Pulmonary oxidative stress response in young children with cystic fibrosis

Jeremy Hull, Peter Vervaart, Keith Grimwood, Peter Phelan

Abstract

***Background* – It has been suggested that oxidative stress contributes to lung injury in cystic fibrosis. There is, however, no direct evidence of increased pulmonary oxidative stress in cystic fibrosis nor of the effects of inflammation on the major pulmonary antioxidant, glutathione. A study was undertaken to measure these parameters in infants and young children in the presence or absence of pulmonary inflammation.**

***Methods* – Thirty two infants and young children with cystic fibrosis of mean (SD) age 21.4 (15.3) months (range 2–54) and seven non-cystic fibrosis control subjects of mean (SD) age 21.0 (21.2) months (range 2–54) were studied using bronchoalveolar lavage (BAL). On the basis of the BAL findings the cystic fibrosis group was divided into those with (CF-I) and those without pulmonary inflammation (CF-NI). Levels of lipid hydroperoxide, total glutathione, and γ-glutamyl transpeptidase (γ-GT) were then measured in the BAL fluid.**

***Results* – The concentrations of lipid hydroperoxide and γ-GT in the epithelial lining fluid were significantly increased in the CF-I group compared with the control and CF-NI groups, each of which had similar values for these parameters (ratio of geometric means for CF-I group versus control for lipid hydroperoxide 5.4 (95% confidence interval (CI) 1.8 to 15.8) and for γ-GT 5.2 (95% CI 1.4 to 19.4)). The glutathione concentration tended to be lower in the CF-I subjects but the difference did not reach statistical significance.**

***Conclusions* – These results demonstrate that the airways in patients with cystic fibrosis are exposed to increased oxidative stress which appears to be a consequence of pulmonary inflammation rather than part of the primary cystic fibrosis defect. The increase in γ-GT in the CF-I group suggests a mechanism by which extracellular glutathione could be utilised by airway epithelial cells.**

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Lung disease in cystic fibrosis is responsible for most of the morbidity and mortality associated with this disease. It is characterised by endobronchial infection, which is often intermittent in early life but which becomes persistent in older patients, particularly after they become infected with *Pseudomonas aeruginosa*. The endobronchial infection is accompanied by an intense neutrophil dominated inflammatory response.

Evidence to support the involvement of reactive oxygen species as mediators of tissue damage in lung disease in cystic fibrosis has come largely from studies measuring the products of lipid and protein oxidation in plasma. These studies have shown that, in older children and adults, increased plasma concentrations of oxidation products can be found in patients with cystic fibrosis compared with control subjects.[1 2] One study has shown a correlation between the plasma level of a marker of lipid oxidation (malondialdehyde) and lung function.[3] The origin of these oxidation products is not clear. Although it is possible that they are the result of oxidative stress associated with pulmonary inflammation, other studies have also shown that there is a defect in intracellular oxygen metabolism in cystic fibrosis cells[4 5] resulting in increased levels of reactive oxygen species leaking from mitochondria. It may be this second mechanism that is predominantly responsible for the observed increased plasma levels of oxidation products in patients with cystic fibrosis.

In the lung the major local antioxidant is glutathione which is present at high concentration in the epithelial lining fluid.[6] Cells can be protected against oxidative stress by extracellular glutathione through its degradation catalysed by the exo-enzyme γ-glutamyl transpeptidase (γ-GT) and its de novo synthesis within the cytosol via the γ-glutamyl cycle.[7] Levels of glutathione have been shown to be reduced in cystic fibrosis, both in the epithelial lining fluid of the lung and in plasma.[8] It has been suggested that the systemic deficiency of glutathione in cystic fibrosis may be a direct consequence of the basic genetic defect and predisposes patients with cystic fibrosis to oxidative tissue damage.[8]

In this study we have tested the hypothesis that there is evidence of increased oxidative stress present in the lungs of patients with cystic fibrosis and that this increased stress is in response to inflammation associated with infection, rather than part of the primary defect. We have also hypothesised that the reduced glutathione levels in the epithelial lining fluid of patients with cystic fibrosis are a response to pulmonary oxidative stress, mediated via induced γ-GT, and that in the absence of pulmonary inflammation they do not differ from normal.

Table 1 Analysis of bronchoalveolar lavage (BAL) fluid for evidence of infection and inflammation in each of the three study groups.

