Alveolar Fluid Glutathione Decreases in Asymptomatic HIV-Seropositive Subjects Over Time*

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Background: Initial investigations demonstrated a deficiency of glutathione (GSH) in the epithelial lining fluid (ELF) of HIV-seropositive patients. In a recent study, our laboratory was unable to document such a deficiency. The current study was performed in an attempt to reconcile those disparate findings.

Study objectives: To determine if ELF GSH decreases over time in asymptomatic HIV-seropositive subjects.

Design: Prospective, longitudinal study.

Setting: Major university medical center.

Patients or participants: Thirty-three asymptomatic HIV-seropositive volunteers.

Interventions: None.

Measurements and results: BAL was performed on 33 asymptomatic HIV-seropositive subjects at baseline, 6 months later, and 12 months later. The volume of ELF and the concentration of GSH and oxidized GSH were determined. The concentration of total GSH in ELF was 689.0±100.4 μM. This significantly decreased when measured 6 and 12 months later (355.9±41.7 μM, and 397.9±52.7 μM, respectively, p=0.01, compared with baseline, both comparisons). Significant decreases were also noted in the HIV-seropositive subjects who smoked cigarettes (baseline—762.6±142.4 μM; 6 months—373.7±45.9 μM; 12 months—450.3±73.8 μM, p<0.03, for baseline vs 6 months, and baseline vs 12 months). In nonsmoking HIV-seropositive subjects, there was a decrease in ELF GSH over time, but it did not reach statistical significance (baseline—589.1±138.2 μM; 6 months—335.3±74.1 μM; 12 months—345.8±74.0 μM, p>0.1, all comparisons). The percentage of total GSH in the oxidized form was similar at all three time points (baseline—3.8±0.5%; 6 months—3.1±0.5%; 12 months—3.9±0.9%, p>0.1, all comparisons).

Conclusions: The current study demonstrates that the GSH level in ELF is significantly decreased in HIV-seropositive subjects 6 and 12 months after the initial determination.

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Key words: antioxidants; epithelial lining fluid; glutathione; human immunodeficiency virus; oxidant injury

Abbreviations: ELF=epithelial lining fluid; GSH=glutathione; GSSG=oxidized glutathione; SSA=5-sulfosalicylic acid

Glutathione (GSH), which is the most abundant nonprotein thiol in living organisms, is essential for a number of vital biological functions, including synthesis of proteins and DNA, transport of amino acids, enzyme activity, and protection of cells.1,2 This

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tri peptide (L-gamma-glutamyl-L-cysteinyl-glycine) can function as an antioxidant by acting as a reductant in the presence of GSH peroxidase to reduce H₂O₂ or other hydroperoxides to less toxic substances.1-3 GSH is also able to react with and scavenge reactive oxygen species such as H₂O₂,4 and therefore may be important in lung cell protection. GSH is also believed to be important in host defense in a number of ways, including the initiation and progression of lymphocyte activation, proper functioning of natural killer cells, and lymphocyte-mediated cytotoxicity.5,7

Two recent studies have measured GSH in the epithelial lining fluid (ELF) of asymptomatic HIV-seropositive subjects.5,8 The initial study demon-
strated a deficiency of GSH in the ELF of asymptomatic HIV-seropositive subjects, while our laboratory found that the concentration of GSH was not significantly different than in normal volunteers. In an attempt to reconcile the differences between these two studies, our laboratory decided to examine the hypothesis that ELF GSH decreased over time in HIV-seropositive subjects. It seemed possible that the two studies examined patients at different stages of their HIV infection, and that a deficiency of ELF GSH could theoretically develop over time, possibly contributing to the deleterious pulmonary consequences seen in HIV-infected individuals.

**Materials and Methods**

**Subject Groups and Study Protocol**

Thirty-three HIV-seropositive subjects with no history of AIDS-related pulmonary complications were recruited for this study. All subjects, except one, were men and their mean age was 35.1±1.7 years. There were 19 current smokers (mean pack-years, 19.7±2.8) and 14 current nonsmokers (two subjects had a remote history of smoking cigarettes). All subjects were studied under an approved protocol of The Ohio State University Human Subjects Review Committee.

All subjects underwent pulmonary function testing followed by simultaneous collection of BAL fluid and serum at baseline, 6 months later, and 12 months later (see below).

**Pulmonary Function Tests**

Pulmonary function tests were performed (on a pulmonary function analyzer; S&M Instrument Co, Doylestown, Pa). The steady-state helium dilution technique was used to obtain the functional residual capacity. Standards of the American Thoracic Society were used for all testing procedures and results compared to established normal values for spirometry, lung volumes, and diffusing capacity. Diffusing capacity was normalized for distribution volume and hemoglobin concentration. All tests were performed by the same two laboratory technicians.

