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Proteasomes in human bronchoalveolar lavage fluid after burn and inhalation injury

Joslyn M. Albright, MD¹, Jacqueline Romero, MS¹, Vikas Saini, MD, PhD¹, Stephan U. Sixt, MD², Melanie D. Bird, PhD¹, Elizabeth J. Kovacs, PhD^{1,3}, Richard L. Gamelli, MD¹, Jürgen Peters, MD², and Matthias Majetschak, MD, PhD^{1,4}

¹ Burn and Shock Trauma Institute, Department of Surgery, Loyola University Chicago Stritch School of Medicine

² Klinik für Anästhesiologie und Intensivmedizin, Universitätsklinikum Essen, Universität Duisburg-Essen, Germany

³ Department of Cell Biology & Neuroanatomy, Loyola University Chicago Stritch School of Medicine

⁴ Department of Pharmacology and Experimental Therapeutics, Loyola University Chicago Stritch School of Medicine

Abstract

Objective—To determine whether 26S proteasome is detectable in human bronchoalveolar-lavage fluid (BALF) and whether burn and inhalation injury is accompanied by changes in BALF proteasome content or activity.

Methods—BALF was obtained on hospital admission from 28 patients with burn and inhalation injury (controls: 10 healthy volunteers). Proteasome concentrations were quantified by ELISA and their native molecular mass was assessed by gel filtration. Proteasome peptidase activity was measured employing a chymotryptic-like peptide substrate in combination with epoxomicin (specific proteasome inhibitor).

Results—BALF protein was increased in patients ($p < 0.001$) and correlated positively with the degree of inhalation injury. 20S/26S proteasomes were detectable in all BALF by ELISA. Gel filtration confirmed the presence of intact 20S and of 26S proteasome which was stable without soluble ATP/Mg²⁺. In all BALF chymotryptic-like activity was detectable and could be inhibited with epoxomicin by 60–70% ($p < 0.01$). Absolute amounts of 20S/26S proteasomes and proteasome activity were increased in patients ($p < 0.001$ for all). The relative BALF composition after injury was characterized by increased concentrations of 20S proteasome/mg protein ($p = 0.0034$ vs. volunteers), decreased concentrations of 26S proteasome/mg protein ($p = 0.041$ vs. volunteers) and reduced specific proteasome activity ($p = 0.044$ vs. volunteers). 26S proteasome/mg and specific proteasome activity were even further reduced in patients who developed ventilator-associated pneumonia ($p = 0.045$ and $p = 0.03$ vs. patients without VAP, respectively).

Conclusions—The present study supports the novel concept that extracellular proteasomes could play a pathophysiological role in the injured lung and suggests that insufficient proteasome function may increase susceptibility for pulmonary complications.

Keywords

Extracellular proteasomes; 26S proteasome; inhalation injury; protein degradation; ventilator-associated pneumonia

Introduction

Proteasomes are the principal non-lysosomal proteases in all eukaryotic cells and regulate a multitude of important intracellular functions (1, 2). They consist of a cylinder-shaped multimeric protein complex which is termed 20S proteasome core particle or 20S proteasome. This cylinder is composed of four stacked rings, each consisting of seven α - or β -type subunits ($\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$). The proteolytically active sites of the 20S proteasome are located in the β -type subunits. The 20S proteasome can also be singly or doubly capped at its ends by a 19S regulator complex when ATP/Mg²⁺ is present and is then termed 26S proteasome. While the 20S proteasome is involved in the destruction of misfolded and damaged proteins, the 26S proteasome plays important roles within the ubiquitin proteasome pathway of protein degradation and uses ubiquitylated proteins as substrates for degradation (1–4).

Besides the intracellular localization of proteasomes, the 20S proteasome is also a constituent of normal plasma and increased concentrations of circulating 20S proteasomes have been reported under various pathological conditions, including autoimmune diseases, trauma and sepsis (5–11). While evidence for a functional role of circulating 20S proteasomes has as yet not been provided, it is thought to reflect the extent of cell damage and immunological activity in disease states (9–11).

