Characterization of fibroblasts recruited from bone marrow-derived precursor in neonatal bronchopulmonary dysplasia mice

Chun Deng,1 Jin Wang,1 Youfu Zou,1 Qianqian Zhao,1 Jie Feng,1 Zhou Fu,2 and Chunbao Guo3

Departments of 1Neonatology and of 2Respiratory Medicine Children’s Hospital, 3Laboratory of Hepatobiliary Surgery, Chongqing Medical University, Chongqing, China

Submitted 25 February 2010; accepted in final form 30 December 2010

Deng C, Wang J, Zou Y, Zhao Q, Feng J, Fu Z, Guo C. Characterization of fibroblasts recruited from bone marrow-derived precursor in neonatal bronchopulmonary dysplasia mice. J Appl Physiol 111: 285–294, 2011. First published January 13, 2011; doi:10.1152/japplphysiol.00201.2010.—We sought to determine whether the extrapulmonary origin of fibroblasts derived from bone marrow (BM) progenitor cells is essential to lung fibrosis in bronchopulmonary dysplasia (BPD). Neonate mice were durably engrafted with BM isolated from transgenic reporter mice that expressed green fluorescent protein (GFP). Such chimera mice were subjected to 60% O2 exposure for 14 days. A large number of fibroblast-specific protein-1 (FSP1) and GFP-positive fibroblasts were identified in active fibrotic lesions. More surprisingly, however, FSP1+ fibroblasts also arose in considerable numbers from BM-derived alveolar type II cells (AT2) through epithelial-mesenchymal transition (EMT) during lung fibrogenesis. Cultured lung fibroblasts could express the CXC chemokine receptor (CXCR4) and responded chemotactically to their cognate ligand, chemokine (C-X-C motif) ligand 12 (CXCL12), which were elevated in the serum of BPD mice. These data suggest that lung fibroblasts in BPD fibrosis could variably arise from BM progenitor cells. This finding, which suggests the pathophysiologic process of fibrosis, could contribute to a therapy for BPD that is characterized by extensive interstitial fibrosis.

chemokine; type II pneumocyte; epithelial-mesenchymal transition; fibrosis

BRONCHOPULMONARY DYSPLASIA (BPD) is a chronic lung disease, representing the most prevalent of the long-term sequelae of oxygen- and ventilation-mediated injuries following a premature birth. When oxygen injury is constantly present, the host initiates a repeated process of repair and destruction, which subsequently leads to tissue remodeling. Over the years, probably as a consequence of progress in pathophysiological research and the greater survival rates of highly premature infants, impaired alveolarization and widespread interstitial fibrosis have come to be recognized as the prominent features of BPD (3). Fibroblasts could play an important role in exacerbating the fibrotic process of chronic lung disease in premature infants (2, 9) by producing collagens and extracellular matrix proteins. A persistent increase in the number of fibroblasts is found both in the interstitium and in the airspace of the lungs of infants with BPD (27). This occurrence has led to a greater focus on fibroblasts and their direct involvement in the fibrotic pathway.

Fibroblasts are known to be heterogeneous with respect to a number of phenotypic and functional features (8, 12). This heterogeneity may arise not only from the activation and differentiation processes that take place in the cells but also from different cellular origins. Several recent studies have suggested that pluripotent precursor cells in bone marrow (BM) can repopulate distal organs. Possessing tissue fibroblast properties that may participate in the repair process (22, 26), these cells travel to the site of damage and undergo differentiation in the process of promoting structural and functional repair (25). Furthermore, it is possible that BM-derived cells can become alveolar type II (AT2) pneumocytes (15), which may then form local interstitial fibroblasts through epithelial-mesenchymal transition (EMT) (29). Combined with the recent evidence of BM stem cell plasticity cited above, this phenomenon provides a compelling reason to reexamine the origin of the fibroblasts in BPD-associated pulmonary fibrosis. In particular, these fibroblasts may have extrapulmonary origins, possibly having migrated to the lung in response to signals of lung injury and fibrosis. Some of these signals may be chemokines, which play a pivotal role in the regulation of fibroblast trafficking and extravasation through the luminal surface of endothelial cells into sites of tissue inflammation (1). The precise trafficking signals responsible for these cells’ directed migration remain unknown. These led us to investigate the possible relationship between BM-derived fibroblasts and various fibroblast phenotypes in an animal model of BPD and to ask whether BM-derived cells could represent a significant source of lung fibroblasts.

Although the pathogenesis of BPD is incompletely understood, prolonged exposure to sublethal hyperoxia in animal models could recapitulate some of the processes observed during the development of BPD (32). This research model allowed us to assess whether fibroblast engraftment was modulated by tissue damage, to analyze the phenotypes of BM-derived fibroblasts, and to evaluate the functional contributions of new fibroblasts identified as originating from multiple sources. Using BM chimera mice expressing both enhanced green fluorescent protein (GFP; only in BM-derived cells) and red fluorescent protein (GFP; only in BM-derived cells) and responded chemotactically to their cognate ligand, chemokine (C-X-C motif) ligand 12 (CXCL12), which were elevated in the serum of BPD mice. These data suggest that lung fibroblasts in BPD fibrosis could variably arise from BM progenitor cells. This finding, which suggests the pathophysiologic process of fibrosis, could contribute to a therapy for BPD that is characterized by extensive interstitial fibrosis.

