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Biochemical and Biophysical Research Communications 353 (2007) 104–108
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Circulating peripheral blood fibrocytes in human fibrotic interstitial lung disease ☆

Borna Mehrad [a], Marie D. Burdick [a], David A. Zisman [b], Michael P. Keane [b], John A. Belperio [b], Robert M. Strieter [a,b,*]

[a] Division of Pulmonary and Critical Care Medicine, University of Virginia, Charlottesville, VA, USA
[b] Division of Pulmonary and Critical Care Medicine, David Geffen School of Medicine at University of California—Los Angeles, Los Angeles, CA, USA

Received 10 November 2006
Available online 11 December 2006

Abstract

Fibrotic interstitial lung diseases are illnesses of unknown cause characterized by progressive decline in lung function. Fibrocytes are bone marrow-derived, circulating progenitor cells capable of differentiating into diverse mesenchymal cell types. Prior work has shown fibrocytes to traffic to the lung via the CXCL12–CXCR4 chemokine axis in an animal model of pulmonary fibrosis. We therefore assessed the relevance of fibrocytes in patients with fibrotic interstitial lung disease. We found enhanced expression of CXCL12 in both the lungs and plasma of patients with lung fibrosis. CXCL12 levels were associated with an order of magnitude higher number of circulating fibrocytes in the peripheral blood of these patients. Most of the circulating fibrocytes in patients with interstitial lung diseases were negative for the myofibroblast marker α-smooth muscle actin, suggesting a relatively undifferentiated phenotype. Taken together, these data suggest that fibrocytes are involved in the pathogenesis of human lung fibrosis.

Keywords: Idiopathic interstitial pneumonia; Usual interstitial pneumonia; Idiopathic pulmonary fibrosis; Cryptogenic fibrosing alveolitis; Non-specific interstitial pneumonia; Fibroblast; Myofibroblast; Fibroblastic foci; Stem cells; Chemokines

Fibrosis is central to the pathogenesis of many chronic lung disorders, including asthma, pneumoconioses, and many infections. The quintessential fibrotic lung diseases, however, are the fibrotic interstitial lung diseases, usual interstitial pneumonia (UIP) and fibrotic variant of non-specific interstitial pneumonia (NSIP). These illnesses are of unknown cause and are characterized by progressive lung fibrosis, typically culminating in respiratory failure and premature death. No treatment has been clearly effective in altering the clinical course of these diseases, and there is an urgent need for better understanding of their pathogenesis [1].

Dysregulated tissue remodeling is fundamental to the development of fibrotic lung diseases: UIP and fibrotic NSIP share the histologic features of relatively mild leukocyte infiltration but prominent accumulation of extracellular matrix in the form of dense or loose fibrosis [2]. They are distinguished by the variegated pattern of pathology in UIP, in which normal areas are justaposed with areas with leukocyte infiltration and other areas with advanced fibrosis, whereas fibrotic NSIP is homogenous in its distribution [3–5]. In addition, the pathological lesion of fibroblastic foci, which consist of concentrated numbers of fibroblasts and myofibroblasts associated with focal injury and generation of new collagen, is more prominent in UIP than fibrotic NSIP [6–8].

The source of lung fibroblasts and myofibroblasts is a critical question in the pathogenesis of fibrotic lung diseases. While these cells were classically thought to be derived exclusively from resident lung fibroblasts, recent studies

☆ This work was supported by NIH Grants HL73848 and an American Lung Association Career Investigator Award (Mehrad), HL080206 (Belperio), AR055075 (Keane), and CA87879 and HL66027 (Strieter).
* Corresponding author. Fax: +1 434 243 0399.
E-mail address: Strieter@Virginia.edu (R.M. Strieter).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.11.149

indicate that they can differentiate from pulmonary epithelial cells [9] and from a circulating precursor cell, the fibrocyte [10]. Fibrocytes are bone marrow-derived cells with monocytic morphology, that express surface markers of leukocytes and hematopoetic stem cells but also collagen-I; and are capable of differentiating into diverse cell types [11–13]. In this context, we have previously shown that, in a mouse model of bleomycin-induced pulmonary fibrosis, both mouse and human fibrocytes can traffic to the lung and contribute to collagen deposition and accumulation of α-smooth muscle actin (αSMA)-expressing cells in the lung [10]. Furthermore, the recruitment of these cells was mediated via the interaction of the chemokine ligand, CXCL12, in the lung and the receptor, CXCR4, on fibrocytes.

