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Original Article

Oxidized glutathione and uric acid as biomarkers of early cystic fibrosis lung disease

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Abstract

Background: In cystic fibrosis (CF) there is an urgent need for earlier diagnosis of pulmonary infections and inflammation using blood- and urine-based biomarkers.
Methods: Using mass spectrometry, oxidation products of glutathione and uric acid were measured in matched samples of bronchoalveolar lavage (BAL), serum and urine from 36 infants and children with CF, and related to markers of neutrophilic inflammation and infection in BAL.
Results: Oxidation products of glutathione (glutathione sulfonamide, GSA) and uric acid (allantoin), were elevated in BAL of children with pulmonary infections with *Pseudomonas aeruginosa* (PsA) compared to those without ($p < 0.05$) and correlated with other markers of neutrophilic inflammation. Serum GSA was significantly elevated in children with PsA infections ($p < 0.01$). Urinary GSA correlated with pulmonary GSA ($r = 0.42$, $p < 0.05$) and markers of neutrophilic inflammation.
Conclusions: This proof-of-concept study demonstrates that urinary GSA but not allantoin shows promise as a non-invasive marker of neutrophilic inflammation in early CF lung disease.

Keywords: *Pseudomonas aeruginosa*; Glutathione sulfonamide; Allantoin; Neutrophil; Neutrophil elastase; Myeloperoxidase

1. Introduction

Lung disease in cystic fibrosis (CF) begins in early life, is progressive and characterized by neutrophil-dominated inflammation [1–3]. Free neutrophil elastase (NE) activity, detected in bronchoalveolar lavage (BAL) as early as 3 months of age indicates an increased risk of persistent, progressive bronchiectasis [1]. Despite best current therapy approximately 60–80% of children with CF have radiological evidence of bronchiectasis before they reach school age [1,3]. Clearly a better approach aimed at preventing structural lung disease in early life is required.

Studies by the Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF) have provided considerable insight into mechanisms underlying the onset and progress of CF lung disease using a BAL-based program [4]. Major risk factors for progressive lung disease include inflammation and infection, severe CF genotype and free NE activity in the BAL [1,5]. However, all of these can be present in the complete absence of clinically-apparent lung disease [1,2]. Acute pulmonary exacerbations, especially those requiring hospitalization, have been associated with loss of lung function and reduced lung function growth in children [6], but predicting these is problematic.

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The BAL-based program used by AREST CF is too invasive for frequent use and has limited ability to predict acute pulmonary exacerbations. Biomarkers of inflammation, infection or structural lung disease would aid management of young children, indicating who needs more intensive therapy. However, despite extensive efforts (reviewed in [7]) no blood or urine-based biomarker has yet entered clinical practice. In addition, biomarkers validated in older patients with established lung disease may not be valid in young children with early disease [8,9].

Neutrophils infiltrate the epithelium to combat infections [10] but release damaging proteases such as NE [1] and reactive oxygen species (ROS) [11]. Hypochlorous acid is produced through oxidation of chloride by myeloperoxidase (MPO) released by activated neutrophils [12]. Glutathione sulfonamide (GSA) is an oxidative metabolite of glutathione specific to hypochlorous acid (Supplementary Fig. 1A) [13]. Unlike oxidized glutathione (GSSG), GSA is not a substrate of glutathione reductase and provides a stable biomarker of neutrophil oxidant activity. Consequently, we postulated that GSA might be a systemic biomarker of pulmonary inflammation, infection and oxidative stress in CF.

Allantoin is the major oxidation product of uric acid when exposed to ROS including hypochlorous acid, hydroxyl radicals, and methaemoglobin/H_2O_2 (Supplementary Fig. 1B) [14,15] as well as when MPO directly oxidizes uric acid [16]. As allantoin is elevated in serum of patients with acute gout [17], we proposed that allantoin would be elevated during acute pulmonary inflammation in CF.

The aim of the present proof-of-concept study was to determine whether allantoin and GSA were present in BAL from the lungs of young children with CF, reflect current lung disease and whether they are elevated in serum and/or urine when children are infected.

2. Methods

Full details of the study population and methods used are provided in the online data supplement.

2.1. Study population

Matched urine, serum and BAL samples were obtained from a previous study investigating the potential of YKL-40 as a serum and urinary biomarker for inflammation in CF [8]. Samples were collected from 36 infants and young children with CF who participated in the AREST CF early surveillance program. Details of the program, collection and measurements for cytokine concentrations, neutrophil elastase activity, cell counts and detection of infection and bronchiectasis have been described previously [1–3,5,8]. During 55 unique annual visits, a total of 55 BAL samples, 52 serum and 41 urine samples were obtained. Nine children visited twice, three children visited three times and one child visited five times. Measurements from the same child showed variability in all the analytes presented here. Variables related to each BAL sample were considered a separate event.

2.2. Measurement of GSA in BAL, serum and urine by LC–MS

GSA content was analysed by liquid chromatography with mass spectrometry (LC–MS) using multiple reaction monitoring on an Applied Biosystems 4000 QTrap as described before [18]. The relative standard deviations for intra-and inter-day precision were <10% and <15%, respectively [18]. Standard deviations were determined from a set of quality control BAL samples covering low, medium and high points on the respective calibration curves that were repeatedly frozen, thawed and analysed on five different days [18]. The lower limit of quantification for GSA (S/N > 10) in standard samples was 2 nM.

2.3. Measurement of allantoin in BAL, serum and urine by LC–MS

Allantoin was measured as described previously [19]. The relative standard deviations for intra-day and inter- day precision was <7% [19]. Standard deviations were determined from a set quality control plasma samples that were repeatedly frozen, thawed and analysed on four different days [19]. Artefactual production of allantoin from urate during sample preparation was also ruled out in this study [19]. The lower limit of quantification (S/N > 10) for allantoin in standard samples was 0.2 nM.

2.4. Measurement of 3-chlorotyrosine and methionine sulfoxide in BAL by LC–MS

The protein oxidation products 3-chlorotyrosine and methionine sulfoxide were measured as described in the online supplement. Chlorotyrosine concentrations were reported as chlorotyrosines per 1000 tyrosines. Methionine sulfoxide was reported as the % of total methionine species (methionine and methionine sulfoxide).

2.5. Measurement of MPO activity and protein by sandwich ELISA

MPO was determined by ELISA as described previously [20].

2.6. Measurement of urine creatinine

Urine creatinine was determined by Jaffe's reaction, where creatinine produces an orange coloured product with picric acid in alkaline medium [21]. The absorbance at 520 nm was measured and urine creatinine concentrations were determined using a standard curve.

2.7. Specific gravity analysis and normalization of urine concentrations of GSA and allantoin

Specific gravity of urine was measured on a refractometer (American Optical Corporation, Southbridge, MA), on which the specific gravity could be read directly and used to normalize urine analyte concentrations. To compare the effect of normalization by