More recently, it was shown that small concentrations of enzymatically active 20S proteasomes are also present in normal bronchoalveolar lavage fluids (BALF) and that significantly increased 20S proteasome BALF concentrations are detectable after blunt chest trauma in animals and in patients with acute respiratory distress syndrome (ARDS) (12–15). Furthermore, studies in animals suggested that 26S proteasomes also occur in BALF after blunt chest trauma and that proteasomes contribute to extracellular degradation of natural BALF proteins in the injured lung (13).

However, it is not known whether 26S proteasomes exist in human BALF and alterations in BALF proteasome content and activity in a homogenous population of patients with direct lung injury have not been studied. Therefore, we analyzed BALF from healthy volunteers and burn patients with inhalation injury for the presence of 20S and 26S proteasomes, and evaluated in a prospective observational study whether possible alterations in proteasome content and activity after burns and inhalation injury are associated with injury severity and outcomes.

Methods

Patients and volunteers

This study was approved by the Internal Review Boards of the Loyola University Medical Center and the Universitätsklinikum Essen, University of Duisburg-Essen, Germany. Burn patients admitted to the Burn Intensive Care Unit (ICU) service of the Loyola University Medical Center requiring bronchoscopy for diagnosis of inhalation injury were recruited between 08/25/2007 and 07/14/2008. Patients were excluded from the study for the following reasons: age less than 18 years, malignancy, immunosuppressive medications or known autoimmune or chronic inflammatory diseases. Thirty-two consecutive patients were

eligible for study entry, of which four patients could not be enrolled due to logistical problems (n=2) and clinical instability not permitting bronchoscopy on admission (n=2).

All patients were intubated, mechanically ventilated with a positive end-expiratory pressure (PEEP) of 5 cm H₂O, and all bronchoalveolar lavages (BAL) were performed within 14 hours after injury. The following clinical variables and outcomes were documented: age, sex, time point of BAL, percentage of total body surface area burned (%TBSA), grade of inhalation injury, PaO₂/fraction of inspired oxygen (P/F) ratio at the time of BAL collection, development of ventilator-associated pneumonia (VAP), results of microbial isolates from BALF cultures, and mortality.

The degree of inhalation injury was determined using a standard scoring system based on the morphology of the airways during bronchoscopy (Grade 0: absence of carbonaceous deposits, erythema, edema, bronchorrhea or obstruction, Grade 1: minor or patchy areas of erythema, carbonaceous deposits in proximal or distal bronchi, Grade 2: moderate degree of erythema, carbonaceous deposits, bronchorrhea, or bronchial obstruction, Grade 3: severe inflammation with friability, copious carbonaceous deposits, bronchorrhea or obstruction, Grade 4: evidence of mucosal sloughing, necrosis, endoluminal obliteration), as described in detail previously (16, 17).

Ventilator-associated pneumonia (VAP) was defined using the American Burn Association Consensus Conference criteria (18). In brief, diagnoses were made clinically when two of the following were present: (i) chest x-ray revealing a new and persistent infiltrate, consolidation, or cavitation, (ii) sepsis and/or (iii) a recent change in sputum or purulence in the sputum. Gram's stain and quantitative BAL cultures were performed on all bronchoscopic samples, and suspected pneumonias were evaluated by a standard ICU protocol using blind BAL (19). The patients' epidemiological and clinical characteristics are shown in table 1.

BALF from ten healthy adult volunteers (7 men, 3 women, age: 30 ± 4.7 (mean ± SD) years) who were recruited at the Universitätsklinikum Essen served as controls. Volunteers were free of pulmonary, cardiac, infectious, and allergic disease, had no history of chemotherapy or radiation therapy and were non-smokers. In these individuals, BALF sampling was performed under local anesthesia, as described (20). The recovery of BALF (volume of fluid recovered/volume of fluid instilled) from volunteers ranged from 45–70%.