In the present study, we sought to evaluate the contribution of fibrocytes to human fibrotic interstitial lung disease. We therefore compared human lung tissue and peripheral blood from patients with UIP and fibrotic NSIP to normal controls, for the expression of the fibrocyte-attracting chemokine, CXCL12, and presence and number of circulating fibrocytes and their expression of the CXCL12 receptor, CXCR4. Our findings support the notion that the number of circulating fibrocytes may represent a novel biomarker in patients with fibrotic lung disorders.

Methods

Patients and samples. Patients were recruited from the interstitial lung disease clinic at UCLA and all work was approved by the Institutional Review Board. Lung tissue samples from patients with fibrotic lung disease were obtained during open lung biopsy for the diagnosis of interstitial lung disease, and normal samples were obtained from patients who underwent lobectomy for bronchogenic carcinoma, at a site distant from the malignancy. Samples were homogenized and sonicated in complete buffer (Roche Diagnostics, Indianapolis, Indiana, USA) as previously described [14]. Homogenates were centrifuged at 900*g* for 15 min, filtered through 1.2 μm sterile Acrodiscs (Gelman Sciences, Ann Arbor, Michigan, USA). We also obtained 10 ml of heparinized venous blood from 5 normal volunteers and 5 patients with fibrotic interstitial lung disease. Of the patients with fibrotic interstitial lung disease, 4 were diagnosed with UIP on the basis of clinical, radiographic, and histopathologic features, and 1 was diagnosed with fibrotic NSIP on the basis of histopathology. Samples were processed to isolate the buffy coat leukocyte populations for FACS analysis by centrifugation, as previously described [15,16]. Contaminating red blood cells were removed then the cells were washed and brought up to a concentration of 1×10^7/ml in PBS containing 0.1% FBS. Lung homogenates and plasma samples were frozen at −70 °C until processed for ELISA. Samples were assayed for CXCL12 levels as previously described [10].

Immunohistochemical staining. Paraffin embedded tissues were processed for immunohistochemical localization of CXCL12 as previously described [17]. Briefly, tissue sections were dewaxed with xylene and rehydrated through graded concentrations of ethanol. The slides were fixed for 30 min in 1:1 absolute methanol and 3% H_2O_2, rinsed in PBS and then non-specific binding sites were blocked with universal blocking reagent (Biogenex, San Ramon, CA) by incubation at room temperature for 30 min. Following the blocking step, a 1:500 dilution of either control (goat) or goat anti-hCXCL12 serum was added as a primary antibody, and slides were incubated for 30 min at room temperature. Slides were then rinsed with PBS, overlaid with biotinylated anti-goat IgG (Vector ABC Elite Kit, Vector Laboratories, Burlingame, CA) and incubated for an additional 30 min. Slides were rinsed 2 times with PBS, and were then treated with streptavidin-conjugated peroxidase for 30 min at room temperature. Following, three washes with PBS, the slides were subjected to colorimetric detection using the substrate chromogen 3,3′-diaminobenzidine (DAB, Vector Laboratories). Slides were incubated for 5–10 min in DAB solution at room temperature to allow color development, and rinsed with distilled water to quench the reaction. Mayer's hematoxylin was used as a counterstain.

Flow cytometry. Circulating fibrocytes were identified by flow cytometry according to previously published methods [10]. Briefly, leukocytes were stained with anti-CXCR4-FITC (R&D systems), and anti-CD45-PerCP (BD Biosciences). Next, the cells were permeabilized using cytofix/cytoperm (BD Biosciences) prior to intracellular staining of collagen-I (Col I, Rockland) and αSMA (R&D Systems). Col-I and αSMA were then stained with unconjugated rabbit anti-human Col-I and mouse anti-human αSMA Abs followed by Alexa Fluor 610-R-phycoerythrin goat anti-rabbit or mouse (Molecular Probes). Samples were processed on a FACS Calibur flow cytometer using Cellquest software.

Statistical analysis. Data were analyzed on a Dell PC (Dell, Round Rock, Texas, USA) computer using Statview statistical package (Abacus Concepts, Berkeley, California, USA). Comparisons were evaluated by Student's unpaired *t* test. Results were considered statistically significant if *p* values were less than 0.05.

Results

Given the role of the chemokine ligand, CXCL12, in recruiting fibrocytes to the lung in animal models of pulmonary fibrosis, we began by comparing the lung expression of CXCL12 in biopsy tissue in archived samples obtained from patients with fibrotic lung disease. We found extensive accumulation of CXCL12 in lung tissue from patients with the clinical and histologic diagnosis UIP, as detected by immunohistochemistry (Fig. 1a). To assess this finding quantitatively, we compared protein levels of CXCL12 in lung tissue from patients with UIP and fibrotic NSIP, and compared them to levels in normal lung tissue (Fig. 1b). We noted a 64% and 77% increase in lung CXCL12 levels in lungs of patients with fibrotic NSIP and UIP, respectively, as compared to normal lungs. Interestingly, the levels of CXCL12 did not differ between NSIP and UIP.

