The Role of Neutrophils in the Pathogenesis of Idiopathic Pulmonary Fibrosis*

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Study objectives: The prognostic value of the neutrophil count in BAL fluid (BALF) has been controversial. The role of neutrophils in this inflammatory lung disease, therefore, was evaluated in this study by additional measures.

Materials and methods: We performed BAL in 22 patients with idiopathic pulmonary fibrosis (IPF) diagnosed by open lung biopsy specimen. Percent polymorphonuclear leukocyte (PMN) in BALF and absolute neutrophil counts were compared with those of normal nonsmokers. Elastase complexed to alpha-1-proteinase inhibitor (α1-PI) in plasma and BALF was measured as a marker of elastase burden, and neutrophil distribution in 22 lung tissues was observed by immunohistochemistry using antineutrophil elastase antibody.

Results: Percent PMN and absolute neutrophil counts in BALF did not increase in patients with IPF as compared with normal nonsmokers (n=15); the plasma elastase-α1-PI complex value (mean±SE) of patients with IPF (668.5±112.4 ng/mL) was significantly high as compared with that of normal nonsmokers (130.3±21.3, p<0.001). In addition, the BALF elastase-α1-PI complex value (mean±SE) of patients with IPF was also significantly high (333.1±87.0 ng/ml albumin) as compared with that of normal nonsmokers (83.1±29.3 ng/mg albumin, p<0.05). Immunohistochemistry demonstrated considerable numbers of neutrophils infiltrating the lung parenchyma in biopsy specimens obtained by open lung biopsy.

Conclusions: These results suggested that although the neutrophil count in BALF could not represent the distribution of neutrophil in the lung, high levels of neutrophil elastase were demonstrated in lung parenchyma and also in both BALF and sera. Therefore, neutrophils might indeed play an important role in the pathogenesis of IPF.

Key words: idiopathic pulmonary fibrosis; immunohistochemistry for neutrophil elastase; neutrophil elastase

Abbreviations: α1-PI=alpha-1-proteinase inhibitor; BALF=BAL fluid; HPF=high-power field; IPF=idiopathic pulmonary fibrosis; PMN=polymorphonuclear leukocyte

Idiopathic pulmonary fibrosis (IPF), an inflammatory lung disease of unknown etiology, is characterized by the accumulation of inflammatory cells, followed by the progressive deposition of collagen within the interstitium and subsequent destruction of lung airspaces.1,2 Increasing scientific evidence defines the importance of neutrophils in the pathogenesis of pulmonary fibrosis. Increases in polymorphonuclear leukocytes (PMNs) on BAL fluid (BALF)3-8 and in lung tissue9 have been demonstrated in patients with IPF. However, it has been reported that the number and proportion of PMNs in BALF do not correlate with the activity of alveolitis and have limited prognostic value.8,10-12

Therefore, we hypothesized that the percentage PMNs in BALF may not represent the distribution and activation of neutrophils in lung specimens. To examine this hypothesis, we measured elastase complexed to alpha-1-proteinase inhibitor (α1-PI) (elastase-α1-PI complex) in plasma and BALF in patients with IPF, and these results were compared with the percentage of PMNs in BALF. In addition, to evaluate the site of neutrophil accumulation in
patients with IPF, specimens obtained by open lung biopsy were examined by immunohistochemistry using antihuman neutrophil elastase antibody.

Materials and Methods

Subjects

The protocols of this study were approved by the institutional review board for human studies, and informed written consent was obtained from the subjects.

A diagnosis of IPF required the following:12,13 (1) no clinical history of exposure to environmental agents known to cause interstitial lung disease, no history suggestive of extrinsic allergic alveolitis, and no history of chronic lung infection or left ventricular failure; (2) evidence of interstitial infiltrates on chest radiograph or physiology consistent with a restrictive ventilatory defect, including decreased lung volumes and normal flow rates; and (3) histologic specimens obtained by open lung biopsy show interstitial pneumonia with varying degrees of interstitial fibrosis without evidence of granulomas, vasculitis, or inorganic material visualized by polarized light microscopy. Histologic confirmation was obtained in all cases by open lung biopsy specimens.

The average age of 22 patients was 64 years (median age, 67 years), with a range of 47 to 77 years. There were 12 men and 10 women in the group. There were 13 nonsmokers, six ex-smokers, and three current smokers at the time of the study. In all patients, the diagnosis of IPF was confirmed by CT. All studies were performed before the initiation of treatment and the pulmonary disease was presumed to be active instead of end staged.

The 15 normal nonsmokers, 6 women and 9 men with an average age of 55 years, had no history of lung diseases, and no clinical findings suggesting lung diseases. They all had normal chest radiographs and their pulmonary function test results were within normal range.

Blood samples with and without edetic acid were obtained from 17 patients with IPF before breakfast. After centrifugation at 1,000 g for 10 min at 4°C, the plasma was frozen and stored at −70°C until used.

