 10⁹ pfu IGFBP-5-expressing adenovirus (Ad5) or control adenovirus (cAd). Lungs were harvested 8 days after adenoviral administration. Immunohistochemistry was used to detect CD3⁺ cells in cAd and Ad5-treated mouse lung tissues (a and b, respectively; scale bar is 15 μm), and CD4⁺ and CD8⁺ cells in Ad5-treated mouse lung tissues (c and d, respectively; scale bar is 3 μm). (C) Number of CD4⁺ and CD8⁺ cells in mouse lungs expressing IGFBP-5. Three frozen sections were stained with anti-CD4 and CD8 antibodies, respectively, and positive cells were counted in five different high-power fields (×400 magnification). * $P < 0.0003$ by Mann-Whitney *U*-test.

TABLE 2. CD4/CD8 RATIO OF MIGRATING T CELLS (n = 3)

	Vehicle	rBP3	rBP5	RANTES
CD4/CD8 ratio	0.7 ± 0.1	0.4 ± 0.1	1.1 ± 0.1*	0.7 ± 0.1

Definition of abbreviations: RANTES, regulated on activation, normal T cells expressed and secreted; rBP3, recombinant insulin-like growth factor-binding protein-3; rBP5, recombinant insulin-like growth factor-binding protein-5.
* P < 0.05 by Mann-Whitney U-test, compared with vehicle.

in IGFBP-5-expressing mouse lungs by immunohistochemistry using anti-CD4 and CD8 antibodies. In IGFBP-5-expressing mouse lungs, preferential infiltration of CD4⁺ T cells was evident (Figure 3B, panels c and d), confirming and extending the *in vitro* findings.

IGFBP-5 Induces Migration of Monocytes and T Cells Expressing Activation Markers

We also examined the expression of activation markers on the surface of migrated CD4⁺ T cells and monocytes. Expression of HLA-DR on monocytes, and that of CD69, CD45RA, and RO molecules on T cells as activation markers was examined (Figure 4). The ratio of HLA-DR⁺ to HLA-DR⁻ cells in migrating monocytes was significantly increased by IGFBP-5 treatment (Table 3). In CD4⁺ T cells migrating in response to IGFBP-5, the ratio of CD69⁺ to CD69⁻ and CD45RO⁺ to CD45RO⁻ was significantly increased, whereas the ratio of CD45RA⁺ to CD45RA⁻ was significantly decreased. No difference was observed in CD8⁺ T cell subsets (data not shown). To determine whether IGFBP-5 can directly activate immune cells or attract pre-activated cells, PBMCs were incubated with recombinant IGFBP-5 for 4 hours. No up-regulation of activation marker expression or change in the proportion of activated cells was observed (data not shown), suggesting that IGFBP-5 induces the preferential migration of monocytes and CD4⁺ T cells with activation markers.

IGFBP-5-Induced Migration Is Mediated by the MAPK Signaling Pathway

The effect of IGFBP-5 on PBMC migration was IGF-I independent (Figure 1), suggesting that IGFBP-5 is an independent ligand that can trigger intracellular signaling. To identify the signaling pathway regulating IGFBP-5-induced cell migration, we examined the activation status of signaling components by Western blot. As shown in Figure 5A, recombinant IGFBP-5 induced phosphorylation of MEK1/2 and p44/p42 MAPK in PBMCs within 5 minutes after stimulation, but did not induce Akt phosphorylation. We further examined the activation of signaling components upstream of MEK1/2 using monocytes migrating in response to IGFBP-5. IGFBP-5 induced phosphorylation of Raf, a signaling molecule upstream of MEK1/2 (Figure 5B). Whereas IGF-I induces activation of both MAPK and PI3K pathways, IGFBP-5 only induced the activation of MAPK signaling, suggesting that its signaling differs from that of IGF-I. To determine if the chemoattractant activity of IGFBP-5 requires activation of MAPK signaling, migration assays were done in the presence of the MEK1/2 inhibitor, U0126. As shown in Figure 5C, IGFBP-5-induced migration of PBMCs was blocked by the MEK1/2 inhibitor U0126 in a dose-dependent manner.

