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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ABSTRACT

Background: ARDS is characterized by inflammation of the lung parenchyma and alterations of the alveolar haemostasis with extravascular fibrin deposition. Factor VII-activating protease (FSAP) is a recently described serine protease in plasma and tissues, known to be involved in haemostasis, cell proliferation and migration.

Methods: We investigated FSAP protein (western blotting/ELISA/immunohistochemistry) and activity (coagulation/fibrinolysis assays) in plasma, bronchoalveolar lavage (BAL) fluids and lung tissue of mechanically ventilated patients with early ARDS as compared to patients with cardiogenic pulmonary oedema and healthy controls. Cell culture experiments were performed to assess the influence of different inflammatory stimuli on FSAP expression by various cell populations of the lung.

Results: FSAP protein level and activity were markedly increased in ARDS plasma and BAL fluids, thus significantly contributing to the increased alveolar procoagulant activity. In ARDS lungs, immunoreactivity for FSAP was observed in alveolar macrophages, bronchial epithelial and endothelial cells, as compared to controls, in which immunoreactivity for FSAP was restricted to alveolar macrophages. Only a low basal FSAP expression was detected in these cell populations. However, LPS and IL-8 induced FSAP-specific mRNA expression in human lung microvascular endothelial and in bronchial epithelial cells. We further demonstrated that FSAP is taken up by alveolar macrophages and degraded within the lysosomal compartment.

Conclusions: Increased levels and an altered cellular expression pattern of FSAP are found in ARDS lungs. This may represent a novel pathological mechanism contributing to pulmonary extravascular fibrin deposition. In addition, this may modulate inflammation in the acutely-injured lung via haemostasis-independent cellular activities of FSAP.
INTRODUCTION

The acute respiratory distress syndrome (ARDS) is characterized by an acute inflammation of the lung parenchyma and is caused by a variety of direct (e.g. pneumonia) and indirect (e.g. sepsis) insults.[1, 2] The acute phase of ARDS is associated with severe injury of the endothelial and epithelial barrier of the lung, leading to influx of protein-rich oedema fluid into the alveolar and interstitial compartment.[1, 2] The disturbed integrity of the capillary alveolar barrier and a marked alteration of the alveolar haemostatic balance are the underlying mechanisms for extravascular fibrin deposition in the alveolar space that is characteristic of diverse forms of ARDS.[3-5] Procoagulant activity is significantly increased in bronchoalveolar lavage (BAL) fluids of ARDS, and fibrinolytic activity is decreased. Tissue factor (TF) associated with factor VII and inhibition of urokinase-type plasminogen activator (u-PA) by plasminogen activator inhibitor (PAI)-1 are major factors that are responsible for the switch of the alveolar haemostatic balance.[3-5] These alterations of the alveolar haemostasis are observed in extrapulmonary as well as in pulmonary ARDS.[3] Tissue fibrin as well as coagulatory and fibrinolytic intermediates may influence inflammation and lung function in multiple ways: they can increase vascular permeability, influence the expression of inflammatory mediators, and alter the migration and proliferation of inflammatory cells.[6-9] Moreover, fibrin and its derivatives are strong inhibitors of surfactant function, contributing to alveolar collapse and gas exchange disturbances in the injured lung.[10] In addition, persistent fibrin deposition has been suggested to play a considerable role in the development of post-inflammatory lung fibrosis, providing a matrix on which fibroblasts can migrate and produce collagen.[11]

On this basis, several animal and clinical studies investigated the use of anticoagulants or fibrinolytic agents in the treatment of acute lung injury (ALI) and ARDS. In animals, an improvement of gas exchange, a reduction of inflammation and, at least in some studies, an increased survival have been demonstrated with the administration of anticoagulants such as TF pathway inhibitor (TFPI) [12], site-inactivated factor VIIa [13], heparin [14], recombinant hirudin [15], antithrombin [16], and activated protein C [17]. In addition, the aerosolization of heparin or urokinase [18], or overexpression of u-PA in the distal respiratory epithelium [19] effectively prevent the development of pulmonary fibrosis following inflammation in bleomycin-induced lung injury. In humans, administration of activated protein C has been shown to improve survival and lung function in patients with severe sepsis.[20]

