Online data supplement

Elevated protein levels and altered cellular expression of factor VII-activating protease (FSAP) in the lungs of patients with acute respiratory distress syndrome (ARDS)

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METHODS

Materials

Human FSAP was purified from whole plasma by affinity chromatography as recently described.[1] Rabbit polyclonal and murine monoclonal antibodies against FSAP (#677 against light chain of FSAP, #1189 against heavy chain of FSAP and #570 as inhibitory antibody) were provided by Aventis Behring (Marburg, Germany). Single chain human urokinase-plasminogen activator (sc u-PA) was obtained from ZLB-Behring (Marburg, Germany). Heparin was purchased from Ratiopharm (Ulm, Germany). Fibrinogen was purchased from Kabivitrum (Munich, Germany). Plasminogen was purified from human plasma by lysine-Sepharose adsorption, followed by gel-filtration.[2] Thrombin was purchased from Sigma-Aldrich (Taufkirchen, Germany). LPS from *E.coli* was obtained from Sigma-Aldrich. IL-1β, IL-6, IL-8 and TNF-α were purchased from R&D Systems (Wiesbaden, Germany). Goat polyclonal antibody against IL-8 was obtained from R&D Systems. FSAP-deficient plasma was provided by Aventis Behring.

Study population

BAL fluids were obtained from the following patient groups:

Extrapulmonary ARDS without pulmonary infection (ARDS; n=15)

Diagnosis was settled on the basis of the ARDS American-European Consensus Criteria.[3] Criteria included the presence of a typical initiating nonpulmonary catastrophic event (sepsis (11 patients) or polytrauma (4 patients)), Pa_{O2}/Fi_{O2} < 200mmHg, diffuse bilateral alveolar infiltrates on chest X-rays, and pulmonary artery wedge pressure less than 18 mm Hg, or no clinical evidence of acute or chronic left heart failure. BAL was performed within the first 120 h after onset of disease (early ARDS). Patients with primary or secondary lung infection were not included in this group.

ARDS with primary lung infection (ARDS + Pneu; n=8)

Patients were included with a typical clinical history of primary lung infection (fever, tachycardia, dyspnea, tachypnea, typical auscultatory findings, circumscript lung infiltrates on chest X-rays, microbiological identification of pathogens in the lower respiratory tract by bronchoscopy), requiring mechanical ventilation and displaying the above listed ARDS criteria of the American-European Consensus Conference in course of the disease.

Cardiogenic pulmonary oedema (CLE; n=5)

This group consisted of patients requiring mechanical ventilation with radiographic and clinical signs of pulmonary congestion due to left heart failure in the absence of ARDS and lung infection. Proof of a pulmonary capillary wedge pressure > 18 mm Hg was mandatory for inclusion of these patients.

All patients required mechanical ventilation. Respirator settings were chosen according to the individual requirements. General therapeutic approaches included intravenous volume substitution, low-dose heparin application, parenteral nutrition, antibiotic drug therapy, and administration of vasoactive or inotropic drugs, when indicated. Patients with proven or suspected malignancy of the lung, or with any preexisting lung disease with a FEV_1 or FVC less than 65% predicted were excluded from the study. Pa_{O2}/Fi_{O2} values, duration of mechanical ventilation, sex, age, and smoking history did not differ substantially among the different patient groups. The main demographic and clinical data are summarized in Table 1.

Table 1. Demographic and clinical data of the patient groups

	Controls (n=15)	CLE (n=5)	ARDS (n=15)	ARDS + Pneu (n=8)
Age (years)	47.3±4.1	57.3±5.1	52.6±6.8	49.5±4.2
Male/female	9/6	4/1	11/4	5/3
Never smoker (n)	15	1	10	6
Ex smoker (n)	0	1	3	1
Current smoker (n)	0	3	2	1
Pa_{O2}/Fi_{O2} (mmHg)	418±17	201±16	182±14	187±11

CLE = patients with cardiogenic pulmonary oedema; ARDS = ARDS patients without pulmonary infection; ARDS + Pneu = ARDS patients with primary lung infection.

BAL was performed within the first 72 h after the beginning of mechanical ventilation. One segment of the lingula or the right middle lobe was lavaged with a total volume of 200 ml of sterile saline in 10 aliquots with a fluid recovery ranging between 50 and 70%. All BAL fluid fractions were pooled, filtered through sterile gauze, centrifuged at 200 x g (10 min, 4 °C) to remove cells and membraneous debris, and stored at -80°C for further investigation.