Bronchoalveolar Lavage

To sample the lower respiratory tract, flexible fiberoptic bronchoscopy and BAL were performed in 15 of 22 patients with IPF. BAL was performed by infusing three 50-mL aliquots of sterile saline solution at the site of the anterior segment of the right lower lobe. The last two aliquots were saved for evaluation. Absolute cell counts and cell differential counts were also examined. Cells were separated from alveolar lavage fluid by centrifugation (300 g for 10 min). BALF was frozen and stored at −70°C until used.

Measurement of Elastase-α1-PI Complex Levels in Plasma and BALF

Elastase-α1-PI complex concentration was determined by using an enzyme-linked immunosorbent assay. Briefly, the plasma samples were added to wells coated with rabbit antineutrophil elastase IgG (Calbiochem-Novabiochem Co; La Jolla, Calif; lot B12443). This antibody does not cross-react with cathepsin G or other neutrophil proteinases. After incubation and washing, the solid phase-bound elastase-α1-PI complexes were further incubated with alkaline phosphatase-labeled rabbit anti-α1-PI IgG (Organon Teknika Co; West Chester, Pa; lot 31949). After further washes, p-nitrophenylphosphate was added to measure the amount of solid phase-bound complexes. The assay was calibrated using a standard solution of known elastase-α1-PI complex concentration. The lower detection limit of this assay was 3 ng/mL. Data were expressed as mean values from duplicate determinations. In BALF, data were corrected to albumin.

Immunohistochemistry by Antineutrophil Elastase

To evaluate the site of neutrophil accumulation in patients with IPF, specimens obtained by open lung biopsy were immunohistochemically stained using an enhanced polymer one-step staining (EPOS) reagent conjugated with mouse monoclonal antihuman neutrophil elastase (DAKO Japan; Kyoto; clone NP57). For immunohistochemistry, paraffin sections were deparaffinized, treated with H2O2/methanol, digested with trypsin, and reacted with an EPOS reagent. After reaction with diamobenzidine, sections were counterstained with hematoxylin and observed. Immunohistochemistry using anti-Leu-M1 (CD15) antibody (Becton Dickinson; Mountain View, Calif) was also performed to confirm distinction between neutrophils and other cells. Density of total neutrophil infiltration observed in biopsy specimens was scored as follows: grade 4 (20/high-power field [HPF]), grade 3 (20 to 10/HPF), grade 2 (10 to 5/HPF), and grade 1 (<5/HPF). In addition, density of neutrophil infiltration in each component of lung tissue (endothelium, interstitium, and alveoli) was graded separately from 4 (dense) to 1 (very sparse).

Statistical Methods

All comparisons between groups were made using the nonparametric Wilcoxon-Mann-Whitney rank order test. Correlations were evaluated by the Pearson’s correlation coefficient, and Fisher’s r to z method was used to calculate the p values.

Results

Neutrophils in BALF

There was no significant increase of percentage of PMNs in IPF patients (3.3±1.0% [mean±SE]) as compared with normal nonsmokers (3.5±0.6%, p=0.87, Fig 1A [left]). In addition, there was also no significant difference in the absolute neutrophil counts recovered by BAL in patients with IPF (7.4±2.8 [10^3]/mL) as compared with normal nonsmokers (3.8±0.7 [10^3]/mL, p=0.31, Fig 1B [right]).

Measurement of Human Neutrophil Elastase

Figure 2A (left) shows the concentrations of plasma elastase-α1-PI complex in our study population. Plasma elastase-α1-PI complexes in patients with IPF were significantly high (668.5±112.4 ng/mL) as compared with normal nonsmokers (130.3±21.3 ng/mL, p<0.001).

Figure 2B (right) shows the concentrations of elastase-α1-PI complex in BALF in our study population. The elastase-α1-PI complex value in BALF in patients with IPF (333.1±87.0 ng/mg albumin) was
significantly high as compared with normal non-smokers (83.1±29.3 ng/mL, p<0.01).

**Immunostaining by Human Neutrophil Elastase**

Inflammatory cells infiltrating the interstitium adjacent to dilated airspaces forming a honeycomb structure seemed to be mainly lymphocytes and plasma cells. However, immunohistochemistry for elastase demonstrated considerable numbers of neutrophils intermingled among them. Sometimes the number of infiltrating neutrophils was much higher than had been expected in ordinary hematoxylin-eosin-stained sections. Interestingly, positive elastase stain observed was exclusively intracellular within neutrophils. Neutrophil distribution was observed mainly in the interstitium, especially in the dense fibrotic tissues, as well as in the alveolar septa (Fig 3). Some neutrophils were observed in the alveolar space (Fig 3). Importantly, neutrophil adhesion was observed along the endothelium in pulmonary microvessels in some patients with IPF (Fig 4), suggesting neutrophilic endotheliolitis took place.

Table 1 summarizes the results of immunohistochemistry by antihuman neutrophil elastase in IPF lung specimens. We also stained normal lung tissues and compared them with lung tissues obtained from patients with IPF. As a result, there was no stain of neutrophils in normal lung tissues.

