Presence of cytokeratins in exhaled breath condensate of mechanical ventilated patients

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Proteomics; ARDS; Mechanical stretch; Mechanical ventilation; Exhaled breath condensate

Summary
Exhaled breath condensate (EBC) contains small amounts of protein leaving the lung by aerosol droplet generation. Protein patterns in EBC might be useful in monitoring acute and severe pulmonary disease and in particular monitoring of mechanical stress during ventilation.

EBC (10 ml) was collected from 30 ventilated patients with respiratory failure including 24 patients with acute lung injury/acute respiratory distress syndrome (ALI/ARDS) and from 10 healthy volunteers. Samples were analyzed using gel electrophoresis. Bands were characterized by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF).

In the EBC of mechanically ventilated patients 53.3% exhibited three bands (50–70 kDa), 26.7% two bands, 10% one band, and 10% had no bands. While no bands were detected in volunteers EBC. MALDI-TOF analysis identified these bands as cytokeratins 2, 9 and 10. Cytokeratins 2 and 10 were confirmed by Western blot. The detection rate of cytokeratins was correlated to peak inspiratory pressure, positive endexpiratory pressure and ARDS score, but not with inflammatory markers or smoking status.

Cytokeratins are present in EBC of mechanically ventilated patients. A strong correlation with parameters of ventilatory stress, such as increased distension, presence of lung injury and time of ventilation suggests a relation with ventilator-associated damage to the pulmonary parenchyma.

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Introduction

Exhaled breath condensate (EBC) consists of water vapor and aerosolized droplets. The aerosolized droplet fraction contains small amounts of proteins and other components of the extracellular lining fluid of the lung. The usefulness of these ingredients for a non-invasive monitoring of pulmonary disease has recently been investigated. EBC has been used predominantly to monitor inflammatory lung diseases such as asthma, COPD, interstitial lung disease and the acute respiratory distress syndrome (ARDS). Investigations of EBC most frequently have focused on H2O2, 8-isoprostanates, eosinoids, and cytokines.

Although the quantification of well-known biochemical parameters in various lung diseases is of course worthwhile, the identification of new markers of lung injury is another challenging task. Methods of proteomics have been used to characterize various biological fluids such as serum, urine, bronchoalveolar lavage and have also begun to be applied to EBC. Protein patterns of EBC for example were investigated using two-dimensional electrophoresis. The authors of this study demonstrated a corresponding protein pattern in EBC and in saliva for a majority of the proteins detected. A contamination of EBC by saliva could be excluded via analysis of amylase which was present in saliva samples but not detectable in EBC. EBC analysis of proteins in another study using tandem electro spray mass spectrometry in a pooled sample from 8 healthy non-smokers has identified cytokeratins 9 and 1. These authors have also reported a significantly increased amount of keratins in smokers compared to non-smokers using quantitative evaluation of spot volume of one-dimensional electrophoresis.

The purpose of the present study was to investigate the protein pattern in EBC of mechanically ventilated patients in order to identify possible markers of injury during mechanical ventilation. The identification of markers indicating tissue damage in response to mechanical during mechanical ventilation may provide the opportunity to adopt the ventilation pattern to the lung tissue response in the future. Significant associations between cytokeratin detection frequencies and peak inspiratory pressure (PIP), positive endexpiratory pressure (PEEP) as well as clinical scores of lung injury and the duration of ventilation were observed suggesting that cytokeratins are indicators of lung damage, most likely caused by mechanical ventilation.

Material and methods

Patients

A total of 30 patients mechanically ventilated through an endotracheal tube (16 male, 14 female; age: 54 ± 15) and 10 healthy volunteers (5 male, 5 female; age: 48 ± 11) were included in this study. All ventilated patients suffered from severe pneumonia and acute respiratory failure that required mechanical ventilation for a minimum of 24 h. Patients were included into the study when they were able with respect to hemodynamics (no change in i.v. catecholamines of more than 25% of baseline) and respiration (no alteration in ventilator settings). A time frame of up to 24 h was allowed to reach a stable situation. In the following time 0–72 h EBC collection was performed when ventilator settings were unaltered during the 24 h prior to EBC collection. Thus, all EBC collections were performed in between 48 and 96 h following the onset of ventilation. Ventilatory parameters at the time of EBC collection are listed in Table 1. All patients were ventilated by pressure control mode.

