Human Mast Cell Basic Fibroblast Growth Factor in Pulmonary Fibrotic Disorders

Yoshikazu Inoue, Talmadge E. King, Jr., Sally S. Tinkle, Karen Dockstader, and Lee S. Newman

Mast cells (MCs) are abundant in fibrotic tissue, although their role in fibrogenesis remains obscure. Recent studies suggest MCs may produce basic fibroblast growth factor (bFGF). To evaluate the hypothesis that MC bFGF contributes to the fibrotic response in human interstitial lung disease, we analyzed lung tissue, bronchoalveolar lavage fluid and serum in 1) idiopathic pulmonary fibrosis, 2) chronic beryllium disease and sarcoidosis, 3) control subjects with no disease or who were beryllium sensitized with normal lung histology. Diseased subjects underwent clinical assessments to stage disease severity. We determined that most bFGF+ cells in lung interstitium are MCs and are most abundant in idiopathic pulmonary fibrosis. Distribution of bFGF+ MCs matched that of extracellular matrix deposition and correlated with the extent of fibrosis morphometrically. Only one bFGF isoform (17.8 kDa) was found in idiopathic pulmonary fibrosis and chronic beryllium disease lung tissues and interacted with heparanase-like molecules in the lung. Using a human MC line, we verified that MCs express bFGF mRNA and protein that localizes to cytoplasmic granules. Clinically, bFGF concentrations in bronchoalveolar lavage fluid and serum were highest in disease states and correlated with bronchoalveolar lavage cellularity and severity of gas exchange abnormalities, supporting a role for MC bFGF in the pulmonary fibrotic response and its clinical consequence. (Am J Pathol 1996, 149:2037-2054)

The fibrotic process is important for normal tissue repair and wound healing. However, if unchecked, fibrotic responses may alter organ structure and compromise physiological functions. Pulmonary fibrosis represents the stereotypic final pathological consequence of alveolar injury produced by numerous inciting agents, including infections, drugs, chemicals, organic and inorganic dusts, gases, fumes, vapors, and autoimmune diseases. Unfortunately, the regulatory mechanisms that control pulmonary fibrosis remain enigmatic.

Interestingly, mast cells (MCs) are abundant in fibrotic lungs in both animal models of fibrosis and in diseased humans.1-3 However, the role these cells play in the fibroproliferative process remains obscure. Murine MCs have been shown to produce mediators and growth factors that may contribute to fibrosis, including tryptase4, tumor necrosis factor-α,5,6 interleukin-1,7 and transforming growth factor-β (TGF-β).8 Importantly, studies using MC-deficient mice (WBB6F1-W/Wv) show less silica-induced,9 ozone-induced,10 and fungal-antigen-induced11 fibrosis and inflammation than in normal mice. However, bleomycin, a potent fibrotic stimulus, does induce pulmonary fibrosis even in these WBB6F1-W/Wv mice.12 Recent studies have suggested that human MCs are a potential source of fibrogenic factors.13 Thus, MCs appear to be critical to the development of experimental and human pulmonary fibrosis. However, the exact mechanism of these cells’ involvement remains ill defined.

Several lines of evidence suggest that basic fibroblast growth factor (bFGF; FGF-2) may be important in the fibroproliferative process. bFGF is synthesized by alveolar macrophages, fibroblasts, T lympho-

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cytes, and endothelial cells.\textsuperscript{14–16} It is bound to heparin sulfate proteoglycans in extracellular matrix and basement membrane.\textsuperscript{17} bFGF is a potent mitogenic and chemotactic factor for cells of mesodermal, ectodermal, and endodermal origin,\textsuperscript{18,19} including fibroblasts, smooth muscle cells, and alveolar type II cells\textsuperscript{20,21}, regulating proliferation and extracellular matrix production by these cells \textit{in vitro}.\textsuperscript{20–22}

High levels of bFGF have been found in the lower respiratory tract of patients with acute alveolar injury,\textsuperscript{23} in alveolar macrophages from patients with lung injury,\textsuperscript{14} in the excised fibrotic lesions of Dupuytren’s contractures,\textsuperscript{24} and in carbon-tetrachloride-induced hepatic fibrosis.\textsuperscript{25} \textit{In vivo}, the administration of exogenous bFGF causes a rapid neovascularization in the cornea and kidney and fibroplasia in the dermis.\textsuperscript{15} Recent reports confirm the presence of bFGF in human MCs,\textsuperscript{26,27} leading us to hypothesize that MC bFGF may promote chronic pulmonary fibrosis in humans. To test this hypothesis, we used immunohistochemical, biochemical, and molecular methods to study the contribution of bFGF in chronic interstitial lung disease including 1) idiopathic pulmonary fibrosis (IPF), in which fibrosis is severe and diffuse, and 2) the two granulomatous disorders chronic beryllium disease (CBD) and sarcoidosis (Sar) in which fibrosis occurs in localized bands surrounding granulomas. In addition, to examine the mechanisms of bFGF secretion by human MCs, we studied the human mast cell line HMC-1 \textit{in vitro}.\textsuperscript{28}

\textbf{Table 1. Demographics of Study Subjects}

<table>
<thead>
<tr>
<th>Race</th>
<th>Number of subjects</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Smoking status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-Hispanic,</td>
<td></td>
<td></td>
<td>Current smoker</td>
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<tr>
<td></td>
<td>Caucasian</td>
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<tr>
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<td>Hispanic,</td>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>African American</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n = 18)</td>
<td>34 (21–55)</td>
<td>16</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>BeS (n = 8)</td>
<td>48 (34–62)</td>
<td>6</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>CBD (n = 23)</td>
<td>46 (37–70)*</td>
<td>15</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Sar (n = 10)</td>
<td>39 (27–67)*</td>
<td>8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>IPF (n = 21)</td>
<td>60 (28–75)*</td>
<td>21</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Age data are expressed as median (range).
*\(P < 0.05\) versus control.
*\(P < 0.05\) versus IPF.