In order to examine whether this increased expression of lung CXCL12 in patients with fibrotic lung disease was correlated with fibrocyte trafficking, we next performed a pilot study, in which we prospectively collected peripheral blood from 5 patients with UIP or fibrotic NSIP and compared them to 5 normal volunteers. We began by comparing the plasma CXCL12 levels between the groups, since plasma chemokine levels have previously been shown to correlate with tissue levels in pulmonary fibrosis [18]. The plasma CXCL12 levels were 2.4-fold higher in patients with UIP and fibrotic NSIP than normal patients (Fig. 2), confirming our observation in lung tissues.

Next we compared the number of circulating fibrocytes in the two groups by enumerating collagen-I expressing $CD45^+$ cells [10]. Remarkably, patients with fibrotic lung disease had an order of magnitude higher number of circulating fibrocytes as compared to healthy volunteers (Fig. 3a). We have previously reported that circulating

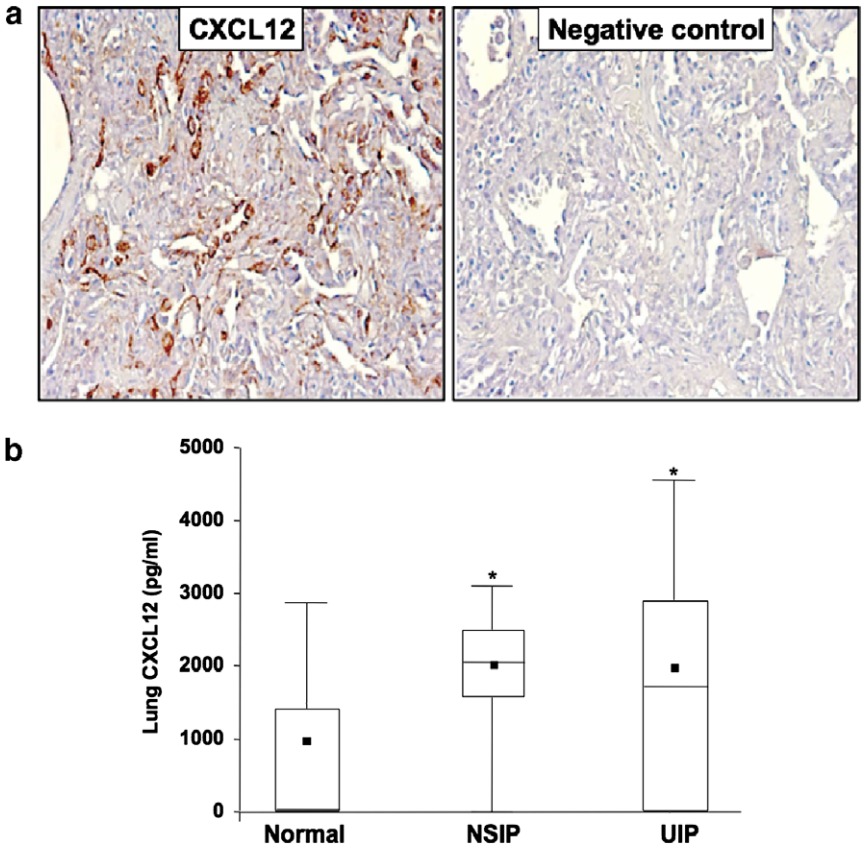


Fig. 1. Lung CXCL12 in fibrotic interstitial lung disease. (a) Immunohistochemical detection of CXCL12 protein in the lung of a patient with UIP. (b) Lung CXCL12 levels measured by ELISA in lung homogenates. Box and whiskers represent 25–75th and 10–90th percentiles, respectively; small squares and transverse lines represent the mean and median, respectively. NSIP, lung biopsy from patients with fibrotic non-specific interstitial pneumonia; UIP, lung biopsy from patients with histologic UIP; $^{*}p < 0.05$ compared to normal lung samples ($n = 92$ for normal lungs, 13 for fibrotic NSIP, 56 for UIP).

