Iron accumulates in the lavage and explanted lungs of cystic fibrosis patients

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Abstract

Oxidative stress participates in the pathophysiology of cystic fibrosis (CF). An underlying disruption in iron homeostasis can frequently be demonstrated in injuries and diseases associated with an oxidative stress. We tested the hypothesis that iron accumulation and altered expression of iron-related proteins could be demonstrated in both the bronchoalveolar lavage (BAL) fluid and explanted lungs of patients with cystic fibrosis. BAL fluid collected from 10 children with CF showed elevated concentrations of protein, iron, ferritin, transferrin, heme, and hemoglobin relative to that obtained from 20 healthy volunteers. Using Perl’s Prussian blue staining, explanted lung from CF patients revealed increased iron in alveolar and interstitial macrophages. Similarly, there was an increased expression of ferritin, the iron importer DMT1, and the exporter ferroportin 1 in lung tissue from CF patients. We conclude that iron homeostasis is disrupted in CF patients with an accumulation of this metal and altered expression of iron-related proteins being evident in the lungs.

Keywords: Iron; Ferritin; Cystic fibrosis; Transferrin; Lung diseases

1. Introduction

The relationships between the presence of a CFTR mutation, cell dysfunction, tissue injury, and the variety of clinical manifestations of cystic fibrosis are not well understood. A current postulate suggests that abnormal ion transport through the CFTR protein leads to paradoxical changes in sodium uptake, dehydrated lining fluid, and a collection of viscous mucus [1]. This mechanistic theory supports a pathway of injury in CF which focuses on respiratory and gastrointestinal obstruction by thickened secretions leading to infection and parenchymal remodeling.

Oxidative stress, resulting from both an increased generation of reactive oxygen species (ROS) by host cells and decreased anti-oxidant concentrations, is believed to participate in the pathophysiology of CF [2,3]. Evidence of such oxidative stress in CF can be observed in elevated measures of plasma malondialdehyde (MDA) levels [4,5] and 8-isoprostanone [4,6] as well as breath pentane and ethane [7]. Factors which contribute to an increased production of ROS in CF patients include an airway inflammation, that can precede the introduction of microbes into the respiratory tract [8,9], and recurrent infections which are associated with a further inflammatory influx that produces high levels of ROS [10]. In addition, individuals with CF can demonstrate an elevated metabolic rate associated with an increase of ROS (superoxide and hydrogen peroxide) continuously generated as mitochondrial products of the respiration process [11]. Regarding anti-oxidants, investigation revealed low levels of plasma antioxidant vitamins in CF patients with malabsorption contributing to a decreased uptake of fat soluble

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vitamin E and beta-carotene [3,4]. Lower concentrations of the anti-oxidant glutathione have been quantified in the epithelial lining fluid and serum of CF patients relative to control subjects [12]. One of the postulated reasons for deficiency of this specific anti-oxidant is a loss of permeability in the CFTR channel for glutathione [13,14]. Accordingly, there are several interacting networks which can produce an increased oxidative stress in CF patient.

In numerous injuries and diseases associated with an oxidative stress, an underlying disruption in iron homeostasis can frequently be demonstrated [15]. There is evidence to support such an accumulation of iron in the respiratory tract of individuals diagnosed to have CF [16–18]. We tested the hypothesis that iron accumulation and altered expression of iron-related proteins can be demonstrated in the lavage and explanted lungs of CF patients.

2. Materials and methods

2.1. Bronchoalveolar lavage of patients and healthy volunteers

Bronchoalveolar lavage fluid was obtained from ten children with CF undergoing clinically indicated bronchoscopy. Details of bronchoscopy have been previously described [19,20]. Patients were anesthetized with inhaled sevoflurane and intravenous propofol. Lidocaine was applied to the vocal cords and carina for topical anesthesia. Generally, lavage was directed at areas of either radiographic abnormality or visibly heavy secretion. For each lavage, 1 mL/kg aliquots (up to a maximum of 20 mL) of normal saline were instilled and suctioned back through the bronchoscope. Parents and, as appropriate, patients gave consent to use of the BAL fluid for research based on an IRB-approved protocol.

Twenty healthy non-smoking volunteers (18 to 40 years of age) also underwent fiberoptic bronchoscopy with bronchoalveolar lavage (BAL). Lidocaine only was employed in anesthesia for healthy volunteers [21]. The fiberoptic bronchoscope was wedged into a segmental bronchus of the lingula and then the right middle lobe. Fifty mL aliquots of sterile saline were instilled and suctioned back through the bronchoscope. Parents and, as appropriate, patients gave consent to use of the BAL fluid for research based on an IRB-approved protocol.