**BAL and BAL Fluid Processing**

BAL was performed as previously described. The BAL fluid was filtered immediately through coarse surgical gauze and centrifuged (300 g, 10 min) to separate cellular and noncellular elements. The supernatant was acidified to a pH of 5.5 using 4 \( \mu \)L/mL of 50% 5-sulfosalicylic acid (SSA) and immediately assayed for GSH and oxidized GSH (GSSG) (see below). Once the supernatant was decanted, the cell pellet was suspended in 10 mL of Hank's balanced salt solution without calcium or magnesium. Total cell count was determined by hemocytometer. A small aliquot was cytocentrifuged (35 g, 20 s), air-dried, and stained by a Giemsa-type stain (Diff-Quik). A differential cell count was performed on a minimum of 300 cells.

**Measurement of ELF Volumes**

The volume of ELF was estimated by use of the urea dilution technique.

**Measurement of BAL GSH**

Total GSH (GSH plus GSSG) was measured in unconcentrated BAL fluid immediately following bronchoscopy using the recycling assay of Sies and Akerboom. BAL fluid and diluent (0.9% saline solution acidified with 4 \( \mu \)L/mL 50% SSA) were preincubated in a water bath at 30°C for 5 to 7 min. Then, a 1.5-mL cuvette with 140 \( \mu \)L of sodium phosphate-EDTA buffer with NADPH (1.24 mg/mL) was placed in the water bath for 1 to 2 min. Subsequently, 50 \( \mu \)L of 12 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) was added to the cuvette and allowed to incubate for 1 min. Quickly, these cuvettes were then loaded with 810 \( \mu \)L of diluent and 100 \( \mu \)L of acidified BAL fluid and placed in the spectrophotometer. The reaction was initiated by adding 12 \( \mu \)L of GSSG reductase (250 U/mL) and the conversion of DTNB to 5-thio-2-nitrobenzoic acid was followed at 412 nm. A standard curve was constructed with known concentrations of GSH.

The determination of GSSG required several additional steps. During the acidification of the BAL fluid (see above), a portion of the BAL fluid supernatant had both SSA and 2 \( \mu \)L/mL of 2-vinyl pyridine added in order to bind all the reduced GSH, and the pH adjusted to between 6.0 and 7.0 with 50% triethanolamine. This mixture was incubated at room temperature for 2 h to allow for complete binding. The recycling assay, as described above, was performed on these samples and standard curves were constructed using GSSG processed with 2-vinyl pyridine. All assays were performed in duplicate with the final result reported as the average of the two values.

**Statistical Methods**

All data were expressed as the mean and SEM. Data were compared using the paired \( t \) test and considered significant if \( p<0.05 \). Clinical correlations were determined using a simple linear regression model with analysis of variance.

**Total GSH in ELF**

The concentration of total GSH in ELF was 689.0±100.4 \( \mu \)M. This significantly decreased when measured at 6 months, but appeared to stabilize at 12 months (355.9±41.7 \( \mu \)M, and 307.9±52.7 \( \mu \)M, respectively, \( p=0.01 \), compared with baseline, both comparisons, Fig 1). Significant decreases were particularly noted in the HIV-seropositive subjects who smoked cigarettes (baseline—762.6±142.4 \( \mu \)M; 6 months—373.7±45.9 \( \mu \)M; 12 months—459.3±73.8 \( \mu \)M, \( p<0.03 \), for baseline vs 6 months, and baseline vs 12 months, \( p>0.1 \), for 6 months vs 12 months, Fig 1). In nonsmoking HIV-seropositive subjects, there was a decrease in ELF GSH over time, but it did not reach statistical significance (baseline—589.1±138.2 \( \mu \)M; 6 months—335.3±74.1 \( \mu \)M; 12 months—345.8±74.0 \( \mu \)M, \( p>0.1 \), all comparisons, Fig 1). The percentage of total GSH in the oxidized form (GSSG) was similar at all three time points (baseline—3.8±0.5%; 6 months—3.1±0.5%; 12 months—3.9±0.9%, \( p>0.1 \), all comparisons).
The concentration of total glutathione in ELF was 689.0±100.4 μM. This significantly decreased when measured 6 and 12 months later (355.9±41.7 μM, and 397.9±52.7 μM, respectively, p=0.01 compared to baseline, both comparisons). Decreases were also noted in the HIV-seropositive subjects who smoked cigarettes (baseline—762.6±142.4 μM; 6 months—373.7±45.9 μM; 12 months—459.3±73.8 μM, p<0.03, for baseline vs 6 months, and baseline vs 12 months). In nonsmoking HIV-seropositive subjects, there was a decrease in ELF GSH over time, but it did not reach statistical significance (baseline—589.1±138.2 μM; 6 months—335.3±74.1 μM; 12 months—345.8±74.0 μM, p>0.1, all comparisons).