BM chimera mice and intervention. Donor mice expressing a GFP transgene (1 mo old) were purchased from the Shanghai Research Center for Biomodel Organisms. The strain-matched, recipient wild-
type C57BL/6 mice were purchased from the experimental animal center of Chongqing Medical University. Animal studies were conducted according to protocols approved by the Animal Use Committee of Chongqing Medical University, and we adhered strictly to Chongqing Medical University’s Institutional Animal Care and Use Committee guidelines on the use and care of experimental animals. BM chimeras were prepared using a slightly modified version of the method that has been described previously (10). In brief, whole BM was harvested from 1-mo-old donor GFP transgene or wild-type C57BL/6 mice by flushing their femurs, tibia, and humeri with ice cold Dulbecco’s Modified Eagle Medium (DMEM; Gibco, Grand Island, NY). The recipient mice were strain-matched, 1-day-old C57BL/6 wild-type mice. Recipient mice were sublethally irradiated with two doses of 5 Gy, administrated 3 h apart using a 60 Co-γ irradiator. After irradiation, 4 × 10^6 BM cells from the GFP transgene mice were injected retro-orbitally under anesthesia (endogenous BM cells). No radiation with BM cells injection controls were defined as exogenous BM cells administration. Radiation controls were prepared simultaneously to confirm the adequacy of the sublethal irradiation dose. Two days after durable BM engraftment had been established, recipient mice were maintained in paired chambers (filled with either air or 60% oxygen) for 14-day exposure periods. The day of the oxygen administration was designated as BMT day 0/oxygen day 0.

Equal litter sizes (10–12 pups) were maintained in each of the paired chambers. No deaths occurred as a consequence of the exposure to 60% O_2.

Histological and morphometric analyses. BM recipients were killed at predetermined times by means of cervical dislocation. The lungs were thoroughly perfused with saline to remove blood from the lung vascular beds and then removed from the thoracic cavity and cleared of extraneous tissue. The lungs were perfused for 30 min via the pulmonary arteries with 4% formaldehyde at a pressure of 65 cmH_2O. Both lungs were first inflated to an airway pressure of 30 cmH_2O for 1 min and then deflated to 10 cmH_2O for the rest of time. Representative lung tissue blocks from all lung lobes were embedded in paraffin. Serial sections (4 μm thick) were then either stained with hematoxylin and eosin or left unstained for fluorescence microscopy to evaluate the distribution or localization of GFP^+ cells. Because of regional variations in lung maturation, all morphometric assessments were performed on the right middle lobe. These tasks were performed in a blinded manner so that investigators would not be able to identify the treatments and outcomes of the animals until completion of the measurement. Morphometric assessments were performed on coded images designed to mask the treatment category. Digitized images were captured at ×40 magnification using a Nikon Labphot camera (Nikon, Tokyo, Japan). A total of 8–20 ×20 images were taken randomly from 10 non-overlapping fields at 3, 6, 9, and 12 o’clock on each slide, with three slides for each animal’s lung lobe and five animals per group. Mean linear intercepts were measured as previously described (5). In brief, using a predetermined grid with randomly distributed lines totaling 1 mm in length, the mean linear intercept (MLI) was calculated as follows: L/(no. air-tissue interfaces)×1,000, yielding the average distance between two air-tissue interfaces in micrometers. With the images at ×200 magnification, ~60–80 lines per field were drawn perpendicular to the narrowest segment of the primary and secondary alveolar septa. The mean length of the lines crossing the septa, representing alveolar wall thickness, was determined using Scion Image (Scion, Frederick, MD).

Isolation and culture of primary lung fibroblasts. The lung tissue from BM chimera mice after 14 days of oxygen exposure was removed under sterile conditions following the exsanguination of the animals. The lung parenchyma was cut into 2- to 3-mm³ pieces, plated onto dry culture dishes, and incubated at 37°C for 30 min to ensure attachment. Eagle’s MEM, containing 10% FCS, 1% l-glutamine, and 1% penicillin/streptomycin, was then added, and the tissue was left for 7 days without further handling. After 7 days, fibroblasts were seen to be growing out of the tissue. Released cells were cultured on 6-well plates in complete medium, which was composed of DMEM (supplemented with 10% plasma-derived serum), insulin, transferrin, selenium liquid media supplement, and antibiotics to confluence in 25-cm² flasks (BD Biosciences, Sandy, UT). Growth factors were included in the suspension culture at the following concentrations: human recombinant platelet-derived growth factor (PDGF)-BB (5 ng/ml; R&D Systems, San Diego, CA), recombinant human epidermal growth factor (10 ng/ml; R&D Systems). Where indicated, confluent cell monolayers were harvested to assess GFP or FSP expression by flow cytometry. The procedure for analyzing FSP in whole lung cells is described below.

