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Factor VII-Activating Protease Is Activated in Multiple Trauma Patients and Generates Anaphylatoxin C5a

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Olivier Rannou,* Stephanie Denk,‡ Michael Etscheid,¶ Guenter Lochnit,*
Marcus Krueger,# and Markus Huber-Lang‡

Severe tissue injury results in early activation of serine protease systems including the coagulation and complement cascade. In this context, little is known about factor VII-activating protease (FSAP), which is activated by substances released from damaged cells such as histones and nucleosomes. Therefore, we have measured FSAP activation in trauma patients and have identified novel FSAP substrates in human plasma. Mass spectrometry-based methods were used to identify FSAP binding proteins in plasma. Anaphylatoxin generation was measured by ELISA, Western blotting, protein sequencing, and chemotaxis assays. Plasma samples from trauma patients were analyzed for FSAP activity and nucleosomes, C5a, and C3a. Among others, we found complement components C3 and C5 in FSAP coimmunoprecipitates. C3 and C5 were cleaved by FSAP in a dose- and time-dependent manner generating functional C3a and C5a anaphylatoxins. Activation of endogenous FSAP in plasma led to increased C5a generation, but this was not the case in plasma of a homozygous carrier of Marburg I single nucleotide polymorphism with lower FSAP activity. In multiple trauma patients there was a large increase in circulating FSAP activity and nucleosomes immediately after the injury. A high correlation between FSAP activity and C5a was found. These data suggest that activation of FSAP by tissue injury triggers anaphylatoxin generation and thereby modulates the posttraumatic inflammatory response in vivo. A strong link between C5a, nucleosomes, and FSAP activity indicates that this new principle might be important in the regulation of inflammation.

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Immediately after severe tissue trauma the coagulation system is massively activated, resulting in an extensive fibrin deposition and a concomitant activation of fibrinolysis (1, 2). Uncontrolled systemic inflammatory response develops, as exemplified by systemic mediator release and complement activation (3). Among others, factors released by necrotic cells such as nucleic acids, histones, high mobility group box 1, and other danger-associated molecular patterns contribute to the inflammatory spiral (4, 5). Early defense mechanisms of both innate immunity and the hemostasis system share some common factors as reviewed in detail (6, 7).

Factor VII-activating protease (FSAP) is a circulating serine protease that can activate factor VII as well as prourokinase (8). A single nucleotide polymorphism (SNP) in the FSAP gene (Marburg I [MI] SNP, G534E, 1601G/A) results in a protein with reduced enzymatic activity (9). Moreover, the MI-SNP was found to be strongly linked to carotid stenosis (10), cardiovascular disease in general (11), stroke and its related mortality (12), and plaque thickness and calcification (13, 14). Recent studies show that FSAP zymogen in plasma can be activated by histones and nucleosomes arising from necrotic and/or apoptotic cells (15, 16). This leads to the concept that cell injury may result in FSAP activation and that FSAP functions as a sensor of cell injury (16).

To extend this concept we have investigated the activation of FSAP over time in polytrauma patients and have correlated this with circulating nucleosomes as a marker of tissue necrosis. Furthermore, to gain more insight into the functions of FSAP we have used a proteomics-based approach to identify potential FSAP-interacting partners in the plasma. Using this strategy we have discovered that complement proteins form complexes with FSAP and that FSAP can cleave C3 and C5 to generate anaphylatoxins C3a and C5a. Hence, FSAP functions as a sensor of tissue injury and in turn activates anaphylatoxin production and the regulation of the inflammatory process. FSAP may be considered as a novel circulating danger-associated molecular pattern receptor that can modulate the inflammatory response.

Materials and Methods

Human plasma/FSAP interactions

To identify proteins in the circulation that form complexes with FSAP, citrate plasma samples from three different healthy normal subjects were subjected to immunoprecipitation with anti-FSAP (mAb 677; American
Diagnostica, Pfungstadt, Germany) and control mAb. After SDS-PAGE, the whole gel was cut into small bands and subjected to in-gel digest with trypsin. Reverse-phase nano-liquid chromatography and nanospray ionization mass spectrometry was performed by using an Agilent 1100/1200 nanoflow LC system (Agilent Technologies, Karlsruhe, Germany) equipped with a nanoelectrospray source (Proxeon, Schwerte, Germany). Mass spectrometry data were analyzed with MaxQuant and Mascot soft-ware for peptide and protein identification. The results were arranged according to the number of recovered peptides from each protein as well as the intensity of the peptides as described before (17). For identification of peptides and proteins a false discovery rate of 1% was used, and only peptides with a minimum of 6 aa length were considered for identification. After statistical analysis to determine which peptides show the highest difference between immunoprecipitation with the FSAP Ab compared with the control Ab across the three samples, a ranked list of proteins was obtained. Western blotting was performed to confirm the coimmunoprecipitation of various proteins with FSAP, and direct binding studies with purified proteins were performed to further characterize these interactions.