IGFBP-5 Also Induces Fibroblast Migration

Our previous studies showed that IGFBP-5 expression induces production of ECM components by primary fibroblasts (15) and the development of pulmonary fibrosis (17). We thus hypothesized that fibroblasts might also migrate in response to IGFBP-5. To determine if IGFBP-5 exerts chemoattractant activity on fibroblasts, primary human lung fibroblasts were used in migration assays (Figure 6). Since fibroblasts are adherent cells, cells penetrating through the filter pores and adhering to the bottom side of the membrane were defined as migrated cells, stained with hematoxylin, and counted (Figure 6A). As shown in Figure 6B, the number of migrated fibroblasts was significantly in-

Figure 4. IGFBP-5 preferentially induces migration of activated monocytes and CD4⁺ T cells. Lymphocytes and monocytes were gated using forward and side scatter, and activated monocytes (CD14⁺HLA-DR⁺, upper left), activated CD4⁺ T cells (CD4⁺CD69⁺, lower left; CD4⁺CD45RO⁺, upper right), and naïve CD4⁺ T cells (CD4⁺CD45RA⁺, lower right) were detected using flow cytometry. Representative results from a healthy donor are shown. The numbers denote percentage of cells in each quadrant in gated monocytes or lymphocytes, respectively.

TABLE 3. RATIO OF MONOCYTES AND CD4⁺ T CELLS WITH AND WITHOUT ACTIVATION MARKERS (n = 3)

	Pre-Migration	Vehicle	rBP5
Monocytes (CD14 ⁺)			
HLA-DR ⁺ / HLA-DR ⁻	0.04	0.5 ± 0.2	1.1 ± 0.0*
CD4 ⁺ T cells (CD4 ⁺)			
CD69 ⁺ / CD69 ⁻	0.04	0.1 ± 0.1	0.3 ± 0.1*
CD45RO ⁺ / CD45RO ⁻	0.2	0.7 ± 0.1	1.2 ± 0.1*
CD45RA ⁺ / CD45RA ⁻	1.4	1.6 ± 0.2	0.6 ± 0.1*

Definition of abbreviation: rBP5, recombinant insulin-like growth factor-binding protein-5.

* $P < 0.05$ by Mann-Whitney U-test, compared with vehicle.

creased by IGFBP-5 compared with recombinant IGFBP-3 or vehicle, suggesting that IGFBP-5 also induces migration of primary lung fibroblasts.

Monocytes Infiltrating IGFBP-5-Expressing Lung Tissues Have a Mesenchymal Phenotype

We recently reported that IGFBP-5-triggered pulmonary fibrosis *in vivo* was preceded by prominent infiltration of mononuclear cells (17). This observation raised the question whether infiltrating monocytes can acquire a mesenchymal phenotype

and are a secondary source of ECM in IGFBP-5-induced fibrosis. To examine this possibility, the acquisition of mesenchymal cell characteristics was assessed by monocyte expression of α -SMA and fibronectin *in vivo* and *in vitro*. As shown in Figures 7A and 7B, monocytes infiltrating IGFBP-5-expressing lungs co-expressed α -SMA and MAC-1. The number of double-positive cells expressing both MAC-1 and α -SMA was significantly increased in AdIGFBP-5-treated compared with cAd-treated lungs (33.0 ± 3.6 versus 3.7 ± 0.6 , $P < 0.05$) (Figure 7C). Furthermore, IGFBP-5 induced expression of α -SMA and fibronectin in human monocytes cultured *in vitro* with recombinant IGFBP-5 in a time-dependent (Figure 7D) and dose-dependent manner (Figure 7E). Monocytes stimulated with IGFBP-5 acquired a spindle-like morphology and expressed both α -SMA and fibronectin (Figure 7F). At 72 hours, monocytes treated with IGFBP-5 did not have detectable collagen type I expression, nor did their expression of CD34 increase in response to IGFBP-5 (data not shown). Thus IGFBP-5-treated monocytes do not express typical fibrocyte markers after 72 hours in culture. Longer-term cultures are not feasible, as monocytes are deprived of serum to avoid introducing growth factors such as IGF and thus cell viability decreases after 72 hours. Together, our findings suggest that a subpopulation of monocytes have acquired phenotypic characteristics of mesenchymal cells after stimulation with IGFBP-5.