\textbf{Materials and Methods}

\textbf{Study Population}

The population for our studies included 80 subjects: 21 with IPF, 23 with CBD, 10 with Sar, 8 beryllium-sensitized subjects without lung disease (BeS), and 18 normal control subjects described in Table 1. These subjects were enrolled in a study supported by a Specialized Center of Research Grant from the National Institutes of Health. Informed consent was obtained from each patient, and the protocol was approved by our Institutional Human Subjects Review Board.

\textbf{Idiopathic Pulmonary Fibrosis}

Lung tissues from 11 patients with IPF were used for morphometry. Lung tissue was obtained by open or thoracoscopic lung biopsy in 7 subjects and at autopsy in 4 subjects. Bronchoalveolar lavage fluid (BAL) was obtained from 10 IPF subjects. The diagnosis of IPF was established according to previously described clinical and histological criteria.\textsuperscript{29,30} Patients with connective tissue disease, drug, or chemical exposure or other possible etiologies of interstitial lung disease, such as bronchiolitis obliterans and organizing pneumonia, diffuse alveolar damage, lymphocytic interstitial pneumonia or nonclassified forms of chronic interstitial pneumonia, were excluded from this study group.

\textbf{Chronic Beryllium Disease}

Lung tissue was obtained from 13 patients with CBD by transbronchial lung biopsy (TBLB). BAL and sera were obtained from 18 patients with CBD. The diagnosis of CBD was based on the following criteria:\textsuperscript{31} 1) history of beryllium exposure, 2) positive BAL beryllium lymphocyte proliferation test, 3) transbronchial lung biopsy yielding pathological changes consistent with CBD, ie, noncaseating granulomas and/or mononuclear cell infiltrates, and 4) respiratory symptoms, pulmonary function test abnormalities, or chest radiographic small opacities profusion score greater than or equal to 1/0 by the International Labour Organization classification system.\textsuperscript{32} In addition, none of the individuals had other detectable causes of granulomatous inflammation and all had
negative cultures and special stains for acid-fast bacilli, fungi, and bacteria.

**Sarcoidosis**

Lung tissue was obtained from 10 patients with Sar, including 6 by TBLB and 4 by open-lung biopsy. The diagnosis of Sar was based upon a compatible clinical history and chest x-ray abnormalities and lung biopsy demonstrating noncaseating epithelioid granulomas in the absence of other detectable causes of granulomatous inflammation and negative culture and special stains for acid-fast bacilli, fungi, and bacteria.

**Beryllium-Sensitized Subjects without Lung Disease**

Lung tissues were obtained from 8 BeS subjects by TBLB. Individuals included in this category were required to meet the following criteria: 1) a history of beryllium exposure, 2) abnormal blood beryllium lymphocyte proliferation test, 3) negative BAL beryllium lymphocyte proliferation test, and 4) no pathological evidence of CBD on TBLB.

**Controls**

Eighteen subjects served as controls. Lung tissues obtained from ten controls included lung allograft donors and five TBLBs from subjects who had a history of beryllium exposure but without evidence of either BeS or CBD. BAL and sera were obtained from eight controls.

**Sample Collection and Preparation**

BAL was performed by previously published methods using four 60-ml aliquots of normal saline in a subsegment of the right middle lobe. The cellular and BAL fluid components were separated by centrifugation and stored at −70°C until use. Total cell counts and cell differentials were obtained by hemocytometer and Diff-Quik staining, respectively. Serum was obtained from study subjects on the same day as BAL and stored at −70°C until use. Lung tissue was fixed with 10% formalin and embedded in paraffin. One paraffin block was used for each subject. Each block contained two to three specimens obtained by open-lung biopsy, lung transplantation, or autopsy or a minimum of six specimens when obtained by TBLB. Each block was cut into 2-μm-thick serial sections and laid on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA). All samples were stained using hematoxylin and eosin H&E, Movat's pentachrome, and one or more immunohistochemical stains discussed below.

**Clinical Measurements**

**Pulmonary Physiology**

Lung function was assessed in all subjects with IPF, CBD, or Sar. We measured forced vital capacity and forced expiratory volume in 1 second with a recording spirometer or a pneumotachograph, reporting maximal values obtained from three satisfactory maneuvers. Normal predicted values were derived from the work of Morris and co-workers. We measured total lung capacity, residual volume, and thoracic gas volume in a constant-pressure body plethysmograph using the predicted normal values of Goldman and Becklake. We used the single-breath method of Ogilvie and co-workers to determine the diffusing capacity for carbon monoxide. Carbon monoxide diffusing capacity data are presented as the percentage of predicted normal values of Crapo and Morris. We evaluated these subjects' maximal exercise capacity and gas exchange during exercise using a Seimens Elema 380B cycle ergometer, with continuous monitoring of cardiac rhythm and oxygen saturation (Hewlett-Packard, Waltham, MA). A mass spectrometer (1100 medical gas analyzer; Perkin-Elmer Medical Instruments, Pomona, CA) was used to measure inspired air and expired oxygen and carbon dioxide concentrations. Using an ABL-2 blood gas analyzer (Radiometer, Copenhagen, Denmark), we measured arterial blood gases at rest and after each minute of graded exercise, obtained through an indwelling arterial catheter in the radial or brachial artery.