**Cleavage of C3 and C5 in vitro by FSAP**

In vitro experiments were performed by incubating native C3 (100 µg/ml) or C5 (100 µg/ml) (EMD, Darmstadt, Germany) in Dulbecco’s PBS in the absence or presence of FSAP at 37°C in a dose- and time-dependent manner. Single-chain FSAP zymogen was isolated from human plasma as described before (9). Dilution of FSAP into the end buffer leads to its rapid autoactivation into the enzymatically active two-chain form (9). Cleavage was followed by Western blotting with C3a and C5a Abs as well as ELISA using commercially available ELISA kits (Quidel, San Diego, CA and DRG Diagnostics, Marburg, Germany). For Western blotting, samples were separated by SDS-PAGE under reducing conditions and transferred onto a polyvinylidene difluoride membrane (Schleicher & Schuell, Keene, NH). The blots were incubated overnight at 4°C, using either polyclonal rabbit anti-human C3a IgG (EMD) or rabbit anti-human C5a IgG (EMD), and immunoreactive proteins were visualized using appropriate secondary Abs (Jackson ImmunoResearch Laboratories, West Grove, PA) and chemiluminescence detection kit (GE Healthcare, Heidelberg, Germany) or an alkaline phosphatase substrate color development buffer (Bio-Rad Laboratories, Hercules, CA).

For determining the cleavage sites, the nonreducing SDS-PAGE gels were run and blotted onto a polyvinylidene difluoride membrane, stained with Coomassie blue, and the protein bands were cut out for sequencing. The N-terminal sequences of the fragments were determined by automated Edman degradation using an Applied Biosystems 492 pulsed liquid phase sequencer equipped with an on-line 785A phenylthiohydantoin derivative analyzer (Applied Biosystems, Darmstadt, Germany). Seven cycles of Edman degradation were performed and the amino acids detected at each cycle were aligned with the C3/C5 sequence.

**Activation of endogenous FSAP in plasma and the concomitant generation of C3a/C5a**

Human plasma was incubated for 90 min at 37°C with various test substances that potentially activate FSAP. After incubation, the samples were precipitated of various proteins with FSAP, and direct binding studies with purified proteins were performed to further characterize these interactions.

Human plasma was incubated for 90 min at 37°C with various test substances that potentially activate FSAP. After incubation, the samples were precipitated of various proteins with FSAP, and direct binding studies with purified proteins were performed to further characterize these interactions.

**Results**

For neutrophil isolation, whole blood from healthy human volunteers was labeled with BCECF (2′,7′-bis(2-carboxylethyl)-5-(and 6)-carboxyfluorescein acetoxymethyl ester; Molecular Probes, Eugene, OR) for 30 min at 37°C. HMC-1 (mast cell line) was cultivated in basal Iscove’s medium containing 10% FCS, l-glutamine, antibiotics, and monothio-glycerol.

**Chemotaxis assays**

To determine the chemotactic activity of the C3a and C5a generated by FSAP, mast (HMC-1) cells or human neutrophils were used. BCECF-labeled neutrophils (5 × 10⁶ cells/ml) were loaded into the upper chamber of a 96-well device (Neuro Probe, Gaithersburg, MD) and separated by a 3-µm polycarbonate filter. The lower chambers were loaded with recombinant human C5a (50 ng/ml, positive control) or with indicated samples e.g., C5 in absence or presence of increasing concentrations of FSAP. After incubation at 37°C for 30 min, the number of cells that migrated through the polycarbonate membrane was determined by cytofluorometry (Cytofluor II; PerSeptive Biosystems, Framingham, MA). Similarly, to evaluate C5a-dependent chemotaxis activation, HMC-1 cells (mast cell line HMC-1 cells) and a 5-µm filter was used. Recombinant human C3a (100 ng/ml) served as a positive control.