Figure 5. IGFBP-5 induces activation of the MAPK signaling in PBMCs and monocytes. (A) Western blot analysis using cellular lysates of PBMCs stimulated with recombinant IGFBP-5. PBMCs (2×10^6) were treated with 500 ng/ml recombinant IGFBP-5 (rBP5), and cellular lysates were harvested at 5, 10, 15, and 30 minutes after treatment. Phosphorylated and total proteins were detected by Western blot. Cellular lysates of 3×10^5 cell-equivalent were applied to each lane. Glyceraldehyde phosphate dehydrogenase (GAPDH) is shown as a loading control. (B) Western blot analysis using cellular lysates of monocytes stimulated with recombinant IGFBP-5. PBMCs (2×10^6) were plated onto 35-mm dishes, and nonadherent cells were removed after 1 hour of incubation. Adherent cells were treated with 500 ng/ml recombinant IGFBP-5, and cellular lysates were harvested at 5, 10, 15, and 30 minutes after treatment. Phosphorylated and total proteins were detected by Western blot. Cellular lysates of 4×10^4 cell-equivalent were applied to each lane. GAPDH is shown as a loading control. (C) IGFBP-5-induced migration is blocked by MEK1/2 inhibition. Migration assays were performed in the presence of 2 and 10 μ M MEK1/2 inhibitor, U0126, or equivalent volumes of vehicle (DMSO). PBMCs were pre-treated with increasing concentrations of U0126 or DMSO as a vehicle control in the upper chamber. IGFBP-5 (500 ng/ml) was added to the lower chamber. After a 4-hour incubation, cells were harvested from both chambers and the percentage of migrated cells was calculated. Data shown represent mean three independent experiments; values represent mean $\pm$ SD. * $P < 0.05$ by Mann-Whitney U-test.

Figure 6. IGFBP-5 induces migration of primary human lung fibroblasts. Primary human lung fibroblasts from a normal donor were used in a migration assay with vehicle as a control, 500 ng/ml recombinant IGFBP-3 (rBP3), 500 ng/ml recombinant human IGFBP-5 (rBP5), or 20 μ g/ml fibronectin (FN) as a positive control. (A) Images of fibroblasts migrating through the trans-well membrane. Cells were stained with hematoxylin. Scale is 15 μ m. (B) Graphical presentation of fibroblast migration. Data represent mean $\pm$ SD of 12 independent fields. * P < 0.0003 by Mann-Whitney U-test.

DISCUSSION

We previously reported that intratracheal administration of IGFBP-5-expressing adenovirus into mouse lungs results in the development of pulmonary fibrosis (17). In IGFBP-5-treated mouse lung, prominent infiltration of mononuclear cells and interstitial fibrosis were observed. Lung-infiltrating cells were predominantly T lymphocytes 8 days after administration (17). In this study, we extend our findings and demonstrate that IGFBP-5 plays a role in the inflammatory phase preceding and coinciding with the fibrotic phase. IGFBP-5-induced migration of monocytes, NK cells and T cells, and migrating cells expressed activation markers. Of the T lymphocytes, CD4⁺ T cells were predominant both *in vitro* and *in vivo*, suggesting that CD4⁺ T cells are preferentially involved in the early phase of IGFBP-5-induced fibrosis. IGFBP-5-induced migration of immune cells was mediated by an MAPK-dependent and IGF-I-independent pathway. Moreover, IGFBP-5 triggered migration of primary lung fibroblasts and induced the transition of monocytes into mesenchymal cells, suggesting that infiltration of these cells into the interstitium may also contribute to the early stages of fibrosis.

IGFBP-5 has been shown to induce migration of mouse embryonic cells (22), porcine vascular smooth muscle cells (23), and rat glomerular mesangial cells (24). However, the chemo-attractant activity of IGFBP-5 on human cells and specifically fibroblasts and immune cells has not been previously described. Our study is the first to demonstrate that IGFBP-5 has a chemo-

attractant activity for immune cells and fibroblasts *in vivo* and *in vitro*. Moreover, we show that IGFBP-5 induces cell migration in an IGF-I-independent manner, which parallels previous findings in vascular smooth muscle cells (23) and glomerular mesangial cells (24). Thus, IGFBP-5 can act independently to modulate immune and pro-fibrotic responses.