Lavage fluid was analyzed using ELISA (R&D Systems, Minneapolis, MN) for interleukin (IL)-8, a pro-inflammatory mediator whose release strongly correlates with oxidative stress [22–25].

2.6. Statistics

Data are expressed as mean value ± standard error unless otherwise specified. T tests of independent means were used to compare lavage results from the healthy volunteers and patients with cystic fibrosis. Two-tailed tests of significance were employed. Significance was assumed at P < 0.05.
3. Results

3.1. CF subjects

BAL fluid was obtained from 10 children with CF and a mean age of 10.8±1.9 years. Four of the CF samples only had oral flora or no bacterial growth and six showed infection with typical CF pathogens (Pseudomonas aeruginosa, Burkholderia multivorans, Staphylococcus aureus and Haemophilus influenzae). One patient had infection with P. aeruginosa and Mycobacterium massiliense and had the highest heme and hemoglobin measures. Patients who did not have infection in BAL were younger than those with infection (5.9±1.4 vs. 14.0±2.3 years). Total cell counts (305×10^4 cells/mL) and percent neutrophils (mean 49±9%) indicated increased inflammation which was higher in those with current infection (64.8±8.8%) vs. those currently not infected (25.6±12.9%). Iron indices, iron and iron related proteins did not differ between the groups nor show a correlation with current infection in this sub-sample.

3.2. Bronchoalveolar lavage iron and iron-related proteins

Lavage urea nitrogen values from healthy volunteers and CF patients showed no significant differences (1.3±0.4 and 1.6±0.5 mg/dL respectively). The lavage protein concentration among CF patients was significantly increased relative to those from healthy volunteers (Fig. 1A). This difference approximates a 6-fold increase in lavage protein concentration and likely reflects a tissue injury attributable to inflammation associated with both CF and complicating infections [16]. Iron levels were elevated several folds in CF patients (Fig. 1B). Similarly, lavage concentrations of the metal storage protein ferritin and the transport protein transferrin were increased among CF patients (Fig. 1C and D respectively). Finally, both lavage heme and lavage hemoglobin levels were higher in CF patients which could suggest micro-bleeds into the lower respiratory tract among patients with CF (Fig. 1E and F). Changes in iron, ferritin, transferrin, heme, and hemoglobin concentrations support an accumulation of iron in the lower respiratory tract among CF patients. While the lavage heme and hemoglobin concentrations significantly correlated with each other (with an R greater than 0.95), neither showed significant relationships with lavage iron, ferritin, and transferrin suggesting that the disruption in metal homeostasis did not result from hemorrhage alone. Finally, concentrations of IL-8 in lavage fluid from normal subjects and patients with cystic fibrosis ranged from 8 to 41 and from 277 to 3130 pg/mL respectively (with means and standard errors of 19±8 and 453±81 pg/mL). Levels of this pro-inflammatory mediator supported increased oxidative stress in the lower respiratory tract among patients with CF. IL-8 concentrations significantly correlated with lavage protein, ferritin, and iron (R=0.65, 0.81, and 0.67 respectively).
3.3. Iron and ferritin in lung tissue

Lung tissue from controls (those patients treated for lung cancer with pneumonectomy) showed neither retained particle nor metal on Perl’s Prussian blue stain for iron (Fig. 2A). Airway epithelium did not stain positively for iron in CF patients. However, the most distal respiratory tract of the explanted lungs showed positive Perl’s stain by macrophages either in the alveolar lumen (Fig. 2B) or in the interstitium (Fig. 2C and D).

