Carbonylated proteins in bronchoalveolar lavage of patients with sarcoidosis, pulmonary fibrosis associated with systemic sclerosis and idiopathic pulmonary fibrosis

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Oxygen-derived free radicals produced by phagocytes have been postulated to contribute to lung tissue damage occurring during diffuse lung diseases (DLD). The two-dimensional electrophoretic (2-DE) analysis of bronchoalveolar lavage (BAL) protein composition revealed different protein profiles in sarcoidosis (S), idiopathic pulmonary fibrosis (IPF) and systemic sclerosis (SSc) with a significant increase of low molecular weight proteins in IPF. Some of these proteins are involved in antioxidant processes. The aims of this report were to analyse the oxidative stress occurring in patients with DLD through determination of BAL protein carbonyl content and to identify target proteins of oxidation by a proteomic approach (2-DE combined with immunoblotting with specific antibodies for carbonyl groups). Carbonylated proteins detected by enzyme-linked immunosorbent assay (ELISA) were increased in BAL of patients with S, IPF and SSc compared to healthy controls with a significant difference for S and IPF. The proteomic approach to the analysis of BAL revealed that protein carbonylation was a process involving specific carbonylation-sensitive proteins and that in IPF a greater number of proteins target of oxidation were present. In conclusion, to our knowledge, this is the first report providing a database of proteins target of oxidation in BAL of patients with sarcoidosis, idiopathic pulmonary fibrosis and systemic sclerosis.

Keywords:
Bronchoalveolar lavage / Carbonyl groups / Diffuse lung diseases / Oxidised proteins / Proteome

1 Introduction

The oxygen-derived free radicals, produced by phagocytes, are postulated to contribute to lung tissue damages occurring in diffuse lung diseases (DLD) [1, 2], a heterogeneous group of diseases with a different pathogenesis but a common evolution towards pulmonary fibrosis of various degrees of severity [3]. A defence mechanism of the lungs against...
oxidative stress is the production of antioxidants, such as vitamins, glutathione and specific proteins [4]. In a previous paper we found that the proteomic analysis of bronchoalveolar lavage (BAL) revealed different protein profiles in sarcoidosis (S), idiopathic pulmonary fibrosis (IPF) and pulmonary fibrosis associated with systemic sclerosis (SSc). Interestingly, the three diseases showed statistically different levels of antioxidant proteins [5, 6], allowing to hypothesise different pathways according to their pathogenetic mechanism to counteract tissue damage due to oxidant-antioxidant imbalance. These DLD are characterised by different pathogenesis, being S and SSc systemic immunoinflammatory diseases, whereas IPF is a disease limited to the lung and is considered a prevalently epithelial/fibroblastic disorder with a very severe prognosis [2, 6–9]. Therefore, the role of oxidative stress in the pathogenesis of these diseases is an interesting topic and has been studied by different methodologies [1, 10–14]. Damage and alteration of alveolar epithelial cells is one of the hallmarks of interstitial lung fibrosis [8, 15]. Recently, it has been demonstrated that the presence of oxidative stress may lead to the damage, activation and/or apoptosis of alveolar epithelial cells, either directly, through intracellular redox imbalance, or indirectly, by activating redox-sensitive effector pathways [16]. In addition, a recent study has shown that oxidative stress may affect immune responses as an initiator of cytokine release [17]. There are clinical studies aimed to correct oxidant-antioxidant imbalance and among these Behr et al. [18] reported high-dose N-acetylcysteine that seems to prevent epithelial cell injury mediated by oxygen radical, in patients with IPF reversed extracellular glutathione deficiency, present in this disease, and oxidative damage.

There are many different physiological and non-physiological sources of oxidising agents. Air pollutants, such as ozone and cigarette smoke, and inflammatory cells may damage BAL proteins by oxidative stress [19]. Macrophages, neutrophils and eosinophils produce reactive oxygen species (such as superoxide anion or hydroxyl radical) in large amounts during inflammation [20]. These cells dominate the inflammatory response in DLD and are particularly elevated in BAL of patients with IPF, S and SSc [1, 2–4, 8]. The most widely studied oxidative stress-induced modification of proteins is the formation of carbonyl groups on lysine, proline, arginine and threonine residues. The amount of carbonyls is considered a biomarker of oxidative stress and it is used to quantify the level of oxidative damage in polypeptide chains [13, 21]. The analysis of protein carbonyl groups may have some advantages in comparison with other methods: their formation is considered a common product of protein oxidative reactions, they are early produced, relatively stable and induced by almost all types of reactive oxygen species [13]. In S and IPF patients, an increase in the BAL protein carbonyl content has been described [2, 10]. The identification of carbonylated proteins could be a relevant goal to evaluate the presence of distinct protein targets in DLD in relation to their different pathogenic mechanisms. It could be important to know the protein targets of carbonylation in BAL because the reactive oxygen species may inactivate certain proteins or alter certain protein functions and in turn oxidised proteins may be dangerous for lung tissues by activating cytokines and other mediators [1, 17].