**Polytrauma studies**

The study was approved by the Independent Local Ethics Committee of the University of Ulm (approval no. 44/06). A written informed consent was obtained from all volunteers and patients where possible. If the patient was unable to consent due to the injury pattern, sedation, or altered mental status, informed consent was obtained after recovery. The polytrauma cohort consisted of 12 patients (10 men, 2 women) with a median age of 36 y (range, 19-74 y) suffering from multiple injuries (injury severity score, 30.3 ± 2.9). Ten patients (85%) had sustained fractures to extremities, pelvis, or spine. Eight multiple injured patients were diagnosed to have a blunt chest trauma (67%), four patients sustained abdominal injuries (33%), and one patient had a major vascular injury. Eleven patients survived their injuries (92%) for at least 28 d. Four patients developed pneumonia (33%) and three patients showed signs of the sepsis syndrome (25%), whereas seven patients developed signs of multiple organ dysfunction syndrome (58%). Sex and age (distributions of the healthy volunteers (n = 12; medium age, 33 y) displayed a similar pattern in comparison with the trauma cohort. All enrolled trauma patients showed clinical signs of hemorrhagic shock requiring RBC transfusions (average, 12 RBC concentrates) and catecholamine support within the first 24 h (11 of 12 patients). Patients received standardized critical care and surgical damage control principles. All patients were followed up for a 28-d observation period. Blood was collected from a central line in citrate at the indicated time points and immediately centrifuged at 2000 rpm for 10 min to obtain plasma. Plasma was frozen and stored at −80°C.

**ELISA analysis of C3a, C5a, and FSAP Ag, FSAP activity, FSAP/α2-antiplasmin complex, and nucleosomes**

For quantitative analysis of C3a and C5a, commercially available ELISA kits (Quidel, San Diego, CA and DRG Diagnostics, Marburg, Germany) were used according to the manufacturers’ instructions. FSAP/α2-antiplasmin complexes and nucleosomes were measured as described before (16). FSAP Ag and activity were measured by ELISA as previously described (19).

**Statistical analyses**

Data are shown as means ± SEM. All in vitro experiments were performed in at least three independent replicates. Multiple significance testing was performed by ANOVA followed by a Tukey or Dunn posttest. For correlation analysis, the Spearman rank correlation coefficient was determined. Results were considered statistically significant when p < 0.05.

**Results**

Identification of FSAP-interacting proteins in human plasma

To further understand the functions of circulating FSAP, we used a hypothesis-free screening strategy to search for FSAP-interacting proteins in normal human plasma. For this we used the method of coimmunoprecipitation followed by mass spectrometry to identify the proteins. Immunoprecipitation of FSAP from plasma of healthy subjects (n = 3) led to the coimmunoprecipitation of a number of other proteins such as α2-macroglobulin, α1-trypsin inhibitor, heparin cofactor-2, C1 inhibitor, and α2-antiplasmin. Apart from
Role of FSAP in complement activation and C3a and C5a anaphylatoxin generation

The interaction of FSAP with a number of complement proteins in the plasma led us to hypothesize that FSAP could interact with the classical or alternative pathway of complement activation. Using an in vitro test, dependent on the generation and measurement of the C5b-9 complex (20), we found that the activation of the classical as well as the alternative pathway in serum was not influenced by the addition of exogenous FSAP or blocking endogenous FSAP by using a specific mAb or aprotinin. Similarly, no difference was observed in complement activation in the serum of a homozygous MI-SNP carrier in comparison with the normal genotype (data not shown). Because C3a and C5a anaphylatoxins can be generated by serine proteases of the coagulation and fibrinolysis system such as thrombin and plasmin (21), we tested whether FSAP also had a similar activity. Incubation of C3/C5 with FSAP led to the generation of the corresponding anaphylatoxin in a time- and dose-dependent manner as determined by ELISA and Western blot analysis (Fig. 2). The generation of C5a was faster and achieved with lower concentrations of FSAP as compared with that of C3a. Blocking the enzymatic activity of FSAP with a specific FSAP-blocking mAb could diminish C3a/C5a generation (Fig. 3).

To check the precise nature of C3a and C5a generated by FSAP, N-terminal sequencing was performed. Both C3 and C5 are composed of an α- and β-chain, and internal cleavage of the α-subunit releases the anaphylatoxin and the remainder is denoted C3α′ or C5α′, respectively. Cleavage of C3 with FSAP led to the generation of an ~10–14 kDa peptide with the N terminus of the expected C3α, whereas the C5α was truncated at the N terminus by 4 aa (Fig. 4A).