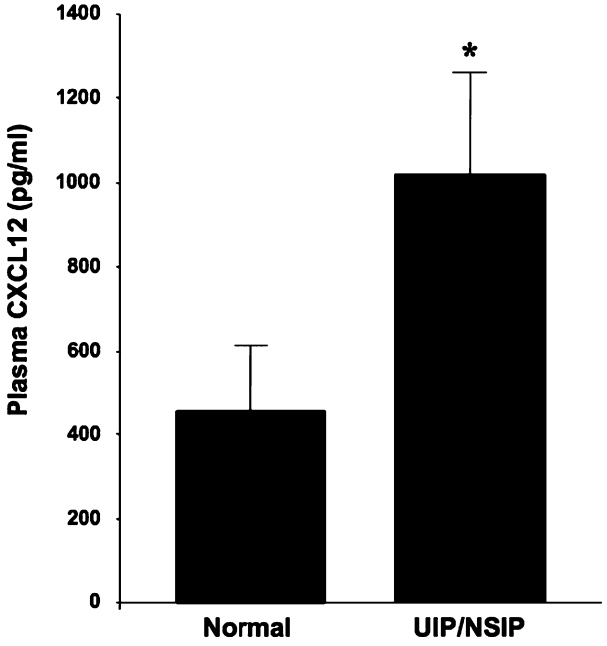
fibrocytes are composed of a larger CXCR4-positive and a smaller CXCR4-negative subsets [13]. The expanded circulating fibrocyte pool was most notable in the CXCR4-expressing subset, but was also noted in the CXCR4-negative subset (Fig. 3a).

Many of the lung fibroblasts in pulmonary fibrosis have myofibroblast phenotype and the contractile properties of myofibroblasts have been hypothesized to be important in the progression of lung fibrosis [6,19,20]. Since circulating fibrocytes are capable of differentiating into myofibroblasts both in culture and in vivo [21,22], we also compared the degree of differentiation of the circulating fibrocytes in the two groups by assessing them for the expression of the myofibroblast marker, α-SMA (Fig. 3b). The αSMA-expressing fibrocytes constituted a small subset of total circulating fibrocytes in both groups. Although we observed no statistically significant difference between total αSMA^{+} fibrocytes or the $CXCR4^{+}$ or $CXCR4^{-}$ subsets of αSMA^{+} fibrocytes between patients and normal volunteers, there appeared to be a trend toward higher numbers of circulating of αSMA^{+} cells in patients with fibrotic interstitial lung diseases. This suggests that the greater numbers of circulating fibrocytes in patients with fibrotic interstitial lung diseases may, in addition, show early evidence of differentiation to myofibroblast phenotype.

Discussion

Fibrocytes are a recently identified population of bone marrow-derived circulating cells that express markers for both hematopoetic cells and fibroblasts. Prior evidence has linked circulating fibrocytes to the biology of wound repair, scleroderma, and asthma, via their differentiation into fibroblasts and myofibroblasts (reviewed in refs. [11,12]). Fibrocytes are, in addition, capable of differentiating into diverse cell types, including adipocytes and antigen-presenting cells [13,23].

We recently identified fibrocytes as contributing to lung scarring in the mouse model of bleomycin-induced pulmonary fibrosis and demonstrated that both mouse and human fibrocytes can traffic to the lung via the CXCL12–CXCR4 axis in the context of this model [10]. While intrapulmonary administration of pro-fibrotic agents represent the dominant animal models for the study of fibrotic lung diseases, they differ from the human disease in several key respects, including temporal progression and histology [24,25]. This prompted us to examine the relevance of fibrocytes in patients with fibrotic interstitial lung diseases in the current study. We found increased expression of the fibrocyte-attracting chemokine, CXCL12, in the lungs and plasma of patients with fibrotic interstitial lung disease. In



Fig, 2. Plasma CXCL12 level in fibrotic interstitial lung disease. Data represent means ± SEM of n of 5 patients per group. "UIP/NSIP", patients with UIP or fibrotic NSIP; $^{*}p < 0.05$ compared to samples from normal volunteers.

addition, we found as well as a marked expansion of circulating fibrocyte pool in these patients: fibrocytes, which normally constitute ~0.5% of circulating leukocytes [11,12], comprised 6–10% of leukocytes in our cohort. Indeed, these cells may have contributed to a peripheral blood monocytosis observed in this cohort (absolute monocyte count $4.54 \pm 0.44 \times 10^9$ cells/L in this cohort; reference range $0.10–1.10 \times 10^9$ cells/L, $p = 0.004$). To our knowledge, this is the first reported evidence of the involvement of fibrocytes in human interstitial lung disease.

