Fibrogenic Activities in Bronchoalveolar Lavage Fluid of Farmer’s Lung*

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Hyaluronic acid (HA), type III procollagen, fibronectin, and fibroblast growth factors (FGF) were measured in 43 bronchoalveolar lavage fluid (BALF) specimens obtained from 38 patients with farmer’s lung (FL) and in BALF of 9 nonexposed normal control subjects. Bronchoalveolar lavage was done in 21 farmers with acute FL (acute) and in 22 with a history of previous FL (Ex) who were still in daily contact with dairy barns. All farmers from the acute and Ex groups had a lymphocytic alveolitis, respectively, 62.7 (3.5) percent (mean [SEM]) and 48.1 (4.3) percent. Hyaluronic acid, type III procollagen, fibronectin, and FGF were all highly increased in acute disease. These substances were also increased in the BALF of subjects of the Ex group who had no clinical symptoms or signs of acute disease at the time of lavage, but were actively farming. The increase in type III procollagen, however, was less in this group than in the subjects with acute disease. These observations suggest that the fibrosing activities and potentialities of the allergic alveolitis of FL are fully expressed at the time of clinical presentation and also in the subclinical phase of the disease in susceptible farmers who remain exposed after an initial acute phase of the disease.

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FGF = fibroblast growth factors; FL = farmer’s lung; HP = hypersensitivity pneumonitis

Farmer’s lung (FL) and other forms of hypersensitivity pneumonitis (HP) can lead to interstitial fibrosis. The susceptibility of an individual patient to develop lung fibrosis is extremely variable; some subjects with HP will completely recover after one or even multiple attacks of acute disease, while others will have residual functional alterations even after one acute episode. Some farmers may develop progressive lung fibrosis without ever having had the acute phase of the disease, which usually leads to medical consultation. Also, some patients with FL progress to a more obstructive form of chronic disease. In addition, exposed farmers can present precipitin antibodies and/or alveolitis but remain asymptomatic.

In recent years, several factors implicated in the pathogenesis of lung fibrosis have been identified in the lung tissues and bronchoalveolar lavage fluids (BALF). These include type III procollagen and hyaluronic acid, both matrix materials implicated in the production of fibrosis, and fibronectin and fibroblast growth factors (FGF), molecules released mainly by activated alveolar macrophages, that are participating in the fibroblast attraction, deposition, accumulation, and proliferation in the lung.

Farmers with acute FL have increased levels of type III procollagen and hyaluronic acid in their BALF. The BALF levels of hyaluronic acid have been suggested to differentiate farmers with disease from those with an asymptomatic alveolitis. These molecules decreased after treatment with corticosteroids and after contact cessation associated with the remission of the disease. The long-term significance of these substances in FL are currently unknown; however, in sarcoidosis, the level of these substances in BALF can predict outcome. Studies in asbestosis have shown that fibronectin and procollagen type III, were increased in the presence of early fibrosis due to asbestos exposure, but not in the absence of a fibrotic process in exposed subjects, suggesting a significant role of these molecules in the characterization of a fibrotic lung process.

This study was carried out to further evaluate in acute FL the alterations of matrix materials associated with lung fibrosis activity, as assessed by hyaluronic acid and type III procollagen, and to assess their levels in subjects in clinical remission of their acute disease but still in daily contact with the farm environment. We also evaluated the ability of extracellular milieu of our patients to stimulate fibroblast growth. This aspect was tested by measuring FGF and fibronectin in the BALF of subjects.