The extent of acute lung injury (ALI) was estimated at the time of EBC collection using the criteria of the American-European Consensus Conference on ARDS (AECC) as well as Murray’s Lung Injury Severity Score (LISS). Approval for this investigation was received from the ethics committee of the University of Leipzig.

EBC collection

EBC was collected by inserting a special conduit (FILT Lung and Chest Diagnostics Ltd.; Berlin Germany) into the expiratory limb of the ventilator tubing. Collecting time for EBC was 30 min. All EBC was filtered and stored at −20°C until analysis. EBC analysis of proteins was performed in a pooled sample from 8 healthy non-smokers using tandem mass spectrometry.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Number of patients</th>
<th>PEEP (mbar)</th>
<th>PIP (mbar)</th>
<th>BF (/min)</th>
<th>Vt (ml/kgBW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No lung injury</td>
<td>n = 6</td>
<td>6.5 ± 3.7</td>
<td>16.0 ± 6.1</td>
<td>23.2 ± 2.5</td>
<td>6.3 ± 1.1</td>
</tr>
<tr>
<td>ALI criteria</td>
<td>n = 17</td>
<td>10.6 ± 3.2</td>
<td>22.4 ± 4.6</td>
<td>23.8 ± 3.1</td>
<td>5.8 ± 1.0</td>
</tr>
<tr>
<td>ARDS criteria</td>
<td>n = 7</td>
<td>15.6 ± 5.6</td>
<td>29.1 ± 7.0</td>
<td>28.3 ± 7.1</td>
<td>6.0 ± 1.5</td>
</tr>
<tr>
<td>Murray’s lung injury score</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No lung injury (Score: 0)</td>
<td>n = 4</td>
<td>5.0 ± 0.0</td>
<td>14.0 ± 1.8</td>
<td>22.5 ± 2.9</td>
<td>5.9 ± 1.0</td>
</tr>
<tr>
<td>Mild-to-moderate lung injury (Score: 0.1–2.5)</td>
<td>n = 17</td>
<td>9.6 ± 2.9</td>
<td>21.6 ± 5.0</td>
<td>23.9 ± 3.1</td>
<td>5.9 ± 1.0</td>
</tr>
<tr>
<td>Severe lung injury (Score: &gt; 2.5)</td>
<td>n = 9</td>
<td>16.1 ± 4.3</td>
<td>28.6 ± 6.5</td>
<td>27.2 ± 6.5</td>
<td>6.0 ± 1.4</td>
</tr>
</tbody>
</table>

PEEP: positive end-expiratory airway pressure, PIP: peak inspiratory airway pressure, BF: breathing frequency, Vt: tidal volume adapted to body weight.

For control purposes, EBC was also collected from 10 healthy volunteers breathing spontaneously through a standard mouthpiece of the EcoScreen\textsuperscript{16} system using a nose clip for an equivalent collection period of 30 min as was previously described.\textsuperscript{15}

In order to determine cytokeratin background as a source of contamination by environmental air five condensate samples were collected from an artificial lung setup ventilated with identical humidification conditions and ventilator setups.

**Mediators and serum markers**

In order to exclude contamination by saliva the EBC samples were examined for amylase activity (alpha-Amylase ESP1491300 kit; detection limit 0.05 \( \mu \)mol/l/s (\( \approx 0.003 \) U/ml); Boehringer Mannheim, Germany).

Five milliliters aliquots of EBC were lyophilized, reconstituted in 500 \( \mu \)l and used in IL-6 and IL-8 ELISA assays (Quantikine HS human IL-6, Quantigene human IL-8, R&D Systems, USA). In addition, IL-6, IL-8 (Immulite, DPC Biermann, Germany), procalcitonin (LUMItest PCT, BRAHMS Diagnostica, Germany), and C-reactive protein (Tina-quant CRP, Roche Diagnostics, Germany) were measured from serum concomitantly. Protein concentration in EBC was measured using the Micro-BCA Protein Assay (Pierce, Rockford, USA).