FSAP antigen and activity assay

A Maxisorp microtiter plate (Nunc, Wiesbaden, Germany) was coated with rabbit polyclonal antibody against FSAP at a concentration of 10 μg/ml overnight at 4°C in 50 mM $NaHCO_3$, pH 9.5. The plate was blocked with 3% (wt/vol) BSA in TBS-T (25 mM TRIS-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 1 h at room temperature and then incubated with the cell-

free BAL fluid or plasma. Fifty μl of BAL fluid or 50 μl of plasma were added to each well. The plasma samples were prediluted 1:100 with TBS-T. After 2 h incubation at 37°C, the plate was extensively washed and then incubated with a mixture of monoclonal antibodies against FSAP (#677 and #1189), followed by peroxidase-linked secondary antibody (Dako, Gostrup, Denmark). Final detection was performed with TMB Substrate Kit (Pierce, Rockford, Il), according to the manufacturer´s instruction. A standard curve was generated with purified FSAP. Standards and probes were run in triplicates.

FSAP activity was assessed by investigating its single chain urokinase activating potency. Microtiter plates were coated overnight at room temperature with mouse monclonal antibody #1189 raised against heavy chain FSAP at a concentration of 10 μg/ml in 50 mM $NaHCO_3$, pH 9.5. Subsequently, the plate was washed with PBS, 0.02 % Tween 20, pH 7.4 (washing buffer) and blocked with 0.02 M Na-citrate, 0.15 M NaCl, 2 % BSA, 0.1 M Arginine, pH 6.0. After 1 h incubation at 37°C, the blocking solution was discarded and 100 μl of BAL fluid prediluted 1:1 with dilution buffer (0.02 M Na-citrate, 0.15 M NaCl, 1% BSA, 0.1% Tween 80, 100 U/ml heparin, pH 6.0) or 100 μl of plasma prediluted 1:100 with dilution buffer were added to each well. After 1 h incubation at 37°C, the solution was removed and the plate was washed three times with washing buffer. Thereafter, 50 μl single chain urokinase (10 μg/ml) as well as 50 μl 0.05 M TRIS, 0.15 M NaCl, 0.2% Tween 80, 0.03 M $CaCl_2$, 100 U/ml heparin, pH 7.2 were added to each well. Standards and probes were run in triplicates. After 2 min incubation at room temperature, 100 μl S-2444 (L-Pyroglutamyl-glycyl-L-arginine-p Nitroaniline hydrochloride; Chromogenix, Molndal, Sweden) dissolved in TBS-T buffer was reacted at 0.6 mM for 1 h at 37°C, after which the reaction was stopped by addition of 50 μl 50% acetic acid. The change in absorbance at 405 nm was quantitated with the help of a standard curve, set up with a Standard Human Plasma pool. The protease content of this plasma pool was defined as one plasma equivalent unit per ml (PEU/ml).

Similarly, after capturing FSAP on the plate, FSAP activity in ARDS and control BAL fluids was also assessed by a direct chromogenic assay using the chromogenic substrate S-2288 (H-D-Isoleucyl-L-prolyl-L-arginine-p-nitroaniline dihydrochloride; Chromogenix). These experiments were performed both in the presence and absence of heparin.

Western blotting for the detection of FSAP

For Western blot analysis of the lung homogenate, lung tissue was homogenized in ice-cold lysis buffer (20 mM TRIS, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100, 2.5 µM Na-pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, pH 10, 1 mM PMSF, 1 µg/ml complete). After 30 min incubation on ice, the lung homogenate was centrifuged at 14,000 rpm for 15 min and the protein content in the supernatant was determined using the bicinchoninate method (BCA Assay, Pierce, Rockford, Il). Forty µg of protein each was separated on a 12% SDS polyacrylamide gel under reducing conditions, followed by electrotransfer to a PVDF membrane. After blocking with 5% non-fat dry milk in TBS-T buffer, the membrane was incubated overnight at 4°C with a mixture of both murine monoclonal antibodies against FSAP (#677 and #1189), followed by incubation with peroxidase-labelled secondary antibody (Dako, Gostrup, Denmark). Final detection of protein was performed using ECL Plus Kit (Amersham Biosciences, Freiburg, Germany). The membrane was stripped using stripping buffer (2% SDS, 100 mM β-mercaptoethanol in TBS) and reprobed with mouse anti-ß-actin antibody (Sigma-Aldrich). For Western blot analysis of BAL fluid and plasma, 15 µl lavage and 10 µl plasma (prediluted 1:10 with 0.9 % NaCl), respectively, were used. For the detection of FSAP uptake in cultured mouse alveolar macrophages, cells were lysed in SDS sample buffer and directly subjected to electrophoresis.