In a next step the biologic activity of the anaphylatoxins C3α/C5α generated by FSAP was tested in a chemotaxis assay. Incubation of both C3 and C5 with FSAP led to an increase in the chemotactic activity that paralleled the generation of the respective anaphyla-

FIGURE 1. Immunoprecipitation of FSAP and FSAP/protein complexes from plasma and its analysis by proteomics. (A) Normal human plasma was subjected to immunoprecipitation with a mAb against FSAP as well as a control mAb. Immunoprecipitates were run on SDS-PAGE and stained with Coomassie blue. (B) The immunoprecipitates were analyzed by Western blotting with Abs against FSAP, C3, C5, plasminogen (PLSG), C1 inhibitor (C1-Inh), and α2-Antiplasmin (α2-AP). (C) The gel from (A) together with two other samples was then cut into pieces and trypsinized and the peptides were eluted. Reverse-phase nano-liquid chromatography and nanospray ionization mass spectrometry was performed and was coupled to a LTQ Orbitrap instrument equipped with a nanoelectrospray source. Mass spectroscopy data were analyzed for peptide and protein identification. The ratio between immunoprecipitation with the FSAP Ab compared with the control Ab (x-axis) as well as the significance (p) value of this ratio (~log thereof represented on the y-axis) gave a ranked list of proteins. In the scatter plot, black dots indicate proteins that were significantly coimmunoprecipitated with FSAP (p < 0.01) and the others are indicated with gray dots. Only those proteins that showed a >2-fold log10 ratio of FSAP mAb/control mAb and a high significance (p < 0.002) are annotated.

Role of FSAP in complement activation and C3α and C5α anaphylatoxin generation

The interaction of FSAP with a number of complement proteins in the plasma led us to hypothesize that FSAP could interact with the protease inhibitors, other proteins found in the complexes included plasminogen, clusterin, ceruloplasmin, serum paraoxanase, as well as the central complement proteins C3 and C5 (Fig. 1, Supplemental Data 1). Western blotting for FSAP, C3, C5, plasminogen, C1 inhibitor, and α2-antiplasmin in the immunoprecipitates was performed to confirm these interactions (Fig. 1). Robust coimmunoprecipitation of FSAP with C3, plasminogen, C1 inhibitor, and α2-antiplasmin was observed whereas the coimmunoprecipitation of C5 was weaker. Strong direct binding between FSAP and immobilized plasminogen as well as fibrinogen was observed but there was no direct binding between FSAP and C5 or C3 (data not shown).

FIGURE 2. Concentration-dependent effect of FSAP on the generation of C3α and C5α. C5 (A) and C3 (B) were incubated with varying concentrations of FSAP for 90 min at 37°C and the generation of active C5α/C5α was measured with a specific ELISA. Values are given in ng/ml (mean ± SEM, n = 3). Similarly, the reaction mixtures were analyzed by Western blotting with specific Abs against C5α (C) and C3α (D). Values are given in ng/ml (mean ± SEM, n = 3). Statistical significance was determined by ANOVA and a Tukey posttest. Similar results were obtained in three independent replicates. *p < 0.05.
toxin as measured by ELISA (Fig. 4B, 4C). Hence, active C3a and C5a were generated by FSAP from the precursor proteins.

**Activation of endogenous FSAP in plasma and generation of C3a and C5a**

In the next step we investigated the effect of activating endogenous FSAP in human plasma on C3a and C5a generation. Moreover, we also tested plasma from a subject homozygous for MI-SNP (9) in which FSAP has very low enzymatic activity and is akin to a human “knockdown.” Addition of factors released from dead cells such as histones and model polyanionic molecules such as dextran sulfate into plasma could activate endogenous FSAP as determined by the formation of the 130-kDa FSAP/inhibitor complex as described before (15, 16) (Fig. 5A). The activation of FSAP was inhibited by aprotinin and it was weaker in plasma of a subject homozygous for MI-SNP (Fig. 5A). High-molecular mass heparin did not activate FSAP in plasma. FSAP activation was also inhibited by a specific FSAP-blocking mAb (no. 570) but not a control mAb (Supplemental Data 2), whereas other general inhibitors such as corn trypsin inhibitor or ε-aminocaproic acid, which inhibit factor XII or plasmin, respectively, did not influence FSAP activation. Although both FSAP and factor XII can be activated by negatively charged polyanions in vitro, factor XII was not activated by histones or nucleosomes (Supplemental Data 3). Hence, with respect to histones, FSAP activation is completely independent of factor XII or plasmin activation.