In patients, BAL were performed based on standardized ICU protocols (21, 22). In brief, a bronchoscope was directed into the left lower lobe and wedged into a subsegmental bronchus. Twenty mL of 0.9% saline solution were instilled and discarded. The bronchoscope was then repositioned into another subsegmental bronchus in the same lobe and another 20 mL were instilled followed immediately by gentle wall suction into a sterile Leuken's fluid trap. One to four subsequent 20 mL aliquots were collected from the other four lobes, as tolerated by the patient. The recovery of BALF in patients ranged from 30–60%. The samples were then placed on ice and filtered using a cell strainer (BD Falcon, 100 µm membrane, BD, Franklin Lakes, New Jersey). Following centrifugation at 250 × g for five minutes, the cell free fluid was then aliquoted, total protein measured according to Lowry (23), and stored at –80°C until further analysis.

20S and 26S proteasome enzyme linked immunosorbent assays (ELISA)

20S and 26S proteasome concentrations were quantified with newly developed ELISAs, as described in detail previously (24). In brief, microtiter plates (Nunc Maxisorb, Nalge Nunc International, Rochester, NY) were coated with the capture antibodies (20S: anti-20S subunit α6 (PW8100, Biomol, Plymouth Meeting, PA); 26S: anti-19S subunit Rpn2

(AP-104, Boston Biochem, Boston, MA)) diluted in phosphate buffer saline (PBS), pH 7.4 over night at 4°C. After coating plates were blocked with PBS, 1% bovine serum albumin (Sigma, PBS-BSA). Standard curves were prepared employing highly purified 20S (PW8270) and 26S proteasomes (PW 9310, both from Biomol) diluted in PBS-BSA. 100 μ L of standards and samples were placed in the wells and incubated for two hours. After washing the plates, the secondary antibodies (20S: anti-20S “core subunits” (α 5, α 7, β 1, β 5, β 5i, β 7) (PW8155, Biomol); 26S: anti-20S subunit α 6 (PW8100, Biomol)) were added to the wells and incubated for 1 hour at room temperature. The plates were washed again and HRP labeled anti-rabbit or anti-mouse (NA934V and NA931V, GE Healthcare, Piscataway, NJ) were added. After 1 hour of incubation, plates were washed again and the bound antibodies were detected using tetramethylbenzidine (TMB, Sigma-Aldrich, St. Louis, MO). The reaction was stopped by addition of 50 μ L 2N HCL and the optical densities (OD) were determined at 450/540 nm in a microplate reader (Synergy 2, Biotek Instruments, Winooski, VT). For the 26S ELISA, all buffers contained 5 mM ATP and 5 mM Mg^{2+} (Sigma). To assess the influence of ATP/ Mg^{2+} on the detectable 26S concentrations, all measurements were repeated in the absence of ATP/ Mg^{2+} . While the 20S proteasome ELISA detects free 20S proteasomes and 20S proteasomes within the 26S proteasome complex, the 26S proteasome ELISA shows no cross reactivity with free 20S proteasomes. For the calculation of the molar concentration of 20S proteasomes, a molecular mass of 700 kDa was used. For the 26S proteasome, a molecular mass of 1.7 MDa was assumed to account for a mixture of 20S proteasomes that are capped with either one or two 19S regulator complexes (24). The lower detection limits were 1.5 ng/mL and 11.7 ng/mL for the 20S and 26S proteasome ELISA, respectively.

Proteasome peptidase activities

Proteasome peptidase activities were measured employing the fluorogenic chymotryptic-like peptide substrate *N*-Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC, Biomol), as described (13, 25). Reaction mixtures contained 10 mM Tris/HCl, pH 7.5, 100 μ M peptide substrate and 30 μ L of BALF. Mixtures were incubated for 60 minutes at 37°C. Ethanol (2:1; volume:volume) was added, mixtures placed on ice for 10 minutes and centrifuged at 16000 $\times$ g, 5°C for 6 minutes. Supernatants were transferred into microplates (Corning, Acton, MA) and free 7-amino-4-methylcoumarin cleaved from the substrates measured in a microplate reader (Synergy 2, Biotek, $\lambda_{excitation/emission}$ =340/440 nm) against standard curves of 7-amino-4-methylcoumarin (Sigma). To differentiate the proteasome from other peptidase activities, the epoxomicin (specific proteasome inhibitor) sensitive proportion was determined by addition of 7 μ M epoxomicin (Boston Biochem) to the mixtures (26). Peptidase activities were determined as mol of 7-amino-4-methylcoumarin cleaved per hour. Proteasome peptidase activity was calculated as total peptidase activity minus peptidase activity in the presence of epoxomicin and expressed as activity per mL BALF. Because the proteolytic active sites are located in the β subunits of the 20S, the specific proteasome activity was calculated as activity per ng of 20S.