BAL fluid procoagulant and fibrinolytic activity

For measuring the recalcification clotting time of BAL fluids, 40 µl of a human plasma pool were mixed with 40 µl citrated BAL fluid (adjusted to a phospholipid concentration of 10 µg/ml) and were incubated for 5 min at 37°C. Clotting was initiated upon addition of 40 µl 20 mM $CaCl_2$, and clotting times were measured in triplicate samples using a KC10A microcoagulometer (Amelung, Lemgo, Germany). Clotting tests were performed in the absence or presence of the inhibitory antibody against human FSAP (#570) or isotype-matched mouse IgG, which were added to each BAL fluid sample at a final concentration of 1 µg/ml 16 h prior to the recalcification assay. The recalcification assay has also been performed with FSAP-deficient plasma.

The extent of BAL fluid-induced fibrin clot lysis was determined by a fluorogenic assay, whereby a solution of FITC-labeled fibrinogen (100 nM) and plasminogen (50 nM), diluted in a buffer containing 0.05 M TRIS-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, 10 µM $ZnCl_2$, pH 7.4, was mixed with 5 nM thrombin and then incubated for 2 h at 37°C. Thereafter, 50 µl of BAL fluid was added and the degree of clot lysis was quantified in the absence or presence of the inhibitory antibody against human FSAP (#570) with the help of a fluorescent plate reader and compared to baseline fluorescence of clots incubated with 0.9% NaCl.

Factor VII activation

Factor VII activation in BAL fluids was assessed by incubating 25 µl BAL fluid in the presence of 2.5 µl factor VII (0.04 U/µl; American Diagnostica, Stamford, CT) and 0.4 mM of a chromogenic substrate specific for factor VIIa (Spectrozyme®FVIIA; American Diagnostica). The change in absorbance at 405 nm was followed and factor VIIa generation was quantitated with the help of a factor VIIa protein reference (American Diagnostica). Baseline factor VIIa activity of the BAL fluids were substracted. Factor VII activation was determined in the absence or presence of an inhibitory antibody against human FSAP (#570)

or isotype-matched mouse IgG, which were added to each BAL fluid sample at a final concentration of 1 µg/ml 16 h prior to the assay.

Immunohistochemistry and immunocytochemistry for the detection of FSAP

Lung tissue specimens were fixed with 4% formaldehyde in PBS and subsequently embedded in paraffin. Five µm sections were mounted on poly-L-lysine-coated slides, deparaffinized in xylene and rehydrated through graded ethanol washes. Immunohistochemistry was performed using Histostain-SP Kit according to the manufacturer´s instruction (Zymed Laboratories Inc., San Francisco, CA). A mixture of anti-FSAP antibodies #677 and #1189 was diluted 1:800 in 1% BSA in TBS-T. Controls were performed by substituting the primary antibody by a non-specific antibody. For safe and reliable identification of FSAP positive cells, immunohistochemical staining was performed on serial sections using antibodies directed against CD68 (alveolar macrophages), von Willebrand factor (endothelial cells), vimentin (fibroblasts), and pro-surfactant protein C (alveolar type II cells). For the detection of FSAP uptake in cultured mouse alveolar macrophages, cells were incubated with human FSAP, washed twice with TBS buffer and then incubated for 10 min with 4% paraformaldehyde. After three washes with TBS, the cells were permeabilised for 5 min with 0.5% Triton X-100 in TBS, blocked for 2 h with 3% BSA in TBS-T, and then incubated for 1 h with a mixture of FITC-conjugated anti-FSAP antibodies #677 and #1189. Finally, the slides were washed three times with TBS buffer and mounted with fluorescence vectashield mounting medium (Vector, Burlingame, VE). In all cases, cell nuclei were counterstained with DAPI (Sigma-Aldrich). For microscopic inspection, a Leica DMR microscope was used.