Measurements of C5a in these plasma samples showed that FSAP activation with histone and dextran sulfate, but not heparin, led to higher C5a generation, which was blocked in the presence of aprotinin (Fig. 5B). These activators or aprotinin did not interfere with the C5a ELISA, but mAbs interfered with the ELISA and, hence, FSAP-blocking mAbs could not be used in these experiments. The generation of C5a was much lower in the plasma of an MI-SNP homozygous subject (Fig. 5B). Zymosan, which is a potent activator of anaphylatoxin generation, did not influence the FSAP activation status (data not shown), and its influence on C5a generation was not affected by either aprotinin or the use of plasma of different genotypes (Fig. 5B). Hence, in MI-SNP plasma, C5a generation via FSAP was reduced but another FSAP-independent pathway was intact. C5a generation was also measured in FSAP-depleted plasma. In this experiment FSAP was depleted with anti-FSAP Ab beads as well as control Ab beads. We observed a strong C5a generation due to the process of immunodepletion with both Abs, and hence this experiment did not provide unambiguous results. Generation of C3a after activation with FSAP activators was low (<1.5-fold) but could also be reduced by aprotinin and was lower in MI-SNP plasma (data not shown). These results show a clear generation of C5a in plasma after activation of endogenous FSAP.

**FSAP activity, nucleosomes, and anaphylatoxins in the circulation of polytrauma patients**

In the above experiment endogenous plasma FSAP was activated with exogenously added histones. To further consolidate our hypothesis we investigated whether, in humans, activation of endogenous FSAP correlates with nucleosome release and C5a generation. We determined whether FSAP is activated in situations of severe tissue injury such as in polytrauma patients. Hence, plasma at different time points after trauma from 12 polytrauma patients as well as 12 normal healthy controls was systematically analyzed for evidence of FSAP activation, including release of nucleosomes that are associated with cellular injury as well as anaphylatoxins C5a/C3a. Immediately after injury there was a ~6-fold increase in FSAP activity (Fig. 6A) and a ~2-fold decrease in FSAP Ag
compared with normal controls (Fig. 6B). This drop in Ag may be explained by the loss of blood after multiple injury followed by RBC transfusion and fluid replacement. The activity to Ag ratio was 12-fold higher above controls within the first hour after trauma. The FSAP Ag and activity levels slowly returned to normal levels during a period of 24–48 h.

It has been recently shown that FSAP activation in plasma leads to an increase in FSAP/α2-antiplasmin complexes (15, 16) and that these serve as a surrogate marker for FSAP activation. In polytrauma patients the initial concentration of these complexes was high and decreased to normal levels after 48 h (Fig. 6C). These results were further confirmed by performing Western blot analysis of circulating FSAP. Authentic single chain C5a was measured with a chemotaxis assay with human neutrophils (B) and HMC1 mast cells (C), respectively (mean ± SEM, n = 6). *p < 0.05.

The above data support the concept that tissue injury releases factors that influence FSAP activation. In fact, it has been shown that in trauma patients there is an increase in circulating nucleosomes (22) as well as nucleic acids (23), so we also measured nucleosomes in our samples to determine the correlation with FSAP activity. Nucleosome concentration was high immediately after trauma and returned to baseline level at 24 h (Fig. 6D).

Circulating C5a level was increased 10-fold immediately after trauma and decreased progressively over time (Fig. 7A). In contrast, C3a concentrations showed a 2- to 3-fold increase that remained elevated during 5–10 d (Fig. 7B). Correlation analysis revealed that C5a, but not C3a, correlated strongly with FSAP activity and nucleosomes (Fig. 7C, 7D) (Table I).

Discussion

The hemostasis as well as the complement system show very high organizational similarities. Both consist of zymogens that are linked in a cascade leading to sequential activation of proteolytic activities and are often dependent on surface-mediated reactions.
Particularly, the kallikrein/kininogen arm of the contact pathway of the coagulation system might share activators as well as inhibitors with the complement system (24). Using a completely hypothesis-free screen to elucidate the function of FSAP in the circulation, we found evidence for a possible interaction with the complement pathway. Detailed analysis revealed that FSAP could generate anaphylatoxins from the precursor complement proteins C3 and C5. Furthermore, we provide quantitative evidence for massive activation of the FSAP zymogen in the circulation of patients with polytrauma. These findings fit well into our previously described concept of a noncanonical pathway for anaphylatoxin generation based on the activation by coagulation and fibrinolysis factors (21, 25).