**EBC preparation and gel electrophoresis**

For each sample, 10 \( \mu \)l of condensate was freeze-dried. Pellets were reconstituted in 30 \( \mu \)l Tris/HCl buffer pH 7.4. Protein concentrations were measured using the Bradford method,\textsuperscript{16} with bovine serum albumin as a standard. Protein samples were then diluted in Laemmli buffer (50 mM Tris–Cl pH 6.8; 2\% w/v SDS; 0.1\% Bromophenolblau; 10\% (v/v) Glycerol, and 5\% (v/v) \( \beta \)-mercaptoethanol) and denatured at 95 °C for 10 min. SDS–polyacrylamide gel electrophoresis (PAGE) was performed at room temperature using 12\% acrylamide gels (16 \( \times \) 20 \( \times \) 0.1).\textsuperscript{17} Protein bands were stained with a SilverQuest kit (Invitrogen, Karlsruhe, Germany).

**Preparation of proteins and proteolytic fragments for matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF)-MS**

The in-gel digestion of the proteins was performed according to Dihazi et al.\textsuperscript{18} Silver-stained proteins were destained with chemical reducers to remove the silver according to the protocol provided by the manufacturer (Invitrogen). When the gel bands become colorless, gel pieces were washed twice with water, dehydrated in acetonitrile (ACN) and finally dried in a vacuum centrifuge. Dithiothreitol (DTT) (10 \( m \)M) in 100 \( m \)l NH\textsubscript{4}HCO\textsubscript{3} was added to cover the gel pieces, and the protein was reduced for 1 h at 56 °C. After cooling to room temperature, DTT was replaced by 100 \( m \)l of the same buffer without DTT and finally dried in a vacuum centrifuge. Dithiothreitol (DTT) (10 mM) in 100 mM NH\textsubscript{4}HCO\textsubscript{3} was added to cover the gel pieces, and the protein was reduced for 1 h at 56 °C. After cooling to room temperature, DTT was replaced by 100 \( m \)l of the same buffer without DTT and finally dried in a vacuum centrifuge.

**Database search**

Protein identification was carried out using MALDI-MS, based on peptide mass fingerprinting.\textsuperscript{19} Database searches of peptide masses were done in the MSDB and NCBI\textsuperscript{1}r databases using the Mascot peptide mass fingerprint software provided by Matrix Science (Oxford, UK).\textsuperscript{20} Carboxamidomethylation and methionine oxidation were considered as variable modifications. A database search was performed so that each hit was inspected visually following identification in order to match as much spectral information as possible. Criteria for selection were: optimized mass accuracy (ca. 50 ppm), minimal mass deviation (in the mDa range), maximized sequence coverage, highest possible probability score, and maximal number of intense ion signals had to be assigned to the identified protein.

**Western blot analysis**

Western blot analyses were performed according to Towbin et al.\textsuperscript{21} Fifteen microliter per lane of EBC sample (7.5 \( \mu \)g protein) were loaded after denaturation with Laemmli buffer on 10\% SDS–gel. After SDS–PAGE, blotting was performed on nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) at 250 mA for 24 h in transfer buffer (25 mmol/l Tris–HCl pH 8.4, 192 mmol/l glycine, 0.5\% SDS, 20\% methanol). Membranes were blocked in 5\% non-fat dry milk in PBS-buffer containing 0.1\% Tween-20 for 2 h at 37 °C. Primary monoclonal antibodies against cytokeratin (CK) 2E (Clone Ks2.342.7.1, NH\textsubscript{4}HCO\textsubscript{3}, and shrunk again by addition of the same volume of ACN. The liquid phase was removed, and the gel pieces were dried in a vacuum centrifuge. The gel pieces were swollen in digestion buffer containing 50 \( m \)M NH\textsubscript{4}HCO\textsubscript{3}, 5 \( m \)M CaCl\textsubscript{2}, and 10 \( ng/ \mu l \) of trypsin (sequencing grade, Sigma) on ice. After 45 min, the supernatant was removed and replaced by 100 \( m \)l of the same buffer without trypsin to keep the gel pieces hydrated during the enzymatic cleavage at 37 °C overnight. Peptides were extracted by adding 5\% formic acid in 50\% ACN for 20 min at room temperature. After drying down, the resulting peptides were reconstituted in 0.1\% TFA and desalted using a ZipTip C18 ion exchange column (Millipore, Eschborn, Germany).