**Radiology**

We scored the radiographic severity of interstitial lung disease using standard posteroanterior chest radiographs according to the International Labour Organization classification system. All films were scored by a certified B-reader, trained to score the severity of interstitial lung disease, who was blind to diagnosis. Normal profusion of small opacities was defined as a profusion score less than or equal to 0/1. Data were analyzed on a 0- to 10-point ordinal scale in which profusion ranks 0/1- and 0/0 were combined.
Immunohistochemical Staining of bFGF, Tryptase, and CD68

To examine the relationships among MCs, macrophages, bFGF, and fibrosis, we employed a combination of immunohistochemical and conventional staining methods. We used two different monoclonal antibodies directed against bovine bFGF, type I (1 μg/ml, IgG1κ, neutralizing antibody) and type II (5 μg/ml, IgG3κ, non-neutralizing antibody) from Upstate Biotechnology (Lake Placid, NY) for immunostaining of bFGF, principally using the type II antibody. These antibodies are highly specific for bovine, human, rat and mouse bFGF.40 Anti-human MC tryptase monoclonal antibody (1 μg/ml, subclass IgG1κ; Dako, Carpinteria, CA) and anti-human CD68 monoclonal antibody (3.6 μg/ml, PG-M1, IgG3κ; Dako) were used to identify MCs and macrophages, respectively. Immunohistochemistry was performed by avidin-biotin complex (ABC) peroxidase methods using VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, CA). After incubation in 0.3% H2O2 dissolved in methyl alcohol for 30 minutes, sections were treated with 1 mg/ml hyaluronidase (Sigma Chemical Co., St. Louis, MO) for 30 minutes at 37°C. After blocking with 1.5% normal horse serum for 30 minutes, we incubated sections with primary antibody at the appropriate dilution in 1.5% normal horse serum for 60 minutes, washed in Tris-buffered saline, pH 7.3, and incubated in 200X diluted biotinylated horse anti-mouse IgG antibody for 30 minutes at room temperature. After washing in Tris-buffered saline, sections were incubated in horseradish-peroxidase-conjugated ABC for 30 minutes. We used 3′-diaminobenzidine (Vector Laboratories) as the substrate for peroxidase. Counterstaining was performed with either hematoxylin or Movat’s pentachrome. Diaminobenzidine and nickel were used as the substrate of peroxidase with our Movat’s pentachrome counterstains.

Specificity of immunolabeling of bFGF was tested under various conditions. We omitted the first antibody, second antibody, or ABC. We used normal mouse serum, normal nonimmune mouse IgG (Sigma), anti-human platelet-derived growth factor (PDGF-BB) monoclonal antibody (IgG2b, Upstate Biotechnology), and anti-human TGF-β monoclonal antibody (IgGk, Genzyme Corp., Cambridge, MA) in lieu of primary antibody. Sufficient human recombinant bFGF (Upstate Biotechnology) was incubated for 60 minutes at room temperature in the presence of anti-bFGF antibody, type II, to preabsorb the antibody. To inhibit nonimmunological binding of monoclonal antibody to the heparin in MCs, the buffers for dilution of antibody and washing solution were acidified to pH 6.0 using 2-(N-morpholino)ethanesulfonic acid (Sigma) buffer containing 150 mmol/L NaCl, pH 6.0, and with high-salt-containing washing buffer (400 mmol/L NaCl).41

To identify bFGF-immunopositive cell types (bFGF+ cells), we double labeled sections with anti-bFGF plus anti-tryptase or with anti-bFGF plus anti-CD68 using combined ABC-peroxidase and ABC-alkaline phosphatase methods (Vectastain ABC-AP kit, Vector Laboratories). Diaminobenzidine was used as the substrate of horseradish peroxidase and Vector Red (Vector Laboratories) as the substrate of alkaline phosphatase. Levamisole (1.25 mmol/L; Vector Laboratories) was applied to the alkaline phosphatase substrate. For this experiment, we used several controls: 1) anti-bFGF antibody first and anti-tryptase or anti-CD68 antibody later, 2) anti-tryptase or anti-CD68 antibody first and anti-bFGF antibody later, and 3) omission of the first antibody and/or secondary antibody.

To stain bFGF in the human MC line HMC-1, cells were fixed in 4% paraformaldehyde and attached to slides by cyto spin. The slides were stained using anti-bFGF monoclonal antibody, type II, as described above. Normal mouse IgG was used in lieu of anti-bFGF antibody as a negative control.

To test for interactions of bFGF with heparin-like molecules, the following histochemical studies were performed: 1) in the presence of 1000 U/ml heparin (Sigma), we preincubated anti-bFGF antibody for 60 minutes at room temperature before use as the first antibody in our immunohistochemistry procedure; 2) lung tissue sections were incubated in 1000 U/ml heparin for 60 minutes at room temperature, washed, and then immunostained with anti-bFGF antibody as above; and 3) lung tissue sections were incubated in solution containing 20 U/ml heparinase (heparinase I, Sigma), 100 mmol/L 3-[N-Morpholino]propanesulfonic acid, and 5 mmol/L CaCl₂ at pH 7.0 for 3 hours at 37°C before immunostaining of bFGF.

Morphometric Analysis

We performed quantitative morphometric analysis of all lung histology specimens using computer-assisted videomicroscopy as described previously.42,43 By this method we can determine the volume of each cell type or tissue component of interest relative to the total volume of lung tissue examined. In brief, to determine the volume density (δv) of interest (eg, bFGF+ cells), microscopic images of each histology section were captured and stored in the computer. A grid (42 or 125 points) was super-
imposed on each image, points of the interest intersecting the grid counted, and the \( V_r \) calculated. We counted 12 random fields from each tissue section from which we calculated a mean value for each section. The number of fields in each section was determined before quantitation, by preliminary counting using TBLB and open-lung biopsy sections and calculating the minimal number of images needed for minimal variance.\(^4\) To normalize for potential artifacts created by compression or expansion of lung specimens due to fixation or biopsy technique, the \( V_r \) of each component was corrected for the \( V_r \) of parenchymal lung tissue measured on H&E-stained sections at \( \times 10 \) magnification with a 42-point grid by the following formula:\(^4\) Normalized \( V_r \) = (\( V_r \) of the component of interest \( \times 100 \))/\( V_r \) of lung tissue.

The \( V_r \) of bFGF\(^+ \) cells and \( V_r \) of tryptase-immunopositive cells were calculated by counting the grid points that hit immunopositive cells (histiocyte-like cells, described below) in 1) the interstitium and 2) the alveolus at \( \times 400 \) magnification using a 125-point grid. The \( V_r \) of total immunopositive cells was calculated by summing the \( V_r \) of interstitial cells and the \( V_r \) of alveolar immunopositive cells. To determine the degree of lung fibrosis, we quantified the \( V_r \) of collagen/reticular fibers and the \( V_r \) of elastic fibers on sections stained by Movat’s pentachrome\(^3\) measured at \( \times 40 \) magnification with a 42-point grid. The \( V_r \) of granuloma was quantified in CBD and Sar cases at \( \times 10 \) magnification using a 42-point grid.

