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Antoine Ménoret  
*University of Connecticut School of Medicine and Dentistry*

Sanjeev Kumar  
*University of Connecticut School of Medicine and Dentistry*

Anthony T. Vella  
*University of Connecticut School of Medicine and Dentistry*

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Cytochrome b5 and Cytokeratin 17 Are Biomarkers in Bronchoalveolar Fluid Signifying Onset of Acute Lung Injury

Antoine Ménoret, Sanjeev Kumar, Anthony T. Vella

University of Connecticut Health Center, Farmington, Connecticut, United States of America

Abstract

Acute lung injury (ALI) is characterized by pulmonary edema and acute inflammation leading to pulmonary dysfunction and potentially death. Early medical intervention may ameliorate the severity of ALI, but unfortunately, there are no reliable biomarkers for early diagnosis. We screened for biomarkers in a mouse model of ALI. In this model, inhalation of S. aureus enterotoxin A causes increased capillary permeability, cell damage, and induces protein and cytokine concentration in the lungs. We set out to find predictive biomarkers of ALI in bronchoalveolar lavage (BAL) fluid before the onset of clinical manifestations. A cutting edge proteomic approach was used to compare BAL fluid harvested 16 h post S. aureus enterotoxin A inhalation versus BAL fluid from vehicle alone treated mice. The proteomic PF 2D platform permitted comparative analysis of proteomic maps and mass spectrometry identified cytochrome b5 and cytokeratin 17 in BAL fluid of mice challenged with S. aureus enterotoxin A. Validation of cytochrome b5 showed tropic expression in epithelial cells of the bronchioles. Importantly, S. aureus enterotoxin A inhalation significantly decreased cytochrome b5 during the onset of lung injury. Validation of cytokeratin 17 showed ubiquitous expression in lung tissue and increased presence in BAL fluid after S. aureus enterotoxin A inhalation. Therefore, these new biomarkers may be predictive of ALI onset in patients and could provide insight regarding the basis of lung injury and inflammation.

Introduction

Pulmonary biomarkers are needed to predict the clinical course of lung disease, status, progression, and response to treatment [1,2]. A key aspect in biomarker discovery is uncovering molecules that appear early during disease initiation, when the natural history of the disease can be modified. During acute lung injury (ALI), several factors correlate with tissue damage such as recruitment of neutrophils and macrophages, and also increases in IFNγ in BAL fluid [3]. However, these events occur late in the disease process, once ALI is fully initiated. Therefore, it is critical to find new biomarkers that occur prior to tissue damage [4] and perhaps even before cytokines are produced.

In humans, a major cause of ALI is the immune response to a lung infection [5,6]. Naturally there are other causes such as exposure to aerosolized toxic chemicals, aspiration, and multiple trauma. However, even in these cases, a secondary infection may trigger the inflammatory response that leads to ALI [6]. Although no animal model is a perfect representation of ALI in humans [7], bacterial lipopolysaccharide (LPS) does induce key features such as alveolitis, neutrophil recruitment, and induction of IFNγ that mimic symptoms of human ALI. However, unlike infection with whole organisms, LPS does not specifically stimulate T cells, which likely make a substantial contribution to disease in humans.

Therefore, pathogen byproducts that stimulate T cells add a critical dimension to the modeling of human ALI. Staphylococcus aureus secretes enterotoxins proteins that are pathogenic in humans, can cause toxic shock syndrome [4], and are implicated in Chronic Obstructive Pulmonary Disease (COPD) [8]. Importantly, S. aureus has been detected in nasal polyps of patients suffering from chronic rhinosinusitis, and IgE antibodies specific to S. aureus enterotoxin A (SEA) have been detected in patients with severe asthma, suggesting that SEA is involved in the pulmonary immune response [9,10]. In our previous mouse study we showed that inhalation of SEA induced T cells to migrate into lung, become effectors, and prime innate cells [11]. This response was rapid, marked by neutrophil recruitment and increases of protein in BAL fluid along with high levels of IFNγ and alveolitis. Consequently, the SEA inhalation model approximates many aspects of human ALI.

