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Increased oxidation of extracellular glutathione by bronchoalveolar inflammatory cells in diffuse fibrosing alveolitis

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ABSTRACT: An unbalanced oxidative stress is thought to be an important element in the pathogenesis of diffuse fibrosing alveolitis (DFA). The purpose of our study was to investigate the role of reactive oxygen metabolites (ROMs) released from cultured bronchoalveolar inflammatory cells (BA-cells) on glutathione oxidation. We studied bronchoalveolar lavage samples from 10 healthy controls and from 20 patients with diffuse fibrosing alveolitis (all were nonsmokers).

BA-cells obtained by bronchoalveolar lavage (BAL) were incubated with 50 μM of reduced glutathione (GSH). Oxidation of GSH to glutathione disulphide (GSSG) by BA-cell derived oxidants was detected as a decline of GSH in the supernatants. Total glutathione (GSH_{tot} = GSH + 2 GSSG) and GSSG in the epithelial lining fluid (ELF), and methionine sulphoxide (Met(O)) content of BAL proteins were determined.

In diffuse fibrosing alveolitis the oxidative activity of BA-cells was enhanced, GSH_{tot} and GSH were decreased, whereas the GSSG:GSH ratio was increased. The oxidative activity of BA-cells correlated positively with the GSSG:GSH ratio, but not with the methionine sulphoxide content. The methionine sulphoxide content was elevated in diffuse fibrosing alveolitis and inversely correlated with GSH_{tot}. The methionine sulphoxide content also correlated positively with the percentage of BAL neutrophils.

We conclude that BA-cell-derived reactive oxygen species are capable of oxidizing extracellular GSH *in vitro*. The positive correlation between the BA-cell oxidative activity *in vitro* and GSSG:GSH ratio in ELF suggests that a similar oxidative effect on extracellular GSH may also occur *in vivo*. Thus, reactive oxygen metabolites released from BA-cells may contribute to a lowered antioxidant screen, thereby causing oxidative damage of other molecules, as indicated by an increase of methionine sulphoxide content.

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The nicotinamide-adenine-dinucleotide phosphate (NADPH) oxidase of activated inflammatory cells, such as macrophages and neutrophils, produces superoxide anions which are transformed into a variety of reactive oxygen metabolites (ROMs): hydrogen peroxide, hydroxyl radicals and hypohalide anions [1–3]. These reactive oxygen species are capable of inducing cell damage as well as lesions of the extracellular tissue compounds [4–7]. Therefore, toxic oxygen products have been claimed to play a prominent role in the pathogenesis of chronic inflammatory lung diseases [8–11]. Moreover, the antioxidant screen of the extracellular compartment of the lung with reduced glutathione (GSH) as the key molecule is impaired in patients with idiopathic pulmonary fibrosis [12–14], and GSH deficiency may aggravate oxidative tissue injury in this disease. However, the antioxidant effect of GSH has not yet been demonstrated in the human lung *in vivo*.

The goal of the present study was to measure extracellular GSH oxidation by ROMs released from inflammatory cells of the lower respiratory tract. The results were compared to glutathione concentrations and redox balance in the epithelial lining fluid (ELF), in order to test the hypothesis that ROMs released from bronchoalveolar inflammatory cells (BA-cells) contribute to an impairment of the antioxidant capacity of the lung, thereby potentially aggravating oxidant injury. Oxidative stress was assessed by quantifying the methionine sulphoxide content of proteins contained in ELF [11, 15, 16].

Table 1. – Clinical and lung function characteristics of control subjects and patients with DFA

	Control group n=10	DFA patients n=20
Age yrs	30±3	64±2*
Gender M/F	5/5	7/13
Vital capacity % pred	103±3	77±4*
Total lung capacity % pred	98±5	75±4*
Transfer factor % pred	104±5	51±3*
Resting P_{a,O_2} kPa	11.4±0.3	9.9±0.3*
mmHg	85.5±2.5	74.1±2.3*
Exercise P_{a,O_2} kPa	12.0±0.3	8.7±0.6*
mmHg	89.9±2.1	65.4±4.8*

Values are presented as mean±SEM. M: male; F: female; DFA: diffuse fibrosing alveolitis. P_{a,O_2}: partial pressure of oxygen in arterialized blood. *: $p<0.05$ *vs* control group.