Immunofluorescence and colocation assays. In preparation for immunofluorescence, both paraffin-embedded 4-μm sections of lung tissue and isolated whole lung cells were stained with primary antibodies, including rabbit polyclonal anti-GFP Alexa Fluor 488 (1:500, Molecular Probes, Eugene, OR) and rabbit polyclonal anti-fibroblast-specific protein-1 (FSP1; 1:500, Cell Signaling Technology, MA). After being washed with PBS, slides were incubated with affinity-purified FITC- or rhodamine-conjugated secondary antibodies (mouse anti-rabbit IgG, 1:4,000, Jackson Immuno Research, West Grove, PA). In separate experiments, lung sections and whole lung cells were stained for GFP expression, using goat FITC-conjugated anti-GFP antibody and FSP1 (Cell Signaling Technology). Type I collagen (COL I; Santa Cruz Biotechnology, Santa Cruz, CA), Pro-SP-B (BD Pharmingen), thyroid transcription factor-1 (TTF-1; BD Pharmingen, San Diego, CA), and α-smooth muscle actin (α-SMA; Santa Cruz Biotechnology, Santa Cruz, CA) with PE-labeled antibody. FITC-labeled antibody of the surfactant protein B-precursor (Pro-SP-B) was also subjected to immunofluorescence. Included in each batch were negative control slides, which carried an isotype-matched immunoglobulin in the same concentration as the primary antibody on the other slides. After 1 h incubation, the slides were mounted and analyzed using a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss, Thornwood, NY) equipped with an FITC (green), PE (red), and DAPI (blue) filter set. All digital images were processed and merged using Photoshop 6.0 (Adobe Systems, San Jose, CA).

Analysis of whole lung cells by flow cytometry. On predetermined days after oxygen exposure, the lungs from BM chimera mice were removed, digested with DNAse and collagenase/dispase (Shanghai DMEM Biological Technology, China), and filtered with 40-μm cell strainers to obtain single cell suspensions. After their initial isolation, the cells were stained with FITC-labeled GFP. Next, the cells were permeabilized with the BD Cytofix/Cytoperm Kit (R&D Systems) to facilitate intracellular staining. All single cell suspensions were stained with 2 μg/ml of propidium iodide to exclude dead cells. Cell fragments and erythrocytes were excluded from the analysis by appropriate gating. In separate experiments, the whole lung cells were stained with PC5-CD45 (BD Pharmingen, San Diego, CA), CXCL chemokine receptor (CXCR4; Santa Cruz Biotechnology), PE-labeled TFF-1 (BD Pharmingen), FSP1 (Cell Signaling Technology), and FITC-labeled GFP (BD Pharmingen). Three-color analysis of the stained cells was performed on an FACScan flow cytometer (R&D Systems) using Cellquest 3.2.1f1 software. All antibodies were purchased from commercial sources.

Fibrocyte chemotaxis assay. Chemotaxis assays were performed using Costar Transwell inserts (8-μm pore size), which were precoated with 0.1% gelatin, according to the manufacturer’s protocol. Isolated murine lung fibroblasts were suspended at 1×10⁶ cells/ml in DMEM containing 0.1% BSA. Either the medium alone (negative control) or the medium containing chemokine (C-X-C motif) ligand 12 (CXCL12; CXCR4 ligand; 600 ng/ml) was added to individual wells of a 24-well plate. Transwell devices were then inserted, and the fibroblasts (100 μl) were layered on top of the membrane (n = 3 wells/condition). After 4 h, the cells on the top of the filter were removed by scraping. The filter was fixed with methanol for 10 min and then stained with hematoxylin QS (Vector Laboratories, Burlingame, CA). For the checkerboard analysis of CXCL12-directed che...
motaxis of fibroblasts, CXCL12, at a concentration of 500 ng/ml, was added to either the top chamber, the bottom chamber, or to both. With a light microscope, migration was assessed by counting the number of cells in five high-power fields. These experiments were replicated with separate cultures of cells on separate occasions.

ELISA analysis of lung homogenate and plasma chemokine receptor. The lung tissue from GFP genotype mice were homogenized and sonicated in Complete Buffer (Roche Diagnostics, Indianapolis, IN) using a method as previously described (7). Specimens corresponding to 60 µg of protein were centrifuged and stored at −70°C until they could be thawed for assay. These assays determined lung homogenate and serum CXCL12 concentrations using a human CXCL12 ELISA kit (Santa Cruz Biotechnology) according to the manufacturer’s instructions.