Our goal was to use a proteomic mining strategy to uncover differences in BAL fluid from SEA treated mice versus vehicle alone controls. The PF 2D proteomic platform allows twodimensional liquid fractionation of biological fluid based on isoelectric focusing and hydrophobicity. We used this strategy to detect changes during colon cancer chemoprevention [12], and others have used it for biomarker discovery [13,14]. Recently, this method uncovered a pathway involved in cytokine based...
inflammation [15]. Therefore, we set out to find distinct changes in the BAL fluid proteome of SEA treated mice compared to vehicle alone, and found an unexpected increase in microsomal cytochrome b5 and cytokeratin 17. These data were validated and coincided with rapid changes in lung pathology that included increased lung inflammation. Thus, our mouse model data show that cytochrome b5 and cytokeratin 17 are detected in BAL fluid very early after lung damage, and thus may be potential biomarkers of pulmonary injury in patients with ALI or other life-threatening diseases.

**Results**

**Inhalation of SEA Induces Lung Inflammation and Damage**

We used an *in vivo* mouse model of ALI to uncover molecular biomarkers of lung inflammation. After inhalation of SEA, mice were monitored for signs of acute inflammation. In the current study we first examined lung pathology 2 days post SEA challenge, a time point where T cell effectors start to accumulate [11]. Lung sections stained by hematoxylin and eosin (H&E) showed leakage of red blood cells from blood vessels into the alveoli (Fig. 1A, black arrows in upper right panel), abundant leukocyte infiltration in the peri-vascular tissue, and increased cellularity in the interstitium and alveolar space as we observed previously [11]. Proteinous exudate was visible after SEA (Fig. 1A, white arrows in upper right panel). In contrast, lungs from vehicle-treated mice showed no signs of injury (Fig. 1A lower panels). To visualize the possibility of increased infiltrating leukocytes we performed the same inhalation study with or without lung perfusion. We found that perfusing the lung allowed for visualization of adherent leukocytes in the lumen of blood vessels after SEA treatment in the lung sections (Fig. 1B). In the absence of inflammation (vehicle) leukocyte adhesion was not evident. Secondly, electron microscopy (EM) was used to demonstrate the binding of leukocytes to the vascular lung endothelium. EM showed increased leukocyte adherence to blood vessel endothelium in the lung airways after SEA inhalation whereas vehicle alone injected mice showed no sign of leukocyte infiltration or adhesion to the endothelium (Fig. 1C). Moreover, leukocytes appeared to be transmigrating from the vasculature into lung tissue (Fig. 1C, left panel: L*). Altogether, these pathologic changes are consistent with rapid inflammation and immune-mediated lung damage.

Next, we measured parameters representative of ALI and tested the effect of 2" inhalation of SEA at inducing compounded inflammation. Increased cell number and total protein in BAL was evident (Figs. 2A and 2B). High levels of IFNγ and IL-6 were detected in BAL fluid after SEA inhalation and were strongly augmented after 2" SEA (Figs. 2C and E). Additionally, IFNγ and IL-6 were detected in serum demonstrating a powerful systemic response, which is a key factor in modeling ALI (Figs. 2D and F). Finally, lactate dehydrogenases (LDH), a marker of cell injury detectable in BAL fluid [16,17] was present in BAL fluid but there was no difference after SEA inhalation (Fig. 2G). In fact, this result reinforces the current consensus for the need of new and more sensitive biochemical markers for ALI [1,2].

**Identification of Early Markers of ALI**

To identify biomarkers of lung injury we used a proteomic approach comparing BAL fluid of SEA versus vehicle alone treated mice. Importantly, in order to find early biomarkers of ALI we collected BAL fluid 16 h after SEA inhalation and analyzed the samples using a PF 2D proteomics platform as described before [12,15]. PF 2D proteomics enables protein separation by charge followed by reverse-phase chromatography (Fig. 3). Proteomic maps of BAL fluid SEA versus vehicle alone were similar as illustrated (Fig. 3A), but 2 peaks were spotted as...
Figure 2. Intranasal SEA challenge induces cellular infiltration, protein leakage and inflammation. Mice received 1 µg of SEA or vehicle alone i.n., secondary (2nd) challenge of either vehicle or SEA 48 h after the primary (1st) and were sacrificed 5 h later (53 hrs total). (A) Cells present in BAL fluid were harvested and enumerated and (B) total BAL fluid protein quantified by BCA. (C) IFN-γ, (D) IL-6 in BAL fluid and (E) in serum, and (E) IL-6 in BAL and (F) in serum were quantified by ELISA. (G) LDH in BAL fluid was quantified. The data are representative of two independent experiments using 4 mice per group. The errors bars indicate the standard error of the mean between biological replicates.