CD4 Counts

The mean CD4 count at baseline was 485.4±38.6 cells/mm³. This decreased slightly at 6 months (457.3±40.4 cells/mm³), but significantly decreased at 12 months (399.3±41.7 cells/mm³, p=0.038 compared with baseline). There were no significant differences in CD4 counts over time in either the smoker or the nonsmoker groups (p>0.1, all comparisons), and there were also no significant differences between the smoker and nonsmoker groups at each of the three time points (p>0.1, all three comparisons).

Pulmonary Function Tests

At baseline, the values for FEV₁/FVC, total lung capacity, and the diffusing capacity, corrected for alveolar volume were normal (78.4±1.1%, 96.6±1.9% of predicted, and 84.1±1.8% of predicted, respectively).

Clinical Correlations

There was no significant correlation between the decrease in GSH over time and the fall in the CD4 counts in the HIV-seropositive individuals.

Discussion

The present study demonstrates that in asymptomatic HIV-seropositive individuals, the concentration of GSH in ELF significantly decreases over a 12-month period. Although previous investigators had found a GSH deficiency in plasma, leukocytes, B and T cells, and liver of HIV-seropositive individuals, there was some discrepancy involving the level of GSH in the ELF of HIV-seropositive individuals. Buhl and coworkers demonstrated that asymptomatic HIV-seropositive subjects had a deficiency of ELF GSH compared to that in normal volunteers, while our laboratory has shown that baseline levels of ELF GSH were normal in HIV-seropositive individuals. Although both studies examined asymptomatic HIV-seropositive subjects with similar CD4 counts, it is well known that CD4 counts correlate only roughly with the duration of infection. The current investigation suggests that increasing duration of HIV infection is an important determinant of ELF GSH. Indeed, Delatour and colleagues have previously demonstrated a significant correlation between decreasing plasma GSH concentration and increasing duration of HIV seropositivity. Thus, we hypothesize that the discrepancy between our original report and the investigation of Buhl and colleagues relates to the difference in the natural history of HIV infection between the two populations studied.

The concentration of GSH in the alveolar spaces is likely to be particularly relevant in HIV infection. It has been demonstrated that alveolar macrophages from HIV-seropositive subjects have increased oxygen radical production (superoxide anion) compared to normal subjects. Therefore, in an environment of increased oxidant production, GSH may function as an important and critical antioxidant. Cantin and colleagues have demonstrated that the concentration of GSH in the ELF was sufficient to protect cat AKD cells (diploid feline alveolar epithelial cell with both type I and type II cell characteristics) from H₂O₂-induced injury. Haugen and coworkers demonstrated that rat type II cells were able to take up exogenous GSH and thus provide substantial protection from parquat-mediated oxidant injury. Simon and colleagues demonstrated that GSH was instrumental in protecting type II alveolar epithelial cells from hydrogen peroxide and neutrophil-induced injury. Previous investigations in our laboratory demonstrated that exogenous GSH could prevent H₂O₂-mediated loss of type II alveolar epithelial cell adenosine triphosphate. In a similar study, Chang and coworkers demonstrated that pretreatment with exogenous GSH prevented menedione-induced loss of bovine pulmonary artery endothelial cell adenosine triphosphate and GSH. A second study from the same laboratory demonstrated extracellular GSH could protect alveolar macrophages from sublethal injury (loss of their respiratory burst) due to exposure to hyperoxia. Taken together, these studies suggest that...
ELF GSH may play an important role in the antioxidant defense of the lung epithelial cells. It seems conceivable that the decline in ELF GSH levels over time could contribute to the pathogenesis of the pulmonary complications seen in HIV infection, particularly the noninfectious complications such as an emphysema-like lung injury and pulmonary hypertension.27

In addition to being an important antioxidant in ELF, GSH is critical for cell defense. Prior investigations have shown that adequate concentrations of GSH are important for T-cell proliferation, differentiation of T and B cells, cytotoxic T-cell activity, and natural killer cell activity.5,7,13 Therefore, a deficiency of GSH has significant effects on immune function. Staal and coworkers28 have shown that GSH helps regulate HIV expression and a cellular deficiency permits more rapid growth and replication of the HIV. These studies, in concert with the earlier cited studies documenting a systemic GSH deficiency in HIV-seropositive individuals, have led some investigators to propose that GSH deficiency contributes to the pathogenesis of AIDS.13,29

The role of cigarette smoking is difficult to define. The GSH level decreased in both smoking and nonsmoking HIV-seropositive subjects over time, although the differences did not reach statistical significance in the smoking group. The lack of a statistically significant drop in the nonsmoking group was most likely due to a small number of subjects. However, this needs to be studied in a greater number of individuals before firm conclusions can be made.

The current study provides at least a partial explanation of the disparate results seen in the two prior studies of GSH levels in ELF.5,8 Clearly, further investigations will need to be undertaken to further clarify this important area.

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