Methods

Study population

Control group. The control group consisted of 10 nonsmoking volunteers (five females and five males) without pulmonary disease, who underwent fibreoptic bronchoscopy and bronchoalveolar lavage (BAL) (table 1). The bronchial anatomy and mucosa were macroscopically normal. Differential cell counts in the BAL fluid (BALF) [17] and results of lung function tests [18] were within the normal range in all control subjects.

Patients. Twenty patients (13 females and 7 males) presenting with clinical, functional and radiological signs of diffuse fibrosing alveolitis (DFA) were investigated [19–22]. All patients were nonsmokers. There was no evidence of hypersensitivity pneumonitis and no history of exposure to organic or inorganic dusts. The diagnosis was confirmed histologically by transbronchial biopsy in 12 patients, and by open lung biopsy in five, in whom the transbronchial specimen was inconclusive. Three patients refused open lung biopsy when transbronchial biopsy had not been diagnostic. Although histological confirmation was lacking in these three patients, they were included in the study because there were typical findings of fibrosing alveolitis according to the above cited criteria and during follow-up. There was a significant restrictive ventilation pattern and impairment of the alveolar gas exchange as compared to the control group (table 1). All patients had been off treatment for at least 4 weeks when BAL was performed. The study protocol was approved by the local Ethics Committee. Informed consent was obtained from all control subjects and patients.

Bronchoalveolar lavage

BAL was performed in all controls and patients as described previously [17, 23]. A fibreoptic bronchoscope was wedged in a subsegmental bronchus and five serial infusions and aspirations were performed, each of 20 mL sterile saline (0.9% NaCl). This procedure was performed for the middle lobe, the lingula and the left upper lobe. The fluid recovered was pooled, filtered through sterile gauze, centrifuged at 300×g for 10 min. An aliquot of the supernatant was analysed immediately for total glutathione (GSH_{tot}). The cells were resuspended in phosphate buffered saline (PBS). Total cell counts were measured by means of a Coulter counter. Mean percentages of alveolar macrophages, neutrophils, eosinophils, lymphocytes, and mast cells were determined from slide preparations stained with May-Grünwald Giemsa stain, counting 600 cells.

GSH-oxidation assay

Preparation of bronchoalveolar inflammatory cells (BA-cells). BA-cells obtained by BAL were cultured in a standard medium (RPMI 1640, Sigma) using 6-well, flat-bottomed, plastic culture dishes (Falcon, NJ, USA) as described previously [10, 24–27]. After incubation for 30 min at 37°C with 5% CO_2, non adherent cells were removed by washing five times with Hank's balanced salt solution (HBSS) at 37°C. The fractions of the washing-procedures were pooled and the number of cells removed was counted (MD-Kova Raster 10, Madaus, Köln, FRG). This method allows the generation of BA-cell monolayers consisting mainly of alveolar macrophages [26, 27]. In our experiments, macrophage enrichment was >95%. A more detailed analysis of the residual cells was performed in four controls and six patients. After differentiating the cells in the pooled washing fractions, we were able to calculate the percentages of the residual cells, which did not differ significantly between controls and patients with respect to neutrophils (0.83±0.51 *vs* 1.44±0.77%), eosinophils (0.21±0.11 *vs* 0.56±0.31%), and lymphocytes (1.41±0.72 *vs* 1.01±0.62%).