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differential fingerprints (Fig. 3A dotted circle). After careful analysis of three independent experiments using Proteoview software, two fingerprints were consistently identified at specific coordinates present in the BAL fluid map from 16 h SEA treated mice but absent or reduced in vehicle alone samples (Fig. 3B, right boxes). To identify the proteins contained in the differential fingerprint fractions, a third dimension was used. The fractions of interest were lyophilized, resolved by SDS-PAGE, and detected by fluorescent dye staining (Fig. 3C). The bands uniquely detected in the SEA BAL fluid samples were cut out of the gel, digested with trypsin and proteins identified by LC/MS/MS (Fig. 3D). Using this strategy cytochrome b5 and cytokeratin 17 were identified as differentially present upon SEA inhalation. Importantly, the sequences of cytochrome b5 and of cytokeratin 17 indicated the proteins were derived from mouse and were not contaminants of human origin. Interestingly, the molecular weight of cytochrome b5 corresponds to the migrating size (15 kDa) of the band on SDS-PAGE suggesting that the full length protein was isolated (Fig. 3D). All the other identified proteins had a higher molecular weight suggesting a breakdown product of these proteins were isolated. Several proteins like albumin and haptoglobin, known to be associated with inflammation and injury [18,19] serve as a validation component of our analysis.

Cytochrome b5 is a Marker of Lung Damage

We performed immunohistochemistry analysis of the proteins with the greatest spectral count as assessed by mass spectrometry (Fig. 3D) to validate their expression and to pinpoint from which site in the lung these proteins were derived. Staining for cytochrome b5 showed strong expression restricted to the epithelium of lung airways (Fig. 4A, top panels). Interestingly, the staining was quite evident in the airways of the vehicle alone treated group (Fig. 4A upper left panel), but weaker and punctuated in the 3 groups that received SEA (Fig. 4A the three upper right panels). This observation is consistent with the notion that cytochrome b5 may be released from epithelial cells of lung airways [11,21,22], increased vascular permeability (Fig. 1) [22], endothelial and epithelial damage [21] (Fig. 1), production of nitric oxide [23], and increase protein and cytokine concentration in the lungs [11,21,22,23] (Fig. 2). Hence, SEA inhalation in mouse provides a useful animal model of ALI that complements other experimental approaches [7]. In this report we specifically show rapid onset of protein deposition, leakage of red blood cells, increased adhesion of leukocytes in small blood vessels, leukocyte infiltration, and accumulation of proteins and cytokines in BAL fluid (Figs. 1–2). These data firmly establish that SEA inhalation can induce ALI.

The search for biomarkers of lung injury has provided candidates expressed in different anatomical compartments [24]. The von Willebrand factor (vWF) and ICAM-1 have been proposed as predictive markers of endothelial injury but with mixed results [25,26,27]. Surfactant proteins SP-A and SP-D expressed by type II pneumocytes, the receptor for advanced glycation end-product (RAGE), and KL-6 have been associated with pulmonary epithelial cell damage [28,29,30,31]. Desmosine, a stable fragment of elastin, has been used as a marker for destroyed extracellular matrix in ALI [32,33]. Moreover, the presence of inflammatory cytokines present during ALI is often associated with a negative prognosis. For example, IL-1β, IL-6, IL-8, and IL-10 are associated with morbidity and mortality in ALI patients [34,35]. However, the etiology of the disease and the presence of infectious agents promoting inflammation add to the complexity of finding the appropriate biomarker [25]. Although biomarkers of ALI have been identified, no single biomarker has been successfully used to diagnose and predict the clinical course of the disease [1,2]. However, the combination of a panel of multiple biomarkers has been shown to be an improvement for predicting mortality in acute lung injury [36] and reinforces the idea that finding additional new early biomarkers will increase the accuracy of diagnosis.