The current report has a number of implications for future work. First, the mechanism of fibrocyte function in the pathogenesis of pulmonary fibrosis is now of great interest. In addition to fibrocyte generation of extracellular matrix and contractile properties as fibroblasts/myofibroblasts, fibrocytes may conceivably play a role in angiogenesis or on-going immune responses in these illnesses [23,26]. Second, the relative contribution of fibrocytes, local precursor cells, and pre-existing lung fibroblasts to the final lung pathology of human pulmonary fibrosis is yet to be established. Third, the role of $CXCR4^+$ and $CXCR4^-$ fibrocyte subsets in the progression of these diseases should be determined. Finally, the study of mechanisms of fibrocyte traffic, including their egress from bone marrow, endothelial

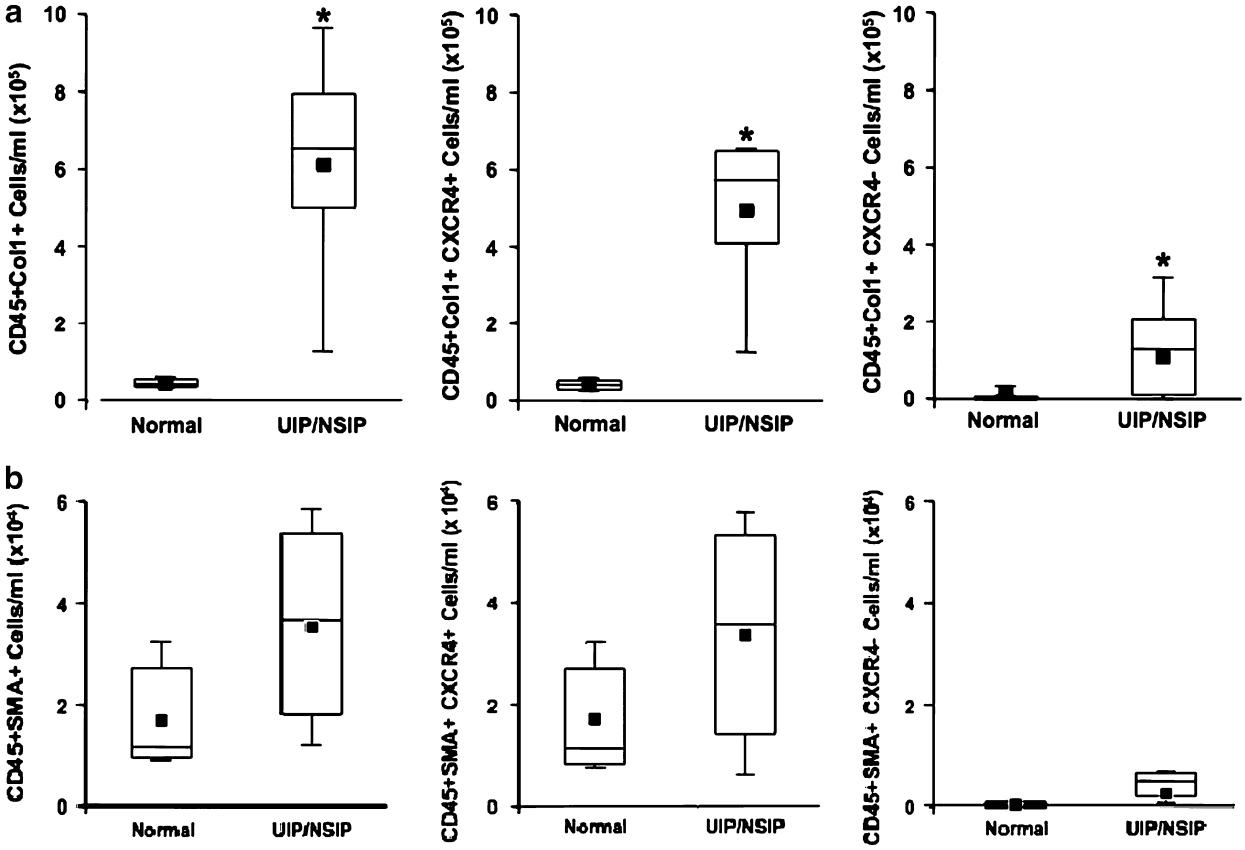


Fig. 3. Circulating fibrocytes in fibrotic interstitial lung disease. Panels (a) and (b) represent numbers of circulating $CD45^+$ collagen-I^+ cells and $CD45^+$ collagen-I^+ αSMA^+ cells, respectively. Box and whiskers represent 25–75th and 10–90th percentiles, respectively; small squares and transverse lines represent the mean and median, respectively. "UIP/NSIP", patients with UIP or fibrotic NSIP; $^{*}p < 0.05$ compared to normal volunteers ($n = 5$ patients per group).

adhesion and egress from the circulation, and response to chemokine gradients, may represent novel targets for therapeutic intervention in these devastating disorders.

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