Measurement of GSH-oxidation. The BA-cell cultures were incubated in HBSS (Sigma, USA) containing 50 µM GSH ±160 nM phorbol myristate acetate (PMA) (Sigma, USA) at 37°C with 5% CO_2. Blanks, *i.e.* incubation medium without cells present, were processed in exactly the same way. After 1 h, the supernatant was removed and catalase (Sigma, USA) was added to a final concentration of 80 $U \cdot ml^{-1}$ to stop H_2O_2-dependent reactions [28]. The samples were mixed with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Serva, Heidelberg, FRG), final concentration 250 µM, and after 5 min of incubation the absorption was measured at 412 nm (Lambda 19 Spektrometer, Perkin Elmer, Überlingen, FRG). The GSH concentration was calculated using a 4-point standard curve (75, 56.25, 37.5 and 18.75 µM). From the decrease of the GSH concentration in the presence of BA-cells (+/-PMA) as compared to the blanks (no cells present) we calculated the nMoles of GSH which were oxidized by 10^6 BA-cells$\cdot h^{-1}$ (nMol$\cdot 10^{-6}$ BA-cells$\cdot h^{-1}$). For the calculations the number of adherent cells was used.

All measurements were performed in duplicate, variation was less than 2%.

Quantification of ELF

The volume of epithelial lining fluid was quantified by the urea method [29]. Concentrations of urea in BALF and serum were measured with the Urea Nitrogen 65-UV Kit (Sigma Chemical, St. Louis, MO, USA).

Measurement of total glutathione

Total glutathione (GSH_{tot} = GSH + 2 glutathione disulphide (GSSG)) was measured with modifications as described previously [30–33]. Immediately after BAL, 100 µl of the BALF supernatant (centrifuged at 3,000 ×g for 10 min) was mixed with 1.1 ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM ethylenediamine tetra-acetic acid (EDTA), 0.2 mM NADPH, 63.5 µM DTNB, and 4 $U \cdot mL^{-1}$ glutathione reductase (all chemicals from Sigma, St. Louis, MO, USA). The rate of reduction of DTNB was recorded spectrophotometrically at a wavelength of 412 nm. The GSH_{tot} concentration within the BALF sample was calculated by using an internal standard of 0.84 µM GSH. All measurements were performed in triplicate, the variation was less than 3%.

Measurement of glutathione disulphide (GSSG)

BALF GSSG was determined according to the method described by ADAMS *et al*. [31]. BALF supernatants (centrifuged at 3,000×g for 5 min) were mixed with an equal volume of 10 mM N-ethylmaleimide (NEM) in 0.1 M potassium phosphate buffer, pH 6.5, containing 17.5 mM EDTA. Two hundred and fifty microlitres of the mixture was passed through a SEP-PAK C_{18} cartridge (Waters Associates, Milford, MA, USA) that had been prewashed with 3 ml methanol followed by 3 mL aqua bidest. GSSG was eluted from the column with 1 mL of 0.1 M potassium phosphate buffer, pH 7.5, 5 mM EDTA. Seven hundred and fifty microlitres of the eluate were added to 250 µL potassium phosphate buffer, pH 7.5, with 5 mM EDTA, 800 µM DTNB, 2 $U \cdot mL^{-1}$ glutathione reductase, and 1 mM NADPH. The rate of reduction of DTNB was recorded spectrophotometrically at 412 nM. Standards of GSSG (Boehringer, Mannheim, FRG) of known concentrations (0.25–4 µM) were processed exactly as the BALF samples and were used to generate standard curves.

Methionine sulphoxide content of BAL proteins

The relative content of oxidized methionine residues (methionine sulphoxide, Met(O)) of BALF proteins was determined as described previously [11, 15, 16]. Briefly, 2 mL aliquots of BALF were dialysed and lyophilised, then dissolved in 75% (vol/vol) formic acid. By cyanogen bromide (CNBr) treatment, methionine (Met) residues are converted to homoserine and homoserine lactone, whereas Met(O) is stable. The CNBr-treated proteins were hydrolysed with 6 M HCl. In the presence of 5 mM dithioerythritol at 110°C under vacuum for 48 h. Under these conditions, Met(O) was quantitatively reduced to Met. After precolumn derivatization with orthophthalaldehyde (Pierce, Rockford, IL, USA), the samples were subjected to amino acid analysis by reversed-phase high-performance liquid chromatography (LKB, Freiburg, FRG). Results are presented as Met(O) in percentage of total methionine (=Met+Met(O)).