Our results demonstrate that IGFBP-5 preferentially induces migration of CD4⁺CD45RO⁺ T cells and CD14⁺ HLA-DR⁺ monocytes. CD45RO⁺ T cells are increased in lung interstitium of patients with SSc with fibrosing alveolitis (25) and of patients with IPF (26). Since IGFBP-5 is highly expressed in pulmonary fibrosis in IPF and SSc (15), IGFBP-5-induced migration can explain, at least in part, the phenotype of T cells detected *in vivo* in human diseases. Infiltration of these cells may contribute to the development of fibrosis by expression of cytokines such as TGF- β . On the other hand, monocytes are also thought to play an important role in the development of fibrosis. CD14⁺ monocytes are described as mesenchymal progenitors (27), or fibrocytes (28), which can express ECM components such as collagen type I. Moreover, HLA-DR-expressing monocytes can transform into neo-fibroblasts (29). Thus, CD14⁺ HLA-DR⁺ monocytes might be an additional source of ECM and contribute to the development of fibrosis. In this study, we examined HLA class II expression on monocytes as an activation marker on the surface of monocytes/macrophages based on previous reports (30). More recently, HLA-DR expression on the surface of CD14⁺ cells was suggested as a phenotypic marker of a specific subset of monocytes (31). Since circulating monocytes are heterogeneous, it is possible that monocytes migrating in response to IGFBP-5 represent a specific subset of monocytes rather than activated monocytes.

In lung tissues of patients with IPF, α -SMA-expressing myofibroblasts form fibroblastic/myofibroblastic foci (32). The source of these cells is believed to be resident fibroblasts activated by cytokines and/or chemokines. Our results suggest that α -SMA is also expressed by IGFBP-5-stimulated monocytes. Since we have shown that IGFBP-5 is increased *in vivo* in IPF lung tissues (17), and that IGFBP-5 induces α -SMA expression in primary fibroblasts (16, 17), it is likely that the source of α -SMA-expressing cells in lung tissues expressing IGFBP-5 includes fibroblasts and monocytes that have acquired a myofibroblastic phenotype. In culture, IGFBP-5-treated monocytes did not express collagen I, nor did their expression of CD34 increase, suggesting that either they are not fibrocytes, or that treatment of monocytes for 72 hours is not sufficient to induce fibrocyte markers. Thus cells that contribute to ECM production and remodeling in fibrosis include fibroblasts (32), fibrocytes (28), bone marrow-derived mesenchymal cells (33), or multi-potential peripheral blood monocytes (27).

IGF-I also induces migration of many cell types, including T lymphocytes and human mesenchymal progenitor cells (34), and IGFBPs may enhance the effect of IGF-I (19). The signaling cascades activated by IGF-I via IGF-I receptor have been thoroughly examined. However, information on signaling induced by IGFBP-5 has been limited to studies in mouse and human osteoblasts and smooth muscle cells. In these cells, IGFBP-5 has been shown to activate p38 kinase and P44/P42 MAPK (21, 35). IGF-I, acting via IGF-I receptor, activates the MAPK pathway, including P44/P42 MAPK, JNK, p38 kinase, and the PI3K pathways (36). However, based on our results, IGFBP-5 only activates the MAPK pathway in human mononuclear cells, and paracrine/autocrine IGF-I did not affect IGFBP-5-induced PBMC migration, suggesting that IGFBP-5 exerts its effect independently of IGF-I and the IGF-I receptor.

We show that IGFBP-5-induced migration requires activation of the MAPK signaling cascade. These findings support

Figure 7. IGFBP-5 induces up-regulation of mesenchymal markers in monocytes. (A and B) Immunofluorescence staining of mouse lung infected with IGFBP-5-expressing adenovirus (Ad5) or control (cAd). Ad5 or cAd was administered intratracheally into wild-type C57BL/6J mice, and lung tissues were harvested on Day 14. Tissue sections were stained with anti- α -smooth muscle actin (α -SMA) (FITC, green) and Mac-1 (Texas Red, red) antibodies. Co-localization of α -SMA and MAC-1 is shown in yellow (broken arrows). Strong yellow homogeneous staining (solid arrows) indicates autofluorescence of red blood cells. Scales are (A) 15 μ m and (B) 3 μ m. (C) Quantitation of MAC-1/ α -SMA double-positive cells. Cells staining for both MAC-1 and α -SMA were counted in three independent fields; * $P < 0.05$. (D) Western blot analysis using cellular lysates of monocytes stimulated with recombinant IGFBP-5. PBMCs (2×10^6) were plated onto 35-mm dishes, and adherent cells were treated with 500 ng/ml recombinant IGFBP-5. Cellular lysates were harvested at 0, 6, 48, and 72 hours after treatment. Expression of fibronectin and α -SMA was detected by Western blot. Cellular lysates of 4×10^4 cell-equivalent were applied to each lane. GAPDH is shown as a loading control. (E) Monocytes were treated as in D, except that concentrations of rIGFBP-5 ranging from 50–500 ng/ml were used for 72 hours. RecIGFBP-3 at 500 ng/ml was used for comparison. (F) Monocytes were treated with vehicle, recIGFBP-3, or recIGFBP-5 for 72 hours. Immunofluorescence was used to detect fibronectin and α -SMA. DAPI was used to detect nuclei.