To better understand the onset of lung injury the PF 2D was used to detect proteins in BAL fluid. Comparative proteomic mapping of BAL fluid taken from mice after SEA inhalation uncovered cytochrome b5 as a putative biomarker. Among the...
different isoforms of cytochrome b5, we identified the isoform bound to the ER membrane, called microsomal cytochrome b5 [37]. Following PF 2D fractionation, LC/MS/MS resolved 8 unique peptides covering 43% of microsomal cytochrome b5 sequence with perfect homology. However, a truncated soluble form of cytochrome b5 encoded by a second gene lacking the membrane binding carboxy terminus and expressed in erythrocytes, lung, gallbladder, adrenal gland, and bone marrow [38] matches the peptide sequences. Nevertheless, the molecular size of the protein isolated by PF 2D fractionation corresponded to the membrane bound microsomal and not soluble cytochrome b5. Additional genetic studies will be needed to confirm with absolute certainty which form of cytochrome b5 has been identified in our study. However, we ruled out O Mb, the mitochondrial isoform of cytochrome b5 [39], which has only 58% amino acid conservation with microsomal cytochrome b5 [40]. Cytochrome b5 is a highly conserved protein involved in electron transfer between NADH/NADPH and cytochrome P450, participating in the oxidation of a wide array of endogenous and xenobiotic substances [37,41]. The use of model substrates and drugs in conditional hepatic knock-out cytochrome b5 mice demonstrated that P450-mediated metabolism is dependent on cytochrome b5 [42]. Interestingly, systemic LPS administration decreases cytochrome P450 mRNA expression in lungs [43], which support the finding that inhibitors of cytochrome P450 activity exacerbate LPS-mediated inflammation while compounds known to induce cytochrome P450 reduce inflammation [44]. Thus, cytochrome b5 may influence inflammatory responses by impacting P450 function. Additionally, some of the immunomodulatory proteins released from lung epithelial cells, such as CC16 [45], surfactant proteins [46,47], and galectin-3 [48] are considered damage-associated molecular-patterns (DAMPs) [49]. Therefore, cellular damage could induce release of DAMPs and aggravate ALI. We identified cytochrome b5 and cytokeratin 17 by focusing on the most prominent and reproducible differences apparent on our proteomic maps. However, it is possible that known DAMPs were present in the BAL fluid but not identified. Some of the biomarkers identified in our study could potentially be DAMPs and worsen lung injury by promoting the inflammatory response. For example, cytokeratin 17, which we detected in the BAL fluid after SEA inhalation, can trigger cytokine production and inflammation \textit{in vivo} [50]. Thus, the presence of proteins in BAL fluid after SEA inhalation may enhance lung inflammation. To the best of our knowledge the immunomodulatory effect of cytochrome b5 is unknown, but our results show that cytochrome b5 is modulated during the early stages of lung cell injury and perhaps represent a new target for therapeutic intervention.

Materials and Methods

Mice

C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD) or the Jackson Laboratory (Bar Harbor, ME). All mice were maintained in the central animal facility at the University of Connecticut Health Center (UCHC) in accordance with federal guidelines. The present study was...
approved by the University of Connecticut Health Center’s Animal Care Committee.

Antibodies and ELISA
IFNγ and IL-6 ELISA kits were purchased from Pharmingen (Mountain View, CA). Anti-cytochrome b5 rabbit polyclonal antibody was purchased from Abcam (Cambridge, MA). Anti-cytokeratin 17 rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). LDH detection kit was purchased from Cell Sciences (Canton, MA).