Statistics

Data are expressed as the arithmetic mean and standard error of the mean (SEM). For statistical analysis the Mann-Whitney test for independent samples was employed. Correlation coefficients were determined by Pearson's test. For differences between groups and for correlation coefficients between variables, a p-value of less than 0.05 was considered significant with the two-tailed test. The statistical computations were performed with the aid of SPSS/PC+ software.

Results

BAL total and differential cell counts

The results of BAL differential cell counts are shown in table 2. There was a significantly elevated total cell count in the DFA group and the differential cell count showed an intense, predominantly neutrophilic alveolitis as compared with the controls. The percentage of eosinophils was also increased. Lymphocytes and mast cells did not differ significantly from the control group.

BA-cell oxidative activity

To test whether the decrease of the GSH concentration in the incubation medium was due to oxidation, parallel measurements of GSH and GSSG concentrations in the supernatants were performed in a series of 13 individuals. As shown in figure 1, the decrease of GSH and increase of GSSG corresponded to the equation (2 GSH → GSSG) indicating that extracellular GSH

Table 2. – BAL differential cell counts of controls and patients with diffuse fibrosing alveolitis (DFA)

	Control group n=10	DFA patients n=20
Total cell count $\times 10^6$	22.9±4.3	39.0±8.6*
Alveolar macrophages %	91.9±0.7	67.2±4.5*
Neutrophils %	2.7±0.5	15.5±5.8*
Eosinophils %	0.7±0.2	6.0±2.7*
Mast cells %	0.0±0.0	0.4±0.2
Lymphocytes %	4.6±0.4	10.9±4.7

Values are presented as mean±SEM. *: $p<0.05$ *vs* control group.

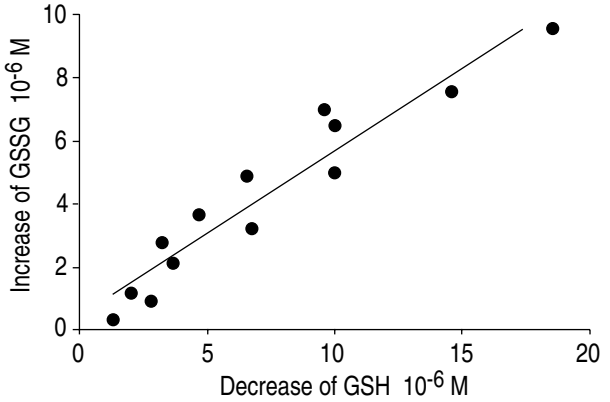

Fig. 1. – Relationship between the decrease of the GSH and increase of the GSSG concentration within the incubation median (r=0.97; n=13; p<0.001). GSH: reduced glutathione; GSSG: glutathione disulphide.

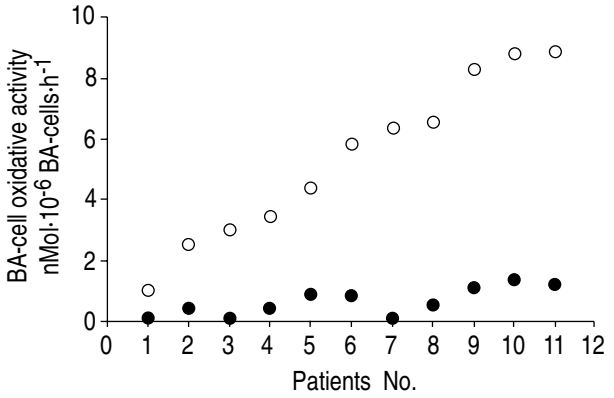

Fig. 2. – Eleven individuals were tested and ranked according to the spontaneous oxidative activity of their BA-cells (○). If catalase (160 U·mL^{-1}) was added to the assay, oxidative activity was almost completely inhibited in all cases (●). BA-cell: bronchoalveolar inflammatory cells.