	Controls (n=7)	*CF-NI (n=19)*	*CF-I (n=13)*
Lavage fluid recovered (%)	30.0 (29–43)	42.5 (32–55)	36.0 (33–44)
Volume of ELF recovered (ml/100 ml recovered lavage fluid)	1.8 (1.6–2.1)	1.9 (1.1–3.1)	2.3 (1.9–4.4)
Neutrophils in BAL fluid (%)	7 (4–30)	12 (7–23)	70 (58–82)
IL-8 in BAL fluid (pg/ml)	10 (4–23)	75 (30–182)	1800 (436–4713)
No. of subjects with $\geq 10^5$ cfu	0	1	9

Results are given as median (interquartile range). CF-NI = subjects with cystic fibrosis without pulmonary inflammation; CF-I = subjects with cystic fibrosis with pulmonary inflammation; ELF = epithelial lining fluid; IL-8 = interleukin 8; cfu = colony forming unit.

Methods

SUBJECTS AND CONTROLS

The state of Victoria, Australia (66 000 births/year) has a newborn screening programme for cystic fibrosis. The diagnosis is confirmed either by establishing homozygosity for the ΔF508 mutation or by sweat chloride concentrations of more than 60 mmol/l. All patients are managed at the Royal Children's Hospital Cystic Fibrosis Clinic. Samples of bronchoalveolar lavage (BAL) fluid were collected from asymptomatic infants with cystic fibrosis identified by the screening procedure and from infants and young children with cystic fibrosis during episodes of respiratory exacerbations. A total of 32 infants and young children with cystic fibrosis of mean (SD) age 21.4 (15.3) months (range 2–54) were studied. Seven children undergoing bronchoscopy for stridor formed a disease control group with a mean (SD) age of 21.0 (21.2) months (range 2–54). These patients had no signs of respiratory infection or a history of antibiotic use during the previous 14 days.

BAL fluid from all subjects was analysed for the presence of infection by quantitative culture and for inflammation by cytological examination and interleukin 8 (IL-8) analysis as described previously.[9] Infection was defined as $>10^5$ colony forming units (cfu)/ml BAL fluid. Levels of IL-8 above 250 pg/ml BAL fluid, associated with the proportion of neutrophils present of more than 50%, were taken to indicate inflammation. On the basis of the IL-8 and percentage neutrophils present in the BAL fluid, the subjects with cystic fibrosis were divided into two groups, one group with evidence of pulmonary inflammation (CF-I, n = 13) and a second group in which these markers were negative (CF-NI, n = 19).

The study was approved by the human ethics committee of the Royal Children's Hospital and written informed consent was obtained from the parents of each child before bronchoscopy.

BRONCHOALVEOLAR LAVAGE

BAL was performed under general anaesthesia. Following topical application of lignocaine to the vocal cords, a flexible bronchoscope (Olympus model BF3 C20; external diameter 3.6 mm, suction channel 1.2 mm) was introduced into the lower airway through a laryngeal mask, avoiding the use of the suction channel until the tip of the bronchoscope was below the carina. The tip was wedged in the right middle lobe bronchus and, to optimise sampling from endobronchial sites, a single small volume lavage was performed by instilling 1 ml/kg of sterile non-bacteriostatic normal saline at room temperature through the suction channel of the bronchoscope for 3–5 seconds. Using negative suction pressures of 100–150 mm Hg the saline was immediately aspirated into a suction set over 10–20 seconds. The bronchoscope was then wedged into the lingula bronchus and, using an identical technique, a further single aliquot lavage was performed. The BAL fluid from both lavages was pooled for analysis.

ANALYSIS OF LIPID HYDROPEROXIDES, GAMMA-GLUTAMYL TRANSPEPTIDASE, AND TOTAL GLUTATHIONE

Lipid hydroperoxides, markers of free radical associated lipid peroxidation, were measured by the FOX1 assay.[10] Gamma-GT was measured using a micro-adaptation of a standard kit (MPR2, Boehringer Mannheim Australia, Castle Hill, NSW, Australia). Total glutathione was assayed by the glutathione reductase recycling assay.[11]

All results were expressed as concentrations per ml epithelial lining fluid using the urea method.[12] Urea concentrations in the BAL fluid were determined by a micro-adaptation of a standard urease/glutamate dehydrogenase kit (Sigma 66-UV, Sigma Chemical Company, St Louis, Missouri, USA) adapted to analyse urea at micromolar quantities. Plasma concentrations of urea were determined on an Ektachem 750 Analyser (Johnson and Johnson Clinical Diagnostics Inc, Rochester, New York, USA).