Ferritin is the intracellular site for iron storage and a major iron-related protein whose expression increases with metal accumulation. Airway epithelial cells demonstrated positive staining for ferritin in control lungs. Uptake for the ferritin antibody by epithelium in the control lungs was less likely in the more distal airways. Macrophages within the airways from controls were found to stain positively for ferritin regardless of whether it was a cartilaginous bronchus, membranous bronchus, terminal bronchiole, or respiratory bronchiole. There was minimal uptake for the ferritin antibody in the small airways of the distal respiratory tract in control subjects (Fig. 3A). In the airways of explanted lung from patients with cystic fibrosis, there was an increased ferritin expression compared to control and this staining pattern showed regional heterogeneity. Several different patterns of uptake for the ferritin antibody were evident. In the first, staining for this storage protein was observed at the base of columnar epithelium of the large airways in cystic fibrosis patients (Fig. 3B) and in inflammatory cells in the submucosal region of the airway showed uptake of the ferritin antibody (Fig. 3B). In the second pattern of uptake for ferritin, epithelial cells immediately adjacent to significant inflammation frequently demonstrated a total lack of ferritin staining while the inflammatory cells, both in the airway and in the submucosa showed high levels of expression (Fig. 3C). In a third pattern of uptake for this antibody, other regions of the CF tissue could display high expression of ferritin both in the epithelial cells and in the influxed inflammatory cells within the submucosa (Fig. 3D). In the more distal units of the lung, ferritin expression was increased among samples from CF patients. The control lungs demonstrated occasional staining for ferritin but this was almost exclusively restricted to intra-alveolar macrophages (Fig. 4A). In explanted CF lung, ferritin stain of the most distal portion of the respiratory tract showed positive uptake by macrophages and inflammatory cells within the alveoli (Fig. 4B and C). In addition, there could be uptake of the ferritin antibody by macrophages in the interstitium of the distal respiratory tract (Fig. 4D). While not uniform, there was an increased expression of ferritin in both the airways and the periphery of all lung samples from CF patients.

3.4. Divalent metal transporter 1 and ferroportin 1 in lung tissue

Divalent metal cations must be transported across cell membranes by metal carrier proteins. DMT1 is a major iron importer and subsequently its expression was evaluated. In the control lungs, there was uptake for the DMT1 antibody at both the apical surface of the epithelial cells and at the basement
membrane (Fig. 5A). In the periphery of control lungs, airway epithelium continued to stain positively for this importer (Fig. 5B). In addition, alveolar macrophages were observed to show significant uptake of the DMT1 antibody (Fig. 5B). In the large airways of CF explanted lung, DMT1 expression was significantly increased in the epithelial layer and in inflammatory cells of the lamina propria and the submucosa (Fig. 5C). In the alveolar region of the explants, DMT1 expression was increased and localized to intra-alveolar and interstitial macrophages (Fig. 5D).

The expression of the iron exporter FPN1 was the final protein examined. There was little expression of FPN1 in the control lungs in the airways (Fig. 6A) and distal respiratory tract (Fig. 6B). Explanted lung from CF patients showed elevated levels of staining for FPN1, specifically in epithelial cells in distal airways (Fig. 6C). Additionally, inflammatory cells in the lamina propria and the submucosa of the airways in explanted tissue stained positive for FPN1 (Fig. 6C). In the alveolar region, there was a positive FPN1 staining in alveolar and interstitial macrophages (Fig. 6D).

4. Discussion

Measurement of iron and ferritin in BAL fluid revealed elevated levels of both in patients with CF relative to healthy volunteers. These results confirm prior investigation which similarly showed elevations in iron and ferritin in respiratory samples. Compared with control subjects, concentrations of iron and ferritin in sputum were observed to be increased in patients with CF [18]. In a second study utilizing sputum samples collected from CF patients, iron and ferritin (as well as microalbumin) were significantly elevated relative to normal controls and patients with COPD [16]. Further, sputum iron and ferritin concentrations correlated with each other (as well as with sputum cytokines) and ferritin levels were further elevated in CF patients during disease exacerbation. In addition to sputum, BAL fluid from patients with CF was previously demonstrated to have elevated concentrations of iron and iron-related proteins [17]. Iron was detected in BAL fluid from patients with CF but not healthy non-smokers. Ferritin was present in all BAL fluid with higher total ferritin in patients with CF relative to healthy non-smokers. While iron and ferritin levels have been repeatedly observed to be elevated in CF patients, the concentration of transferrin in respiratory samples from this group has varied. Relative to healthy volunteers, we observed an elevated transferrin level in the lavage fluid collected from our CF cohort while other studies have shown reduced sputum and equivalent lavage transferrin concentrations [17,18]. While changes in transferrin can reflect a disruption in the iron homeostasis of the respiratory tract, a major source of this glycoprotein is its translocation from the blood to the lower respiratory tract [29]. Increases in lavage transferrin observed in our study may reflect an injury to the alveolar-capillary membrane and elevated vascular permeability among CF patients.
patients comparable to the protein levels [30]. Finally, there were elevated concentrations of both heme and hemoglobin in the lavage fluid from CF patients. This may reflect bleeding in the lower respiratory tract of these individuals which will further affect an accumulation of iron following the action of heme oxygenase in resident cells. Additionally, the presence of heme, an essential nutrient for many bacteria, can enhance bacterial growth.

