Elevated Surfactant Protein A in Bronchoalveolar Lavage Fluids From Sarcoidosis and Hypersensitivity Pneumonitis Patients*

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Interstitial lung diseases often are accompanied by histopathologic evidence of alveolar type 2 cell alterations. In the alveolar milieu, the surfactant-specific protein A (SP-A) is a secretory product of alveolar type 2 cells. Therefore, we measured SP-A levels in bronchoalveolar lavage (BAL) fluids from patients with untreated sarcoidosis (n=35) and hypersensitivity pneumonitis (HP [n=10]) and compared the results with those from 21 healthy control subjects. In sarcoidosis patients, SP-A was markedly higher than in control subjects with a mean of 8.0 μg/ml of recovered BAL fluid ± 0.7 SEM (p<0.0001 compared with control subjects). In HP, SP-A values were comparable with those in sarcoidosis with a mean of 9.0 μg/ml ± 1.7 SEM. Mean SP-A in the control group was 4.0 μg/ml ± 0.3 SEM. These results suggest that SP-A secretion is stimulated in sarcoidosis and HP. Further studies seem justified to investigate the role of the surfactant system in interstitial lung diseases as well as the potential clinical usefulness of SP-A measurements in BAL. (Chest 1994; 106:1766-70)

In the alveolar milieu, surfactant specific proteins are synthesized and secreted by alveolar type 2 cells and represent important constituents of the alveolar surfactant complex. In concert with surfactant phospholipids, they support the reduction of the alveolar surface tension down to levels that are required for alveolar stability during the ventilatory cycle and thus for an adequate alveolar gas exchange.

Surfactant specific protein A (SP-A) is the major surfactant protein in regard to relative abundance in the surfactant complex and has been intensively studied in recent years. In the alveoli, it is secreted by alveolar type 2 cells but in the terminal airways it may also stem from Clara cells. The SP-A appears to enhance the surface activity of the surfactant complex and may also increase the resistance of surfactants against inhibitory proteins. Furthermore, secreted SP-A probably plays an important role in the regulation of the alveolar surfactant concentration by inhibiting the secretory activity of alveolar type 2 cells and by enhancing the reuptake of surfactant lipids (reviewed, eg, in Hamm et al). Evidently, SP-A also may play a role in the activation of alveolar macrophages. Several in vitro studies have demonstrated that the presence of SP-A enhances the phagocytosis of various antigens and microorganisms by alveolar macrophages. This process may be important to the understanding of local host defense mechanisms and immunomodulation in the alveolar milieu.

It is known that interstitial lung diseases often are accompanied by histopathologic evidence of alveolar type 2 cell alterations. We were, therefore, interested in possible changes of the surfactant system in human interstitial lung diseases like sarcoidosis and hypersensitivity pneumonitis (HP). The SP-A has the advantage of being a characteristic secretory product of alveolar type 2 cells, with presumably only a minor portion being secreted by Clara cells in the terminal airways. Therefore, we hypothesized that SP-A levels in bronchoalveolar lavage (BAL) fluids may reflect possible changes in the secretory activity of these cells. In a previous immunocytochemical study, we had already shown that alveolar macrophages of patients with untreated active HP have a markedly higher SP-A content than healthy control

Key words: Pulmonary surfactant; surfactant protein A (SP-A); bronchoalveolar lavage (BAL); alveolitis; sarcoidosis; hypersensitivity pneumonitis.

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Elevated Surfactant Protein A in BAL Fluid from Sarcoidosis and Pneumonitis Patients (Hamm et al)

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METHODS

Patients and Control Subjects

Sarcoidosis: Diagnosis of sarcoidosis was based on consistent clinical features, along with biopsy evidence of noncaseating epithelioid cell granulomas or the characteristic increase of the CD4/CD8-lymphocyte subset ratio in BAL fluid or both. Radiologically, 22 patients had type I sarcoidosis and 13 had type II disease. At the time of BAL, no patient received steroids or other immunosuppressive agents. Thirty patients were nonsmokers and five were smokers. The mean age was 59 years (range, 24 to 60 years).