is not oxidized to products other than the disulphide. GSH was not imported into or exported from the cells because total glutathione remained constant. Moreover, in a series of 11 patients, we found that GSH oxidation was almost completely inhibited by adding 160 U·ml^{-1} catalase to the assay before starting the incubation (fig. 2). These experiments suggest that the observed GSH-oxidation is almost completely due to hydrogen peroxide released from cultivated human BA-cells. Only a small amount of catalase-insensitive GSH oxidation occurred, which might be ascribed to reactive oxygen metabolites (ROMs), such as hypochlorous acid released from residual polymorphonuclear leucocytes (PMNs) within the BA-cell cultures.

Significantly increased amounts of extracellular GSH were oxidized by cultivated BA-cells from patients with DFA as compared with the control group (fig. 3). The spontaneous rate of GSH oxidation was 0.9±0.2 nMol·10^{-6} BA-cells·h^{-1} in the control group and 4.0±1.1 nMol·10^{-6} BA-cells·h^{-1} in the DFA group (p<0.05). With PMA stimulation, the absolute difference between controls and patients was even more pronounced: 1.8±0.3 *vs* 6.3±1.8 nMol·10^{-6} BA-cells·h^{-1} (p<0.05).

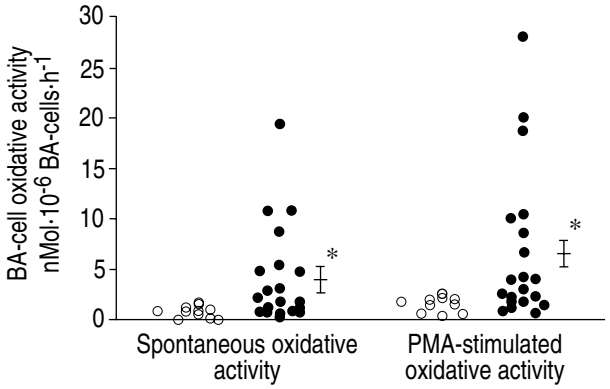

Fig. 3. – Spontaneous and PMA stimulated oxidative activity of BA-cells from patients with DFA (n=20) (●) was significantly (p<0.05) increased compared with the control group (n=10) (○).⊣ : mean±SEM for DFA patients. *: p<0.05 *vs* control. DFA: diffuse fibrosing alveolitis; PMA: phorbol myristate acetate; BA-cells: bronchoalveolar inflammatory cells.

Table 3. – Glutathione concentration and form in ELF

	Control group (n=10)	DFA patients (n=20)
ELF recovered mL	0.86±0.04	1.24±0.08*
Total GSH µM	568±45	285±20*
GSH reduced µM	506±53	224±19*
GSSG µM	31.2±6.5	30.7±6.3
GSSG:GSH ratio	0.06±0.02	0.14±0.03*

Values are presented as mean±SEM. ELF: epithelial lining fluid; DFA: diffuse fibrosing alveolitis; GSH: reduced glutathione; GSSG: glutathione disulphide. *: p<0.05 *vs* control group.

Glutathione in the ELF

Table 3 summarizes the results of the GSH and GSSG measurements calculated for the recovered ELF volume, which was increased in the patient group. The levels of total GSH and reduced GSH were significantly decreased in the DFA patients, whereas the GSSG concentration was similar in controls and patients. The GSSG: GSH ratio was significantly increased in the patient group. Moreover, there was a significant positive correlation between the BA-cell oxidative activity and the GSSG: GSH ratio in the ELF (fig. 4). Interestingly, neither the percentage of BAL neutrophils (r=0.29; n=20; p=0.21) nor the percentage of BAL alveolar macrophages correlated significantly with the GSSG:GSH ratio (r=0.29; n=20; p=0.21 and r=0.25; n=20; p=0.29; respectively).