Explanted lung has not previously been examined for evidence of a disruption in iron homeostasis. Histologic and immunohistologic assessment of the explanted lung supported an accumulation of iron in the CF lung. Iron was stainable in the macrophages of the distal respiratory tract (both alveolar and interstitial). This specific metal can catalyze electron exchange with the subsequent oxidant generation which damages host tissues. The sequestration of iron with control of its capacity to support oxidant generation requires storage within ferritin [31,32]. The increased expression of ferritin observed in the explanted lungs of CF patients can reflect increased concentrations of biologically available iron. In the staining for ferritin, elevations in the expression of this protein were observed in the airways and the alveolar region in the explanted lungs of CF patients. Airway epithelial cells, inflammatory cells in the lamina propria, submucosa, and bronchus lumen itself, and alveolar and interstitial macrophages in the distal lung units all demonstrated significant uptake of the ferritin antibody. The heterogeneity of ferritin localization in explanted CF lungs cannot be explained with certainty; it may reflect the heterogeneity of the lung injury in cystic fibrosis or other factors such as exposure to microbes and particles [33,34].

Staining for the iron importer DMT1 and exporter FPN1 demonstrated comparable elevations in expression in explanted CF lung tissue. DMT1 is a member of the natural resistance-associated macrophage proteins (Nramp) present in most tissues and cell types as an integral membrane protein (with a molecular weight of 90 to 100 kD) modified by glycosylation [35]. Its ubiquitous expression suggests that it might be involved in transferrin-independent iron uptake [36]. In tissues which do not function to meet the nutritional requirements of the living system such as the lung, DMT1 may serve to diminish the oxidative stress and injury which iron presents. Accordingly, expression would be expected to be increased. With intracellular transport, iron could then be stored in ferritin and free radical generation would diminish. While cellular uptake of excess iron can limit the degree of toxicity in the lung, storage in this intracellular site is vulnerable to mobilization. FPN1 functions as such an exporter in lung cells and iron can augment its expression [37]. Both DMT1 and FPN1 participate in iron recycling and detoxification [38] and, comparable to ferritin, changes in the expression of these proteins support a disruption in iron homeostasis in CF.

In this study, concentrations of iron and iron-related proteins were observed to be altered in CF patients. The issue of whether...
this is a non-specific response or centrally involved in the pathogenesis of this disease is not addressed by this investigation. Iron accumulation could be a process directly associated with the pathophysiology of cystic fibrosis; the disruption of its homeostasis could participate in initiating inflammation and infection [39]. Alternatively, the accumulation of the metal could be the result of the infection. Regardless, elevated concentrations of iron in the lower respiratory tract of patients with cystic fibrosis correlate with the risk for infection [16,40]. However, it has been previously described that children dying with CF as early as 3 months of age can show an abnormal accumulation of the metal [41]. This observation would suggest that the disruption in iron homeostasis observed in this and other studies is not the result of chronic inflammation and infection. It is possible that iron concentrations are increased in the respiratory tract of CF patients through an abnormality of metal transport inherent to this disease. Possible mechanisms through which iron transport might be altered in CF include a disruption of chloride and sodium import pathways. Interactions between chloride, sodium and iron transport have been suggested by prior investigations [42,43]. If the basic abnormal chemistry of the defect in CF were to include an abnormal transport of iron, the relationship between an increased incidence of infection (e.g. *P. aeruginosa*) and its dependence on available metal could be better appreciated [44]. Furthermore, descriptions of anemia in CF could subsequently be appreciated as a possible secondary manifestation of the disruption in iron homeostasis comparable to an anemia of chronic disease [45,46]. Treatment with iron would be avoided until absolutely required possibly decreasing both worsening of CF and risk for infection.

We conclude that iron homeostasis is disrupted in the lungs of patients with cystic fibrosis both in early and end-stage lung diseases. There is an accumulation of iron in the respiratory tract in CF associated with altered expression of several iron-related proteins including ferritin, DMT1, and FPN1. Future studies, likely utilizing animal models, are required to examine whether these changes are secondary to inflammation and infection or centrally involved in the pathogenesis of this disease. In addition, further human investigation into systemic and lung iron homeostasis may be possible utilizing non-invasive measures such as magnetic resonance imaging.

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**Fig. 5. Immunohistochemistry for DMT1.** Tissue from non-CF patients revealed staining for the iron importer at the most apical portions of airway epithelial cells and at the basement membrane (A; orange to brown color). These control lungs also showed uptake for DMT1 in the smaller airways and alveolar macrophages (B). CF explanted lung demonstrated greatly elevated expression in both the respiratory epithelium (including the epithelial and inflammatory cells) and inflammatory cells in the alveolar region (C and D). Magnification approximates 100×.
References


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