Analytical gel filtration

Gel filtration experiments were performed on a Superose 6 HR 30/10 column (inner diameter: 10 mm, lengths: 300 mm; GE Healthcare) in PBS, pH 7.4, at 5°C using a computer controlled fast protein liquid chromatography (FPLC) system (BioLogic DuoFlow Maximizer 20, Bio-Rad, Hercules, CA). V_{total} , as determined with vitamin B12 (1.35 kDa, Bio-Rad), was 21.9 mL. Five hundred μ L BALF were injected onto the column and filtered at a flow rate of 0.25 mL/min. Fractions of 0.5 mL were collected. The column was calibrated using proteins of known molecular mass (thyroglobulin (670 kDa), bovine γ -globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa) (all from Bio-Rad) and ubiquitin (8.6 kDa; from Boston Biochem)).

Statistics

Data are described as mean with standard deviation and median with 25th and 75th percentiles (in parenthesis) for normally and non-normally distributed data, respectively. To assess normal distribution, the Komolgorov-Smirnov test was used. For data that passed the normality test ($\alpha = 0.05$) the Levene's test for equality of variances was calculated and comparisons between groups were analyzed using the Student's t-test or unequal variance t-test, respectively. The Mann-Whitney U test was used to compare groups when normal distribution of the data could not be assumed. Fisher's exact test was used for dichotomic categorical variables. Correlations were assessed using partial correlation coefficients (r_p) that control for the effects of one or more additional variables. Statistical analyses were calculated with the SPSS for Windows release 16.0 program (SPSS Inc., Chicago, IL). Regression analyses were calculated with the GraphPad Prism program (GraphPad Software Inc., La Jolla, CA). A two-tailed $p < 0.05$ was considered significant.

Results

As compared with volunteers total BALF protein content was significantly increased in burn patients with inhalation injury (patients: 902 (578/1640) $\mu\text{g/mL}$, volunteers: 107 (83/122) $\mu\text{g/mL}$, $p < 0.001$; Fig. 1A). 20S and 26S proteasomes were detectable in all BALF by ELISA. 20S and 26S contents were significantly increased in BALF after inhalation injury (20S: volunteers: 36 (23.5/48.5) ng/mL , patients: 590 (338/2035) ng/mL , $p < 0.001$; 26S: volunteers: 117 (73/137) ng/mL , patients: 727 (287/986) ng/mL , $p < 0.001$; Fig. 1B/C). When specific proteasome concentrations were expressed as proteasome content per mg of BALF protein, specific 20S proteasome concentrations were significantly increased (20S: volunteers: 330 (228/450) ng/mg , patients: 889 (434/1821) ng/mg , $p = 0.0034$), whereas 26S proteasome concentrations were significantly decreased after burn and inhalation injury (26S: volunteers: 1213 (579/1690) ng/mg , patients: 675 (397/1169) ng/mg , $p = 0.041$; Fig. 1D/E).

To confirm the presence of intact 20S and 26S proteasomes in BALF, the fluid was gel-filtered and the eluted fractions were analyzed for 20S proteasome immunoreactivity by ELISA. The overall recovery of 20S proteasomes in the eluted fractions was 53%. Although gel filtration was performed in the absence of ATP/Mg^{2+} , 20S proteasomes were detectable at elution positions corresponding to the native molecular masses of the 20S proteasome (700 kDa) and the 26S proteasome (>1.5 MDa) (Fig. 2). In agreement with the proteasome measurements in the BALF before gel filtration (molar ratio between 20S and 26S proteasomes: 11), the molar ratio between 20S proteasomes that eluted at 700 kDa and >1.5 MDa was 6.1.