Factor VII-activating protease (FSAP) is a recently described serine protease in plasma and tissues.[21, 22] FSAP is produced as a 64 kDa single chain zymogen (scFSAP), which is converted by autoactivation to the proteolytically active two chain form (tcFSAP).[23-25] tcFSAP consists of a 46 kDa heavy chain and a 29 kDa, active-site bearing light chain, connected by a disulfide bridge.[23-25] Once generated, tcFSAP is subjected to rapid autodegradation.[23-25] FSAP is mainly expressed in the liver, but the protein was detectable in various organs such as lung, kidney, placenta and pancreas.[21, 26] The precise role of FSAP in different physiological and pathophysiological states is presently not fully understood. A dual role of FSAP in haemostasis was recently discussed: FSAP is a potent activator of coagulation factor VII, thus promoting in vitro coagulation independently of TF.[22] Moreover, FSAP activates prourokinase to promote clot lysis in vitro.[27] In addition to its role in haemostasis, FSAP, like other haemostatic serine proteases, expresses cellular activities related to cell migration and proliferation. FSAP inhibits the proliferation and migration of vascular smooth muscle cells (VSMC) and endothelial cells in vitro.[28, 29] Locally applied FSAP was recently shown by our group to be a potent inhibitor of neointimal thickening in a mouse model of wire-induced injury of the femoral artery. This protective effect was mediated by a decrease in cell proliferation and a reduction in the number of VSMC and inflammatory cells in the neointima, pointing to a possible anti-inflammatory role for FSAP (Sedding et al., unpublished observations and [30]).
Up to now, FSAP has never been investigated in the context of pulmonary diseases. The objective of the present study was to investigate if FSAP contributes to the alterations of the alveolar haemostatic balance in the lungs of ARDS patients, thereby serving as a potential target molecule for therapeutic interventions. In addition, the cellular expression of FSAP under inflammatory conditions was investigated.

METHODS

Study population
All investigational measures were approved by the local ethics committee, and written informed consent was obtained from either the patients or their closest relatives.

BAL fluids were obtained by flexible fiberoptic bronchoscopy from 15 spontaneously breathing healthy volunteers without any history of cardiac or lung disease and with normal pulmonary function testing (medical students at the Medical School of the Justus-Liebig-University, Giessen, Germany) and from a total of 28 patients. All patients included in this study were recruited from the Intensive Care Unit of the Department of Internal Medicine at the Justus-Liebig-University Giessen, Germany, between 1999 and 2003. The following patient groups were investigated: extrapulmonary ARDS without pulmonary infection (ARDS; n=15), ARDS with primary lung infection (ARDS + Pneu; n=8), and cardiogenic pulmonary oedema (CLE; n=5). Patients fulfilling the inclusion criteria for the different groups as detailed in the online data supplement were included in the study.

All patients required mechanical ventilation. BAL was performed within the first 72 h after the beginning of mechanical ventilation. PaO₂/FiO₂ values, duration of mechanical ventilation, sex, age, and smoking history did not differ substantially among the different patient groups. Details on the demographic and clinical data of the patient groups and on the BAL procedure are outlined in the online data supplement.

In addition to BAL fluids, lung specimens from 7 patients with ARDS were obtained by autopsy. All patients met the clinical American-European Consensus Conference criteria [1] and died in the early phase with a median duration of mechanical ventilation of 92 h. Four patients had ARDS due to pneumonia, 3 patients had ARDS of extrapulmonary origin (sepsis). All lung specimens showed the histopathological pattern of diffuse alveolar damage that is characteristic of ARDS. As control, lung specimens were obtained by autopsy from 5 individuals who died of myocardial infarction (4 patients) or drug intoxication (1 patient). In each case, pathological conditions of the lung were ruled out by histological examination of lung tissue sections.

FSAP antigen and activity assay
A recently described ELISA technique [31] was used to determine FSAP antigen level in BAL fluids and plasma. FSAP activity was assessed by investigating its single chain urokinase activating potency as recently described [31] and by a direct chromogenic assay, as outlined in the online data supplement.

Western blotting
Western blotting for the detection of FSAP was performed using a mixture of two murine monoclonal antibodies directed against light and heavy chain of FSAP, respectively. Additional information is provided in the online data supplement.

BAL fluid procoagulant and fibrinolytic activity
The recalcification clotting time of BAL fluids in the absence or presence of an inhibitory antibody against human FSAP was measured using a microcoagulometer. The extent of BAL fluid-induced fibrin clot lysis was determined by a fluorogenic assay as detailed in the online data supplement.
Factor VII activation
Factor VIIa generation in BAL fluids in the absence or presence of an inhibitory antibody against human FSAP was assessed with a factor VIIa-specific chromogenic substrate as outlined in the online data supplement.