Hypersensitivity Pneumonitis: The diagnosis was based on the individual patient’s history with adequate exposure to compatible antigens and exposure-related symptoms, serologic evidence of precipitating antibodies against the offending materials, abnormal chest x-ray films with signs of interstitial lung disease, restrictive patterns in lung function tests, and a typical lymphocytic alveolitis with low or normal CD4/CD8 ratios in BAL fluids. All ten patients were nonsmokers. Seven suffered from budgerigar breeder’s lung, two from pigeon-breeder’s lung, and one had farmer’s lung. None had received steroid or other immunosuppressive therapy prior to diagnostic bronchoscopy. The mean age was 44 years (range, 6 to 60 years).

Control Subjects: To qualify as a control subject, an individual had to have absence of clinical or radiologic evidence of interstitial lung disease, normal lung function tests, and no abnormalities in BAL and differential cytologic studies. Control subjects were patients who underwent diagnostic bronchoscopy for unexplained pulmonary symptoms, eg, chronic cough, without evidence of interstitial lung disease (n=16 [Dept of Pulmonary Medicine, Hannover Medical School]) and healthy volunteers who underwent bronchoscopy after informed consent (n=5 [Dept of Pulmonary Medicine, Ruhrlandklinik Essen]). None of the control subjects was receiving steroid or other immunosuppressive therapy. Mean age was 48 years (range, 28 to 78 years), 10 subjects were lifetime nonsmokers, and 11 were current cigarette smokers.

Techniques

Bronchoscopy and Bronchoalveolar Lavage: All investigated subjects underwent fiberoptic bronchoscopy. After premedication with atropine and codeine and local anesthesia with 2 percent lidocaine, the bronchoscope was wedged in a segment or subsegment of the middle lobe or lingula, and BAL was carried out with 100 ml of 0.9 percent saline solution (5 aliquots of 20 ml each). The mean recovery did not significantly differ among groups (Table 1). Recovered BAL fluids were strained through gauze, and an aliquot was submitted for cytologic examination. The remaining fluid was centrifuged at 500 g for 10 mins to remove cells and debris. The cell-free supernatant was stored at −28°C for further analysis.

Analysis of Lavage Cells: The lavage fluid was centrifuged at 500 g, and the cell pellet was resuspended. Cytologic preparations were stained with May-Grünwald-Giemsa stain, and differential cell counts were performed on 600 cells. Lymphocyte subsets were analyzed by a peroxidase-antiperoxidase assay performed on glass slides. Monoclonal antibodies (Ortho Diagnostics, New Jersey) OKT4 were used to identify T-helper/inducer cells (CD4+) and OKT8 for T-suppressor/cytotoxic cells (CD8+).

Total Protein Measurement: The total protein content of the supernatants of centrifuged BAL fluids was determined using the standard method of Lowry et al.

Surfactant Specific Protein A Analysis: We developed an enzyme-linked immunosorbant assay technique to quantify SP-A in unconcentrated supernatants of BAL fluids using a monoclonal antibody raised against highly purified recombinant human SP-A which recognizes the globular domain of SP-A. As a standard, we used SP-A obtained by recombinant DNA technology from a human DNA matrix. Bronchoalveolar lavage samples were diluted with phosphate-buffered saline (PBS [pH value, 7.2]) according to their total protein content (1:100 for samples with total protein content <100 µg/ml; 1:200 for <200 µg/ml, etc). The SP-A standard was portioned to 20-µl aliquots (containing 1.1 µg SP-A) and stored at 80°C. For each assay, one aliquot was diluted in PBS at a ratio of 1:200, and the standard curve was prepared to yield standards in the range of 1 to 15 ng per well of microtiter plates (Nunc-Immuno plates Polysorb F96-4, Intermed Co., Bikerød, Denmark) corresponding to 1 to 15 µg/ml of diluted BAL samples. The wells were loaded with 100 µl of diluted BAL fluids and standard solutions and incubated overnight at 4°C. After washing (PBS with 0.1 percent Tween 20 [Sigma Chemicals, Deisenhofen, Germany]), free sites were blocked with PBS solution containing 3 percent bovine serum albumin (weight per volume) for 3 h. After washing, the plates were incubated with the monoclonal SP-A antibody (1:10,000 in 0.3 percent bovine serum albumin solution, 100 µl) at 4°C overnight. The plates were washed again and incubated with HPO-conjugated antimouse IgG (Dianova, Hamburg, Germany), 1:2,000 in 0.3 percent bovine serum albumin solution, 100 µl, 1 h, at room temperature). After washing, the enzyme activity was measured with ABTS (2, 2-azino-bis-(3-ethylbenthiazoline-6-sulfonic acid) diammonium salt) (Sigma Chemicals, Germany) as substrate. Developed color was read at 405 nm (Microplate reader Dynatech MR 700, Dynatech, Guernsey, United Kingdom). Results were quantified by parallel measurement of the known SP-A standard. The coefficient of variation between all assays was 9.25 percent.