**Culture of HMC-1 and Human Liver Adenocarcinoma (SK-HEP-1) Cell Lines**

To test for MC production of bFGF in vitro, we cultured HMC-1 (generously provided by Dr. Joseph H. Butterfield, Mayo Clinic and Mayo Foundation, Rochester, MN), a cell line established from the peripheral blood of a patient with MC leukemia.\(^4\) We used the human liver adenocarcinoma cell line SK-HEP-1 (American Type Culture Collection, Rockville, MD) as a positive control for bFGF.\(^4\) HMC-1 cells were cultured in Iscove’s medium (BioWhittaker, Walkersville, MD) with 10% defined iron-supplemented calf serum (HyClone, Logan, UT), 1.2 mmol/L \( \alpha \)-thioglycerol (Sigma), \( \gamma \)-glutamline, penicillin, and streptomycin (BioWhittaker) at 5% \( CO_2 \) and 37°C. SK-HEP-1 cells were cultured in Dulbecco’s modified Eagle’s medium (BioWhittaker) with 10% defined iron-supplemented calf serum containing \( \gamma \)-glutamline, penicillin, and streptomycin at 5% \( CO_2 \) and 37°C.

After 24 and 48 hours in culture, HMC-1 cells were centrifuged and lysed in extraction buffer consisting of 400 mmol/L NaCl, 50 mmol/L Tris/HCl pH 7.5, containing 1% Nonidet P-40, 4 mg/ml aprotinin, 220 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L leupeptin, and 2 mmol/L prostatin A (all from Sigma). bFGF concentrations in supernatants and in cell lysates were measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN). The concentrations of bFGF protein in cell lysates and supernatants are expressed as picograms per 10\(^6\) cells. This ELISA system detects both the recombinant 17.8-kd human bFGF and high molecular weight 24-kd bFGF, with a published detection limit of 0.25 pg/ml bFGF.

**Western Blot Analysis**

Western blotting was performed to identify specific isoforms of the bFGF protein in frozen lung tissue specimens from subjects with IPF and CBD and controls and in both freshly cultured HMC-1 and the SK-HEP-1 line, which is known to express four isoforms of bFGF. One gram (weight) of lung tissue was placed in 2.5 ml of extraction buffer and homogenized for 30 seconds, four times, at 4°C. The homogenized samples were frozen once, thawed quickly, sonicated at 50 W at 4°C, and centrifuged at 20,000 \( \times g \) for 30 minutes at 4°C, and the supernatants were collected. HMC-1 or SK-HEP-1 cells (10\(^7\)) were placed in 1 ml of extraction buffer, sonicated, and centrifuged, and the supernatants were collected.

Crude extracted samples were diluted twice with 400 mmol/L NaCl, 50 mmol/L Tris/HCl, pH 7.5, and incubated with 250 \( \mu l \) of prewashed and equilibrated heparin acrylic beads (Sigma) in an end-over mixer overnight at 4°C. The gels were then washed with 10 ml of 600 mmol/L NaCl, 10 mmol/L Tris/HCl, pH 7.2.\(^4\) We eluted the bFGF fraction with Laemmli buffer,\(^4\) boiled samples for 3 minutes with dithiothreitol (Sigma), and applied them to 15% polyacrylamide gels containing sodium dodecyl sulfate (SDS). The proteins were transferred to nitrocellulose membranes (BioRad Laboratory, Hercules, CA), blocked with 3% dry milk in Tris-buffered saline containing 0.05% Tween 20 overnight at 4°C, and incubated with 5 \( \mu g/ml \) anti-bFGF monoclonal antibody, type II, for 4 hours at room temperature. Membranes were washed, incubated with sheep anti-mouse IgG and peroxidase-linked F(ab'\(^2\)) fragment (2000X; Amersham, Arlington Heights, IL), treated with enhanced chemiluminescence solution (Amersham), and exposed to autoradiography film.
Analysis of Mast Cell bFGF mRNA

To determine whether human MCs express bFGF mRNA, we performed reverse transcription and polymerase chain reaction (RT-PCR) analysis of HMC-1 RNA. Total RNA was isolated from 10^6 HMC-1 cells and from 10^6 SK-HEP-1 cells using 4 mol/L guanidinium isothiocyanate, 25 mmol/L sodium citrate, pH 7.0, 0.5% sodium N-lauroylsarcosine, 0.1 mol/L 2-mercaptoethanol, and the glass MAX RNA microisolation spin cartridge system (Gibco BRL, Grand Island, NY). We performed reverse transcription with avian myeloblastosis virus reverse transcriptase and oligo(dT) on total RNA as previously described. The bFGF primers used were 5'-CG-GCTGTACTGCAAAAACGGG-3' and 5'-GCTCTTAGCAGACATTGGAAG-3'. These primers encompass a 375-bp nucleic acid region encoding amino acids 31 to 37 (oligonucleotide) and amino acids 149 to 155 (3'-oligonucleotide), respectively. Primers for the housekeeping gene β-actin were purchased from Clontech Laboratory (Palo Alto, CA). The complementary DNA was amplified in a 50-μl reaction volume containing 0.2 mmol/L dNTP mix, 2.0 U of AmpliTaq DNA polymerase, 0.4 μmol/L of each primer, 1.5 mmol/L MgCl2, 50 mmol/L KCl, and 10 mmol/L Tris/HCl (Promega, Madison, WI). The reaction mixture was overlaid with 40 μl of mineral oil and denatured (at 94°C for 45 seconds), annealed (at 60°C for 45 seconds), and extended (at 72°C for 2 minutes) for 45 cycles in a DNA thermal cycler (Perkin Elmer, Norwalk, CT). A 25-μl volume of each PCR product was resolved by electrophoresis on a 2% agarose gel containing ethidium bromide and photographed.