Immunohistochemistry
Immunohistochemistry for the detection of FSAP in formalin-fixed paraffin-embedded lung tissue was performed using Histostain-SP Kit according to the manufacturer’s instruction (Zymed Laboratories Inc., San Francisco, CA), with the same mixture of antibodies against FSAP as described for the western blot experiments. Controls were performed by substituting the primary by a non-specific antibody. For safe 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). Details are outlined in the online data supplement.

Cell culture
Lung microvascular endothelial cells (LMVEC) were purchased from Clonetics (San Diego, CA). Human primary bronchial airway epithelial cells (PBEC) were isolated from non-utilized donor lungs without history of pulmonary disease at the time of lung transplantation as recently described.[32] BAL fluid alveolar macrophages (AM) from healthy volunteers were purified by adherence to plastic tissue culture dishes as recently described.[33] Details on the cell culture conditions are provided in the online data supplement.

Cell stimulation, RNA isolation and reverse transcriptase (RT) reaction
Subcultures of LMVEC, PBEC and AM were either unstimulated or stimulated with various concentrations of LPS from Escherichia coli (0.01-1 µg/ml) for 4 h 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. Total cellular RNA was extracted using QIAzol™ lysis reagent (Qiagen, Hilden, Germany), and 1 µg of total RNA was reverse transcribed as detailed in the online data supplement.

Relative FSAP mRNA quantification by real-time PCR
The regulation of FSAP mRNA expression in stimulated cells was analysed by real-time quantitative PCR using the ∆∆ CT method for the calculation of relative changes.[34] Real-time PCR was performed by the Sequence Detection System 7700 (PE Applied Biosystems) as outlined in the online data supplement. ß-actin was used as reference.

Uptake of FSAP by AM
Mouse alveolar macrophages were pretreated with 70 nM LysoTracker (Cambrex Bio Science, Walkersville, MA) and subsequently incubated with 2 µg/ml human FSAP for 10, 30 or 60 min, followed by immunostaining and western blot analysis for the detection of FSAP at each time point, as described in the online data supplement. In some experiments, cells were preincubated with 100 µM chloroquine 2 h prior to the addition of human FSAP.

Statistics
The statistical analyses were performed in R, Version 2.3.1.[35] Deviations from the normal distribution were tested using the Shapiro-Wilk-test. All in vitro data were normally distributed, therefore, these data are presented as mean and standard deviation. Clinical data
are given as median and interquartile range. 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. Differences between two groups were tested with the Student's t- and Wilcoxon rank sum test, according to the distribution of the data. All tests were performed with an undirected hypothesis (two-sided). The level of statistical significance was set at 5%.”

RESULTS

ELISA experiments revealed highly elevated FSAP antigen levels in the BAL fluids of patients with extrapulmonary ARDS, as compared to healthy controls. Accordingly, FSAP activity in the BAL fluids of these patients was found to be significantly increased, regardless of whether FSAP activity was assessed by its single chain urokinase activating potency (Figure 1) or by a direct chromogenic assay (see Figure 1 of the online data supplement). In addition, a significant increase in FSAP antigen level and activity in the plasma of these patients was observed. FSAP antigen level and activity were also elevated in the BAL fluids of patients with pulmonary ARDS, but to a lesser extent. Only a slight increase in FSAP antigen and activity were noted in the plasma of this patient group. No significant change in FSAP antigen level and activity were found in BAL fluids and in plasma of patients with cardiogenic pulmonary oedema (Figure 1). In line with these observations, western blot experiments detected significant amounts of the 46 kDa heavy chain and the 29 kDa light chain of the proteolytically active two chain form of FSAP in plasma and BAL fluids of ARDS patients, but not in patients with cardiogenic pulmonary oedema (Figure 1). For the detection of the 29 kDa light chain in ARDS BAL fluids, BAL fluids have been concentrated prior to western blotting (inset of Figure 2B). Increased levels of heavy and light chain of the active two chain form of FSAP were also detected in the lung tissue of ARDS patients (Figure 2C). Interestingly, in contrast to BAL fluids heavy and light chain FSAP were detected in comparable quantities, but no single chain FSAP was detectable in the lung tissue. As a possible explanation, two chain FSAP may be stabilized when bound to the cell surface and/or extracellular matrix. Accordingly, we observed strong binding of two chain FSAP but not single chain FSAP to the extracellular matrix protein vitronectin (Wygrecka et al., unpublished observations).