Statistical Analysis: Results were defined as statistically significant by probability values less than 0.05 (Student’s t test).

RESULTS

All SP-A results are given as micrograms per milliliter of recovered BAL fluids. Adjustment of SP-A values to the amount of fluid recovery did not substantially change the results. The detailed data is

<table>
<thead>
<tr>
<th>Clinical Data</th>
<th>Control Subjects (n=21)</th>
<th>Sarcoidosis Patients (n=35)</th>
<th>HP Patients (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluid recovery, %</td>
<td>55 ± 2</td>
<td>56 ± 1.5</td>
<td>57 ± 3.2</td>
</tr>
<tr>
<td>Lymphocytes, %</td>
<td>12 ± 1</td>
<td>46 ± 27</td>
<td>67 ± 4.1</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>Not done</td>
<td>6.07 ± 0.57</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Total protein content, mg/ml</td>
<td>82 ± 16</td>
<td>250 ± 62</td>
<td>401 ± 178</td>
</tr>
<tr>
<td>SP-A, µg/ml</td>
<td>4.0 ± 0.3</td>
<td>8.0 ± 0.7</td>
<td>9.0 ± 1.7</td>
</tr>
</tbody>
</table>

*Data are expressed as mean ± SEM.
not shown, but mean fluid recoveries in the individual groups were not significantly different (Table 1). We did not attempt to calculate the epithelial lining fluid recovered with the BAL fluids, since none of the existing methods has been reported to be sufficiently reproducible or reliable.17,18

Sarcoidosis

The mean SP-A in the BAL fluids of this group (n=35) was 8.0 ± 0.7 (SEM) µg/ml, with a range of 2.4 to 17.4 µg/ml (p<0.0001, compared with control subjects [Fig 1]). The mean protein content of the fluids was 250 ± 62 (SEM) µg/ml, with a range of 19 to 2,242 µg/ml, and the mean lymphocyte percentage of recovered cells, 46 ± 2.7 (SEM) percent, with a range of 11 to 85 percent. The mean CD4/CD8 lymphocyte ratio (performed in 25 patients) was 6.07 ± 0.87 (SEM), with a range of 0.8 to 18, as also shown in Table 1. In patients with type I sarcoidosis (n=22), the mean SP-A was 8.3 ± 0.7 (SEM) µg/ml, with a range of 3.3 to 15.6 µg/ml. In type II sarcoidosis (n=13), the mean SP-A was 7.6 ± 1.3 (SEM) µg/ml, with a range of 2.4 to 17.4 µg/ml.

Hypersensitivity Pneumonitis

The mean SP-A in this group (n=10) was 9.0 ± 1.7 (SEM) µg/ml of BAL fluid, with a range of 3.0 to 19.1 µg/ml (p<0.0001, compared with control subjects [Fig 1]). The mean protein content of the fluids was 401 ± 178 (SEM) µg/ml, with a range of 39 to 1,888 µg/ml. The mean lymphocyte percentage of recovered cells was 67 ± 4.1 (SEM) percent, with a range of 45 to 85 percent. The mean CD4/CD8 lymphocyte ratio (performed in 9 patients) was 1.1 ± 0.2 (SEM), with a range of 0.3 to 2.6 (Table 1).