Isolation and culture of cells

Lung microvascular endothelial cells (LMVEC) were purchased from Clonetics (San Diego, CA), seeded in T25 flasks and maintained according to the manufacturer´s specification in Microvascular Endothelial Growth Medium (CellSystems, Remagen Germany) supplemented with 5% FBS, 10 ng/ml human epidermial growth factor, 4 ng/ml human fibroblast growth factor, 2 ng/ml vascular endothelial growth factor, 75 µg/ml ascorbic acid, 0.2 µg/ml hydrocortisone, 1 µg/ml heparin, and 5 ng/ml insulin. Characterisation of LMVEC was performed on the basis of a positive staining for uptake of acetylated LDL, Factor VIII related antigen and CD31 expression, and negative staining for α-smooth muscle actin.

Human primary bronchial airway epithelial cells (PBEC) were isolated from non-utilized donor lungs or from parts of donor lungs that were not implanted due to lack of compatibility (for instance oversized grafts) as recently described.[4] Donors were without history of pulmonary disease at the time of lung transplantation, and histopathological evalulation did not forward inflammatory processes in the donor lungs. Lungs were explanted at the Department of Cardiothoracic Surgery of the Medical University of Vienna, Austria (Director: Prof. Dr. W. Klepetko). PBEC were maintained in keratinocyte serum-free medium (Invitrogen, Carlsbad, CA) supplemented with 0.2 ng/ml epidermal growth factor, 25 µg/ml bovine pituitary extract, 1 µM isoproterenol, 200 U/ml penicillin, and 200 µg/ml streptomycin. Identity and purity of isolated PBEC was verified by positive staining for cytokeratins 5 and 8, and negative staining for α-smooth muscle actin.

Human alveolar macrophages (AM) were obtained by bronchoalveolar lavage from healthy volunteers. The BAL cells were pelleted, washed twice with PBS (pH 7.4), and resuspended in RPMI 1640 medium (Pan Biotech, Aidenbach, Germany) supplemented with 10 % fetal bovine serum, 10 U/ml penicillin, 10 µg/ml streptomycin, and 2 mM L-glutamine. AM were purified by adherence to plastic tissue culture dishes for 60 min at 37°C as recently

described.[5] Identity and purity of AM was verified by Wrights-Giemsa stain and by immunostaining for CD68.

Cell stimulation, RNA isolation and reverse transcriptase (RT) reaction

Subcultures of human AM, LMVEC and PBEC were seeded in 6-well plates and either unstimulated or stimulated with various concentrations of LPS from *Escherichia coli* (0.01-1 μg/ml) for 4 hours or with 0.5 μg/ml LPS for 2-12 h. Furthermore, LMVEC were stimulated for 2-12 h with IL-6 (10 ng/ml), TNF-α (20 ng/ml), IL-8 (25 ng/ml) and IL-1β (5 ng/ml), respectively, or for 8 h with IL-8 (25 ng/ml) or LPS (0.5 μg/ml) in the absence or presence (1 μg/ml) of an anti-IL-8 antibody. All experiments were carried out with cells from passages 2-4. Cellular toxicity of the test substances was assessed by lactate dehydrogenase (LDH) cytotoxicity colorimetric assay according to the manufacturer´s instructions (Roche Applied Science, Indianapolis, IN).

Total cellular RNA was extracted using QIAzol^{TM} lysis reagent according to the manufacturer´s instruction (Qiagen, Hilden, Germany). One μg of RNA was reverse transcribed in a reaction containing 4 μl 5x First Strand Buffer, 2 μl dNTP (10 mM each; Finnzymes, Finland), 1 μl random hexamers (50 μM; Applied Biosystems, Foster City, CA), 1 μl DDT (0.1 M), 1 μl RNase inhibitor (40 U/μl; Applied Biosystems), and 1 μl Murine Leukemia Virus (MuLV) reverse transcriptase (200 U/μl; Applied Biosystems) in RNase-free water (final volume 20 μl). Reverse transcription was performed for 1 h at 39°C followed by heat deactivation for 2 min at 94°C.