Measurement of bFGF in Serum and BAL Fluid

We measured the concentrations of bFGF in serum and BAL fluid of all study subjects by ELISA (R&D Systems). BAL fluid was measured directly as neat lavage supernatant, without prior concentration.

Statistical Analysis

Dunn’s nonparametric multi-comparison procedure or Wilcoxon nonparametric rank sum procedure for ranked data were used to compare differences among all or two groups. For tests of association, we calculated Spearman’s correlation coefficient (r_s). All data are expressed as median (minimum, maximum). Statistical significance is defined as P < 0.05.

Results

Immunohistochemical Localization of bFGF

In lung tissue of all subjects, we observed anti-bFGF antibody binding to MCs in the lung interstitium, to basement membrane, to endothelial cells, and more weakly to smooth muscle cells, with no immunostaining of either lymphocytes, epithelial cells, or alveolar macrophages. The strongest immunostaining was associated with interstitial MCs (Figure 1, A–C). The...
margins of these MCs were irregular and their cytoplasmic granules showed antibody binding. The extracellular matrix, especially surrounding these cells, displayed weakly positive antibody staining. These cells were found throughout the interstitium in cases of IPF, circumferentially in the periphery of granulomas in both CBD and Sar, and infrequently in the interstitium of control and BeS. We identified these cells as MCs by double immunohistochemistry showing that these bFGF⁺ cells also bound MC-specific anti-tryptase antibody and not the macrophage-specific anti-CD68 antibody (Figure 2. A and B). We observed compatible loss of immune labeling in several negative controls (without primary and/or secondary antibodies). Similar granular patterns of antibody binding in the MC cytoplasm were observed with both anti-bFGF and anti-tryptase (data not shown).

bFGF immunoreactivity was eliminated by preabsorption of antibody with recombinant human bFGF. We observed no specific immunostaining when we omitted primary antibody, secondary antibody, or ABC. We saw no specific staining on MCs with normal mouse serum or normal mouse IgG or when anti-TGF-β monoclonal antibody was used instead of primary antibody. There was no extinction or reduction of bFGF immunostaining in the presence of high-salt-containing buffer or pH 6.0 buffer, whereas nonspecific immunostaining of PDGF-BB on MCs was extinguished in the pH 6.0 buffer. The same staining patterns were observed using either type I or type II anti-bFGF monoclonal antibodies.

To assess the association of bFGF with heparin-like molecules, we preincubated the anti-bFGF antibody or lung tissue sections with heparin. Heparin inhibited all bFGF immunostaining. Treatment of the lung tissue with heparinase blocked the bFGF immunostaining of basement membrane but not the binding to tissue MCs (data not shown).

**Quantification of bFGF⁺ Cells and Mast Cells in Lung Tissue**

As shown in Figure 3, the V₅ of total bFGF⁺ cells in each of the three disease groups were significantly higher compared with control and was significantly higher in IPF than in BeS (P < 0.05). We observed no significant difference between BeS and control. We did not observe a significant difference between IPF and CBD or Sar. However, there was significant difference between IPF (median 4.0; range, 2.3 to 5.9) and granulomatous disease (CBD plus Sar) (2.2; 1.1 to 3.6), between granulomatous disease and control (0.7; 0.4 to 1.3), and between granulomatous dis-
ease and BeS (1.3; 0.4 to 1.6; \( P < 0.05 \)) with highest values in IPF tissue.

Because lung extracellular matrix deposition occurs in the interstitium principally, we quantified bFGF+ cells in both interstitial and alveolar compartments. The \( V_v \) of bFGF+ cells was significantly higher in the interstitium of CBD (2.1; 0.9 to 2.4), Sar (2.0; 1.4 to 3.1), and IPF (3.8; 2.1 to 5.4) lung tissue than in control (0.5; 0.3 to 1.3; \( P < 0.05 \)), whereas in the alveolus, only IPF (0.3; 0.1 to 0.9) showed a significantly higher median value than control (0.1; 0 to 0.5). Similarly, as shown in Figure 4, the \( V_v \) of total tryptase-positive cells was significantly higher in CBD, Sar, and IPF than in control (\( P < 0.05 \)), with no significant difference between BeS and control. The \( V_v \) of total tryptase-positive cells in IPF was significantly higher than in BeS or CBD (Figure 4). The \( V_v \) of tryptase-positive cells was significantly higher in the interstitium of CBC (2.0; 0.9 to 2.8), Sar (2.2; 1.3 to 2.9), and IPF (3.0; 2.0 to 5.0) than in control (0.4; 0.2 to 0.9; \( P < 0.05 \)), whereas in the alveolus, there was no significant difference, as we had observed in bFGF+ cells.

As can be seen in Figure 5, there was a statistically significant correlation between the \( V_v \) of bFGF+ cells and the \( V_v \) of tryptase-positive cells (\( r_s = 0.87, P < 0.001, n = 52 \)) reflecting the co-localization of bFGF and tryptase staining. The ratio of tryptase-positive cells to bFGF+ cells in the groups (\( V_v \) of tryptase-positive cells/\( V_v \) of bFGF+ cells) are 0.81 (0.43 to 1.40) in control, 0.86 (0.33 to 1.80) in BeS, 0.96 (0.72 to 1.77) in CBD, 1.14 (0.63 to 1.89) in Sar, and 0.88 (0.64 to 1.26) in IPF.

**Quantification of Collagen/Reticular Fibers and Elastic Fibers and Correlation with bFGF+ Cells**

Consistent with previous studies,\(^52\) we observed more collagen/reticular fibers in CBD, Sar, and IPF than control and more elastic fibers in IPF than control. More collagen/reticular fibers were observed than elastic fibers in the lung (Figure 6, A and B). As illustrated in Figure 7, A and B, we observed two patterns of distribution of bFGF+ cells. In IPF, in which collagen and reticular fiber deposition is diffuse, bFGF+ MCs were scattered throughout the interstitium, particularly in the area of collagen/reticular fiber deposition. In contrast, in the two disorders in which the fibrosis was confined principally to the rim surrounding granulomas, bFGF+ MCs accumulated preferentially in that circumferential zone. The \( V_v \) of bFGF+ cells was positively associated with the \( V_v \) of granuloma in Sar and CBD (\( r_s = 0.53, P < 0.009, n = 23 \); Figure 8).