Control Subjects

The mean SP-A in this group (n=21) was 4.0 ± 0.3 (SEM) µg/ml of BAL fluid, with a range of 2.0 to 6.3 µg/ml. The mean total protein content was 82 ± 16 (SEM) µg/ml, with a range of 28 to 327 µg/ml. The lymphocyte percentage was 12 ± 1 (SEM) percent, with a range of 6 to 22 percent. Table 1 summarizes the results in this and the patient groups. Nonsmoking control subjects (n=10) had a mean SP-A of 3.4 ± 0.4 (SEM) µg/ml, with a range of 2 to 5.2 µg/ml and a total protein content of 95 ± 29 (SEM) µg/ml, with a range of 28 to 337 µg/ml. The smokers (n=11) had a mean SP-A of 4.5 ± 0.4 (SEM) µg/ml, with a range of 2.2 to 6.3 µg/ml (p=0.056, in comparison with nonsmokers [not significant]) and a total protein content of 91 ± 26 (SEM) µg/ml, with a range of 31 to 327 µg/ml.

Correlations of Surfactant Specific Protein A With Bronchoalveolar Lavage Lymphocyte Percentages

Although elevated SP-A levels in sarcoidosis and HP patients were associated with higher BAL lymphocyte percentages, when the group means were examined (Table 1), there was no significant individual correlation between these two factors in any group (control subjects, r=0.112; sarcoidosis patients, r=0.062; HP patients, r=0.213).

Correlations of Surfactant Specific Protein A With Bronchoalveolar Lavage Total Protein Contents

The percentage of SP-A per total protein content of BAL fluids (SP-A/total protein ratio) was similar in all three groups: it was 6.8 ± 1 in control subjects, 5.3 ± 0.7 in sarcoidosis patients, and 6.3 ± 2.2 in HP patients. There was a positive and significant correlation between these two factors in HP patients (r=0.733; p=0.016), but not in sarcoidosis patients (r=0.056) or control subjects (r=0.146).

DISCUSSION

We found significantly elevated SP-A levels in BAL fluids of 35 patients with untreated types I and II sarcoidosis and in 10 patients with untreated HP as compared with healthy control subjects (Fig 1). In a preliminary report by Ishii and coworkers,19 SP-A levels also were raised in sarcoidosis patients, while van de Graaf et al20 found only a slight, but not significant increase of SP-A in 20 sarcoidosis patients with untreated disease. The differences between our results and those of van de Graaf and coworkers20 are difficult to interpret, since no information on disease activity, BAL lymphocyte percentages, or disease stage is given in this publication.

In sarcoid alveolitis, not only T lymphocytes but
also alveolar macrophages are activated.\textsuperscript{21} Both cell populations are involved in the formation of the characteristic noncaseating granuloma. Several lines of \textit{in vitro} evidence\textsuperscript{6-10} suggest that SP-A has a role in the activation of alveolar macrophages. Therefore, it may be speculated that enhanced alveolar SP-A secretion could contribute to macrophage activation in sarcoidosis.

Surfactant phospholipid levels in sarcoidosis do not seem to be significantly altered in comparison with healthy individuals, and there is no clearly confirmed evidence of changes in the phospholipid profile.\textsuperscript{20,22-24} Furthermore, it has to be kept in mind that phospholipids are not surfactant specific materials but may in part stem from other sources like upper airway epithelial cell secretions\textsuperscript{25} or from cell membranes, which seems of particular importance to consider in lung diseases with high cell turnover rates, \textit{eg}, as noted by Puchelle et al.\textsuperscript{26} Thus, presently available evidence suggests that phospholipid analysis in BAL fluids of sarcoidosis patients is only of limited scientific or clinical value.