Material and Methods

Forty-three BAL specimens were obtained from 38 farmers: 21 in subjects with acute FL (acute) and 22 in subjects with a history of acute disease (Ex). No patient was receiving any treatment for FL at the time of the study. Five farmers were lavaged twice. Of these, three were lavaged once during their acute phase and once in the group of subjects with a history of FL. In the other two, both lavages were obtained in the Ex state. In all cases, a minimum of 1 year separated the 2 lavages. Subjects included in the Ex group had been free of acute febrile reactions for at least 1 year although they were still in daily contact with the farm environment. Five of these subjects had demonstrated progressive worsening of their clinical status and/or deterioration in their lung functions over the year preceding the study. Since these five represented a small group and

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were similar in all other aspects to those in clinical remission, they were included in the Ex group. The mean (SEM) ages for the farmers were as follows: acute, 43.6 (2.7) years; Ex, 47.6 (2.6) years. Nine nonexposed normal subjects were also lavaged as control subjects (age, 33.7 [1.9] years).

All farmers had pulmonary function measurements, including lung volumes, single-breath diffusivity capacity (Dco), and forced expiratory flows. All tests were done before the BAL and according to standard procedures. Predicted values used were those of Grimby and Soderholm for lung volumes, Cotes and Hall for Dco, and Berglund et al for forced expiratory flows.

Bronchoalveolar lavage was done with a 300-ml instillation of normal saline solution, usually in the middle lobe (6 aliquots of 50 ml). The BAL return was kept on ice until processing (within 1 h of the bronchoscopy). Total cell count was determined with a hemocytometer and differential (on Diff Quik) and nonspecific esterase stained cytospinflupe cell preparations. Cell viability was verified by trypan blue exclusion. Differential cell counts were calculated by counting at least 600 cells. Aliquots of BAL fluids were frozen at −70°C until analysis.

The following were measured on the BAL fluids: hyaluronic acid, type III procollagen antigens, fibronectin, FGF, and albumin. Hyaluronic acid was analyzed by radioassay;[14] type III procollagen aminoterminal peptide-related antigens were measured using a radioimmunoassay kit (Behringwerke AG, Marburg, Germany), according to the methods described by Rohde and coworkers.[15] The antibodies against the bovine type III procollagen aminoterminal peptide domain did not cross-react with type I collagen, type I procollagen, 7S collagen, and laminin (product profile: Behringwerke AG, Marburg, Germany, 1983), nor with human fibronectin. With this method, the lower limit of detectability corresponded to propeptide concentrations of 0.1 ng/ml.[15] Fibronectin was measured by an enzyme-linked immunosassay.[16] Fibroblast proliferation assay was done as previously described.[17] In preparation for this analysis, BALF was centrifuged, 15,000 g for 15 min and the supernatant dialyzed extensively against distilled water. The dialyzed fluid was frozen (−70°C), lyophilized, resuspended in sufficient DMEM to yield a final albumin concentration of 500 mg/L and passed through a 0.22-μm sterile filter (Millipore Corporation, Bedford, Mass). The ability of BALF to induce fibroblast proliferation in the absence of serum was evaluated, because serum is a potent source of both competence and progression-type FGF. Fibroblasts were prepared at a seeding density of 5 × 10⁵ well in 24-well culture plates, incubated in DMEM containing 0.4 percent calf serum for 4 days, and washed 5 times with DMEM. The cells were then incubated with 500 μL DMEM (without serum) and varying amounts of BALF for 3 days in an atmosphere of 10 percent CO₂, 37°C. At the end of the incubation period, the cells were counted as previously described.[17] Bronchoalveolar lavage fluid levels of albumin were measured by nephelometry.

Data Analysis

Three approaches were used to verify if relationships exist between markers and lung functions. First, the least squares method was used to estimate the parameters of the models and statistically test for the individual terms, with diagnostic methods for detecting inadequacies (like curvature, heteroscedasticity, normality, and outliers). Second, we used robust techniques to reduce the influence of outlying responses.[18] Finally, to bridge the gap between the first two approaches, a canonical analysis was performed. We obtained similar results with all three approaches. For markers (type III procollagen, hyaluronic acid, fibronectin, and FGF), we used multivariate techniques on observations replaced by their rank because the normality assumption and homoscedasticity were unjustified. To obtain 95 percent confidence intervals, the Bonferroni inequality was applied and a Scheffe's procedure was used at 0.985 for each marker. A nonpaired Student t test was used for comparing lung functions between the two groups of farmers.