Met(O) content of alveolar proteins

The Met(O) content of the BAL derived proteins was significantly increased in the patients with DFA (7.93± 0.73%) compared to the control subjects (4.18±0.74%) (p<0.05). There was no correlation between Met(O) and the spontaneous or PMA-stimulated oxidative activity of cultured BA-cells, measured in terms of GSH oxidation (p>0.05). However, significant inverse correlations existed between the Met(O) content of the alveolar proteins and the concentrations of GSH$_{tot}$ (r=-0.71; n = 20; p<0.05) (fig. 5) and of GSH (r=-0.69; n=20; p<0.05) in

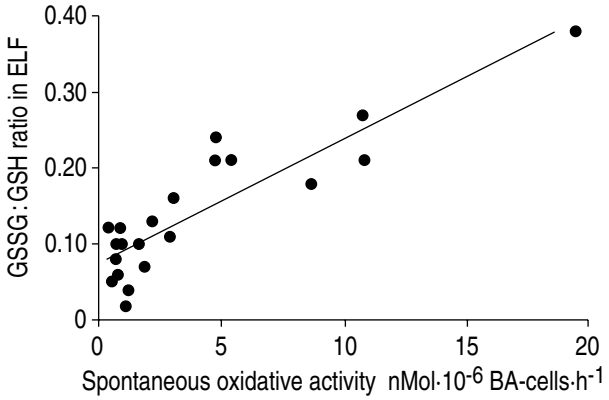

Fig. 4. – The spontaneous oxidative activity of cultured BA-cells correlates positively with the GSSG:GSH ratio within the epithelial lining fluid (ELF) in the DFA group (r=0.87; n=20; p<0.01). For abbreviations see legends to figures 1–3.

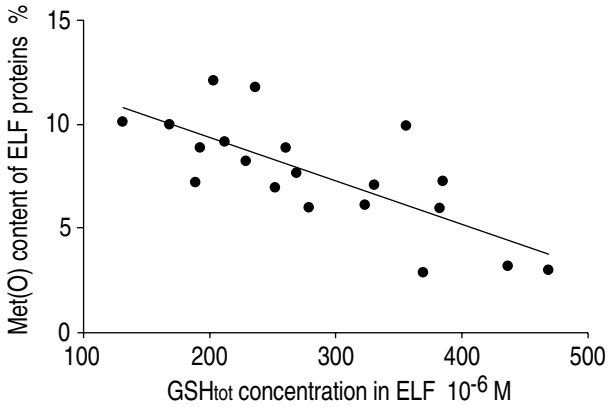

Fig. 5. – Significant inverse correlation between the GSH_{tot} concentration in ELF and the Met(O) content of the ELF proteins in DFA (r=-0.71; n=20; p<0.01). GSH_{tot}: total glutathione; Met(O): methionine sulphoxide; ELF: epithelial lining fluid; DFA: diffuse fibrosing alveolitis.

the ELF. A significant positive correlation was observed between Met(O) and the percentage of BAL neutrophils in the DFA group (r=0.57; n=20; p<0.05).

Discussion

Fibrosing alveolitis is a chronic inflammatory disease of the lower respiratory tract characterized by accumulation of mononuclear and polymorphonuclear phagocytes within the alveolar walls [21, 22]. The release of highly reactive oxygen metabolites from these cells is known to be markedly increased, resulting in an excess oxidant stress [9–11, 34]. This oxidant burden faces a diminished antioxidant defence, because reduced glutathione, the major antioxidant molecule within the ELF of the lower respiratory tract [33, 35], is significantly decreased in the DFA patients (table 3). This finding of our present study confirms reports from other investigators describing a glutathione deficiency in the ELF of patients with idiopathic pulmonary fibrosis [12–14]. However, little is known about the interaction of oxidants released from alveolar phagocytes and extracellular GSH *in vivo*.