The extracted peptides were then co-crystallized with matrix (\( \alpha \)-cyano-4-hydroxycinnamic acid) on a stainless steel target using 1 \( \mu \)l matrix and 1 \( \mu \)l sample. A Bruker Biflex III time-of-flight mass spectrometer (Bruker Daltonik, Germany), operating in reflector mode with an accelerated voltage of 20 kV was used to generate peptide mass maps. Mass spectra were obtained by averaging 50 individual laser shots. All samples were externally calibrated with a peptide mix consisting of des-Arg-bradykinin ([M+H]\textsuperscript{+} 904.46), angiotensin I ([M+H]\textsuperscript{+} 1296.68), Glu1-fibrinopeptide B ([M+H]\textsuperscript{+} 1570.67), ACTH (1–17) ([M+H]\textsuperscript{+} 2093.08), ACTH (18–39) ([M+H]\textsuperscript{+} 2465.19) and the resulting mass spectra were internally calibrated with trypsin autolysis products (\( m/z \) 842.50 and \( m/z \) 2211.10). Monoisotopic peptide masses were assigned and then used in database searches.

The in-gel digestion of the proteins was performed according to Dihazi et al.\textsuperscript{18} Silver-stained proteins were destained with chemical reducers to remove the silver according to the protocol provided by the manufacturer (Invitrogen). When the gel bands become colorless, gel pieces were washed twice with water, dehydrated in acetonitrile (ACN) and finally dried in a vacuum centrifuge. Dithiothreitol (DTT) (10 \( m \)M) in 100 \( m \)l NH\textsubscript{4}HCO\textsubscript{3} was added to cover the gel pieces, and the protein was reduced for 1 h at 56 °C. After cooling to room temperature, DTT was replaced by 60 \( m \)l iodoacetamide in 100 \( m \)l NH\textsubscript{4}HCO\textsubscript{3}. Following 45-min of incubation at room temperature in the dark, gel pieces were washed with 100 \( m \)l NH\textsubscript{4}HCO\textsubscript{3} for 10 min, dehydrated by addition of ACN, swollen by rehydration in 100 mM NH\textsubscript{4}HCO\textsubscript{3}, and shrunk again by addition of the same volume of ACN. The liquid phase was removed, and the gel pieces were dried in a vacuum centrifuge. The gel pieces were swollen in digestion buffer containing 50 \( m \)M NH\textsubscript{4}HCO\textsubscript{3}, 5 \( m \)M CaCl\textsubscript{2}, and 10 \( ng/ \mu l \) of trypsin (sequencing grade, Sigma) on ice. After 45 min, the supernatant was removed and replaced by 100 \( m \)l of the same buffer without trypsin to keep the gel pieces hydrated during the enzymatic cleavage at 37 °C overnight. Peptides were extracted by adding 5\% formic acid in 50\% ACN for 20 min at room temperature. After drying down, the resulting peptides were reconstituted in 0.1\% TFA and desalted using a ZipTip C18 ion exchange column (Millipore, Eschborn, Germany).

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1:1000) and CK 10 (clone DE-K10, 1:100) were purchased from RDI (Research Diagnostik, Inc.). Proteins were visualized with peroxidase-coupled secondary antibody (Amer sham Bioscience), using enhanced chemoluminescence (ECL) for detection (Amer sham Bioscience).