previous reports showing that MAPK signaling and c-Raf activation play an important role in the regulation of cell migration (37, 38). Our findings identify MAPK activation as a key signaling pathway in IGFBP-5-induced cell migration. We observed preferential migration of activated immune cells in response to IGFBP-5. It is conceivable that these cells may have increased number of IGFBP-5 receptors compared with non-activated cells. A specific receptor for IGFBP-5 has been suggested (39), but not identified. Identification of the IGFBP-5 receptor should facilitate studies designed to determine the mechanism mediating IGFBP-5-induced migration of activated cells.

The regions of IGFBP-5 that mediate its chemoattractant activity are still unclear. One previous report showed that a peptide identical to amino acids 201–218 of IGFBP-5 induced migration of rat glomerular mesangial cells (40). The IGFBP family of proteins contains a series of conserved cysteines (41), and IGFBPs are cleaved by several proteases in the extracellular milieu (42). It is thus possible that IGFBP-5 in the extracellular milieu is degraded by proteases, and its degraded products exert chemoattractant activities. Chemokines, which

also have conserved cysteine motifs, and their receptors, are essential components of Th1- and Th2-mediated responses (43). Moreover, chemokine levels are increased in the serum and BAL of patients with SSc-associated pulmonary fibrosis (44, 45). Recently, up-regulated expression of CCL11 was reported to induce fibrosis (46). Furthermore, CXCR12- and CCR2-mediated fibrocyte recruitment was associated with fibrosis (47, 48). Thus, certain chemokines and their receptors exert both chemoattractant activity and profibrotic effects. Since we previously reported that IGFBP-5 also induces infiltration of inflammatory cells and fibrosis (17), IGFBP-5's effects may parallel those of chemokines with respect to migration and fibrosis. The effects of IGFBP-5 on cell migration appear to be direct as IGFBP-5 did not induce expression of 84 different chemokines or chemokine receptors profiled using Oligo GEArray human chemokines and receptors microarray (SA Biosciences Frederick, MD) (data not shown).

The association between fibrosis and inflammation in IPF is controversial. In SSc-associated pulmonary fibrosis, alveolitis usually precedes chronic fibrosis, suggesting that inflammation contributes to the development of lung fibrosis (49). It has been

proposed that infiltrating inflammatory cells play an important role in the progression of fibrosis (50); however, the significance of the interaction between inflammatory cells and fibroblasts is not clear. Several studies have suggested that a shift to a T helper 2 (Th2) response is associated with the development of fibroproliferative diseases (51). Our results suggest that CD4⁺ T cells preferentially migrating in response to IGFBP-5 do so before and during the development of fibrosis (17). Furthermore, IGFBP-5-treated monocytes acquire mesenchymal properties, which, in concert with the activation of fibroblasts by IGFBP-5 to produce ECM, further implicate IGFBP-5 in the development of inflammation and fibrosis. Together, our findings suggest that infiltration of immune cells and their concomitant activation may contribute to the development and/or progression of fibrosis.

In summary, we have shown that IGFBP-5 induces migration of immune cells and fibroblasts. IGFBP-5 also results in the acquisition of mesenchymal markers by monocytes. Our previous findings describing IGFBP-5's profibrotic effects *in vitro* and *in vivo* (15–17), and our current observations, identify new mechanisms by which IGFBP-5 triggers fibrosis and establish IGFBP-5 as an important mediator of inflammation and fibrosis.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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