FSAP has a dual role in haemostasis, involved in both, procoagulant and fibrinolytic pathways. Therefore, we investigated both these properties in BAL fluids from ARDS patients and healthy controls. We observed a significant contribution of FSAP to the increased procoagulant activity in the ARDS BAL fluids, as evident by a prolonged clotting time of these samples in the presence of a neutralizing antibody against FSAP (Figure 3A). Since the neutralizing anti-FSAP antibody may be also directed against activated FSAP in the standard human plasma used in these experiments, the coagulation assay has also been performed utilizing FSAP-deficient plasma. However, clotting times were not significantly altered using FSAP-deficient instead of standard human plasma (Figure 3A). In contrast to FSAP’s contribution to increased procoagulant activity, FSAP did not significantly alter fibrinolytic activity of the ARDS BAL fluids (Figure 3B). To further support a potential role for FSAP in coagulation processes in ARDS BAL fluids, we investigated the activation of factor VII in ARDS BAL fluids in the absence or presence of an inhibitory antibody directed against human FSAP. Factor VII activation in ARDS BAL fluids was significantly reduced by the anti-FSAP antibody (Figure 4). Taken together, these data indicate that FSAP contributes to procoagulant activity in ARDS BAL fluids via generation of factor VIIa.

Immunohistochemical studies showed a wider distribution of FSAP in ARDS lungs as compared to control lungs (Figure 5). FSAP positive staining in control lungs was restricted
to alveolar macrophages (Figure 5 d-f). Whereas, in ARDS lungs FSAP was present in macrophages, endothelial and bronchial epithelial cells (Figure 5 a-c). FSAP was also detected in BAL fluid alveolar macrophages of ARDS subjects and healthy controls (not shown).

To further characterize the cellular expression pattern of FSAP under inflammatory condition, cells that stained positive for FSAP in ARDS lungs were stimulated with LPS, and the expression of FSAP-specific mRNA in these cells was assessed by real-time RT-PCR. Only a low basal expression of FSAP was observed in these cell populations, but in lung microvascular endothelial cells (LMVEC) as well as in bronchial airway epithelial cells FSAP expression was inducible by LPS in a dose- and time-dependent manner. In endothelial cells, the effect of LPS on FSAP mRNA expression was dose dependent over the range of 0.01-0.5 µg/ml after 4 h treatment (Figure 6A). Higher LPS doses resulted in significant cell death as assessed by lactate dehydrogenase (LDH) cytotoxicity assay (not shown). Maximal stimulation was seen at 0.5 µg/ml (7-fold increase in FSAP mRNA expression as compared to unstimulated control; Figure 6A). This concentration was chosen to investigate time-dependent expression of FSAP mRNA in these cells. The expression of FSAP mRNA was doubled after 2 h treatment, and the maximal effect was seen at 8 h (8.5-fold increase; Figure 6B). In contrast to endothelial cells, bronchial epithelial cells were less sensitive to LPS stimulation. Maximal stimulation was seen at 0.5 µg/ml LPS after 8 h treatment (2.8-fold increase; Figures 6 C and D). Once again, higher LPS doses resulted in significant cell death. Since the strongest induction of FSAP mRNA expression was observed in LMVEC, we further examined FSAP mRNA expression in this cell type after treatment with various inflammatory mediators. No induction of FSAP expression was detected after IL-1β stimulation (Figure 7D), FSAP production was slightly increased by IL-6 (3-fold at 4 h; Figure 7A) and TNF-α (2-fold at 2 h; Figure 7B). Whereas, IL-8 treatment strongly upregulated FSAP mRNA level (7-fold at 8h; Figure 7C). We could further demonstrate that LPS-induced expression of FSAP in LMVEC was markedly inhibited by an anti-IL-8 antibody, indicating that LPS-dependent FSAP production in this cell type is at least partially mediated by endogenously produced IL-8 (Figure 8).

In contrast to endothelial and bronchial epithelial cell, FSAP mRNA expression was not inducible in alveolar macrophages by any of the inflammatory mediators (not shown). This observation led us to propose that the strong immunoreactivity for FSAP in alveolar macrophages is rather due to uptake and degradation of FSAP than to FSAP expression. The uptake of human FSAP by cultured mouse alveolar macrophages was subsequently investigated. After 10 and 30 min incubation, human FSAP was detected in mouse alveolar macrophages as assessed by immunocytochemistry (Figure 9B) and by western blotting of the macrophage cell extracts (Figure 9A), but a time-dependent loss of human FSAP in mouse alveolar macrophages was noted. Furthermore, co-localization of human FSAP and lysosomes in the cytoplasm of mouse alveolar macrophages revealed that FSAP was directed to the lysosomal compartment (Figure 9B). Addition of 100 µM chloroquine to the medium prevented the time-dependent loss of human FSAP in mouse alveolar macrophages by blocking lysosomal degradation (Figure 9A). These data indicate that FSAP is taken up by alveolar macrophages and degraded in the lysosomal compartment.