It was surprising that the immunoprecipitation of FSAP led to the coimmunoprecipitation of a large number of proteins including many protease inhibitors. There could be many reasons why protease inhibitors were found in complex with FSAP: 1) even though the vast majority of circulating FSAP is in the zymogen form, a small fraction may be active and form complexes with inhibitors; 2) the zymogen FSAP could be activated by incubation with beads ex vivo; and 3) FSAP, in its zymogen form, may also bind to inhibitors. A further explanation as to why so many proteins coprecipitated with FSAP could be due to binding of FSAP to proteins such as plasminogen and fibrinogen, which then form a macromolecular complex responsible for binding other proteins in the plasma. There is a significant overlap between proteins in the FSAP complex and those known to bind to fibrinogen (26). In fact, direct binding assays showed a robust binding of FSAP to plasminogen and fibrinogen but not to C5 and C3. The screen could be refined by performing coprecipitation studies by using tagged FSAP. Nonetheless, the striking presence of complement proteins in the coprecipitates led us to investigate these interactions in further detail. Detailed analysis of the other candidates might also lead to further insight into the functions of FSAP in the circulation.

Comparison of C5a and C3a generation consistently showed that C5a was produced more rapidly (5 min) and at lower concentrations of FSAP (0.1 mg/ml) than was C3a. Both C5a and C3a generation was dose- and concentration-dependent and was inhibited by an FSAP-blocking Ab. Anaphylatoxin generation could be shown by ELISA, Western blotting, chemotaxis assays, as well as protein sequencing. FSAP-generated C3a showed two species in the range 10–14 kDa with an identical N-terminal sequence indicating cleavage at two different sites in the C-terminal region of C3a. Only one species of C5a was produced with FSAP with a N-terminal sequence 4 aa downstream of the classical C5a. Because the domain in C5a is responsible for binding and activation of C5aR residues in the C-terminal region (27), this would be expected to be a fully active ligand. The effect of FSAP was compared with that of plasmin (data not shown), which in our previous study was shown to be the most effective protease in anaphylatoxin generation (21). Comparisons carried out under the same conditions has shown that FSAP shows not only a similar activity as plasmin with respect to concentrations required but also the stronger propensity to generate C5a compared with C3a (21) (data not shown).

Table I. Correlations between FSAP activity, FSAP/α2-antiplasmin complex, nucleosomes, and C5a/C3a in human polytrauma patients

<table>
<thead>
<tr>
<th></th>
<th>FSAP Activity</th>
<th>Nucleosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSAP activity</td>
<td></td>
<td>0.71 (p &lt; 0.0001)</td>
</tr>
<tr>
<td>FSAP/α2-antiplasmin</td>
<td>0.95 (p &lt; 0.001)</td>
<td>0.76 (p &lt; 0.001)</td>
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<tr>
<td>C5a</td>
<td>0.72 (p &lt; 0.0001)</td>
<td>0.64 (p &lt; 0.0001)</td>
</tr>
<tr>
<td>C3a</td>
<td>0.12 (p = 0.322)</td>
<td>-0.09 (p = 0.488)</td>
</tr>
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</table>

FSAP activity and nucleosome concentrations were correlated with FSAP/α2-antiplasmin complexes, C5a, and C3a. A Spearman rank correlation coefficient and p values were determined.
This activation could be inhibited by aprotinin as well as a specific FSAP-blocking Ab. Activation was strongly reduced in the plasma of a MI-SNP homozygous carrier. This activation of endogenous FSAP led to the generation of C5a in the plasma, and this was inhibited in the presence of aprotinin as well as in MI plasma. Activation of C5a generation with zymosan, which does not activate FSAP, was identical in the presence of aprotinin and in MI plasma. These results confirm the linkage between FSAP activation and C5a generation in plasma. In these experiments in plasma as well, C3a generation showed the same pattern but was much weaker. This highlights a consistent difference between C3a and C5a generation in the in vitro experiments as well as in the plasma experiments.

Previous studies have shown that in patients with various infectious diseases or after LPS application in mice that there is an increase in FSAP/inhibitor complexes (15, 16). However, these assays used did not allow for a quantitative analysis of FSAP activation in these situations. We have now cross-validated the assay to measure FSAP activity in plasma with the presence of FSAP/inhibitor complexes and Western blotting and there is a very high correlation between all three parameters. In polytrauma patients there was a robust increase in FSAP activity in the face of decreased FSAP Ag in the immediate aftermath of trauma, and these values reached their respective basal levels after 24 h. There was a high correlation between FSAP activity and circulating nucleosome concentrations, indicating that both of these parameters are causally linked. This pattern fits well with our observation in patients with bacterial infections (15, 16). Plasma C5a levels correlated strongly with FSAP activity, but this was not the case with C3a. It is possible that activated FSAP also generates C3a in vivo but that the high turnover of C3a does not allow for a significant increase in its circulating levels.