Western blotting. Lung tissue lysed using protein extraction buffer, then homogenized in 250 µl of lysis buffer. Cytoplasmic extracts (10 µg of total protein) were electrophoresed on 4–20% gradient SDS-PAGE gels (Shanghai Sangon Biotech, Shanghai, China) and transferred to nylon membranes (Shanghai Sangon Biotech, Shanghai, China). Blots were probed with various specific primary antibodies to recognize their respective proteins. The secondary antibody, horse-radish peroxidase-coupled mouse anti-rabbit immunoglobulin (Jingmei Biotech, Shenzhen, China) was subsequently incubated for 1 h at room temperature. The subsequent analysis of protein was performed with chemiluminescence using an ECL Western blotting kit (Amer sham Biosciences) following the manufacturer’s recommendations.

Statistical analysis. The results were analyzed using the Mann-Whitney test for comparison between any two groups and by non-parametric equivalents of ANOVA for multiple comparisons. P < 0.05 was considered to indicate statistical significance.

RESULTS

Histological pattern of fibrosis in BPD model. To measure alveolar development and damage during exposure to oxygen, we first evaluated the lung histology of newborn mice, which were exposed to 60% oxygen for 14 days. The histological characteristics of the lungs exhibited decreased alveolarization of the interstitial fibrosis, characterized by a loss of normal alveolar architecture; prominent disorganized thickening of the alveolar septa; and collapse of the alveolar space by organizing inflammatory infiltrate and fibroblasts. In contrast, we found essentially normal lung architecture without fibrosis in air-exposed control mice, which experienced only mild perivascular inflammatory cell infiltration (Fig. 1A). We also measured alveolar wall thickness in a rigorous and exhaustive fashion, restricting the analysis to lobes that were well inflated. Compared with air-exposed controls, there was a significant increase (25–30%) in alveolar wall thickness in the mice exposed to 60% oxygen (Fig. 1B). Because the deposition of collagen is a key characteristic associated with tissue fibrotic processes, and fibroblasts are known to be major producers of this molecule, we focused on fibroblasts as playing pivotal roles in fibrosis. As expected, there was a significant increase in the percentage of FSP1+ and Col I+ lung cells in the 60% oxygen exposure mice relative to the control mice (Fig. 1C). As shown in Fig. 1D, the expression of FSP1 and Col I increased significantly with a 14-day exposure to oxygen. These data are highly significant and closely correlated with increased alveolar wall thickness. We further measured lung weights and volumes, which were then normalized for the total body weight of each pup. There were no significant differences between the groups (data not shown), but there was considerable variability within each group. Some of this variability could be related to the difficulty of measuring accurate lung volumes in the small P14 lungs.

Localization of the transplanted BM cells in the oxygen-induced recipient lung. In this research, we used the GFP BM chimera mice to mark the BM precursor cells and measured its recruitment within the lung. First, histological examination of the lung sections of the control group revealed no evidence of inflammation or fibrosis at day 30 after BMT, whereas mice with 60% oxygen exhibited obvious fibrosis (Fig. 2A) at day 14 after oxygen exposure (BMT day 30/oxygen day 28), a result that supported the essentially normal lung finding HE examination following BMP.

To identify localization of the transplanted BM cells in the recipient lung, we evaluate presence of GFP+ donor cells under fluorescence microscopy by staining the sections of lungs with an anti-GFP-specific antibody. In serial sections of the lungs from control animals (not receiving BMT), there are no GFP+-positive cells shown. Donor-derived cells could be detected in the lungs of animals that received BMP as early as 14 days after receiving O2 exposure (BMT day 16/oxygen day 14), whereas lungs from mice that received BMP without receiving O2 exposure showed few GFP+ cells in the lung under fluorescence microscopy (Fig. 2, B and C).

Also it would be expected that endogenous stem cells (myelosuppress and BM) contributed to more persistent lung repair and exogenously administered BM cells (non-myelosuppress and BM) might have initially lodged in the lung have subsequently been cleared from the lung.

We further identified donor cells by staining whole lung cells with an anti-GFP-specific antibody and then performing a flow cytometry assay. A marked increase in donor-derived cells was detected in the lungs of animals 14 days after oxygen exposure (BMT day 30/oxygen day 28). More than 71% of the oxygen-treated lung cells were donor derived, as opposed to less than 25% of the cells that were donor-derived in air-treated animals receiving bone marrow transplantation (Fig. 2D). These percentages of GFP+ cells remained essentially unchanged by day 56 after BMT (BMT day 58/oxygen day 56) in mice with endogenous stem cells, while the level decreased to less than 20% in the mice with exogenously BM cells administered. In some animals with exogenously BM cells administered, donor cells were observed within the pulmonary vascularity instead of in the parenchyma as well as in recipient spleens, suggesting that the injected marrow cells did circulate beyond the initially encountered lung capillary bed. But we cannot rule out the possibility that exogenous BM cells have the ability to reconstitute lung architecture.