DISCUSSION

FSAP is a recently described serine protease in plasma and tissues, with a potential role in haemostasis, cell proliferation and inflammation.[21-30] In the present study, we demonstrated markedly increased FSAP protein level and activity in plasma and lungs of ARDS patients. FSAP was found to contribute to factor VIIa generation and to increased procoagulant activity in ARDS BAL fluids. Furthermore, a differential cellular distribution pattern of FSAP in the lung tissue of ARDS patients and healthy controls was observed,
whereby FSAP protein was detected in alveolar macrophages, bronchial epithelial and endothelial cells of ARDS lungs. FSAP expression in the latter cell types was found to be inducible by LPS and IL-8. Moreover, we identified alveolar macrophages to be centrally involved in FSAP metabolism in the lung, as they internalize FSAP followed by lysosomal degradation.

Alterations of FSAP level and activity in the BAL fluids of ARDS patients were compared to patients suffering from cardiogenic pulmonary oedema in the absence of ARDS and lung infection, showing a comparable duration of mechanical ventilation (52 versus 60 h), and a similar disturbance in gas exchange, as evident by similar PaO₂/FiO₂ ratios (184 versus 201 mm Hg). No significant change in FSAP level and activity in the BAL fluids of patients with cardiogenic pulmonary oedema as compared to healthy control was observed. These findings indicate that it is not the mechanical ventilation per se that is responsible for the observed changes in FSAP level and activity in the lungs of ARDS patients. Interestingly, virtually identical changes of FSAP level and activity were observed in the lungs of patients with extrapulmonary ARDS without pulmonary infection, and in ARDS with primary lung infection. This is in line with a previous study showing that altered levels of coagulatory and fibrinolytic intermediates can be observed in the BAL fluids of patients with acute inflammatory lung diseases, whether triggered by extrapulmonary systemic events or primary lung infection.[3]

The origin of increased FSAP level and activity in lungs of ARDS patients is presently not known. FSAP has previously been shown to be mainly expressed in the liver.[21, 26] Increased production of FSAP in the liver with subsequent (auto)-activation of FSAP may largely account for increased plasma levels of proteolytically active FSAP as observed in ARDS of extrapulmonary origin. Increased endothelial and epithelial permeability may then favour leakage of proteolytically active FSAP from plasma into the alveolar compartment. Extracellular RNA was recently identified to serve as a negatively charged surface to promote the (auto)-activation of FSAP [36], and may therefore be involved in the (auto)-activation of FSAP in the systemic circulation of ARDS patients. Accordingly, elevated RNA levels have been recently observed in the plasma of ARDS subjects (Wygrecka et al, unpublished observations). In contrast, only a slight upregulation of proteolytically active FSAP was observed in the systemic circulation of ARDS induced by primary lung infection. Therefore, in this patient entity as well as in extrapulmonary ARDS, leakage of the single chain zymogen from the blood into the alveolar space with subsequent activation to the proteolytically active two chain form in the alveoli may also contribute to increased FSAP levels and activity in ARDS lungs. Moreover, our data indicate that the lung itself, in particular endothelial and bronchial epithelial cells, may also represent a source of FSAP in ARDS.