Statistical analysis

Statistical analysis was performed with the SPSS software package (SPSS 11.0, SPSS Inc.; Chicago, IL, USA) and tests were chosen according to a professional statisticians advice. Results are medians and interquartile ranges. The degree of association between parameters was investigated by Spear man’s rank correlation (Spearman rank correlation coefficient; Rho). Statistical significance was accepted at the 5% level using a Bonferroni correction in case of multiple testing (5%/N, N—number of tests). The concordance of cytokeratin detection by gel electrophoresis and Western blotting was shown by Cohen’s Kappa.

Results

Identification of keratin isoforms in EBC samples

SDS-electrophoresis of concentrated EBC samples exhibited bands in the molecular range of 50–65 kDa in 87% (26/30) mechanically ventilated patients and in none of 10 controls (sample in Figure 1). Three bands (all cytokeratins) were observed in 14 out of 26 patients (47%), two bands (two cytokeratins: 8 × CK-10, 1 × CK-2, 7 × CK-9) were seen in 8 patients (27%)and only one band (one cytokeratin: 1 × CK-10, 1 × CK-2, 2 × CK-9) was seen in 4 patients (13%). No protein band was observed in the five samples taken from the artificial lung.

Protein band analysis by MALDI-TOF-MS including data base searches resulted in the identification of cytokeratins 2 (CK-2), 9 (CK-9) and 10 (CK-10) (Table 2) equivalent to the three protein bands. In mechanically ventilated patients cytokeratin CK-2 was found in 16 of 30 (53%) cases, cytokeratin CK-9 in 24 of 30 (80%) cases, and cytokeratin CK-10 in 23 of 30 (76.7%) cases.

Detection of EBC cytokeratins and clinical parameters

The frequency of bands detected by gel electrophoresis was compared with individual clinical data. For this purpose patients were grouped into three groups depending on the number of bands detected: no or 1 band, 2 bands, and 3 bands. There was no correlation between the number of bands and gender, age, weight, height, and smoking status (Spearman’s rank correlation). Ventilated patients divided into groups according to the number of protein bands detected (no cytokeratin, CK-1, CK-2, and CK-3 cytokeratin bands detected) were also correlated with (a) ventilatory parameters: PEEP, PIP, tidal volume adjusted to ideal body weight (Vt), breathing frequency (BF); time of ventilation up to EBC collection, (b) with clinical scores describing the extent of ALI: AECC score13 and Murray’s LISS,14 outcome, and finally (c) indicators of inflammation such as IL-6 and IL-8 in EBC and C-reactive protein (CRP), procalcitonin (PCT), IL-6 and IL-8 in serum (Table 3).

With increasing PEEP and PIP more cytokeratin bands were detected (Figure 2). In respect to indicators of severity

![Figure 1](image1.png)

**Figure 1** Sample of SDS-gel electrophoresis in mechanically ventilated patients (A) with characterization of cytokeratins (B) (S: standard; P: patient).

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Summary of the identification results obtained after in-gel-digestion of the 3 protein bands with trypsin, MALDI-TOF MS analysis and data base search.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>Molecular weight (kDa)</td>
</tr>
<tr>
<td>Cytokeratin 2</td>
<td>65.8</td>
</tr>
<tr>
<td>Cytokeratin 9</td>
<td>62.1</td>
</tr>
<tr>
<td>Cytokeratin 10</td>
<td>56.5</td>
</tr>
</tbody>
</table>
of lung injury an increase in the number of EBC cytokeratins was detected with increasing lung injury scores (LISS) and less so for the AECC score (Figure 3). No correlation was found between outcome and detection rate of cytokeratins (Table 3).

None of the indicators of inflammation in EBC or in serum showed any correlation with the number of cytokeratins detected or with the frequency of detection of the cytokeratins in EBC.

Protein concentrations between mechanically ventilated patients (median: 9.3 g/ml) and healthy volunteers (median: 8.3 g/ml) did not significantly differ (Mann–Whitney test: \( p = 0.55 \)). In addition, there was no correlation between detection rate of cytokeratins in EBC and protein concentration in EBC.