DATA ANALYSIS

The measured parameters all showed a positively skewed distribution that became normal following log transformation. Comparisons between groups were therefore made with log transformed data using the independent sample *t* test with a Bonferroni correction for multiple comparisons.

Results

The results of BAL fluid analysis in the controls and subjects with cystic fibrosis are shown in table 1. The percentage lavage return and volume of epithelial lining fluid recovered were similar in all three groups. The percentages of neutrophils in the BAL fluid from the control and CF-NI groups were similar, while in the CF-I group the percentage of neutrophils in the BAL fluid was markedly increased. The IL-8 concentration in the BAL fluid was higher in the CF-NI group than in the control group but the absolute difference was small when compared with the increase seen in the CF-I group. The presence of infection was strongly correlated with inflammation. Nine of the 13 subjects in the CF-I group had infection (three *Staphylococcus aureus*, two *Pseudomonas aeruginosa*, two *Haemophilus influenzae*, one

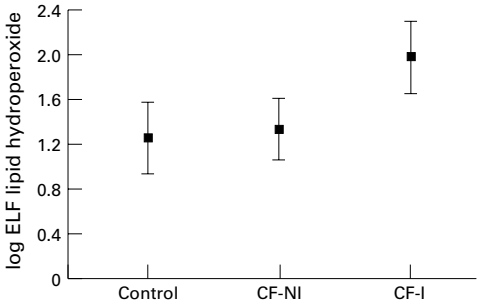

Figure 1 Comparison of the concentrations of lipid hydroperoxide in the epithelial lining fluid (ELF) in each group. Plot shows mean and 95% confidence intervals of log transformed data. CF-NI=subjects with cystic fibrosis without pulmonary inflammation; CF-I=subjects with cystic fibrosis with pulmonary inflammation. The value for the CF-I group was significantly higher than that for the other two groups.

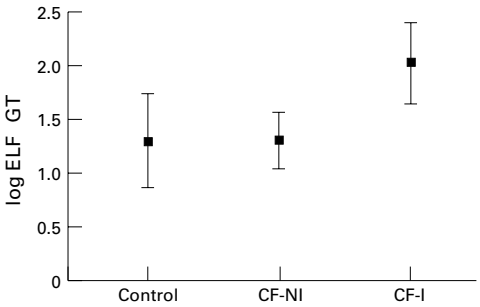

Figure 2 Comparison of the concentrations of gamma-glutamyl transpeptidase in the epithelial lining fluid (ELF) in each group. Plot shows mean and 95% confidence intervals of log transformed data. CF-NI=subjects with cystic fibrosis without pulmonary inflammation; CF-I=subjects with cystic fibrosis with pulmonary inflammation. The value for the CF-I group was significantly higher than that for the other two groups.

Moraxella catarrhalis and one *Stenotrophomonas maltophilia*) whereas infection (*Moraxella catarrhalis*) was only found in one of the 19 subjects in the CF-NI group. None of the controls was infected.

The concentration of lipid hydroperoxide in the epithelial lining fluid was very similar in both the CF-NI group (geometric mean 21.9 μM) and the controls (18.2 μM). In contrast, the concentration in the CF-I group was significantly increased (97.7 μM; $p<0.05$) compared with the other two groups: ratio of geometric means CF-NI versus CF-I 4.5 (95% CI 1.8 to 11.3); control versus CF-I 5.4 (1.8 to 11.3). These data are shown in fig 1.

The concentration of γ-GT in the epithelial lining fluid was also similar in the CF-NI group (geometric mean 20.2 U/l) and the control subjects (20.1 U/l). In the CF-I group the concentration was significantly higher (104.2 U/l) than in the other two groups ($p<0.05$); ratio of geometric means CF-NI versus CF-I 5.2 (95% CI 1.9 to 13.9); control versus CF-I 5.2 (1.4 to 19.4). These data are shown in fig 2.