Another important finding of our study is the observation that alveolar macrophages appear to be centrally involved in the clearance of FSAP from the lung. These investigations were driven by the finding that healthy controls as well as ARDS patients showed strong staining for FSAP protein in alveolar macrophages, despite very minor basal and inducible FSAP-specific mRNA expression in these cells. Our data evidently indicate that FSAP is taken up by alveolar macrophages and degraded within the lysosomal compartment. The detailed mechanism of FSAP uptake into alveolar macrophages needs to be clarified in further investigations. We recently observed complex formation between FSAP and PAI-1 in the BAL fluids of ARDS patients and in a purified system (Wygrecka et al. unpublished observations). Since complexes of PAI-1 with other serine proteases such as urokinase are internalized into cells via the low density lipoprotein receptor-related protein with subsequent degradation in lysosomes [37], this might be a possible mode of FSAP internalization into alveolar macrophages as well.
Increased levels of procoagulant FSAP in the lungs of ARDS patients as observed in the present study may represent a novel pathological mechanism contributing to ARDS progression. In line with these considerations, FSAP activity in ARDS BAL fluids was positively correlated with the Acute Physiology and Chronic Health Evaluation (APACHE) II score, however, the correlation did not reach statistical significance (see Figure 2 of the online data supplement). An increased alveolar procoagulant activity and persistent alveolar fibrin deposition are believed to contribute to the impairment of gas exchange and to the induction of post-inflammatory fibroproliferative processes in ARDS lungs [10, 11, 38]. In previous reports, TF and factor VII had been identified as major contributors to the increased procoagulant activity in the BAL fluids of ARDS patients. [3-5] The current data indicate that at least a part of the extrinsic coagulation pathway activation observed in the alveolar space of ARDS patients may be triggered by FSAP. Therefore, it seems to be reasonable to propose a potential contribution of FSAP to pulmonary fibrin deposition in ARDS, thereby serving as a potential target molecule for therapeutic interventions. On the other hand, FSAP expresses haemostasis-independent activities related to cell migration and proliferation, and a possible anti-inflammatory role for FSAP has recently been proposed via inhibition of inflammatory cell proliferation and migration. These activities are mediated by its interference with different growth factors and/or by its ability to activate prourokinase which is significantly enhanced in the presence of polyanions that can be found on cell surfaces and associated with the extracellular matrix. [27-29] In this context, it was recently demonstrated that the topical application of FSAP can inhibit wire injury-induced neointimal lesion formation in the mouse. This protective effect was mediated by its inhibitory effect on the proliferation and migration of vascular smooth muscle cells and monocytes/macrophages with a significant reduction in the number of inflammatory cells in the neointima of the FSAP-treated vessels (Sedding et al., unpublished observations and [30]). Keeping in mind these observations, it is well imaginable that increased levels and an altered expression of FSAP, as observed in the present study, may modulate inflammation in ARDS-lungs via its haemostasis-independent cellular activities. In this way, FSAP may even exert a beneficial effect in the acutely-injured lung. Exogenous administration and blockage of FSAP, respectively, in animal models of lung injury will further clarify if FSAP plays a critical role in ARDS and if FSAP is harmful or rather protective.

We conclude that increased levels and an altered cellular expression pattern of FSAP can be observed in the lungs of ARDS patients. This may represent a novel pathological mechanism contributing to alterations of the alveolar haemostasis and to extravascular fibrin deposition in ARDS-lungs, suggesting that FSAP may serve as a potential target molecule for therapeutic interventions. In addition, this may modulate inflammation in the acutely-injured lung via haemostasis-independent cellular activities of FSAP.
COMPETING INTERESTS

None of the authors has any financial relationship with a commercial entity that has an interest in the subject matter or materials discussed in the manuscript.
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ETHICS APPROVAL

The study was approved by the local institutional review board/ethics committees and informed consent was obtained from all patients or their legal representatives.
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FIGURE LEGENDS

Figure 1
Quantitation of FSAP antigen and activity in BAL fluid and plasma of ARDS patients as compared to healthy controls and patients with cardiogenic pulmonary oedema
FSAP antigen level, as assessed by ELISA (top panels), and FSAP activity, as assessed by its single chain urokinase activating potency (bottom panels), in BAL fluid (left) and plasma (right) of healthy controls (Control; n=15) and of patients with extrapulmonary ARDS without pulmonary infection (ARDS; n=15), ARDS with primary lung infection (ARDS + Pneu; n=8), and with cardiogenic pulmonary oedema (CLE; n=5) were quantitated. 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 = 0.016, ** p = 0.009, *** p < 0.001 for ARDS versus healthy controls).

Figure 2
Western blot analysis of FSAP protein in plasma, BAL fluid and lung tissue
Western blotting with FSAP specific antibodies was performed to characterize FSAP protein in (A) plasma and (B) BAL fluids of healthy controls (Control) and of patients with extrapulmonary ARDS without pulmonary infection (ARDS), ARDS with primary lung infection (ARDS + Pneu), and with cardiogenic pulmonary oedema (CLE) as indicated. Representative patients for each entity are shown (healthy controls: 2/15, CLE: 2/5, ARDS: 3/15; ARDS + Pneu: 2/8). The inset demonstrates the presence of the 29 kDa light chain of FSAP in concentrated ARDS BAL fluids, but not in controls (healthy controls: 3/3, ARDS: 3/3). Furthermore, FSAP protein was characterized in (C) lung homogenate of ARDS patients and controls (shown: myocardial infarction). Representative patients for each entity are shown (controls: 3/5, ARDS: 3/3; ARDS + Pneu: 3/4). sc FSAP - single chain FSAP; hc - heavy chain of proteolytically active two chain FSAP; lc - light chain of proteolytically active two chain FSAP

Figure 3
Analysis of FSAP procoagulant and fibrinolytic activity in BAL fluids of ARDS patients
(A) The procoagulant activity of BAL fluids from ARDS patients was assessed by plasma clotting assay in the absence or presence of an inhibitory antibody against FSAP (anti-FSAP) or a control antibody (IgG) utilizing standard human plasma (grey boxes) or FSAP-deficient plasma (white boxes). (B) BAL fluid-induced lysis of fibrin clots was assessed by means of a fluorogenic assay and is given for ARDS patients in the absence or presence of an inhibitory antibody against FSAP (anti-FSAP) or a control antibody (IgG) in relation to healthy controls. 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. n = 10. Significance levels are indicated (* p < 0.001 for ARDS versus ARDS plus anti-FSAP antibody).