It has been suggested that the process of hemodilution can nonspecifically activate coagulation factors (28) and support thrombin generation (29). To test the possibility that blood loss and fluid replacement with plasma expanders does not directly lead to FSAP activation, measurements were performed in a rabbit and pig model of blood loss followed by transfusion of erythrocytes and plasma expanders. In both models there was a decrease in FSAP activity. Hence, FSAP activation can be considered to be the result of the original traumatic event rather than hemodilution and the use of plasma expanders.

Therefore, trauma induces an increase in circulating nucleosomes that in turn correlates strongly with increased FSAP activity and C5a levels. Although a strong correlation does not necessarily indicate a mechanistic link between the FSAP, nucleosomes, and C5a, our plasma studies where C5a generation by histones was reduced in situations where FSAP was inhibited provide strong evidence for such a mechanism in vivo. This fits well into the observation that various serine proteases of the coagulation and fibrinolysis system are capable of generating anaphylatoxins C3a and C5a (21).

To the previously known substrates of FSAP such as coagulation factor VII and fibrinolysis factors (prourokinase plasminogen activator), as well as growth regulating factors such as platelet-derived growth factor BB homodimer and basic fibroblast growth factor, we can now add the central complement factors C3 and C5. This indicates that FSAP is a broad-spectrum protease with multiple functions. These multiple functions may contribute to hemostasis, repair and regeneration, as well as inflammation. Anaphylatoxins are potent inflammatory mediators that are largely produced by the activation of various complement pathways after trauma (30) and during the systemic inflammatory response. In trauma patients the activation of anaphylatoxins may be harmful due to perpetuation of inflammation, and their inhibition could be beneficial (31). Based on our results we hypothesize that the outcome of trauma might be different in carriers of the MI-SNP, which is akin to a natural FSAP knockdown. One would predict that generation of C5a might be lower in MI-SNP carriers, which might dampen the inflammatory response in trauma. In this case
routine determination of this SNP may guide the course of therapeutic interventions in trauma patients. This would mirror the situation in patients with meningococcal sepsis where increased levels of C5 (32) as well as nucleosomes and FSAP/inhibitor complexes (16) contribute to poor disease outcome.

Acknowledgments
We acknowledge the excellent technical assistance of S. Tannert-Otto and B. Acker as well as Drs. G. Dickneite and I. Pragst (CSL Behring, Marburg, Germany) for providing plasma samples from the rabbit and porcine models of trauma.

Disclosures
The authors have no financial conflicts of interest.

References
List of proteins co-immunoprecipitated with a FSAP Mab compared to control Mab. Ratios are indicated for those proteins with a significant difference (p <0.01), as well as a positive increase, in FSAP Mab compared to control Mab.