Our aims were to study the oxidative stress occurring in patients with DLDs through determination of the BAL protein carbonyl content and to identify protein targets of oxidation by a proteome approach (2-DE combined with immunoblotting with specific antibodies for carbonyl groups). We showed that carbonylated proteins were increased in BAL of patients with S, IPF and SSc compared to healthy controls with a significant difference for S and IPF. Moreover, by the proteomic analysis, we found that there are distinct protein targets of oxidation in these diseases.

2 Materials and methods

2.1 Patients

The BAL samples were obtained from 16 nonsmoker patients with S (seven men, mean age 58.03 ± 10.43; mean (M ± SD), 13 nonsmokers with IPF/UIP (ten men, mean age 60.32 ± 9.35), 10 nonsmokers with SSc (four men, mean age 59.62 ± 14.20) and 5 nonsmoker healthy controls (three men, mean age 42.16 ± 13.51). BAL was carried out with the informed consent of patients for diagnostic or clinical purposes. The patients were not on therapy at the time of bronchoscopy and the diagnosis was based on international guidelines.

2.2 BAL and total protein assay

BAL was performed in all patients as previously reported [5, 6]. To determine the total BAL protein concentration we used the Bio-Rad Protein assay, based on Bradford’s method [22].

2.3 Quantification of protein carbonyl groups in BAL

The measurement of protein carbonyls in the BAL samples was performed with a commercial test kit (Zentech PC Test) based on ELISA for the detection of oxidised proteins in the biological samples, such as BAL [23]. Samples were reacted with 2,4-dinitrophenylhydrazine (DNPH) and then adsorbed in wells of an ELISA plate before probing with a commercial antibody raised against protein-conjugated DNPH. Absorbance was related to a standard curve prepared for serum albumin containing increasing proportions of the hypochlorous acid-oxidised protein, calibrated colorimetrically. Each sample was assayed in three replicates and intra- and inter-assay coefficients of variation were below 2.1 and 3.2%, respectively. The BAL carbonylated proteins were expressed as nmol carbonyl groups/mg protein.
2.4 2-DE

The BAL fluid samples of patients with S, IPF, and SSc and of healthy controls were dialysed against water, lyophilised and dissolved in lysis buffer (8 M urea, 4% CHAPS, 40 mM Tris-base, 65 mM diethioerythritol and trace amounts of bromophenol blue), so that 100 μL sample contained 45 μg proteins (for silver stained gels) or 60 μg proteins (for gels to be electrotransferred). 2-DE was performed essentially as described by Bjellqvist et al. [24]. The first dimension was run on a nonlinear wide-range immobilised pH gradient IPG (pH 3.5–10, 18 cm long IPG strips; Amersham Biosciences, Uppsala, Sweden). For the detection of the carbonyl group, after IEF the strips were briefly rinsed with water and incubated at room temperature in 5% TCA containing 10 mM phenol blue), so that 100 μL sample contained 45 μg proteins for silver stained gels or 60 μg proteins for gels to be electrotransferred. 2-DE was performed essentially as described by Bjellqvist et al. [24]. The first dimension was run on a nonlinear wide-range immobilised pH gradient IPG (pH 3.5–10, 18 cm long IPG strips; Amersham Biosciences, Uppsala, Sweden). For the detection of the carbonyl group, after IEF the strips were briefly rinsed with water and incubated at room temperature in 5% TCA containing 10 mM DNPH for 20 min [25]. The DNPH-treated strips were washed twice for 5 min with a solution containing 8 M urea, 20% v/v glycerol, 1% w/v SDS and 150 mM Tris-HCl (pH 6.8), and then equilibrated as described [24]. The second dimension was run on 9–16% polyacrylamide linear gradient gels (18 cm x 20 cm x 1.5 mm). The gels were stained with ammoniacal silver nitrate [26]. The proteins were identified by gel matching with 2-D electrophoretical maps of BAL proteins performed in our lab [3, 6] and by MALDI-TOF MS [27].

The electrophoretogram images were obtained with a computing densitometer (Molecular Dynamics 300S) and processed with the Melanie 4 computer system (Genebio). The quantitative variations in proteins were expressed as relative volumes of spots.