Because these data suggested intact 26S proteasome complexes in BALF that are stable during gel filtration in the absence of ATP/Mg^{2+} , we repeated the 26S ELISA in the absence of ATP/Mg^{2+} . The determined 26S proteasome concentrations in the absence of ATP/Mg^{2+} averaged $95 \pm 36\%$ of the measurements in the presence of ATP/Mg^{2+} ($p = 0.11$). In contrast, when highly purified 26S proteasomes from human erythrocytes were assayed in the presence and absence of ATP/Mg^{2+} , its concentration in absence of ATP/Mg^{2+} decreased to $33 \pm 4\%$ of the measurements in the presence of ATP/Mg^{2+} ($n = 4$, $p < 0.001$).

Furthermore, we tested BALF for enzymatic proteasome activity. Chymotryptic-like peptidase activity was detectable in all BALF from volunteers and patients. This activity could be inhibited with the specific proteasome inhibitor epoxomicin in all specimens. The relative inhibition of the chymotryptic-like activity by epoxomicin is shown in Fig. 3A. Based on these data, $69 \pm 29\%$ of the total peptidase activity in BALF from volunteers could

be attributed to the proteasome and 61 ± 22 % in BALF from patients ($p > 0.05$ vs. volunteers), respectively.

BALF from patients contained higher chymotryptic-like proteasome peptidase activity per mL than BALF from volunteers (chymotryptic-like proteasome activity ($\text{pmol} \times \text{h}^{-1} \times \text{mL}^{-1}$), volunteers: 58 (4/151), patients: 736 (409/1345), $p < 0.001$; Fig. 3B). Nevertheless, specific proteasome activities per ng of 20S proteasomes were significantly lower in patients ($\text{pmol} \times \text{h}^{-1} \times \text{ng}^{-1}$ 20S, volunteers: 2.1 (0.57/9.36), patients: 0.73 (0.52/1.56), $p = 0.044$; Fig. 3C).

There were no differences in specific 20S and 26S proteasome concentrations (ng/mg of total protein) or specific proteasome activities (activity per ng of 20S proteasomes) between males and females, and these parameters did not correlate with patients' age (data not shown).

To assess whether total protein, specific proteasome concentrations and specific proteasome activities in BALF depend on the overall burn size or on the degree of inhalation injury, we calculated partial correlation coefficients to control for the effects of differences in total burn size for their correlation with the degree of inhalation injury, and vice versa (Tab. 2). There were no statistically significant associations with %TBSA for any of the measurements. Total protein content correlated significantly positive with the grade of inhalation injury, whereas specific 26S proteasome concentrations correlated significantly negative with the grade of inhalation injury (Tab. 2).

Six of the 28 patients had inhalation injury without cutaneous burns (inhalation injury grade 1: $n=1$, grade 2: $n=1$, grade 3: $n=4$). There were no differences in BALF protein content, 20S and 26S proteasome content per mL or mg of BALF, and proteasome activity per mL or ng 20S proteasome between patients with ($n=22$) and without cutaneous burns ($p > 0.05$ for all parameters, data not shown).

Specific 20S proteasome concentrations in BALF were similar in patients with and without subsequent development of VAP (Fig. 4A). Specific 26S concentrations and specific proteasome activities in BALF from patients who developed VAP were significantly lower than in patients without development of VAP (Fig. 4B/C). Protein, 20S and 26S concentrations per mL of BALF and proteasome activity per mL BALF or mg of protein did not show significant differences between patients with and without VAP development (data not shown). Although mean age and median total burn size were higher in patients with VAP development, these differences were not statistically significant (Tab. 1). There were no significant differences in specific proteasome concentrations and activities between patients who survived ($n=22$) and died ($n=6$) (data not shown).

Discussion

There are several new findings from the present study: *first*, in addition to 20S proteasomes, human BALF fluid also contains 26S proteasomes. *Second*, absolute amounts of 20S and 26S proteasomes as well as proteasome peptidase activities are significantly increased in BALF after burn and inhalation injury. *Third*, BALF contains 26S proteasomes that are stable in the absence of soluble ATP/Mg²⁺. *Fourth*, the specific activity of proteasomes in BALF is significantly reduced after inhalation injury, and even further reduced in patients who develop VAP during their subsequent posttraumatic course.

