Increased Levels of Oxidized Methionine Residues in Bronchoalveolar Lavage Fluid Proteins from Patients with Idiopathic Pulmonary Fibrosis

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Introduction

There is major evidence that oxygen-derived free radicals released from inflammatory cells are involved in the genesis of lung diseases (1-4). In adult respiratory distress syndrome (ARDS) H2O2-dependent reactions have been proposed to contribute to acute lung injury, and bronchoalveolar lavage (BAL) fluid studies have shown that neutrophils are increased in the lower respiratory tract of such patients (5-8). In idiopathic pulmonary fibrosis (IPF) alveolar macrophages and neutrophils dominate the inflammatory cell population in the lower respiratory tract (9). The BAL cells from patients with IPF spontaneously release more oxidants, such as O2•- radicals and H2O2, compared with cells from healthy individuals (10). Both alveolar macrophages and neutrophils are capable of inducing oxidant-mediated lung parenchymal cell cytotoxicity (11, 12). It is still unclear, however, to what extent these inflammatory cells are involved in the process of oxidative alterations of lung-associated proteins. Soluble collagen molecules were shown to be fragmented by reactive oxygen species derived from the xanthine oxidase-xanthine system (13). Degradation of collagen was also observed during treatment with ozone or hydroxyl radicals produced by the Fenton reaction (14, 15). Additionally, modification of proteins by oxidants may increase their vulnerability to proteolytic enzymes (14-16).

There are many open questions concerning oxidative alterations of proteins in the epithelial lining layer and in the connective tissue in inflammatory lung diseases. The role of inflammatory cells, such as alveolar macrophages and neutrophils, in such alterations is of specific interest. This study was designed to obtain more detailed information on the extent of protein modification by oxidation of methionine (met) residues in the BAL fluid from patients with inflammatory lung diseases. To accomplish this, BAL fluid proteins were analyzed for nonoxidized and oxidized met in patients with IPF and compared with those of normal subjects and of patients with sarcoidosis. We found significantly increased levels of methionine sulfoxide [met(O)] in patients with IPF, which correlated with the increased content of neutrophils in BAL fluids.

Methods

Study Population

Patients with idiopathic pulmonary fibrosis. This group consisted of 11 patients (5 men and 6 women; mean age 25 yr; range 21 to 32 yr). All were free of respiratory symptoms and had normal chest radiographs and pulmonary function tests.

Patients with sarcoidosis. This group consisted of 10 patients (7 men and 3 women; mean age 36 yr; range 16 to 56 yr). All were nonsmokers. The diagnosis was based on a compatible clinical picture, biopsy evidence of noncaseating, epithelioid cell granulomas, no evidence of mycobacterial, fungal, or parasitic infection, and no history of exposure to organic or inorganic dusts. By chest radiography 4 patients were Stage I, 5 were Stage II, and 1 was Stage III. In this group 1 patient was receiving prednisone (10 mg/day).

Normal subjects. As control subjects, 11 healthy nonsmoking volunteers were studied (5 men and 6 women; mean age 25 yr; range 21 to 32 yr). All were free of respiratory symptoms and had normal chest radiographs and pulmonary function tests.

Bronchoalveolar Lavage

After informed consent was obtained from...
ine lactone CNBr was added to a final concentration (18, 19). Briefly, aliquots of 2 ml of BAL (about 100 µg/g) to homoserine and homoserine lactone were from Sigma (Thufkirchen, (Oross-Oerau, FRO); homo serine and homoserine lactone were from Baker (HPLC gradient grade) and tetrahydrofuran (HPLC gradient grade) were from Baker (HPLC gradient grade).

diluent buffer (1.0 M potassium borate, pH 10.4), and cyanogen bromide (CNBr) were added to a final concentration of 0.2 M (20). After 24 h incubation under atmospheric nitrogen (in darkness at room temperature) the samples were lyophilized twice. Oxidized met residues are not attacked by CNBr (18, 19). CNBr modifies more than 99% of the nonoxidized met in proteins to homoserine and its lactone under these conditions, as reported by Maier and colleagues (21).