2.5 Western blotting

The proteins were electroblotted onto nitrocellulose membranes (Hybond ECL; Amersham Biosciences) using 25 mM Tris, 192 mM glycine and 20% v/v methanol according to Towbin [28]. Protein transfer was carried out using a tank apparatus (Trans-Blot cell; Bio-Rad, Hercules, CA, USA) for a total of 2 Å at 4°C. Before immunodetection, the membranes were stained in 0.2% w/v Ponceau S in 3% w/v TCA for 3 min and the spot position marked in order to facilitate computer-assisted matching on the silver stained gel [29]. Immunodetection was performed as previously described [25], using anti-2,4-dinitrophenol (DNP) immunoglobulin G (Sigma, St. Louis, MO, USA; dilution 1:10 000). The immuno-reactive spots were detected using goat peroxidase-conjugated antirabbit immunoglobulin G (Sigma, working dilution 1:7000) and a chemiluminescence detection system (Amersham Biosciences).

2.6 Statistical analysis

The results are expressed as M ± SD. Group distribution was normal (Shapiro-Wilk’s test). Comparisons between the two groups were performed by one-way analysis of variance. When a significant difference was found, the t-test for inde- pending groups was applied. A p value <0.05 was considered to be significant. Statistical software (SPSS 11.5 for Macintosh, Chicago, IL, USA) was used for all data analysis.

3 Results and discussion

The BAL protein carbonyl content detected by ELISA, from the patients with S, SSc and IPF and from healthy controls, is reported in Fig. 1. The BAL carbonylated proteins were expressed as nmol carbonyl groups/mg protein instead of nmol/mL of recovered BAL because of the well-known great variability of protein concentration in BAL.

Considering all the patients with DLD, a significantly increased level of carbonylated proteins compared to controls was found (p < 0.03). Moreover, the IPF and S patient groups showed a significantly higher amount of oxidised BAL proteins than the controls (p < 0.02 and p < 0.03, respectively).

For the identification of carbonylated proteins, this study exploited the high resolving power of 2-DE, combined with the high specificity of Western blotting. The immunoblotting analysis with anti-DNP serum was performed on three samples from each disease (S, SSc and IPF) and three from healthy controls, and in Table 1 the frequency of carbonylated proteins is reported. The samples to analyse were selected according to the ELISA results, choosing patients with a protein carbonyl content near to the average value. An example of Western blot analysis of one case for each group and a typical electrophoretic map (considered as reference), which indicates all the carbonylated proteins present at least in one of the samples, are shown in Fig. 2. Albumin and immunoglobulin heavy chains were the only carbonylated proteins present in all gels of the three groups of patients. Moreover, among the group of S patients, complement C3 was the other protein carbonylated in all cases. In IPF patients, complement C3, transferrin, immunoglobulin low chains and immunoglobulin A were the other proteins carbonylated in all patients. Interestingly, in two patients with IPF a group of six plasmatic proteins was also carbonylated, whereas in S and SSc only a limited number of carbonylated proteins were detected (Table 1). Transferrin, α-1-antitrypsin and immunoglobulin light chains were carbonylated in all gels of healthy controls.

Proteins with various functions (antioxidant, anti-protease and anti-inflammatory) were detected in BAL. In our previous papers [5, 6], we demonstrated that some of these proteins are expressed differently in BAL of the S, SSc and IPF patients. In BAL of these patients we also found some proteolytic fragments derived from plasmatic proteins, such as apolipoprotein A1, albumin, haptoglobin β, serotransferrin and α-1-antitrypsin. These findings could be a demonstration of the high proteolytic activity present at the alveolar level in these diseases, a phenomenon also related to the oxidative process [12, 30]. Since oxidative stress produces protein carbonyl derivatives, protein carbonyls are considered a good marker of oxidative injury [13] and the total
amount of oxidised proteins in BAL, as evaluated by carbonyl content, can provide a quantitative measure of oxidative reactions occurring at alveolar level [13, 31]. There is little data on the quantification of carboxyls in BAL of the patients with DLD [2, 10]. In order to contribute to this topic, we performed this study that revealed significantly higher concentration of protein carbonyls in BAL of the DLD patients than in controls, due to respiratory burst activation in alveolar phagocytes present in these diseases. A significant difference was also found for the S and IPF patients with respect to controls. These results were in line with those reported by Lenz in a previous paper [2], but not with those described in a more recent one [10], in which the same author did not find anymore a significant increase of carbonyl groups in S. Apart from technical differences, these discrepancies may be due to different disease stages of the patients studied and the number of patients considered. To our knowledge, in the literature there are no data about BAL carbonyl detection in SSc patients.

Moreover, in this study, we analysed the protein targets of oxidation in BAL of the DLD patients by the proteomic approach because the ELISA detection of carbonyls in BAL do not reveal what the carbonylated proteins are. The 2-DE analysis of BAL showed a greater number of protein targets of oxidation in IPF than in S, SSc and controls, although there were similar levels of carbonylation in the three diseases. This finding may be due to the different pathogenetic mechanisms of IPF with respect to the other two diseases. There is evidence that the recruitment of different inflammatory cells may result in the oxidation of alternative targets [32].