To determine the association between bFGF+ MCs and the extent of pulmonary fibrosis, we examined 1) the correlation between these variables and 2) their geographic distribution. We observed statistically significant positive correlations between the \( V_v \) of bFGF+ cells and the \( V_v \) of collagen/reticular fibers (\( r_s = 0.77, P < 0.001, n = 52 \)) and the \( V_v \) of bFGF+
cells and the \( V_v \) of elastic fibers \( (r_s = 0.43, P < 0.002, n = 52; \text{Figure 9}) \).

Characterization of bFGF Isoforms in Lung Tissue and HMC-1

To determine the predominant isoforms of bFGF in human lung tissue and cell lines, we extracted bFGF and performed Western blotting. As shown in Figure 10, in the extract of our positive control SK-HEP-1 cells, we found four isoforms of bFGF (17.8, 22.5, 23.1, and 24.2 kd) as has been reported previously.\(^\text{46}\) In addition, we observed an approximately
36-kd nonspecific band that was not blocked by preabsorption with recombinant bFGF. In IPF lung tissue, the 17.8-kd bFGF band predominates, compared with a much weaker 17.8-kd band in CBD and no such band in control, consistent with our observation that there are more bFGF+ cells in IPF compared with CBD and control. We did not observe this 17.8-kd bFGF band or any other bFGF isoform in heparin-containing HMC-1 cells after semi-purification using heparin acrylic beads (data not shown). This may be because endogenous heparin in HMC-1 cells may be competing with heparin acrylic beads for bFGF binding. The specificity of each bFGF band was confirmed by extinction of bands using a solution of normal mouse serum or anti-bFGF antibody mixed with recombinant bFGF instead of primary antibody (preabsorption control).

Although bFGF was not found in HMC-1 cell extractions by Western blot, we did detect anti-bFGF antibody binding in a granular pattern suggestive of cytoplasmic granules in HMC-1 cells by light microscopy (Figure 11).

To verify that human MCs can produce bFGF, we examined HMC-1 cells for both bFGF mRNA expression and protein production. As shown in Figure 12, unstimulated HMC-1 cells contain bFGF mRNA, showing the same size PCR product (375 bp) as do bFGF-producing SK-HEP-1 cells.50 HMC-1 cells contained one-fourth as many bFGF transcripts as did SK-HEP-1 cells, normalized by β-actin mRNA.

Under unstimulated conditions, HMC-1 cell lysates contained 1.6 (1.0 to 2.6) pg/10⁶ cells bFGF protein (n = 4). This amount did not significantly change after 48 hours of incubation. No bFGF was
detected in the control culture media, although the supernatants of unstimulated HMC-1 cells contained bFGF: at 0 hour, 0.0 (0 to 0); at 24 hours, 1.1 (0.6 to 1.6); and at 48 hours, 0.7 (0.5 to 1.0) pg/10⁶ cells (n = 4).

Measurement of bFGF Protein in Serum and BAL Fluid

We detected bFGF in both serum and BAL fluid of study subjects, observing significantly higher bFGF concentrations in the unconcentrated BAL fluid of both CBD and IPF, as compared with control (P < 0.05; Figure 13). Serum bFGF levels did not differ among groups (Figure 13). We observed no significant correlation between serum bFGF and BAL fluid bFGF concentrations (r = 0.26, n = 36).

Correlation between bFGF Levels and Clinical Measures of Disease Severity

Table 2 demonstrates clinical measurements in the study subjects, showing that IPF is severe pulmonary fibrosis and CBD is mild fibrosis.

To examine the relationship between bFGF and the severity of pulmonary inflammation and clinical illness in IPF and CBD, we tested the correlation between serum bFGF, BAL fluid bFGF, and the clinical variables shown in Table 3.

Consistent with previous studies, our study subjects with interstitial lung disease had elevated numbers of white blood cells in their BAL.²⁹,³¹,⁵³ Subjects with CBD demonstrated an increase in the proportion of BAL lymphocytes,³¹,⁵³ as shown in Table 2. As shown in Table 3, for CBD, we observed a positive correlation between BAL fluid bFGF and the percentage of lymphocytes and a negative correlation between BAL fluid bFGF and the percentage of macrophages, consistent with previous work showing that the BAL lymphocytosis in CBD reflects the clinical severity of that disease.⁵³ We found no association between bFGF and cellularity or cell differential in IPF, apart from the trends shown in Table 3.

We observed a statistically significant positive correlation between BAL fluid bFGF and the gas exchange parameter alveolar-arterial oxygen gradient (AaPO₂) measured at maximal exercise as well as a
Discussion

The findings in this study support the hypothesis that MCs contribute to the pathogenesis of pulmonary fibrosis, serving as an important source of a 17.8-kd bFGF isofom in the human lung. Quantitatively, MCs that contain bFGF are abundant in fibrotic lung and accumulate preferentially in areas of extracellular matrix deposition. In addition, we found that bFGF may be associated with cytoplasmic granules. We observed release of bFGF by HMC-1 cells into culture supernatant that might occur by non-anaphylactic degranulation, suggesting the possibility of slow, piecemeal degranulation discussed below. These findings identify a potential mechanism by which bFGF contributes to ongoing chronic inflammation. We showed that levels of bFGF in BAL fluid and serum correlate with measures of both pathological and physiological severity of disease.