Relative FSAP mRNA quantification by real-time PCR

The regulation of FSAP mRNA expression was analysed by real time quantitative PCR using the $\Delta\Delta$ C_T method for the calculation of the relative changes.[6] Real time PCR was

performed by the Sequence Detection System 7700 (PE Applied Biosystems). The reactions (final volume: 25 µl) were set up with Platinum SYBR Green qPCR Super Mix-UDG (Invitrogen) according to the manufacturer's protocol using 1 µl of cDNA. The following oligonucleotide primers were used: FSAP, forward primer, 5'-CAGAAACAGGAAAAGGGTCC-3'; FSAP reverse primer, 5'-CAGAGTCA-CCCTGGCAGG-3'; ß-actin, forward primer, 5'-ATTGCCGACAGGATGCAGGAA-3', ß-actin, reverse primer, 5'-GCTGATCCACATCTGCTGGAA- 3'. The reactions were incubated for 2 min at 50°C and then for 6 min at 95°C, followed by 45 cycles of 95°C for 20s, 58°C for 30s, and 73°C for 30s. Due to the non-selective dsDNA binding of the SYBR Green dye, melting curve analysis and gel electrophoresis were performed to confirm the exclusive amplification of the expected PCR product. In addition, identity of PCR products was confirmed by nested PCR and by sequencing.

Uptake of FSAP by mouse alveolar macrophages (AM)

C57/Bl6 mice were killed by intraperitoneal injection of a lethal dose of ketamine and xylazine. After sacrifice, the trachea was cannulated and the lungs were were lavaged with cold, sterile 0.9 % sodium chloride containing 5 mM EDTA until 4.5 ml of BAL fluid were recovered. BAL fluid AM were purified by adherence to plastic tissue culture dishes [5] and subsequently cultured in RPMI medium on cover slips. After overnight culture, cells were washed with HBS buffer (10 mM NaCl, 0.4 mM KCl, 1.0 mM Glucose, 1.8 mM Hepes, pH 7.4) and cultivated for 2 h in RPMI containing 1% FCS and 70 nM LysoTracker (Cambrex Bio Science, Walkersville, Maryland). Thereafter, AM were washed again and incubated with 2 µg/ml human FSAP in RPMI for 10, 30 or 60 min. After the indicated time points, immunostaining and western blot analysis for the detection of FSAP were performed as

described above. In the experiments involving chloroquine treatment, the cells were preincubated with 100 µM chloroquine 2 h prior to the addition of human FSAP.

RESULTS

Determination of FSAP activity in BAL fluids using a direct chromogenic assay

FSAP activity in ARDS and control BAL fluids was also determined using a direct chromogenic assay. These experiments were performed in the absence as well as in the presence of heparin, which is known to promote autoactivation of scFSAP. Utilizing this assay, only very low FSAP activity was detected in controls. In contrast, in ARDS BAL fluids a significant amount of FSAP activity was detectable, even in the absence of heparin. As expected, FSAP activity in ARDS BAL fluids in the absence of heparin was lower when compared to the values measured in the presence of heparin. However, the amounts of FSAP detected in the absence of heparin are assumed to represent primarily the active form of FSAP rather than total FSAP (active FSAP plus FSAP pro-enzyme). These findings give further support for the presence of active FSAP in ARDS BAL fluids (Figure 1).

Correlation of FSAP activity in ARDS BAL fluids and parameter of ARDS disease severity

Although statistically not significant, FSAP activity in ARDS BAL fluids, as assessed by a direct chromogenic assay, was positively correlated with a modified Acute Physiology and Chronic Health Evaluation (APACHE) II score, which further supports a potential role for

FSAP in this disorder (see Figure 2). Due to the use of sedatives, neurologic evaluation could not be performed consistently and was therefore, omitted from this modified score.

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FIGURE LEGENDS

Figure 1

Quantitation of FSAP activity in BAL fluid of ARDS patients as compared to healthy controls using a direct chomogenic substrate assay

FSAP activity in ARDS (n=10) and control (n=10) BAL fluids was assessed by a direct chromogenic assay in the absence (white boxes) or presence (grey boxes) of heparin. FSAP activity is depicted as absorbance at 405 nm. The box-and-whisker-plots indicate the median, 1st and 3rd quartile; the whiskers are extended to the most extreme value inside the 1.5-fold interquartile range. Significance levels are indicated (*** $p < 0.001$ for ARDS versus healthy controls).

Figure 2

Correlation of FSAP activity in ARDS BAL fluids and the Acute Physiology and Chronic Health Evaluation (APACHE) II score

FSAP activity was meassured by a direct chromogenic assay and is presented as absorbance at 405 nm. A modified version of the APACHE II score has been determined without neurologic evaluation that could not be performed consistently due to the use of sedatives.