Phenotypes of BM-derived cells in fibrotic lungs. It is possible that the donor BM cells can assume phenotypic characteristics of the major cell types that compose lung parenchyma, including fibroblasts, as has been suggested in other studies. To assess the phenotype of the donor cells within the recipient lungs, we examined the extent of colocalization of green fluorescence (indicating GFP-positive donor cells) with cell-specific markers. The donor-origin cells were characterized by the presence of CD45 (leukocyte common antigen). FACS-based phenotyping demonstrated that ~65–80% of the GFP+ cells were costained with CD45. Quantitative estimates of the phenotypes of donor cells for animals receiving O2 exposure after endogenous stem cells administered indicated that donor-derived cells could also exhibit fibroblast phenotype. This
result showed that leukocytes had been recruited from circulation into the area of injury, although they were not directly associated with significant fibroblast proliferation. Most of the remaining cells were identified as fibroblasts (FSP1; Fig. 3A). To determine whether GFP+ precursor cells could express fibroblast special markers and contribute to tissue fibrosis, we next examined tissue sections using double immunofluorescence staining. Large numbers of the variably sized cellular elements were found to express GFP within the parenchyma (Fig. 3B), and these were most densely clustered in cellular areas of active fibrosis. A subset of GFP+ cells from the injured lungs demonstrated a distinctly elongated mesenchymal phenotype, suggesting that BM cells are being recruited out of the BM to participate in the lung fibrotic process. Double immunostaining for GFP (green) and FSP1 (red) revealed that GFP+ fibroblasts could express FSP1 or Col I (which appears yellow in Fig. 3B) and that they matched the normal location, size, and orientation of fibroblasts for animals receiving 60% O2 after myelosuppression. An immunoblot of lysate from the different tissues demonstrating lung-specific protein expression is presented in images of Fig. 3C. These data suggest that donor cell seeding of recipient marrow was associated with lung engraftment. To further eliminate the possibility that areas of costaining resulted from overlapping cells rather than from cells expressing both GFP and FSP1, primary cultures of lung fibroblasts were also established. As shown in Fig. 3C, a majority of the lung fibroblasts isolated from O2-treated GFP BM chimera mice’s GFP-expressing cells exhibited normal, primarily spindle-shaped, fibroblast-like morphology under fluorescence microscopy. Double immunostaining for GFP (green) and FSP1 (red) spindle-shaped fibroblasts was detected in most of the cultured fibroblasts (it appears yellow in Fig. 3D) from O2-treated GFP BM chimera mice. Between 68 and 86% of the FSP1+ cells were GFP+ in O2-treated GFP BM chimera mice, indicating that the cells were of donor origins (Fig. 3E). These cells represented between 16 and 31% of the whole lung cells analyzed. In contrast, only 4.5–9.8% of all cells were FSP1+ and GFP+ in the air-treated controls (Fig.

Fig. 1. Histological measurement of the lungs of neonatal mice exposed to 60% oxygen for 14 days. A: arrested alveolarization was measured with mean linear intercept (MLI) in the air- and 60% oxygen-treated animals. B: alveolar wall thickness in the alveoli of newborn mice exposed to either air or oxygen was measured. Black arrow, patchy areas of parenchymal thickening. The morphometry of MLI and alveolar wall thickness are indicated at right. The columns provide average values of at least 3 independent experiments performed in triplicate; the bars, ± SE. *P < 0.01 compared with the corresponding air-exposed control. C: immunostaining for FSP1 (a) and Col I (b) showed that increases of FSP1 (arrow) and Col I were located within the interstitium, consistent with developing septa. Representative figures of at least 3 independent experiments with similar results are shown at the indicated hours after treatment. Original magnification was ×400 for A and C, ×200 for B and D. Representative Western blots from different tissues demonstrate increases in the expression of FSP1 and Col I on day 14 of oxygen exposure.
Oxygen-induced lung injury increased the proportion of GFP* fibroblasts. These results confirmed that BM-derived fibroblasts could directly contribute to the deposition of collagen in pulmonary fibrosis in O2-induced lung fibrosis (Fig. 3D).
Effects of cells from injured lungs on mesenchymal stem cell behavior. To further evaluate the possibility that donor cells may engraft first as AT2 cells and later acquire fibroblast phenotypes, recipients’ lung sections were evaluated at earlier points in time—1 and 2.5 days after oxygen exposure (BMT day 3/oxygen day 1)—stained with GFP antibody. Donor-derived cells could be detected in neither the pulmonary vasculature nor the injured recipient lung parenchyma at that time. On BMT day 22, although clumps of one to three donor cells were found within the parenchyma along the alveolar epithelial surface, these cells also appeared to possess the location and shape of AT2 pneumocytes; they were found to be stained with the AT2 marker, thyroid transcription factor-1 (TTF-1), when examined by fluorescence microscopy (Fig. 4A). To further examine the phenotypes of BM-derived EMT cells, single cell suspensions were isolated from air- and oxygen-treated lungs 22 days after oxygen exposure (BMT day 22/oxygen day 20) and stained with triple colors for colocalization of α-smooth muscle actin (α-SMA) and GFP in lung fibroblasts from 60% oxygen exposure were further evaluated (F). The cells that coexpressed GFP and either FSP1 or α-SMA appear in yellow (arrow). Original magnification, ×200.