In line with previous observations in patients without lung pathologies (12, 14), 20S proteasomes were also detectable in comparable concentrations in BALF from healthy

volunteers (patients without lung pathologies: 61 ± 15 ng/mL (14); healthy volunteers in this study: 36 ± 14 ng/mL).

In agreement with our recent findings in BALF from healthy animals and animals after blunt chest trauma (13), measurements of 26S proteasomes with an ELISA that depends on the integrity of the intact molecule (24) and gel filtration experiments showed consistently that the 26S proteasome is also present in human BALF.

Although it is thought that ATP/Mg²⁺ is required to stabilize the 26S proteasome and that removal of ATP/Mg²⁺ leads to dissociation of the 19S regulator complex from 20S proteasomes (27–29), the mechanisms by which ATP stabilizes 26S proteasomes are not fully understood. Utilizing solid phase affinity immobilization of intact 26S to analyze its stability requirements, we previously provided evidence that at least two proteasomal ATP binding sites are required to fully stabilize the complex between the 20S proteasome and the 19S regulator complex: a low affinity binding site that requires ATP hydrolysis and a high affinity binding site with an equilibrium dissociation constant in the low μ M range that does not require ATP hydrolysis (24). Furthermore, these studies suggested that approximately 10–30% of 26S proteasomes are stable in the absence of soluble ATP/Mg²⁺ (24). The present findings from gel filtration experiments, re-evaluation of the BALF 26S proteasome content by ELISA in the absence of ATP/Mg²⁺ and quantification of 26S proteasomes with and without ATP/Mg²⁺ in highly purified enzyme preparations derived from human cell lysates confirmed that approximately 30% of 26S proteasomes do not dissociate into 20S proteasomes and 19S regulator complexes and suggest that human BALF contains this subset of 26S proteasomes that is stable without soluble ATP/Mg²⁺. Thus, it is likely that the BALF 26S proteasome still contains ATP which is bound to the high affinity binding site and is not used for hydrolysis.

Previously, we reported that significantly increased concentrations of 20S proteasomes are detectable in BALF after blunt chest trauma in an animal model (13). Subsequently, it was shown that 20S proteasome BALF concentrations are also significantly increased in a heterogeneous group of patients with ARDS (14, 30). The findings of the present study confirm our initial observations in an experimental animal model, show that significantly increased contents of proteasomes are also detectable in BALF after direct lung injuries in patients and suggest that increases in BALF proteasome concentrations are independent of the mechanism of acute lung damage.

The determined concentrations of BALF 20S proteasomes in patients after burn and inhalation injury (mean $\pm$ SD: 1414 ± 1743 ng/mL) were in the same range as the concentrations reported previously in patients with ARDS (1070 ± 1194 ng/mL) (14). Due to the heterogeneity of the disease processes that can lead to ARDS, a direct comparison of patients who fulfill criteria of ARDS and burn patients with inhalation injury on hospital admission is difficult. Nevertheless, because 20S proteasome BALF concentrations were found to be significantly lower in patients who were distinguishable from ARDS patients only by a higher P/F ratio (> 200 mmHg and < 300 mmHg) and thus, were grouped as patients with acute lung injury (14, 30), it appears that increases in BALF proteasome concentrations require a certain threshold of acute lung damage, that may not have been reached in patients with ALI. Although the exact cellular origin of proteasomes in the systemic circulation or in BALF is unknown, current findings on proteasomes in BALFs along with previous observations on circulating proteasomes in conditions that are associated with increased cell damage (9, 11, 13) collectively point towards passive release from destroyed or damaged cells and tissues. On the other hand, proteasomes have recently been observed in vacuoles adjacent to cell membrane of pneumocytes type II, and thus, may also be released into the BALF from exocytotic vesicles (14).

After experimental lung contusion, proteasome BALF concentrations and activities peaked 24–48 hours after injury (11). If this release kinetic can be translated to humans, our measurements do not reflect peak proteasome concentrations. This may explain the weak or missing correlation of BALF proteasome concentrations with the grade of inhalation injury in the present study.