The concentration of total glutathione in the epithelial lining fluid was similar in the CF-NI (geometric mean 12.2 μM) and non-CF control (9.0 μM) groups and tended to be lower in the CF-I group (4.4 μM). This difference did not reach statistical significance; CF-NI vs CF-I ratio of geometric means 2.77 (95% CI 0.9 to 8.6); control vs CF-I 2.0 (0.4 to 10.9).

Discussion

This study is the first to demonstrate that increased oxidative stress is present in the lungs of patients with cystic fibrosis. The finding of raised lipid oxidation products in BAL fluid during episodes of pulmonary inflammation suggests that, during these episodes, host antioxidant capability is overwhelmed. A close association between inflammation and infection was observed. We have also shown that in the absence of inflammation there is no evidence of oxidative stress, at least as assessed by lipid peroxidation. It is reasonable to assume that this is a sensitive marker of pulmonary oxidative stress since lipid oxidation has been shown to be associated with severity of lung disease.[3] These results suggest that the increased level of oxidant activity found in this study, and in previous studies using plasma levels, represents an effect of pulmonary inflammation rather than being due to an underlying primary defect in intracellular oxygen metabolism in cystic fibrosis cells.

We have also shown that, in the absence of inflammation, the levels of glutathione in the epithelial lining fluid do not differ from non-cystic fibrosis control subjects. Like others we found a decrease in the total glutathione concentration in the epithelial lining fluid of cystic fibrosis patients with pulmonary inflammation,[8] but in the present study this difference did not reach statistical significance. It is possible that, with larger numbers, the fall in glutathione concentration would become significant. Furthermore, if we had measured reduced glutathione in addition to total glutathione we may have shown a significant fall in the cystic fibrosis patients with inflammation. In the study by Roum and colleagues the fall in total glutathione was almost entirely accounted for by a reduction in reduced glutathione. There was no difference in glutathione concentrations in the epithelial lining fluid between patients with cystic fibrosis without pulmonary inflammation and the non-cystic fibrosis control subjects. This suggests that the reduction in glutathione concentrations in the epithelial lining fluid found in patients with cystic fibrosis is a secondary rather than a primary abnormality of cystic fibrosis.

In an attempt to investigate the mechanism of the previously reported low concentrations of glutathione in the epithelial lining fluid of patients with cystic fibrosis, we measured one of the enzymes involved in glutathione metabolism. Gamma-GT is an exo-enzyme which hydrolyses glutathione, producing glutamate and cysteinyl-glycine. These products may then be taken up by epithelial cells and can be used to resynthesise glutathione inside the cell. Blocking the activity of this enzyme has been found to prevent the increase in intracellular levels of glutathione in a rat cell culture model of oxidative stress.[7] Furthermore, in the same

model, oxidative stress was associated with increased activity of γ-GT associated with increased levels of γ-GT mRNA and protein.[7] Gamma-GT has been found to be expressed in rat alveolar type 2 cells and to be released by this cell into the lung hypophase.[13] Since it has been shown in humans that epithelial lining fluid collected by BAL has high levels of glutathione, we hypothesised that this extracellular source of glutathione could be utilised by airway epithelium to prevent intracellular damage during periods of oxidative stress. Our finding that the levels of γ-GT were significantly increased in the patients with cystic fibrosis with pulmonary inflammation compared with both those with cystic fibrosis without active lung disease and non-cystic fibrosis control subjects supports this hypothesis. Thus, in this model the glutathione in the epithelial lining fluid provides a store of glutathione which is readily available during periods of oxidative stress. The upregulation of γ-GT, presumably induced by the oxidative stress response, provides the mechanism for the increased uptake of the extracellular glutathione. The finding of reduced levels of glutathione in the plasma of patients with cystic fibrosis[8] may, in turn, reflect increased consumption of glutathione in the lung.

In conclusion, we have found direct evidence of increased oxidative stress in the lungs of infants and young children with cystic fibrosis. The increased oxidative stress is associated with the presence of pulmonary inflammation and is not a direct consequence of the genetic defect. We have also shown that pulmonary inflammation in cystic fibrosis is associated with increased concentrations of γ-GT in the epithelial lining fluid which may provide one possible mechanism by which the lung responds to oxidative stress. The results of this study support the hypothesis that oxidative stress contributes to lung injury in cystic fibrosis and encourage the development of strategies to reduce the inflammatory response to airway infection and to enhance host antioxidant capacity. These therapies would need to be started within the first year of life to be most effective.

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