Smoking status (ventilated patients: non-smoker: 17, smoker: 13; healthy volunteers: non-smoker: 5, smoker: 5) did not correlate with cytokeratin detection rate whether in all subjects nor in subgroup of mechanically ventilated patients.

Cytokeratin detection by gel electrophoresis and Western blotting

The results of gel electrophoresis were crosschecked by Western blot analysis for CK-2 (example in Figure 4A) and CK-10 (example in Figure 4B) in a subgroup of 21 patients. For CK-2 we observed concordant results with both methods (\( p < 0.0001 \); Kappa: 1.0). The same was true for cytokeratin CK-10 with the exception of a single case (\( p < 0.0001 \); Kappa: 0.8), in which CK-10 was not detected in the Western blot.

Discussion

We here describe the identification of cytokeratins CK-2, CK-9, and CK-10 in EBC of mechanically ventilated patients.

Cytokeratins are intermediate-sized filaments in human tissues composed of at least 19 polypeptides, which are expressed in cell-type specific combinations related to the state and the differentiation of the specific epithelial cell. Bronchial and alveolar epithelial cells but not alveolar septa, capillaries and other non-epithelial elements were positively stained with fluorescent-labeled anti-cytokeratin antibodies. Several studies have been performed to identify cytokeratin specific for lung cancer. In contrast, there are no reports on the effects of mechanical stress on the pattern of cytokeratin expression in lung parenchyma. Cytokeratins identified in this study have also been described in squamous epithelium or epidermis. In human skin tissue cytokeratin expression changes with elevated levels of physical stress. The data presented in this study do not allow identifying the cells of origin for cytokeratins or the reason for their increased presence in EBC. However, the association of mechanical stress during ventilation and a change in cytokeratin expression in the lung may constitute a similar response to that in skin.

### Table 3

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Detection frequency of cytokeratins</th>
<th>Spearman-Rho</th>
<th>( p )-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(A) Spearman’s rank correlation (Spearman-Rho) of ventilatory parameters and clinical scores with detection frequency of cytokeratins as well as with every individual cytokeratin in the group of mechanically ventilated patients (patients: ( n = 30 ); significance level with Bonferroni correction: (&lt; 0.0005 ))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEEP (mbar)</td>
<td>0.60</td>
<td>0.00049</td>
<td></td>
</tr>
<tr>
<td>PIP (mbar)</td>
<td>0.78</td>
<td>0.00001</td>
<td></td>
</tr>
<tr>
<td>( V_t ) (ml/kg BW)</td>
<td>(-0.19)</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>BF (/min)</td>
<td>0.26</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>( \text{PaO}_2/\text{FiO}_2 )</td>
<td>(-0.59)</td>
<td>0.00067</td>
<td></td>
</tr>
<tr>
<td>Murray’s LISS</td>
<td>0.61</td>
<td>0.00034</td>
<td></td>
</tr>
<tr>
<td>Time of ventilation (hours)</td>
<td>0.57</td>
<td>0.00098</td>
<td></td>
</tr>
<tr>
<td>Outcome</td>
<td>0.33</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parameter</td>
<td>Median</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(B) Spearman’s rank correlation (Spearman-Rho) of inflammatory parameters with detection frequency of cytokeratins as well as with every individual cytokeratin in the group of mechanically ventilated patients (patients: ( n = 30 ); significance level with Bonferroni correction: (&lt; 0.0005 ))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP (serum) (mg/l)</td>
<td>96.8</td>
<td>(-0.02)</td>
<td>0.90</td>
</tr>
<tr>
<td>PCT (serum) (ug/l)</td>
<td>1.0</td>
<td>0.28</td>
<td>0.12</td>
</tr>
<tr>
<td>IL-6 (serum) (pg/ml)</td>
<td>48.2</td>
<td>0.12</td>
<td>0.51</td>
</tr>
<tr>
<td>IL-8 (serum) (pg/ml)</td>
<td>26.2</td>
<td>(-0.05)</td>
<td>0.79</td>
</tr>
<tr>
<td>IL-6 (EBC) (pg/ml)</td>
<td>9.3</td>
<td>0.01</td>
<td>0.95</td>
</tr>
<tr>
<td>IL-8 (EBC) (pg/ml)</td>
<td>11.3</td>
<td>(-0.06)</td>
<td>0.77</td>
</tr>
</tbody>
</table>
alternative explanation for the presence of cytokeratins in EBC, i.e. a contamination of EBC with cytokeratins during collection and preparation does not seem very likely since detection of cytokeratins in this case would also be expected in samples from the spontaneously breathing individuals, or from the artificial lung and in addition would not be expected to correlate with ventilatory parameters.