Partial correlation analyses implied that the presence of cutaneous burns had little effect on BALF proteasome levels and activities in our patient population. Because routine diagnostic bronchoscopy is not performed in burn patients without suspected inhalation injury, we could not compare proteasome levels and activities in patients with and without inhalation injury to further confirm the implications of the correlation analyses. Nevertheless, the observation that proteasome levels and activities were similar in patients with inhalation injury with and without cutaneous burns argues for the lung as the predominant source of BAL proteasomes, and against an additional significant proportion of BALF proteasomes originating from cutaneous cell damage.

Our previous finding that BALF proteasomes contribute to extracellular degradation of natural BALF proteins *in vitro* provided initial evidence for a possible pathophysiological role of proteasomes in the bronchoalveolar space of the injured lung (13). In the present study, specific BALF proteasome activity was significantly lower in patients, as compared with volunteers, and also significantly lower in patients with development of subsequent VAP, when compared with patients without VAP development. Thus, these observations could indicate that insufficient BALF proteasome function delays clearance of proteins that accumulate in the alveolar space of the injured lung, promotes lung edema formation and contributes to development of pulmonary complications.

The finding that BALF proteasome content and activity did not show differences between survivors and non-survivors has limited significance because our patient population was small and only six out of 28 patients did not survive burn and inhalation injuries. Besides the small patient population, other obvious limitations of the present study are that healthy volunteers were younger than patients and not mechanically ventilated, the cross-sectional study design and its descriptive nature. Previously reported normal BALF 20S proteasome concentrations that were obtained from mechanically ventilated patients without lung pathologies with a mean age of 65 ± 4 years were in the same range as the concentrations in volunteers in the present study (14). Furthermore, 20S proteasome concentrations in our patients did not correlate with age and all BALF specimens from patients were obtained within a few hours after injury. Thus, it appears unlikely that age differences and mechanical ventilation were significant confounding factors. While the present and any other clinical observational study cannot address mechanistic aspects, longitudinal studies are hampered by the invasiveness of the BAL procedure and require a strong rationale derived from single time point measurements in specimens that were obtained during standard diagnostic procedures before being justified.

Taken together, in the present study, we show for the first time that not only 20S proteasomes, but also 26S proteasomes are detectable in normal human BALF and that both are significantly increased after burns and inhalation injury. The 26S proteasomes in BALF are stable in the absence of soluble ATP/Mg²⁺ and appear to be a subset of 26S proteasomes that is also detectable in enzyme preparations derived from intracellular sources. Along with previous findings after lung injury in an animal model and in patients with ARDS, the results from the present study further support the concept that BALF proteasomes could have a pathophysiological role in the injured lung and that insufficient proteasome function may increase susceptibility for VAP. Conclusively, the appearance of proteasomes in the bronchoalveolar space may point to a novel pathophysiological mechanism with clinical

relevance after lung injury. Further biochemical characterization of BALF proteasomes and observational longitudinal studies in larger patient populations are required to validate this novel concept.

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Figure 1.

Measurements in BALF from healthy volunteers (n=10, open boxes) and burn patients with inhalation injury (B/I injury, n=28, grey boxes). Boxes extend from the 25th to 75th percentile, the horizontal line shows the median. Error bars show the range of data (min/max). *:p<0.05 vs. volunteers. **A.** BALF protein concentration ($\mu\text{g/mL}$). **B.** 20S proteasome concentration (ng/mL BALF). **C.** 26S proteasome concentration (ng/mL BALF). **D.** Specific 20S proteasome concentration (ng/mg of BALF protein). **E.** Specific 26S proteasome concentration (ng/mg of BALF protein).

Figure 2.

Analytical gel filtration on Superose 6 with BALF from a burn patient with inhalation injury grade 2. 0.5 mL of BALF was injected onto the column and fractions of 0.5 mL were collected (flow rate: 0.25 mL/min). The optical density (OD) at 280 nm was monitored continuously. Fractions were analyzed for 20S by ELISA. ●: 20S (ng/mL). □: elution positions of molecular mass standards (1: 670 kDa; 2: 158 kDa; 3: 44 kDa, 4: 17 kDa, 5: 8.6 kDa, 6: 1.35 kDa).: Standard curve derived from linear regression analysis ($\log \text{ (kDa)}$ vs. elution position (mL); r^2 : 0.99). —: OD at 280 nm. Arrows: Elution of position of 20S corresponding to the native molecular masses of free 20S (700 kDa) and 20S within the 26S complex (>1.5 MDa).