Similar to the results of the sarcoidosis group, we found raised SP-A levels in our ten patients with active, untreated HP. These results correspond with our own earlier immunocytochemical work\textsuperscript{12} in which we demonstrated that the intracellular SP-A content of alveolar macrophages is significantly increased in HP. This may reflect increased phagocytic activity of alveolar macrophages, possibly induced by increased alveolar SP-A levels, since surfactant is known to be ingested by macrophages in the course of phagocytosis.\textsuperscript{27,28} In the literature, we found only one other study\textsuperscript{29} which mentions low SP-A values in three patients with HP but fails to give exact SP-A results or detailed information on activity of the alveolitis at the time of BAL. Apparently, these three patients suffered from more advanced (fibrotic) stages of HP. A recently published animal study\textsuperscript{30} demonstrated that acute immune lung injury (an experimental model of HP) in guinea pigs is augmented in animals with partial surfactant depletion, while surfactant replacement ameliorated the parameters of lung injury. This prompted a rather optimistic comment\textsuperscript{31} that surfactant replacement therapy may prove useful in the therapy of cell-mediated immune diseases of the lung. However, these results may indicate that an increased secretory activity of alveolar type 2 cells in HP might have a protective role in this form of immune lung injury.

The SP-A levels in sarcoidosis or HP do not seem to reach dimensions that have been reported by Honda and coworkers\textsuperscript{32} in BAL fluids of six patients with alveolar proteinosis (mean, 39 \( \mu \text{g/ml} \); range, 25 to 63 \( \mu \text{g/ml} \)). Thus, SP-A measurements may prove useful to exclude this disorder in the differential diagnosis of interstitial lung disease. SP-A levels similar to those observed in sarcoidosis or HP patients in our present study also have been reported in AIDS-related pneumonia.\textsuperscript{33} Only one report has hitherto been published\textsuperscript{29} in which SP-A was determined in BAL fluids from 28 patients with idiopathic pulmonary fibrosis. The SP-A was found to be significantly lower than in normal control subjects. These findings may imply that idiopathic pulmonary fibrosis could be characterized by reduced BAL SP-A levels compared with other interstitial lung diseases. Certainly, further studies including higher patient numbers are needed to clarify this aspect.

The SP-A levels measured in our control subjects were in general agreement with the results obtained by other groups using different BAL procedures and various other enzyme-linked immunosorbent assay techniques with different antibodies and standards. Our ten nonsmoking control subjects had a mean SP-A level of 3.4 \( \mu \text{g/ml} \); in smokers it was slightly higher (4.5 \( \mu \text{g/ml} \); with the difference not significant). Van de Graaf et al\textsuperscript{20} found a mean of 2.82 \( \mu \text{g/ml} \) in their 10 control subjects; Phelps and Rose,\textsuperscript{33} 1.5 \( \mu \text{g/ml} \) in 21 control subjects; McCormack et al,\textsuperscript{32} 0.83 \( \mu \text{g/ml} \) in 25 control subjects; and Honda et al,\textsuperscript{32} 3.5 \( \mu \text{g/ml} \) in 9 smoking and 4 nonsmoking control subjects. Thus, it seems justified to expect normal values of SP-A in human BAL fluids in the range of about 0.5 to 5.5 \( \mu \text{g/ml} \), even when taking into account different methods and the well-known uncertainties concerning the actual quantity of epithelial lining fluid recovered by BAL.\textsuperscript{17,18}

In summary, our results suggest that SP-A levels in BAL fluids are not specific or diagnostic for sarcoidosis or HP. Elevated SP-A levels in these diseases, however, may reflect acute alveolar inflammation with reactive increase of the secretory activity of type 2 cells or probably of lesser importance, Clara cells, or both. It is tempting to speculate that SP-A levels could prove useful as an unspecific marker of disease activity or severity in acute interstitial lung disease like sarcoidosis or HP. This could be clinically useful, since pulmonary medicine is still lacking a reliable marker of disease activity to calculate the risk of impending and potentially irreversible lung damage prior to the deterioration of lung function factors. Furthermore, SP-A measurement could prove helpful in the differential diagnosis of interstitial lung diseases including idiopathic pulmonary fibrosis and alveolar proteinosis.

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