In order to exclude contamination of EBC we used disposable parts and washed those that were not disposable, such as the conduit for the expiratory limb of the ventilatory tubing thoroughly and repeatedly between sample collections. In addition, the cytokeratins detected in EBC from ventilated patients do not represent a typical mixture from skin or hair isoforms as would be expected in case of external contamination. Still, the absence of cytokeratin bands in EBC of spontaneously breathing patients and in samples from the artificial lung setup remains in contrast to the report of Hoffmann et al. who described cytokeratins in spontaneously breathing patients. Significant oropharyngeal contamination is unlikely in our investigation, since no amylase activity could be detected in EBC and no amylase bands were detected on the gels.

Marked differences in total protein concentrations might account for differences in the number of cytokeratins detected in EBC. We therefore measured total protein concentrations in EBC and found that increasing PEEP and/or PIP was associated with a higher detection rate of cytokeratins (classified as no or 1 band, 2 bands, and 3 bands) in SDS–gel electrophoresis.

Figure 2 Increasing PEEP and/or PIP (given in cm H₂O) was associated with a higher detection rate of cytokeratins (classified as no or 1 band, 2 bands, and 3 bands) in SDS–gel electrophoresis.

Figure 3 Increasing disease severity characterized by PaO₂/FiO₂ (according to criteria of the American-European Consensus Conference on ARDS (AECC) and of Murray’s Lung Injury Severity Score (LISS) associated with higher detection frequency of cytokeratins (no or 1 band, 2 bands, and 3 bands) in SDS–gel electrophoresis.

Figure 4 Examples of cytokeratins CK-2 (A) and CK-10 Western blotting (B) in mechanically ventilated patients (S: standard; P: patient; NK: negative control; PK: positive control).
concentrations in all EBC samples. However, we did not observe significant differences in total protein concentration and no correlation of protein concentration and the number of cytokeratin bands was detected.

In this study we were able to identify cytokeratins in mechanically ventilated patients. Our protocol was not designed to explore the mechanisms of cytokeratin release or their origins, but their presence shows that there is some relation to ventilatory aspects, as the number of cytokeratins detected in EBC correlates with ventilatory parameters and indicators of pulmonary (concentration in EBC) and systemic (concentration in serum) inflammation. The inflammatory indicators were not significantly correlated with the EBC cytokeratin content (Table 3). Cytokeratin 19, which was not detectable in EBC from any patient in this study was reported to be increased in bronchoalveolar lavage in chronic inflammatory airway disease. Other authors have described changes of the cytokeratin expression pattern to be a cellular stress response caused e.g. by cigarette smoke in the respiratory tract of rats. Gianazza et al. described detection of cytokeratins CK-1 and CK-9 in EBC of heavy smokers (40 pack-years). These authors were not able to detect protein bands in one-dimensional electrophoresis when using 1–1.3 ml of EBC. In the current study efforts were made to increase the amount of proteins and therefore used 10 ml of EBC that was lyophilized before use in electrophoresis experiments. Nevertheless, we did not observe any influence of the smoking status in this group of mechanically ventilated patients on the number of cytokeratins detected.

In our study, none of the cytokeratins that have been detected by immunohistochemistry in the trachea (CK-15, CK-7), lung (CK-14, CK-15), bronchi and alveoli (CK-7, CK-8, CK-18, CK-19) were detectable in EBC. However, CK-9, one of the cytokeratins observed here, has previously been described by Gianazza et al. using gel electrophoresis. These authors also detected cytokeratin CK-1 in EBC while our experiments revealed cytokeratins CK-2 and CK-10 in addition to CK-9. Different methods of identification (tandem electrospray mass spectrometry vs. MALD-TOF mass spectrometry in this study) were used and different patient groups assessed.