Figure 4
Factor VII activation in ARDS BAL fluids
Factor VII activation in ARDS BAL fluids was assessed by means of a factor VIIa-specific chromogenic substrate assay in the absence or presence of an inhibitory antibody directed against human FSAP, or a control antibody (IgG). Data are presented in relation to ARDS BAL fluids in the absence of antibodies (100 %). 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. n = 10. Significance levels are indicated (* p = 0.03 for ARDS versus ARDS plus anti-FSAP antibody).
Figure 5
Localization of FSAP in lung tissue
Sections from lung tissue of ARDS patients (panels a-c) or controls (shown: myocardial infarction; panels d-f) were stained for FSAP with specific antibodies (brown colour). In ARDS, strong immunoreactivity for FSAP was observed in alveolar macrophages (a) and bronchial epithelial cells (c). In contrast, in controls FSAP protein was detected in alveolar macrophages only (d), but not in other cells such as endothelial (e) or bronchial epithelial cells (f). One representative patient out of 7 (ARDS) and 5 (control), respectively, is shown. Bar: 5 µm.

Figure 6
Dose-response and time-course induction of FSAP mRNA by LPS in lung microvascular endothelial cells and bronchial airway epithelial cells.
Lung microvascular endothelial cells (top panels) or bronchial airway epithelial cells (bottom panels) were stimulated for 4 h with 0.01-0.5 µg/ml of LPS (A and C, respectively), or treated with 0.5 µg/ml of LPS for 2-12 h (B and D, respectively). Total cellular RNA was isolated and reverse-transcribed into cDNA, and cDNA was analyzed for the mRNA expression of FSAP and β-actin by real-time PCR. Results are expressed as a ratio of target gene to β-actin mRNA control and are means ± SD for relative FSAP mRNA levels from five independent experiments. Significance levels are indicated (* p = 0.03 (endothelial cells after 12 h treatment), * p = 0.04 (bronchial airway epithelial cells after treatment with 0.1 µg/ml LPS and after 12 h treatment), ** p = 0.007, *** p < 0.001, all versus non-treated).

Figure 7
Time-course induction of FSAP mRNA by IL-6, TNF-α, IL-8 and IL-1β in lung microvascular endothelial cells
Lung microvascular endothelial cells were stimulated for 2-12 h with 10 ng/ml of IL-6 (A), 20 ng/ml of TNF-α (B), 25 ng/ml of IL-8 (C), and 5 ng/ml of IL-1β (D), respectively. Total cellular RNA was isolated and reverse-transcribed into cDNA. cDNA was analyzed for the mRNA expression of FSAP and β-actin by real-time PCR. Results are expressed as a ratio of target gene to β-actin mRNA control and are means ± SD for relative FSAP mRNA levels from five independent experiments. Significance levels are indicated (* p = 0.03, ** p = 0.02, *** p < 0.001, for stimulated versus non-stimulated cells).

Figure 8
FSAP mRNA expression by lung microvascular endothelial cells after stimulation with either IL-8 or LPS in the presence or absence of an anti-IL-8 antibody
Lung microvascular endothelial cells were stimulated for 8 h with either 25 ng/ml of IL-8 or 0.5 µg/ml of LPS in the presence or absence of an anti-IL-8 antibody. Total cellular RNA was isolated and reverse-transcribed into cDNA. cDNA was analyzed for the mRNA expression of FSAP and β-actin by real-time PCR. Results are expressed as a ratio of target gene to β-actin mRNA control and are means ± SD for relative FSAP mRNA levels from five independent experiments. Significance levels are indicated (* p < 0.001 for LPS versus LPS plus anti-IL-8 antibody and IL-8 versus IL-8 plus anti-IL-8 antibody, respectively).