In the present study, we provide evidence that ROMs released from human bronchoalveolar inflammatory cells are able to oxidize extracellular GSH *in vitro*. In contrast to other assays, which measure the production and/or release of oxidants from phagocytes by reduction of cytochrome C [36] or oxidation of phenol red [37], the GSH-oxidation assay applied in this study was developed to mimic the *in vivo* situation by measuring the oxidative effect using GSH as a pathophysiologically relevant substrate. It remains, however, difficult to directly transfer the *in vitro* results of BA-cell oxidative activity to the *in vivo* situation, because changes of phenotype and function may occur when these cells are cultured [24]. Since the cells from controls and patients were processed in exactly the same way, there is strong evidence that BA-cells are capable of oxidizing increased amounts of GSH in DFA and may, thereby, contribute to the excess oxidative stress *in vivo*. The positive correlation observed between the spontaneous rate of GSH oxidation by cultured BA-cells and the GSSG:GSH ratio in ELF from patients with DFA (fig. 4) supports the concept that ROMs released from BA-cells contribute to the oxidation of extracellular GSH in the human lung. In contrast, the percentages of BAL neutrophils or alveolar macrophages were not significantly related to the GSSG:GSH ratio, indicating that the measurement of cell function with the GSH-oxidation assay is more closely related to biochemical effects *in vivo* than cell numbers.

As recently reported, the Met(O) content of ELF proteins reflects one important aspect of the *in vivo* effects of ROMs in ELF of patients with DFA [11, 16]. In good agreement with previous reports [11, 16], Met(O) was significantly elevated in DFA patients as compared to the control group in this study. However, the Met(O) content of BAL proteins and the oxidative activity of cultured BA-cells measured in terms of GSH oxidation did not correlate significantly. In contrast, Met(O) formation in alveolar proteins was positively correlated with the percentage of BAL neutrophils, confirming similar results of previous studies [11, 16]. There is experimental evidence indicating that methionine residues are efficiently oxidized by products of the myeloperoxidase/H_2O_2/chloride system, such as hypochlorous acid and chloramines, whereas superoxide anions, hydroxyl radicals, and hydrogen peroxide are far less effective [38]. Therefore, the positive correlation between the Met(O) content of BAL proteins and the percentage of BAL neutrophils is probably explained by the neutrophils' ability to release myeloperoxidase, which is necessary to form hypochlorous acid and chloramines, which in turn efficiently oxidize methionine residues of BAL proteins. In contrast, alveolar macrophages, which were the predominant cell type in the BA-cell cultures, are devoid of myeloperoxidase and, thus, unable to oxidize significant amounts of methionine. This may explain the absence of a direct relationship between BA-cell oxidative activity and Met(O) formation.

Interestingly, in this study, Met(O) correlated inversely with GSH_{tot} (fig. 5) and with GSH. Consequently, a decrease of the GSH_{tot} or GSH concentration in the ELF is associated with an increased Met(O) content of

the ELF proteins. This result supports the assumption that GSH acts as an antioxidant at its physiological concentration *in vivo*.

Our data are thus complementary to findings of CANTIN *et al*. [33] indicating that GSH in physiological concentrations protects lung parenchymal cells against oxidative injury. Moreover, because of the inverse correlation between BA-cell oxidative activity and GSSG:GSH ratio in this study, it seems reasonable to assume that BA-cell oxidative activity, which mainly represents hydrogen peroxide release from alveolar macrophages, causes a significant impairment of the antioxidant screen of the lung, allowing oxidative damage as demonstrated by an increase of Met(O). As a consequence of these findings, it seems rational to correct the oxidant/antioxidant imbalance by increasing the GSH concentration in the lung as a therapeutic strategy in patients with diffuse fibrosing alveolitis.

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