During this procedure oxidation of met in the protein was excluded.

The CNBr-digested proteins were hydrolyzed with 6 M HCl (bidistilled) in the presence of 5 mM dithioerythritol at 110°C under vacuum for 48 h. During acid hydrolysis in the presence of dithioerythritol met(O) is quantitatively reduced to met as shown by Shechter and colleagues (19). The originally nonoxidized met was quantitatively recovered as homoserine by subsequent amino acid analysis with reversed-phase high-performance liquid chromatography (RP-HPLC).

Amino Acid Analysis by RP-HPLC

Analysis by RP-HPLC was performed on a 3 µm Spherisorb ODS-2 column (4.6 x 250 mm) from Grom (Herrenberg, FRG). The assays were carried out with a HPLC analyzer from LKB (Freiburg, FRG) at a flow rate of 1.0 ml/min using an autosampler for automatic precolumn derivatization with OPA. For precolumn derivatization the lyophilized hydrolysates were dissolved in 0.2 M potassium borate buffer, pH 10.4, containing 1 to 10 nmol of each amino acid and incubated for 30 min at room temperature. The analyses were performed on an integrator with acetonitrile (21). Peaks were detected by a Hitachi fluorometer analyzer F 1000 (excitation at 330 nm and emission at 450 nm) and evaluated by a Shimadzu integrator.

Protein Determination

The protein content of BAL fluid samples was calculated from the HPLC amino acid analysis.

Statistical Analysis

Statistical analysis was performed by BMDP software from BMDP Statistical Software, Inc. (Los Angeles, CA). Data are expressed as the arithmetic mean ± standard error of the mean (SEM). Data from differential cell counts, met(O)/met ratios, and protein concentrations do not approximate a normal distribution but show positive skewness. Thus the Mann-Whitney test was used to compare the groups. Correlation coefficients were calculated by Spearman rank correlation.

Results

Characterization of BAL Fluids

Comparison of the cell counts (table 1) showed that the absolute numbers of alveolar macrophages in the BAL fluids from IPF patients (9.0 x 10⁴ cells/ml) and from sarcoidosis patients (8.1 x 10⁴ cells/ml) were not markedly different from those of the control group (7.5 x 10⁴ cells/ml). In contrast, the neutrophil counts were significantly increased to 1.95 x 10⁴ cells/ml (p < 0.0005) or 14.7% in IPF patients compared with the controls, with 0.11 x 10⁴ cells/ml or 1.2%. IPF patients also had increased counts of eosinophils (0.75 x 10⁴ cells/ml, p < 0.05, or 5.6%) compared with the controls, with 0.11 x 10⁴ cells/ml or 0.17%, and increased counts of lymphocytes (2.8 x 10⁴ cells/ml, p < 0.05, or 17.7%) compared with the controls, with 1.0 x 10⁴ cells/ml or 7.8%. In the group with sarcoidosis the neutrophils and eosinophils were not significantly different from those in the controls. Lymphocytes were the predominant cells in the sarcoidosis group (9.3 x 10⁴ cells/ml BAL, p < 0.0005), accounting for 49.1% of total BAL cells.

Total protein in the BAL fluid supernatant of the control group amounted to 49.5 µg/ml (table 2). This value was significantly increased in the IPF group (1.5-fold; p < 0.02) and in the sarcoidosis group (5-fold; p < 0.002).

The oxidative status of the BAL fluid proteins was defined as the molar ratio met(O)/met. The results are given in table 2. The molar ratio met(O)/met was significantly increased to 0.223