Figure 3.

Proteasome peptidase activity in BALF. **A.** Chymotryptic-like (CT-L) peptidase activity in BALF measured in the absence (–) and presence (+) of the specific proteasome inhibitor epoxomicin (7 μ M). Activities are expressed as % of the incubation mixtures without epoxomicin. Open circles: Volunteers (n=10). Grey circles: Patients (n=28). *: $p < 0.05$ vs. incubation mixtures without epoxomicin. **B.** BALF chymotryptic-like proteasome peptidase activity (total activity minus activity in the presence of epoxomicin) in pmol $\times$ h⁻¹ $\times$ mL⁻¹ BALF. Open box: healthy volunteers (n=10). Grey box: patients with burn and inhalation injury (B/I injury, n=28). Boxes extend from the 25th to 75th percentile, the horizontal line shows the median. Error bars show the range of data (min/max). *: $p < 0.05$ vs. volunteers. **C.** Specific BALF chymotryptic-like proteasome peptidase activity (total activity minus activity in the presence of epoxomicin) in pmol $\times$ h⁻¹ $\times$ ng⁻¹ 20S. Open box: healthy volunteers (n=10). Grey box: patients with burn and inhalation injury (B/I injury, n=28). Boxes extend from the 25th to 75th percentile, the horizontal line shows the median. Error bars show the range of data (min/max). *: $p < 0.05$ vs. volunteers.

Figure 4.

Specific 20S and 26S proteasome concentrations and proteasome activity in patients with burn and inhalation injury with and without subsequent development of ventilator associated pneumonia (VAP). Open boxes: No VAP (n=17). Grey boxes: VAP (n=11). Boxes extend from the 25th to 75th percentile, the horizontal line shows the median. Error bars show the range of data (min/max). *: p<0.05 vs. no VAP. **A.** 20S (ng/mg BALF protein). **B.** 26S (ng/mg BALF protein). **C.** BALF chymotryptic-like proteasome peptidase activity (total activity minus activity in the presence of epoxomicin) in pmol $\times$ h⁻¹ $\times$ ng⁻¹ 20S.

Table 1

Epidemiological and clinical characteristics of the patient population

	All (n=28)	VAP (n=11)	No VAP (n=17)
Age (years)	53 ± 20	58 ± 19	49 ± 21
Sex (male/female)	17/11	9/2	8/9
%TBSA	15(1/32)	30(4/54)	15(0/19)
Grade of inhalation injury	2(1/3)	2(1/3)	2(1/3)
VAP (%)	39	100	0
Microbial isolates:			
Gram positive (n)		8	n/a
Gram negative (n)		3	n/a
P/F ratio	336 ± 111	314 ± 137	345 ± 93
Mortality (%)	21	27	19
BALF obtained (hours after injury)	7.5(6/12)	7(6/14)	7.5(6/11.5)

VAP: Ventilator-associated pneumonia. Data are means ± standard deviation or median with 25th/75th percentile (in parentheses). %TBSA: Percentage of burned total body surface area. P/F ratio: PaO₂/fraction of inspired oxygen (P/F) ratio at the time point of bronchoscopy. There were no statistically significant differences between patients with and without development of VAP.

Table 2

Correlation of BALF protein concentrations, proteasome concentrations and activities with burn size and grade of inhalation injury

<i>Partial correlation analyses</i>	<i>%TBSA[#] r_p (p)</i>	<i>Grade of inhalation injury^{&} r_p (p)</i>
Total protein (mg/mL)	−0.028 (0.873)	0.634 (<0.001)
20S proteasomes (ng/mg)	0.098 (0.568)	0.247 (0.147)
26S proteasomes (ng/mg)	−0.088 (0.612)	−0.410 (0.013)
Specific proteasome activity	−0.196 (0.252)	−0.256 (0.132)

r_p Partial correlation coefficients. (p): level of statistical significance (in parenthesis).

[#]r_p are controlled for grade of inhalation injury.

[&]r_p are controlled for %TBSA (percentage of total body surface area burned)