RESULTS

Pulmonary function tests for the two groups of farmers (Fig 1) show that Dco and residual volume (RV) were the most abnormal parameters. As expected, Dco was lower in the acute cases. Residual volume was increased and similar in both groups. Results of lavage cell recovery are presented in Figure 2. Both farmer groups had an increased number of cells (acute: 96 [11] × 10⁶; Ex: 59.3 [11] × 10⁶; controls: 14.8 [2.7] × 10⁶) with a high percentage of lymphocytes in their lavages (62.7 [3.5], 48.5 [4.1], and 14.8 [2.7], respectively). Results of the lavage markers of fibrogenesis are presented in Figure 3. Hyaluronic acid was five times higher in both groups of farmers than in the control subjects. Type III procollagen was especially high in the acute phase, at 80 times control values. The Ex FL group had an intermediate level at five times that of normal subjects. Fibronectin levels were similar in both groups of farmers at 5- to 10-fold that of the normal control subjects. Fibroblast growth factors were also similar in both groups of farmers and higher than in the control subjects (×5). In all subjects taken together (including the normal control subjects), a significant correlation was seen among hyaluronic acid, type III procollagen, and fibronectin but not with FGF (Table 1). No correlations were seen be-

**Figure 1.** Results of the pulmonary function tests given in percent predicted values for lung diffusion (Dco), total lung capacity (TLC), and residual volume (RV). The ratio of forced expired volume in 1 s (FEV₁) to forced vital capacity (FVC) is given as such. Dco was significantly lower in the acute phase of farmer's lung than in the farmers with a history of the disease.
of BALF of patients with FL would contribute to better understanding of the pathogenesis of lung fibrosis in this disease and add useful clinical markers of the fibrosing activity of the disease. The present study confirms the prior observations of significant increases in the BALF procollagen III antigens, hyaluronic acid, and adds the finding of elevated FGF and fibronectin in acute FL disease. How these factors get into the lungs is unknown. Since BALF albumin levels were higher in the farmers than in the control subjects, some of these increases could be explained by transudation. Taken together, these observations suggest that in the acute phase of FL, a significant fibrosing activity is ongoing in the lung. Furthermore, the data show that in clinically inactive FL, the potential for lung fibrosis of the subclinical disease of our patients in group Ex is higher than in normal control subjects, but well below the levels of acute FL. It is recognized that the significance of these findings in the BALF has to be interpreted with caution. The persisting high levels of these markers in BALF of subjects in the Ex group suggest that their presence may not necessarily lead to progressive lung fibrosis. Also, the levels found in the BAL of the five farmers in this group who had shown some deterioration in their lung functions over the year preceding the lavage were no different from those levels in those

**FIGURE 2.** Total number of cells recovered by bronchoalveolar lavage and the percentage of lymphocytes for the three groups of subjects.

tween lung functions and the levels of these substances in BALF. The BALF fibronectin level was positively correlated with the total number of recovered cells ($r^2 = 0.75$, $p = 0.001$) and with the percentage of lymphocytes ($r^2 = 0.69$, $p = 0.001$). The BALF albumin levels were as follows: acute, $244 \pm 29$ mg/L; Ex, $124 \pm 21$ mg/L; and control subjects, $57 \pm 8$ mg/L.