### TABLE 1

<table>
<thead>
<tr>
<th>BAL cells</th>
<th>Normal (n = 11)</th>
<th>IPF (n = 11)</th>
<th>SARC (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluid recovery, %</td>
<td>64.1 ± 3.34</td>
<td>60.5 ± 4.06</td>
<td>68.8 ± 2.35</td>
</tr>
<tr>
<td>Total cells/ml x 10⁴</td>
<td>8.83 ± 2.36</td>
<td>14.6 ± 2.54</td>
<td>17.6 ± 2.45</td>
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<tr>
<td>Macrophages</td>
<td>90.8 ± 2.27</td>
<td>61.4 ± 5.15</td>
<td>49.0 ± 6.99</td>
</tr>
<tr>
<td>% Cells/ml x 10⁴</td>
<td>7.47 ± 1.79</td>
<td>9.03 ± 1.88</td>
<td>8.05 ± 1.34</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1.22 ± 0.30</td>
<td>14.7 ± 2.19</td>
<td>1.29 ± 0.51</td>
</tr>
<tr>
<td>% Cells/ml x 10⁴</td>
<td>0.106 ± 0.035</td>
<td>1.95 ± 0.29</td>
<td>0.180 ± 0.077</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.173 ± 0.041</td>
<td>5.59 ± 1.57</td>
<td>0.378 ± 0.116</td>
</tr>
<tr>
<td>% Cells/ml x 10⁴</td>
<td>0.015 ± 0.005</td>
<td>0.748 ± 0.153</td>
<td>0.051 ± 0.012</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>7.82 ± 2.18</td>
<td>17.7 ± 4.70</td>
<td>49.1 ± 7.30</td>
</tr>
<tr>
<td>% Cells/ml x 10⁴</td>
<td>1.04 ± 0.63</td>
<td>2.77 ± 1.07</td>
<td>9.29 ± 2.28</td>
</tr>
</tbody>
</table>

* p Values from the Mann-Whitney test for comparison with normal individuals.
† p < 0.05.
‡ p < 0.0005.
§ p < 0.005.
METHIONINE OXIDATION IN IDIOPATHIC PULMONARY FIBROSIS

**Protein Content of BAL Fluids and Oxidative Status of Proteins Expressed as the Ratio** met(O)/met (MOL/MOL) FROM NORMAL NONSMOKERS AND NONSMOKING PATIENTS WITH IDIOPATHIC PULMONARY FIBROSIS (IPF) AND SARCOIDOSIS (SARC)*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal (n = 11)</th>
<th>IPF (n = 11)</th>
<th>SARC (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL protein, µg/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>49.5 ± 9.41</td>
<td>74.0 ± 8.71†</td>
<td>245 ± 120‡</td>
</tr>
<tr>
<td>Range</td>
<td>18–128</td>
<td>44–129</td>
<td>53–1315</td>
</tr>
<tr>
<td>met(O)/met, mol/mol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>0.046 ± 0.008</td>
<td>0.223 ± 0.053§</td>
<td>0.048 ± 0.010</td>
</tr>
<tr>
<td>Range</td>
<td>0.015 ± 0.104</td>
<td>0.079 ± 0.678</td>
<td>0.011 ± 0.115</td>
</tr>
</tbody>
</table>

* p Values from Mann-Whitney test for comparison with normal individuals.
† p < 0.02.
§ p < 0.002.
‡ p < 0.0002.

The data presented in figure 2 indicate that the met(O)/met ratios and the relative neutrophil counts are closely related when considering the values from the controls and the IPF group (r = 0.86; p < 0.0002; n = 22). The same correlation coefficient (p < 0.0002; n = 22) was calculated between oxidized met and absolute neutrophil counts. In contrast, no significant correlation was found between the met(O)/met ratios and the absolute macrophage number in the BAL fluid (r = 0.22; p = 0.32; n = 22).

**Discussion**

These results demonstrate that met(O) in BAL fluid proteins appears to be a suitable parameter to indicate the oxidative status in the protein fraction. Healthy nonsmoking volunteers showed an average met(O)/met ratio of 0.046, indicating that only a small fraction of the total met of the BAL fluid proteins (4.2%) was oxidized. In contrast, significantly increased levels of met(O) were seen in the BAL fluid proteins of the nonsmoking IPF patients. These results are in favor of pronounced oxidative processes in the epithelial lining layer of the IPF patients. Since the met(O)/met did not correlate with the age of the IPF patients, “aging” as recently proposed as a possible cause for the appearance of oxidized proteins (22–24) seems not to play a remarkable role in the oxidation of met residues in these patients. The level of oxidized met in the BAL fluid proteins from nonsmoking sarcoidosis patients was not different from that of the normal individuals.