Fig. 3. Phenotype of BM-derived cells in fibrotic lung. A: BM-derived GFP+ cells from O2-treated mice were identified for CD45 (left) and FSP1 (middle) and analyzed for CD45 and FSP1 expression after the gating of GFP+ cells (right). B: lung sections from mice with air (Air) or 60% oxygen (O2) exposure were subjected to immunostain. Colocalization of both GFP and FSP1 or both GFP and Col I expression resulted in a yellow color (arrows, double positive cells) in merged staining. All microphotographs were taken at ×200 magnification. C: representative Western blots for FSP1, TTF-1, Col I, GFP, and Pro-SP-B, all from different tissues demonstrating specific expression. Lung fibroblasts were also isolated from mice with air (Air) or 60% oxygen (O2) exposure and analyzed for both GFP and FSP1 staining by fluorescence microscopy (D) and flow cytometric analysis (E). Colocalization of α-smooth muscle actin (α-SMA) and GFP in lung fibroblasts from 60% oxygen exposure were further evaluated (F). The cells that coexpressed GFP and either FSP1 or α-SMA appear in yellow (arrow). Original magnification, ×200.
The results of this procedure showed that, in air-treated mice, ~5.8% of TTF-1\(^+\) cells expressed FSP1, which represented 0.2–2.1% of all GFP\(^+\) BM-derived cells analyzed. In contrast, the lung AT2 from oxygen-exposed mice under otherwise identical culture conditions showed a significant increase in the proportion of such cells, with 16.1% of TTF-1\(^+\) cells expressing FSP1 (Fig. 4B), which represented 2.4–9.1% of all GFP\(^+\) cells. Among the GFP\(^-\) and TTF-1\(^+\) cells, 20% were likely to express FSP1 (data not shown). This finding correlates with the apparent presence of BM-derived FSP1 and positive cells in vivo during oxygen exposure, and it confirmed that EMT could be initiated from BM-derived AT2 cells. We next identified the BM-derived EMT cells with triple color localization, examining the lung sections under fluorescence microscopy (Fig. 4C). On BMT day 22 (oxygen day 20), a few clusters of GFP\(^+\) cells showed up as FSP1 positive and exhibited pro-SP-B, indicating that BM-derived AT2 cells could express the fibroblast marker (Fig. 4C). For comparison, lung sections and single cell suspensions from control mice did not show any GFP-positive cells at either time point.

**Expression of chemokines and chemokine receptors.** In light of the results described above, we next wanted to determine the mechanisms leading to the recruitment of BM-derived progenitor into lungs undergoing a fibrotic response. We undertook biological axis measurement of chemokine stromal-derived factor-1 (CXCL12) and its cognate receptor, CXCR4, in isolated BM-derived lung fibroblasts. Levels of CXCR4-positive cells were significantly higher in the lungs of animals exposed to oxygen for 14 days than in the comparable air control group (Fig. 5A). We next used a Western blot analysis specified for CXCR4 to determine whether this receptor was expressed in BM-derived lung fibroblasts at the protein level. A distinct CXCR4 band was discernable in fibroblasts exposed to O2, while the band for CXCR4 was dark in the control fibroblasts (Fig. 5B). We next triple stained the newly purified fibroblasts

---

**Fig. 4. BM-derived AEC EMT.** A: double immunofluorescent staining of GFP (green) and the type II cell marker, TTF-1 (red), for mice exposed to 60% oxygen (O\(_2\)) 5 days after BM transplantation. As shown, the engrafted cells seem exhibiting the location and shape of type II cells. B: single lung cell suspensions from GFP BM chimera mice exposed to air or oxygen were analyzed by flow cytometry for FSP1 and TTF-1 after gating the GFP\(^+\) cells (left). The data in the right panel summarize the phenotypes of BM-derived (GFP\(^+\)) cells as the percentage of cells both FSP1 and TTF-1 positive in TTF-1-positive cells at the indicated days. \(n = 6\) samples per group. Data represent the mean ± SE. *\(P < 0.05\), significant differences between oxygen and air groups. C: lung sections from GFP BM chimera mice on day 5 after BM injection were subjected to immunofluorescence. Dual-stained lung specimens with type II cells marker (pro-SP-B, red) and AECII derived cells (green) showed a yellow color if they were double-labeled (a). The paired specimens labeled with dual-stained pro-SP-B (red) and FSP1 (green) also demonstrated the yellow color with double-labeled (b) cells. Arrows indicate the same cells in paired images (a/b), illustrating that BM-derived (green) cells could double express both fibroblast and AECII cell markers (arrows). The nuclei were dyed with DAPI (blue) as indicated. Original magnification, \(×200\) for a and b.
chemotaxis in response to CXCL12. *Significant difference (P < 0.05, significant differences between oxygen and air groups.