**DISCUSSION**

It was hoped that this study of fibrogenic activities
Table 1 — Correlations Between the Levels or Activity of the Different Fibrogenic Factors Measured in BAL Fluids*

<table>
<thead>
<tr>
<th>Procollagen</th>
<th>Fibronectin</th>
<th>FGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronic acid</td>
<td>0.422†</td>
<td>0.371</td>
</tr>
<tr>
<td>0.002</td>
<td>0.006</td>
<td>0.079</td>
</tr>
<tr>
<td>Procollagen</td>
<td>0.669</td>
<td>0.110</td>
</tr>
<tr>
<td>0.0001</td>
<td>0.138</td>
<td></td>
</tr>
<tr>
<td>Fibronectin</td>
<td>0.338</td>
<td></td>
</tr>
</tbody>
</table>

*These data are for all subjects (n = 52) for hyaluronic acid, type III procollagen, and fibronectin, and for 50 subjects for fibroblast growth factors (FGF).
† and p values, Spearman correlation coefficients.

subjects without such deterioration, also suggesting that these markers may not be important in the outcome.

There are very few previous data on lavage markers of fibrogenesis in HP. Bjerner et al7 have looked at the levels of hyaluronate and procollagen in ten patients with FL and in asymptomatic farmers. Larson et al8 have shown that BAL hyaluronic acid levels distinguished farmers with FL from those with asymptomatic alveolitis. Studies in lung fibrosis,6 sarcoidosis,8,9 asbestositis,10 irradiated lungs,10 and silicosis11 have shown an increase in these molecules and there was a correlation between some of these markers and altered lung function, lavage cellularity, and prognosis.

In FL, our results confirm those of Bjerner et al7 showing an increase in type III procollagen and hyaluronic acid in acute FL and add information for those with Ex FL who are still active on the farm. We also show that BALF in FL subjects, both acute and Ex, has increased fibroblast enhancement potential as assessed by BALF fibronectin and FGF levels. Our results confirm those of Bjerner et al in that we found a significant correlation between hyaluronate and procollagen levels. Comparisons between the two studies, however, are difficult, given the different study populations. The results in the group of farmers with a history of FL and still on the farm show a persisting BALF fibrogenic activity as opposed to subjects in the study of Bjerner et al7 where hyaluronic acid and type III procollagen returned to normal values on contact cessation. This is interesting since our farmers had minimal or no clinical evidence of disease activity, but BALF evidence of subclinical alveolitis. This suggests that continuing exposure is important, even when exposure does not seem to cause clinically observable disease activity. This group is also different from asymptomatic farmers with a lymphocytic alveolitis who have a small increase of BAL fibronectin and no increase of hyaluronic acid.8

The lack of correlation between FGF and the other markers of fibrogenic activities may appear surprising, considering their suggested role in a common outcome, that is, lung fibrosis. However, the lack of strong correlations in our BALF measurements of indicators of fibrosing activity can be explained at least in part by the ever-increasing complexity of molecular events occurring in the pathogenesis of lung fibrosis, the still poorly understood relationships between the inflammatory and fibrosing activities, and the pertinence of in vitro assays (for FGF, for instance, as opposed to direct measurement of events in lung tissues) to the evaluation of in vivo activities.

The relatively greater sensitivity of the FGF assay has previously been observed in our study of asbestos exposure to enhance the release of FGF even in the exposed animals without asbestosis.28 The increased production of cytokines (including the fibronectin production by alveolar macrophages) upregulating the fibroblast growth in vitro appears to occur in the exposed subjects, even in the absence of overt disease, both in the animal model of asbestos exposure and in the human condition of the present study. Under these circumstances of subclinical disease, it has been suggested that the fibrotic process in the lung tissue itself may also be modulated by the release of opposing molecules such as the prostaglandin E2,23-25 which would then inhibit the fibroblast activities, effectively preventing the development of excessive tissue fibrosis, thus preventing lung function deterioration. In the acute phase of FL, failure of regulation of the fibrotic process may initiate the development of lung fibrosis, with the associated loss of lung function.

In conclusion, the fibrosing activities of the allergic alveolitis of FL are fully expressed at the time of clinical presentation and this alveolitis also has fibrosing potentialities in the subclinical phase of the disease in susceptible farmers who remain exposed after an initial acute phase of the disease. Long-term follow-up studies are needed to evaluate the potential of these markers in BAL to predict the outcome in FL, as suggested for sarcoidosis.9

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