Figure 9
Uptake of FSAP by alveolar macrophages
(A) After incubation of mouse alveolar macrophages with human FSAP in the absence (left panel) or the presence of chloroquine (right panel) for 10, 30 and 60 min, respectively, cell extracts were analysed by western blotting using an antibody specific for human FSAP. In parallel, cell extracts were analysed in the absence of FSAP (control = Co). hc - heavy chain of proteolytically active two chain FSAP. lc - light chain of proteolytically active two chain
FSAP. (B) At the indicated time points, cells were stained for human FSAP (left panels) or for lysosomes (LysoTracker, middle panels), and an overlay of both images was generated in each case (right panels). Bar = 5 µm. One representative experiment out of five independent experiments for each time point is shown. The insets show high power images of single macrophages.
REFERENCES


Figure 1

**BALF**

- FSAP antigen (ng/ml)
  - ARDS
  - ARDS + Pneu
  - CLE
  - Control

- FSAP activity (mPEU/ml)
  - ARDS
  - ARDS + Pneu
  - CLE
  - Control

**Plasma**

- FSAP antigen (µg/ml)
  - ARDS
  - ARDS + Pneu
  - CLE
  - Control

- FSAP activity (mPEU/ml)
  - ARDS
  - ARDS + Pneu
  - CLE
  - Control

*** indicates statistical significance.
Figure 2

A. 

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β-actin

Mr (kDa)
Figure 3

A.

B.

Clot lysis (% of healthy control)

Clotting time (sec)

control anti-FSAP IgG

ARDS
Figure 4
Figure 5
Figure 6

A: Fold induction of FSAP mRNA against LPS (µg/ml).

B: Fold induction of FSAP mRNA against Time (h).

C: Fold induction of FSAP mRNA at different LPS concentrations.

D: Fold induction of FSAP mRNA at different time points.

Legend:
- **": p < 0.01
- ***": p < 0.001
- *": p < 0.05

Bars represent mean ± SD.
Figure 7

A

fold induction of FSAP mRNA

B

fold induction of FSAP mRNA

C

D

Time (h)

Time (h)
Figure 8

The graph shows the fold induction of FSAP mRNA under different conditions:

- **Control**
- **anti-IL-8 antibody**
- **anti-IL-8 antibody**

The conditions are:

- **IL-8**
- **LPS**

The graph indicates a significant increase in mRNA induction with LPS compared to IL-8 and control conditions. The anti-IL-8 antibody treatment results in a reduction in mRNA induction compared to the IL-8 treatment.
Figure 9 A

**Chloroquine**

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</table>

- hc FSAP
- lc FSAP
- β-actin

Mr (kDa)

- 100
- 72
- 55
- 40

Time (min)
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)

Malgorzata Wygrecka, Philipp Markart, Ludger Fink, Andreas Guenther, and Klaus T. Preissner
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)), PaO2/FiO2 < 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, circumscribed 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 FEV₁ or FVC less than 65% predicted were excluded from the study. PaO₂/FiO₂ 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

<table>
<thead>
<tr>
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<th>Controls (n=15)</th>
<th>CLE (n=5)</th>
<th>ARDS (n=15)</th>
<th>ARDS + Pneu (n=8)</th>
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<td>Age (years)</td>
<td>47.3±4.1</td>
<td>57.3±5.1</td>
<td>52.6±6.8</td>
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<td>Male/female</td>
<td>9/6</td>
<td>4/1</td>
<td>11/4</td>
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<td>Never smoker (n)</td>
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<td>Ex smoker (n)</td>
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<td>Current smoker (n)</td>
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<td>PaO2/FiO2 (mmHg)</td>
<td>418±17</td>
<td>201±16</td>
<td>182±14</td>
<td>187±11</td>
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</table>

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 NaHCO₃, 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 monoclonal antibody #1189 raised against heavy chain FSAP at a concentration of 10 µg/ml in 50 mM NaHCO₃, 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 CaCl₂, 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 Na₃VO₄, 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-β-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 CaCl₂, 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 CaCl₂, 10 µM ZnCl₂, 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 epidermal 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 evaluation 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
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™ 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 ΔΔ 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’-CAGAGTCAA-CCCTGGCAGG-3’; β-actin, forward primer, 5’-ATTGCCGACAGGATGCAGGAA-3’, β-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.
REFERENCES


FIGURE LEGENDS

Figure 1
Quantitation of FSAP activity in BAL fluid of ARDS patients as compared to healthy controls using a direct chromogenic 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 measured 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.
Figure 1 (online data supplement)
Figure 2 (online data supplement)

![Graph showing the relationship between modified APACHE II score and FSAP activity absorbance at 405 nm. The graph includes data points and a linear trend line. The correlation coefficient R is 0.55, N is 10, and p is 0.1.](image-url)
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