![Fig. 5. Mechanism of fibroblast chemotaxis when exposed to oxygen. Fibroblasts isolated from GFP BM chimera mice exposed to air (Air) or oxygen (O2) for 14 days were examined for the expression of cell surface CXCR4 protein by fluorescence-activated cell sorting (FACS) analysis (A) and for CXCR4 activation (B) by Western blotting. The isolated fibroblasts were further triple-stained for FSP1, GFP, and CXCR4 and examined by FACS analysis (C). D: kinetics of CXCL12 protein expression in lung tissue as determined by ELISA (n = 8 samples in each group). Data represent the mean ± SE. *P < 0.05, significant differences between oxygen and air groups. E: isolated fibroblasts from oxygen-exposed GFP BM chimera mice were analyzed for chemotaxis in response to CXCL12. *Significant difference (P < 0.05) compared with the 0 ng/ml group. Columns and bars represent the means and ± SE.

with FSP1, GFP, and CXCR4 and analyzed them by flow cytometry (Fig. 5C). Initially, we isolated the GFP+ fibroblasts and then examined them for the dual expression of CXCR4 and FSP1. In this way, we identified a major population of GFP+ FSP1+ CXCR4+ human fibroblasts (Fig. 5C) in the lungs of mice exposed to oxygen. Increased expression of GFP+ FSP1+ CXCR4+ cells began to appear 16 days (BMT day 16) after oxygen treatment, peaked on day 22 (BMT day 24/oxygen day 22), and remained elevated through days 30 and 44. In contrast, the air-treated mice did not show increased GFP+ FSP1+ CXCR4+ fibroblasts (Fig. 5C). We next examined the levels of CXCL12 protein, determined by ELISA, in the lung homogenates prepared from either air- or oxygen-treated animals. CXCL12 levels in the oxygen-treated lungs gradually increased and remained elevated throughout the course of the experiment until day 42 (BMT day 44/oxygen day 42) (an increase of 3.5 times, compared with only a 1.9 times increase in the untreated lungs). By contrast, CXCL12 levels in the control lungs increased slightly at day 21 (BMT day 23/oxygen day 21) and approached the CXCL12 levels observed in the untreated mouse (Fig. 5D). Thus these data suggest that CXCL12 could promote the recruitment of GFP+ FSP1+ CXCR4+ fibroblasts into the fibrotic lung. To confirm that the expression of CXCR4 was actually functional, we performed in vitro chemotaxis assays to look for specific migration. As shown in Fig. 5E, fibroblasts could migrate at both 30 and 50 ng/ml CXCL12 (CXCR4 ligand). checkerboard analysis confirmed that GFP+ FSP1+ CXCR4+ fibroblasts underwent directed movement in vitro due to the presence of a chemotactic gradient.

DISCUSSION

In this study, we examined the possibility that BM-derived precursor cells could serve as a source for fibroblasts in oxygen-induced interstitial fibrosis. While there is no completely satisfactory animal model of human BPD, the oxygen-exposed mice were relatively well characterized, and they did exhibit many of the features found in the human disease. In our studies, mice exposed to 60% oxygen for 14 days developed heterogeneous lung injuries with morphologic similarities to human infants with BPD. This treatment caused epithelial injury to the lungs, which was followed by an inflammatory response over several days. That inflammatory response was then followed by lung fibrosis and an arrest of alveologenesis, both of which are typical of this model. There are clear limitations to this model in terms of the close association with inflammation that accompanies the lung injury. However, our findings from this model may have significant clinical and therapeutic implications for identifying mechanisms and pathogenetic clues that may be relevant to the human disease (11). To clearly distinguish the potential role of BM-derived cells in the establishment of fibrosis from that of resident intrapulmonary fibroblasts, we devised a BMT model in which GFP chimera mice were the source of donor BM cells. Positive GFP staining cannot be attributed to endogenous expression, and so all donor-derived BM cells in the GFP BM chimera mice could easily be distinguished from resident lung cells by their green fluorescence due to GFP expression. The model system described here not only reveals the intrinsic potential of BM cells for BM recruitment, as had been suspected in previous studies (13), but also allows quantification of the process’ robustness and provides insight into the mechanisms involved. Consistent with previous studies (4, 19, 30), successful, complete, and durable BM reconstitution was achieved using this protocol, without any evidence of lung pathology up to day 56 after BMT. The histological appearance of the lungs of animals that were myelosuppressed and treated with BMT tended to be...
worse than in animals not subjected to BMT. We interpret these data to indicate that intact bone marrow serves to limit the severity of the response to oxygen in the lungs.