Pulmonary Interstitial Mast Cells Contain bFGF

Although other hematopoietic cells, including T lymphocytes and alveolar macrophages, may produce bFGF, our immunohistochemical data indicate that the bFGF* cells found in human lung are mainly interstitial MCs and not macrophages or T lymphocytes. In addition, more than 80% of bFGF* cells found in the lungs are MCs. The recent work of Reed and Qu support our conclusion that MCs, not macrophages, are an important source of bFGF in human tissue, especially during chronic inflammation.

Some previously published immunohistochemical data may have overestimated the relationship between human MCs and cytokine/growth factor production. The highly negatively charged glycosaminoglycan heparin is abundant in MC secretory granules. Consequently, this can result in the nonspecific binding of avidin, immunoglobulins, and certain enzymes to MCs, potentially producing false positive antibody binding results in immunohistochemical or in situ hybridization studies of MCs. To address this possibility, we performed a series of immunohistochemical staining controls following the methods of Schlitz and colleagues. We confirmed the immunological specificity of bFGF for MCs. Parallel experiments with growth factor antibodies directed against PDGF-BB showed only nonspecific staining.
Table 2. BAL, Pulmonary Function Test, Gas Exchange, and Chest Radiography Data of Study Subjects

<table>
<thead>
<tr>
<th>BAL parameters</th>
<th>Pulmonary function test, % predicted</th>
<th>Chest radiography, profusion of small interstitial opacities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WBCs x 10^9/ml</td>
<td>% Macrophages</td>
</tr>
<tr>
<td>Control (n = 8)</td>
<td>(0.09-0.28)</td>
<td>(71-96)</td>
</tr>
<tr>
<td>CBD (n = 18)</td>
<td>(0.19-1.42)</td>
<td>(7-64)</td>
</tr>
<tr>
<td>IPF (n = 10)</td>
<td>(0.13-1.01)</td>
<td>(25-89)</td>
</tr>
</tbody>
</table>

Data are expressed as medians (range). WBCs, white blood cells; FVC, forced vital capacity; FEV₁, forced expiratory volume at 1 second; TLC, total lung capacity; NA, not available.

*P < 0.05 versus control.

**P < 0.05 versus CBD.

bFGF Interacts with Heparin-Like Molecules in the Lung

bFGF is a heparin-binding growth factor and requires heparin-like molecules to bind its surface receptor. At low concentrations, exogenous heparin enhances the biological effects of bFGF on endothelial cells and fibroblast proliferation. We hypothesized that MC bFGF would interact with heparin-like molecules in the MCs in a manner analogous to MC tryptase/heparin binding. The intracellular MC bFGF staining pattern we observed is granular and similar to that seen with tryptase bound to heparin within MC secretory granules. Of note, we also observed binding of anti-bFGF antibody to basement membrane, which is known to contain bFGF bound to abundant heparan sulfate. Pretreatment of either the bFGF antibody or the lung tissue sections with heparin extinguished bFGF immunostaining of both MCs and basement membrane. Interestingly, treatment of lung tissue with heparinase eliminated bFGF antibody binding to basement membrane but not to MCs, suggesting either a difference in bFGF concentration, avidity, or binding pattern in the extracellular matrix compared with the MCs. Our data suggest that bFGF on basement membrane is associated with heparin-like molecules and that exogenous heparin binds to heparin-binding sites of bFGF in the lung and blocks anti-bFGF antibody epitopes.

bFGF⁺ Mast Cells Increase in Injured and Fibrotic Lung

Quantitative morphometric analysis of immunohistochemically stained lung showed that, although most interstitial MCs in normal lung contain bFGF, the volume density of bFGF⁺ MCs increases significantly in the lungs of patients with granulomatous and fibrotic lung disease. The increase in these MCs was greatest in IPF, which is the most fibrotic of the lung diseases we studied. As a second reference group, we examined the lungs of BeS individuals who had been occupationally exposed to beryllium and who had mounted a cell-mediated immune response to beryllium in their blood but who had not yet developed either granulomatous or fibrotic dis-

Table 3. Relationship of bFGF Concentration in Serum and BAL Fluid to Measures of Pulmonary Inflammation and Disease Severity

<table>
<thead>
<tr>
<th>BAL cellular constituents</th>
<th>Gas exchange</th>
<th>Chest radiography, profusion of small interstitial opacities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Lymphocytes</td>
<td>% Macrophages</td>
</tr>
<tr>
<td>CBD (n = 18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum bFGF</td>
<td>0.28 (0.25)</td>
<td>-0.19 (0.45)</td>
</tr>
<tr>
<td>BAL fluid bFGF</td>
<td>0.45 (0.05)</td>
<td>-0.52 (0.03)</td>
</tr>
<tr>
<td>IPF (n = 10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum bFGF</td>
<td>0.29 (0.42)</td>
<td>-0.27 (0.45)</td>
</tr>
<tr>
<td>BAL fluid bFGF</td>
<td>-0.59 (0.07)</td>
<td>0.60 (0.07)</td>
</tr>
</tbody>
</table>

All data are expressed as Spearman’s correlation with P values in parentheses.

*P < 0.05.
ease.\textsuperscript{31} These antigen-sensitized individuals did not differ from other control subjects in the density of bFGF\textsuperscript+ MCs found in their lungs.

In the present study, we used quantitative methods to examine tissue from 34 cases of fibrotic and granulomatous lung disease across a wide range of fibrosis severity. This approach permitted us to examine systematically the volumetric and spatial relationship of bFGF\textsuperscript+ MCs to extracellular matrix deposition in diffuse, severe fibrosis (IPF) and in more localized mild fibrotic lung (CBD and Sar). As hypothesized, we observed that bFGF\textsuperscript+ MCs accumulated in areas of dense matrix deposition and correlated with the amount of collagen/reticular fibers and elastic fibers. In CBD and Sar, bFGF\textsuperscript+ MCs accumulate preferentially in the fibrotic circumference of the granuloma, not in the central portion of the lesion and not in areas of normal lung tissue found outside of the granuloma. These findings are consistent with work by others showing significant correlation between the number of MCs in BAL fluid and degree of human pulmonary fibrosis\textsuperscript{56} and levels of type III procollagen propeptide in BAL fluid.\textsuperscript{60} We speculate that, as a byproduct of the cell-mediated immune response to antigen, granulomas produce signals that inhibit fibrosis. Macrophage or lymphocyte-derived cytokines may slow the accumulation of MCs, thereby confining the release of bFGF to the periphery. Whether human pulmonary MCs produce other proinflammatory factors in addition to bFGF will require additional study, although our data suggest that PDGF-BB and TGF-\beta were not detected in these cells by our immunohistochemistry.