Microscopy
PBS/heparin solution was used for perfusion. Lungs were inflated with 10% formalin solution, clamped for 5 min, fixed for 2 h and stored in 70% ethanol before paraffin embedding. Lung sections were stained for H&E or stained with the appropriate antibody for immunohistochemistry (IHC) and counter stained with methyl green (Vector laboratory, Inc. Burlingame, CA). The percentage of bronchiole cells staining positive for cytochrome b5 was quantified using Image-Pro Plus software (Media Cybernetics, Inc. Bethesda, MD).Pixel analysis was performed on 30 representative bronchioles for each condition. Data was reported as the percentage of positive staining area (brown) versus total staining area (all colors) present in each bronchiole. Lung sections for Electron Microscopy were processed at the central microscope facility, University of Connecticut Health Center. Lungs were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer pH 7.2, post fixed in 1% osmium tetroxide, 0.8% potassium ferricyanide in 0.1M cacodylate buffer, en-bloc stained with uranyl acetate, dehydrated in ethanol and embedded in Polybed 812 resin. Thin sections (70 nm) of the lungs were cut and counterstained with uranyl acetate and lead citrate. Thin sections were examined using a Hitachi H-7650 transmission electron microscope.

BAL Fluid Processing and PF 2D Proteomics
BAL was collected by lavage of mouse lung with 5 ml of sterile PBS. BAL was centrifuged at 1,000 rpm at 4°C to separate cells from BAL fluid. BAL fluid was spiked with a protease inhibitor cocktail (SIGMA #P2714), centrifuged at 25,000 x g, 4°C for 10 min, protein concentration was determined by BCA assay (Pierce, Rockford, IL), and processed through a Beckman Coulter ProteomeLab³™ PF 2D platform (Fullerton, CA) as described before [12,15,51]. Fractions were collected every 0.5 pH units. Fractions corresponding to the linear gradient between pH 8.0 through 4.0 were separated on a HPRP column (Beckman Coulter) with a gradient from 0 to 100% of acetonitrile at 50°C. The proteins were detected with...
UV light at 214 nm and collected at 0.5 min intervals and stored at −80°C.

Two Dimensional (2D) Protein Map Analysis

Two dimensional protein expression maps of BAL fluid displaying protein isoelectric point (pI) versus protein hydrophobicity, were generated by the ProteoView/DeltaVue software package as described earlier [12,15].

SDS-PAGE, Immunoblotting, Fluorescence Staining, and LC/MS/MS

The PF 2D fractions of interest were lyophilized and resuspended in denaturing SDS sample buffer and resolved by SDS-PAGE and immunoblotting as described earlier [15]. Gels were stained by ORIOLE (BioRad, Hercules, CA) and proteins were detected by fluorescence. Visible bands were excised from the gel, subjected to trypsin digestion and identified by LC/MS/MS at NextGen Sciences (Ann Arbor, MI). Tandem mass spectra were extracted by Xcalibur (ThermoFisher) rev. 2.0. All MS/MS samples were analyzed using Mascot version 1.0 (Matrix Science, London, UK; version Mascot) assuming digestion by the enzyme trypsin. Protein identifications were accepted if they could be established at greater than 90.0% probability by the Protein Prophet algorithm [52] and contained at least two identified peptides.

Supporting Information

Figure S1 Detection of cytokeratin 17 in BAL fluid after i.n. SEA and BSS.

Figure 5. Detection of cytokeratin 17 in BAL fluid after i.n. SEA. Mice were immunized i.n. as described in legend of figure 4. (A) Immunostaining of the lungs was performed using a rabbit polyclonal antibody against cytokeratin 17 (CK17) and rabbit immunoglobulin control (Ig). The data are representative of three independent experiments, magnification 100X. (B) Immunoblotting of 5x concentrated BAL fluid was performed using a rabbit polyclonal against cytokeratin 17 (CK17) and rabbit IgG control. (C) BAL fluid harvested at different times after i.n. SEA immunization and BAL fluid from naive mice were immunoblotted using anti-CK17 antibody. High exposure of the immunoblot revealed the presence of cytokeratin 17 in most samples including the samples harvested from naive mice but with less intensity. The data show 3 separate experiments using with 3 mice per time point for each experiment.

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Author Contributions

Conceived and designed the experiments: ATV AM. Performed the experiments: AM SK. Analyzed the data: AM SK ATV. Wrote the paper: AM ATV.
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