One concern with the approach of GFP-staining fibroblasts is that contaminated blood cells could be mistaken for engraftment cells. It has been reported that researchers cannot reliably identify either engrafted cells in lung tissue sections after transplantation of BM cells or purified hematopoietic stem cells tracked with ubiquitous labels (6, 14, 18). We carefully isolated the fibroblasts from the injured lung so as to verify their origins. We found that although a significant portion of these GFP+ cells were probably infiltrating leukocytes, as is often the case in this model (16), animals exposed to oxygen are more likely to show lung engraftment of BM-derived cells than animals treated with PBS. Fluorescence microscopy revealed considerable numbers of GFP+ cells in areas undergoing active fibrosis in BPD chimeras. To ascertain the extent to which BM-derived fibroblasts may contribute to the progression of fibrotic lung disease in humans, we further measured these cells through the colocation of GFP+ and fibroblast markers. As has been reported elsewhere (16, 20), cultured fibroblast cells could express FSP1, matrix metalloproteinases 2 (MMP-2), vimentin, type I collagen, and α-smooth muscle actin, but such expression is not seen in vivo, so not all markers suitably identify fibroblasts in tissue. FSP1 expression is found in all resting and activated tissue fibroblasts (21, 28). We currently believe FSP1 is the best, or perhaps the most consistent, marker of fibroblasts in tissue, so we selected FSP1 as the marker to investigate. Flow cytometric analysis of the disaggregated cells from lung tissue confirmed a more than fourfold increase in GFP+ cells in oxygen-injured vs. intact lungs, and 16–31% of cells in fibrotic lung tissue were GFP+ and expressed FSP1. In contrast, less than 10% of cells in the control lungs expressed GFP and FSP1. Thus the increased level of collagen expression in fibrotic lung tissue was due mainly to BM-derived precursor cells, a result that strongly suggests that these cells play a major, direct role in the pathogenesis of fibrosis.

There is also a theoretical possibility of determining the fibroblasts’ source, as current evidence shows that, in cases of lung injury, marrow-derived AT2 could serve as a transient amplifying population and subsequently transform into the fibroblast phenotype. We suspect that fibroblasts formed by EMT differentially could express some residual receptors from their previous life as AT2, and these could theoretically be as heterogeneous as the universe of all fibroblasts. Residual type II cells expressing the GFP marker should be present if type II cell engraftment has occurred. Indeed, Krause et al. (17) reports achieving marrow engraftment with type II cells. These cells were detectable as early as 5 days and as late as 11 mo after BMT (17). To define the phenotypic signatures of the transition from BM-derived AT2 epithelial cells to fibroblast phenotypes in vivo, we used triple immunofluorescence analysis to investigate whether BM-derived fibroblasts within fibrotic lesions belonged to either the TTF-1+ or the TTF-1− subpopulation. We found no evidence of engrafted cells stained with GFP 1 and 3 days after BM injection. However, BM-derived fibroblasts comprised of both TTF-1+ and TTF-1− subpopulations were detectable by day 22 (BMT day 28/oxygen day 26). The proportion of FSP1+ GFP+ TTF-1− cells within wound-healing tissue was found to be ~16.1% of the whole GFP+ and TTF-1+ fibroblasts after 28 days (BMT day 28/oxygen day 26). The presence of a significant number of TTF-1+ BM-derived fibroblasts may be explained by the possibility that BM-derived AT2 come from heterogeneous origins before they become fibroblasts, which may have important consequences for the pathogenesis of fibrotic process.

The influx of BM-derived cells into the lung afflicted with fibrosis requires an induced signal. It has been suggested that the oxidative damage produced by oxygen exposure leads to inflammatory exudation, which then releases effector substances, chemokines, which in turn lead to fibroblast recruitment (23). We assessed this possibility in this study. We demonstrated that CXCL12 (CXCR4 ligand) could serve as the signal for the recruitment of BM-derived precursor cells to the injured lung. Lung fibroblasts from oxygen-treated mice expressed the cognate receptor for this chemokine at a significantly higher level than the control group. Clearly, BM is a sensitive site for CXCL12 activity (31). In this regard, CXCL12 is shown to be involved in directing progenitor cells into the appropriate maturation sites in the BM (24). In addition, CXCL12 may support the colonization of the BM by hematopoietic precursors during embryogenesis. It seems likely that fibroblast recruitment is the result of an active trafficking process that chemokines mediate via the tissue-specific homing of hematopoietic precursors.

In conclusion, we showed that lung fibroblasts in the BPD model could be derived from BM precursor cells. Many cells carrying donor cell GFP+ were also positive for FSP1, indicating their fibroblastic nature. This finding demonstrates the importance of BM-derived fibroblasts in the pathogenesis of BPD. Further studies using phenotypical and functional analyses of BM-derived fibroblasts would help us gain insight into the pathogenesis of the fibrotic process, and they might provide a rationale for the extrapulmonary/BM origins of fibroblasts in fibrosis. The potential therapeutic uses of BM progenitors in the treatment of pulmonary fibrosis conditions are enticing, and further elucidation of the mechanisms involved is needed to develop specific therapies.

ACKNOWLEDGMENTS

We thank Prof. Chunyang Meng for providing statistical support and insightful discussions during the preparation of the manuscript. We thank Dr. Xiaoyong Zhang at the Wistar Institute for help with the linguistic revision of the manuscript.

GRANTS

The research was supported by National Natural Science Foundation of China (No: 30770950) and key project of Chongqing Natural Science Foundation (CSTC, 2008BA0021; CSTC, 2009BB6072).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES

FIBROBLASTS IN BRONCHOPULMONARY DYSPLASIA