**Human Mast Cells Express bFGF mRNA and Produce a 17.8-kd bFGF Protein**

We used HMC-1 cells as our in vitro model of human MCs. This cell line contains tryptase but not chymase,\textsuperscript{26} which is the dominant MC phenotype in human peripheral lung and which is considered to depend on lymphocyte infiltration.\textsuperscript{61} Whereas HMC-1 has been well studied as a model of human MCs and has Kit/ Stem cell factor receptor and CDw32 (FcyRII), it lacks CD16 (FcyRIII), CD23 (FceRII), and FceRI \alpha-chain.\textsuperscript{26}

We have demonstrated the expression of both bFGF protein by ELISA and mRNA by RT-PCR in the human MC line (HMC-1). These findings complement the work of Reed and co-workers who detected bFGF mRNA in human cutaneous MCs by in situ hybridization.\textsuperscript{26} These data are consistent with the hypothesis that human MCs produce bFGF in vivo in the pulmonary interstitium. Four isoforms of bFGF have been identified in humans.\textsuperscript{46} It has been suggested that in disease states the disproportionate induction of one or more bFGF isoforms influences the severity of disease.\textsuperscript{62,63} An emerging body of evidence suggests that the 17.8-kd form of bFGF exhibits a pattern of translocation and distribution within cells that is different from that seen in the three high molecular weight forms of bFGF. Translation of the 17.8-kd protein is initiated at an AUG codon, whereas the high molecular weight bFGF initiates translation at non-AUG codons.\textsuperscript{46} The amino-terminal extension of high molecular weight bFGF contains a nuclear localization signal and is detected abundantly in the ribosomal/nuclear fraction; however, the 17.8-kd isform is abundant in the cytosolic fraction of 3T3 (B3 clone) cells.\textsuperscript{64--67}

In fibrotic human lung we detected only one of four known bFGF isoforms.\textsuperscript{46} Using lung tissue specimens of equal weight, we observed the greatest amounts of the 17.8-kd bFGF in IPF lung, with weaker but positive expression in CBD lung. This is consistent with our morphometric data showing higher amounts of bFGF antibody bound in fibrotic lung tissue. Based on prior studies\textsuperscript{27} and our observation that anti-bFGF antibody stains MC cytoplasmic granules in both lung tissue and in HMC-1 cells, we conclude that the 17.8-kd bFGF is contained in the cytoplasm of MCs and increases in fibrotic lung tissue. Whether there is an increase in both the number of MCs and induction of bFGF production will require additional investigation.

**bFGF Release by Mast Cell Degranulation**

Because bFGF lacks the signal sequence required for vectorial translocation into endoplasmic reticulum and eventual secretion,\textsuperscript{68} the mechanism by which bFGF leaves the cell remains enigmatic. Recently, Reed and colleagues hypothesized that MC degranulation might result in release of bFGF.\textsuperscript{26} Our data provide the first indirect experimental support of this hypothesis. We detected bFGF protein within HMC-1 cell lysates as well as in the cell-free supernatants. Because HMC-1 cells lack complete IgE receptors, they do not undergo IgE-mediated degranulation. Although the quantity detected in the supernatant could have been released by dying or lysing HMC-1 cells in culture, our studies suggest bFGF is secreted into the culture medium by intact MCs. In unpublished preliminary observations using immunoelectron microscopy of bFGF on HMC-1 cells, we have observed focal and geographical loss of bFGF.
from the cytoplasmic granules of HMC-1 cells, in an ultrastructural pattern consistent with piecemeal degranulation.59 These in vitro findings are consistent with an emerging body of literature suggesting that MCs release their contents slowly and partially in chronic inflammatory states, such as in cardiac allografts,70 pulmonary fibrosis,58,73,74 Sar, and collagen-vascular-disease-related lung fibrosis.2,71,72 Although we have not yet studied our fibrotic human lung specimens by electron microscopy, the irregular cellular margins of MCs that we observed by light microscopy offer suggestive evidence of the in vivo degranulation described by Hunt et al.2 In injured lung, such piecemeal MC degranulation offers one potential means of chronic, sustained release of bFGF at sites of inflammation and extracellular matrix deposition. Additional studies will address the evidence for this mechanism.

Alternatively, after being secreted by MCs, bFGF might bind to heparin-like molecules in the basement membrane and be released from tissue stores at a later date as a result of enhanced activity of enzymes produced during inflammation.58,73,74 Interestingly, Buch and colleagues reported that levels of bFGF rise during acute lung injury, using a rat lung oxygen toxicity model.75 The elevated levels of bFGF that we observed in body fluids of patients with chronic lung disease may reflect not only increased production and secretion by MCs but also increased enzymatic activity in the interstitium with increased permeability of the alveolar-capillary wall. Future research should compare the bFGF newly released from MCs with release of basement-membrane-stored bFGF during the course of inflammation.

**BAL Fluid and Serum bFGF Concentrations Correlate with Disease Activity and Severity**

Regardless of the immediate source of bFGF in chronic inflammation, our ELISA data suggest that bFGF concentrations in BAL fluid and in serum reflect the magnitude of the pulmonary inflammatory cell response and correlate with the clinical severity of fibrotic lung disease. In particular, levels of bFGF were associated with the degree of cellularity in BAL and with reduction in the lung’s gas exchange function. Although it is attractive to speculate that bFGF causes this derangement in oxygen delivery, our data are correlational. We consider it likely that bFGF acts in concert with other mediators of inflammation in a complex process that culminates in fibrogenesis and compromised lung function.

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