With the pooled data from the normal subjects and the IPF patients we found a positive correlation between the met(O)/met ratio and the relative/absolute number of neutrophils. In contrast, no positive correlation was seen between oxidized met and the alveolar macrophage number. We therefore suggest that the neutrophil is the major cell type that mediates the oxidation of met in the epithelial lining fluid (ELF). This idea is consistent with data published by Beck-Spieer and colleagues (20) showing that neutrophils are very potent in the oxidation of met in extracellular proteins after stimulation with phorbol myristate acetate or zymosan. The oxidation of met was markedly blocked by catalase, clearly indicating the participation of H_2O_2 in this process (20).

We have recently reported that hypochlorous acid and chlorinated amines are compounds that are highly efficient oxidizers of met residues in α1-protease inhibitor (PI), whereas the superoxide anion and the hydroxyl radical are much less effective (25). Both hypochlorous acid and chlorinated amines are generated by myeloperoxidase (26–28), which occurs in the azurophil granules of neutrophils (26, 29–31). Alveolar macrophages, normally devoid of myeloperoxidase, are not capable of producing such efficient oxidants with respect to the oxidation of met residues. We therefore propose that the oxidation of protein-bound met in the epithelial lining layer of IPF patients is predominantly mediated by neutrophils via the myeloperoxidase-H_2O_2-chloride system.

The oxidation of met in the epithelial lining layer may be regulated by such antioxidants as glutathione, a potent scavenger for hypochlorous acid and chloramines. In this regard Cantin and colleagues (32) found a fourfold lowered glutathione content in the ELF from IPF patients compared with that from normal individuals. Thus the oxidation of met in the ELF of IPF patients may be favored by a deficiency of glutathione.
Taken together it is reasonable to assume that the marked increase in the met(O)/met ratio in the BAL fluid proteins of IPF patients is the result of myeloperoxidase-derived oxidants. This is consistent with the data reported by Cantin and colleagues (10), who found increased levels of myeloperoxidase activity in the BAL fluid of IPF patients. These authors also found a good correlation between the content of myeloperoxidase and the portion of neutrophils in the BAL fluid recovered from these patients. This supports our observation that the levels of met(O) in BAL fluid protein correlate with the neutrophil counts. A marked increase in met(O) in BAL fluid protein points to the existence of a cytotoxic potential present in the ELF. Cantin and coworkers (10) reported that incubation of alveolar epithelial cells with ELF from IPF patients in the presence of H_2O_2 caused significantly increased cellular injury compared with control incubations with ELF from normal subjects. This cytotoxic effect is completely suppressed by met used as an oxidant scavenger (10).

The consequences of oxidation of met residues in ELF proteins are largely unknown. It is well known that the elastase inhibitory activity of α-PI is impaired but oxidation of its reactive site met (33–35), leading to an imbalance of the proteolytic-antiproteolytic system in the respiratory tract. Hypochlorous acid, NH_2Cl, and taurine monochloramine are especially very potent in attacking the reactive site met in α-PI (25). Numerous proteinases, proteinase inhibitors, chemotactic peptides, lysozyme, calmodulin, and cytochrome c are known to be affected in their activity by oxidation of a critical met residue (for review, see reference 36). In this regard met oxidation may be clearly both an asset and a liability for biologic systems. It may be useful for many proteins to retain met residues by which they can be readily rendered inactive (36).

In conclusion, this study demonstrated the increased oxidation of met in BAL fluid proteins from IPF patients and thus lends further support to the concept that neutrophil-mediated oxidative injury to lung parenchymal cells may play a prominent role in the pathogenesis of this chronic progressive lung disorder. The increased met(O)/met ratios found in the BAL fluid proteins from IPF patients may not be restricted to proteins in the ELF. It is reasonable to assume that proteins with a high met content have an increased risk of damage by stimulated neutrophils. In further studies we will focus our interest on such proteins.

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References