Cytokeratins were detected more often and in greater numbers in the ventilated patients with higher PIP and/or higher PEEP pressures. Increased cellular damage following increased alveolar distension by elevated pressures during ventilation may be a reason for the increased release of cytokeratins. Overdistension of lung tissue during mechanical ventilation has indeed been widely recognized. The duration of mechanical ventilation associated stress may also be of influence as suggested by our finding of a weak correlation of ventilatory time with cytokeratin detection rate. Continuing stress acting on the lung in association with mechanical ventilation might explain the unusual pattern of cytokines detected, however it does not easily explain the increase in detection rate observed in our study. Swensson et al. observed specific cytokeratin expression in response to high physical stress in ridged skin. Increased CK-7, CK-8, CK-14, CK-17, and CK-19 were also observed in lung tissue of patients with idiopathic pulmonary fibrosis when compared with normal lung tissue. Smoking has been identified as another cause for increased cytokeratin release in an experimental rat model and in a clinical study of cytokeratins in EBC of heavy smokers. Finally, elevated levels of CK-19 were found in BALF of patients with COPD. The presence of CK-19 was suggested to be useful for assessing the extent of bronchial epithelial damage.

We have reasons to believe, that inflammatory processes are not responsible for the increased cytokeratin detection rate because there was no correlation in pro-inflammatory cytokines in EBC or serum and the number of cytokeratin bands. The fact, that inflammation does not alter cytokeratin expression is supported by observations of unaltered CK-19 expression in BET-1A cells (human immortalized bronchial epithelial cells) following stimulation with TNF-alpha.

High PIP was shown to be particularly damaging to microvascular integrity. We observed here that elevated PIP levels were associated with a higher rate of cytokeratin detection in EBC. In addition a greater number of cytokeratins were also detected when ALI was advanced as indicated by a more severe lung injury score. This latter observation fits well with the hypothesis of mechanical overdistension causing increased cytokeratin detection rates as open lung areas decrease in more severe lung injury and the remaining open lung units are increasingly overdistended. This observation has led to the comparison of injured lungs with “baby lungs” demonstrating their propensity to be overdistended with regular tidal volumes. A comparable association of markers of lung distension with ventilatory parameters was shown for nitrite and tidal volume adapted to ideal body weight.

We have used spontaneously breathing volunteers for control subjects because a suitable ventilated control group is almost impossible to recruit. This of course constitutes a limitation of the study. However, even in surgical patients with e.g. trauma or those undergoing general surgery, an influence of EBC due to the disease process as well as to ventilation would be expected. In addition, the absence of cytokeratins in spontaneous breathing subjects in fact adds to the suggestion of cytokeratins as a marker of ventilator associated stress.

This study did not include repetitive measurements in the same individuals to characterize a time course of cytokeratin occurrence in EBC or to correlate changes in the EBC cytokeratin pattern with changes in the ventilatory pattern. Therefore we cannot conclude that the EBC cytokeratin pattern represents an appropriate clinical tool to monitor mechanical ventilation-induced mechanical stress on the lung tissue. However, EBC cytokeratin pattern seems to be promising candidate that justifies further clinical investigations.

In conclusion, we were able to demonstrate an increased frequency of cytokeratin detection in EBC samples of mechanically ventilated patients with ALI/ARDS as defined by standard criteria. Increased cytokeratin detection rates were correlated with higher PIP and PEEP levels, more severely injured lungs and longer ventilation. These findings suggest that mechanical stress caused by ventilation and aggravated by the extent of concomitant lung damage somehow leads to an increased number of cytokeratins detectable in EBC. Thus, the presence of cytokeratins and their number detected in EBC may serve as an indicator of
lungs damage inflicted by ventilation, and by preexisting lung injury.

Conflict of interest statement

None of the authors have a conflict of interest to declare in relation to this work.

References