**DISCUSSION**

In this study, we evaluated the role of neutrophils in patients with IPF. In our study population, although percentage of PMNs as well as absolute neutrophil counts in BALF did not increase in patients with IPF as compared with normal non-smokers, the mean plasma and BALF elastase-α1-PI complex values were significantly high as compared with values of normal nonsmokers. In addition, we demonstrated the distribution of neutrophils in lung specimens obtained from patients with IPF. Immunohistochemical staining by human neutrophil elastase showed infiltration of considerable numbers of neutrophils, although it was difficult to detect neutrophils intermingled in inflammatory cells that were easily overlooked by hematoxylin-eosin staining.

Several groups have investigated a possible relationship between absolute and relative numbers of
various cell types in BALF samples obtained from patients with IPF and the clinical course. Generally, BALF lymphocytosis is associated with responsiveness to corticosteroid therapy and clinical improvement.\textsuperscript{4,5} Increased eosinophil\textsuperscript{5,6,13} and neutrophil\textsuperscript{4,6,8} counts without increased lymphocyte counts have been associated with failure to respond to treatment; however, this was not confirmed by all investigators.\textsuperscript{10,11,14}

Schwartz et al\textsuperscript{10,11} did not find a relationship between BAL findings and survival in IPF patients, although a higher concentration of lymphocytes ap-

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**Figure 3.** Immunohistochemistry by antihuman neutrophil elastase in IPF lung specimens. Neutrophil elastase stains were detected in areas of honeycombing and the alveolar septa. Some immunopositive cells were observed in the alveolar space.

**Figure 4.** Immunohistochemistry by antihuman neutrophil elastase in IPF lung specimens. Neutrophil adhesion was observed along the endothelium in pulmonary microvessels in some patients with IPF.

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**Figure 2.** Left, A: concentrations of elastase-α\textsubscript{1}-PI complex in plasma in our study population. Right, B: concentrations of elastase-α\textsubscript{1}-PI complex in BALF in our study population.
Table 1—Summary of Results of Immunohistochemistry

<table>
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<tr>
<th>Patients</th>
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*Density of total neutrophil infiltration observed in fibrosis areas of biopsy specimen was scored as follows: grade 4 (>20/HPF); grade 3 (20 to 10/HPF); grade 2 (10 to 5/HPF); and grade 1 (<5/HPF).

Density of neutrophil infiltration in each component of lung tissue (endothelium, interstitium, and alveoli) was graded separately from 4 (dense) to 1 (very sparse).

The absence of a strong relationship between BAL cellularity and survival in IPF raises the possibility that the BAL procedure is not accurately sampling the underlying interstitial process. A major concern related to the use of BAL in patients with IPF is whether a random sample of the lung using BAL is appropriate. This is particularly relevant in IPF in which radiographic and pathologic studies have identified a patchy, uneven distribution of interstitial inflammation and fibrosis that predominates in the basilar and peripheral regions of the lung.

However, our data demonstrated that although increase of percentage of PMNs as well as absolute neutrophil counts in BALF was not demonstrated in our study group, the distribution of neutrophils in the interstitium of lung specimens was demonstrated using immunohistochemistry for neutrophil elastase. This evidence suggested that although neutrophil activation takes place in the interstitium, it might be very difficult to recover neutrophils by the standard BAL procedure.

Using immunohistochemistry, we also demonstrated neutrophils that attached to capillary endothelium in some patients with IPF. It was easily speculated that although neutrophils attached to the endothelium could not be recovered, elastase released from neutrophils and complexed with α-PI could be recovered by the standard BAL procedure. This evidence may explain the discrepancy between a low percentage of PMNs in BALF and high elastase-α-PI values in plasma and BALF. Therefore, although the BAL cells may be representative of the underlying interstitial process, BAL may have misrepresented the degree of interstitial inflammation.

It is generally accepted that chronic alveolar inflammation (alveolitis) is the forerunner of the irreversible alveolar-capillary unit derangement that is characteristic of interstitial pneumonia when a sufficient number of alveolar-capillary units are affected, the lung can no longer maintain gas exchange. In addition, in vitro data suggest that activated neutrophils may exert direct cytotoxic effects against both epithelial and endothelial cells, with much of the effect being assigned to the local...
release of proteases, particularly neutrophil elastase.24 Our results of immunohistochemistry demonstrated that both alveolitis and endotheliolitis took place in IPF,25 suggesting that persistent accumulation of activated neutrophils in the lung could induce a chronic alteration of the endothelial and/or epithelial barrier.26

We also evaluated the correlation between the intensity of neutrophil infiltration of immunohistochemistry for neutrophils and the values of elastase-α1-PI complexes in plasma and BALF. However, we could not demonstrate the significant correlation. This evidence suggested that although open lung biopsy is a most reliable procedure to diagnose pulmonary fibrosis, it represents only a part of the total lung.

In conclusion, our results showed that although the neutrophil count in BALF could not represent the distribution of neutrophil in the lung, high levels of neutrophil elastase were demonstrated in lung parenchyma and also in both BALF and sera. Therefore, neutrophils might indeed play an important role in the pathogenesis of IPF.

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