In our study, comparison of the single gels revealed that BAL protein carbonylation was a process involving a limited number of carbonylation-sensitive proteins. The different sensitivity to carbonylation not only depends on the quantity of proteins present in BAL, but also on their susceptibility to oxidation. This may be determined by metal-binding sites or structural characteristics, such as amino acid sequences or three-dimensional polypeptide structure [33]. Proteins such as transferrin or hemopexin (oxidised in the gels of DLD patients) contain eight and two iron ion binding sites, respectively; albumin and ceruloplasmin (two other target proteins of oxidation in our study) contain one and 12 copper ion binding sites, respectively [33]. The different sensitivity to the carbonylation probably also depends on pathogenetic factors that make proteins more susceptible to oxidation in

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**Table 1.** Carbonylated proteins and their corresponding frequency, observed by immunoblot analysis, in healthy subjects and in patients with S, SSc and IPF (n = 3)

<table>
<thead>
<tr>
<th>Spot number</th>
<th>Carbonylated proteins</th>
<th>Sarco-dosis</th>
<th>Pulmonary fibrosis in SSc</th>
<th>Idiopathic pulmonary fibrosis</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Albumin</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>2</td>
<td>Transferrin</td>
<td>–</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>3</td>
<td>IgG heavy chain α</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>4</td>
<td>Ig light chain κ, λ</td>
<td>1/3</td>
<td>2/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>5</td>
<td>α1-antitrypsin</td>
<td>2/3</td>
<td>–</td>
<td>2/3</td>
<td>3/3</td>
</tr>
<tr>
<td>6</td>
<td>Complement C3 β</td>
<td>2/3</td>
<td>1/3</td>
<td>2/3</td>
<td>3/3</td>
</tr>
<tr>
<td>7</td>
<td>Haptoglobin α</td>
<td>–</td>
<td>–</td>
<td>1/3</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>Haptoglobin β (frag)</td>
<td>–</td>
<td>–</td>
<td>2/3</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>Complement C3</td>
<td>3/3</td>
<td>–</td>
<td>3/3</td>
<td>–</td>
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<tr>
<td>10</td>
<td>Superoxide dismutase</td>
<td>–</td>
<td>–</td>
<td>1/3</td>
<td>–</td>
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<tr>
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<td>Transthyretin</td>
<td>–</td>
<td>–</td>
<td>1/3</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>Hemopexin</td>
<td>–</td>
<td>–</td>
<td>2/3</td>
<td>–</td>
</tr>
<tr>
<td>13</td>
<td>Ceruloplasmin</td>
<td>–</td>
<td>–</td>
<td>2/3</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>Immunoglobulin A S-chain</td>
<td>–</td>
<td>–</td>
<td>3/3</td>
<td>–</td>
</tr>
<tr>
<td>15</td>
<td>Immunoglobulin A heavy chain</td>
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<td>–</td>
<td>1/3</td>
<td>–</td>
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<tr>
<td>16</td>
<td>α2-antiplasmin</td>
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<td>–</td>
<td>2/3</td>
<td>–</td>
</tr>
<tr>
<td>17</td>
<td>Apolipoprotein A-IV</td>
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<td>–</td>
<td>1/3</td>
<td>2/3</td>
</tr>
<tr>
<td>18</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>1/3</td>
</tr>
<tr>
<td>19</td>
<td>Pulmonary surfactant-associated protein A</td>
<td>–</td>
<td>–</td>
<td>1/3</td>
<td>1/3</td>
</tr>
</tbody>
</table>
Figure 2. 2-D immunoblots of carbonylated proteins detected in BAL from a patient with IPF (a), with SSc (b), with S (c), and from a healthy control (d). Carbonylated proteins are detected by chemiluminescence using specific antibodies against DNP and secondary peroxidase conjugated antibodies. (e) Silver-stained 2-D electrophoretic pattern of BAL from a healthy control. The numbers on the immunoblot and on the gel refer to the protein spots shown in Table 1.
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4 Concluding remarks

The increase in oxidatively damaged proteins in BAL of DLD patients suggests changes in pulmonary oxidant/antioxidant balance. The proteomic analysis of BAL showed that protein carbonylation was a selective process and that IPF patients had greater number of protein targets of oxidation in BAL compared to the S, SSC patients and controls. The proteins that have become dysfunctional by oxidation could play a significant role in the pathogenesis of DLD. This is the first report providing a database of target proteins of oxidation in BAL of patients with S, IPF and SSC.

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5 References