<table>
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<th>p value</th>
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<td>Apolipoprotein(a)</td>
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<td>7.8E+06</td>
<td>0.0077</td>
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<tr>
<td>CFHR5; CFHL5</td>
<td>complement factor H-related 5</td>
<td>3.19</td>
<td>1.8E+07</td>
<td>0.0074</td>
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<tr>
<td>CRP; PTX1</td>
<td>C-reactive protein</td>
<td>5.45</td>
<td>8.3E+06</td>
<td>0.0040</td>
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<tr>
<td>SERPING1; C1NH</td>
<td>C1 esterase inhibitor</td>
<td>3.52</td>
<td>1.1E+08</td>
<td>0.0031</td>
</tr>
<tr>
<td>F2</td>
<td>Prothrombin; Coagulation factor II</td>
<td>3.93</td>
<td>2.1E+07</td>
<td>0.0027</td>
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<tr>
<td>PLG</td>
<td>Plasminogen</td>
<td>5.08</td>
<td>2.0E+09</td>
<td>0.0025</td>
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<tr>
<td>FCGBP</td>
<td>IgGFc-binding protein</td>
<td>0.28</td>
<td>7.0E+08</td>
<td>0.0023</td>
</tr>
<tr>
<td>ITIH4; HRP; ITIH1</td>
<td>Inter-alpha-trypsin inhibitor heavy chain H4</td>
<td>3.65</td>
<td>9.1E+08</td>
<td>0.0010</td>
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<tr>
<td>SERPINA3; AACT</td>
<td>Alpha-1-antichymotrypsin</td>
<td>3.34</td>
<td>1.1E+08</td>
<td>0.0009</td>
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<tr>
<td>VTN</td>
<td>Vitronectin</td>
<td>3.48</td>
<td>1.1E+09</td>
<td>0.0009</td>
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<tr>
<td>IGKV A18</td>
<td>Kappa light chain variable region</td>
<td>3.44</td>
<td>9.3E+07</td>
<td>0.0007</td>
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<tr>
<td>APOD</td>
<td>Apolipoprotein D</td>
<td>3.95</td>
<td>1.0E+08</td>
<td>0.0006</td>
</tr>
<tr>
<td>PON1</td>
<td>Serum paraoxonase/arylesterase 1</td>
<td>5.91</td>
<td>1.3E+08</td>
<td>0.0006</td>
</tr>
<tr>
<td>SERPINA4; KST; PI4</td>
<td>Kallistatin; Kallikrein inhibitor</td>
<td>4.06</td>
<td>1.1E+07</td>
<td>0.0006</td>
</tr>
<tr>
<td>C8B</td>
<td>Complement component C8 beta chain</td>
<td>3.98</td>
<td>2.0E+07</td>
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<td>PGLYRP2</td>
<td>N-acetylmuramoyl-L-alanine amidase</td>
<td>6.14</td>
<td>4.9E+07</td>
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<tr>
<td>SERPINF2; AAP; PLI</td>
<td>Alpha-2-antiplasmin</td>
<td>4.76</td>
<td>2.1E+09</td>
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<td>FLJ00382; IGHD</td>
<td>FLJ00382 protein; Ig delta chain C region</td>
<td>6.64</td>
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<td>CLU; APOJ; CLI</td>
<td>clusterin. SP-40.40</td>
<td>3.26</td>
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<td>HABP2</td>
<td>Hyaluronan-binding protein 2; FSAP</td>
<td>2.97</td>
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<tr>
<td>C9</td>
<td>Complement component C9</td>
<td>4.33</td>
<td>3.0E+07</td>
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<td>SERPIND1; HCF2</td>
<td>Heparin cofactor 2</td>
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<td>C3; CPAMD1</td>
<td>Complement C3</td>
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<td>7.4E+09</td>
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<td>PON3</td>
<td>Serum paraoxonase/lactonase 3</td>
<td>5.76</td>
<td>2.8E+07</td>
<td>1 E-05</td>
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<tr>
<td>CP</td>
<td>Ceruloplasmin; Ferroxidase</td>
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<td>5.1E+08</td>
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<td>C5; CPAMD4</td>
<td>Complement C5</td>
<td>5.21</td>
<td>4.9E+08</td>
<td>5 E-06</td>
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<td>A2M; CPAMD5</td>
<td>Alpha-2-macroglobulin</td>
<td>3.10</td>
<td>2.3E+09</td>
<td>1 E-07</td>
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</table>
Activation of endogenous FSAP in plasma: Endogenous FSAP was activated in human pooled plasma to varying degree by the addition of histones, chromatin and dextran sulphate (DS) (μg/ml) for 120 min at 37°C in the presence of a control Mab. or a Mab that blocks the FSAP enzyme activity (#570, 50 μg/ml). Plasma samples were then analyzed by Western blotting with a polyclonal antibody against FSAP to determine the formation of FSAP-inhibitor complexes.
Activation of endogenous FSAP and FXII in human plasma: Endogenous FSAP and FXII was activated in human pooled plasma to varying degree by the addition of histones, chromatin and dextran sulphate (DS) (μg/ml) in the presence of aprotinin (75 μg/ml), corn trypsin inhibitor (CTI) (50 μg/ml) or epsilon amino caproic acid (EACA) (100 mM) for 120 min at 37°C. Plasma samples were then analyzed by Western blotting with a polyclonal antibody against FSAP or FXII to determine their respective activation by formation of complexes with inhibitors. FXII was activated by dextran sulphate but not by histones or chromatin whereas FSAP was activated by all of them. FXII activation by DS was inhibited by CTI and aprotinin whereas FSAP activation was not inhibited by CTI or EACA. FSAP activation through histones is not dependent on the activation of FXII or plasminogen.
Western blot analysis of FSAP antigen in the circulation of patients with trauma:
Serial time-dependent plasma samples from 2 patients with trauma (0.2 μl) were analyzed by Western blotting with a polyclonal Ab against FSAP. FSAP migrates just below 72 kDa and FSAP-inhibitor complexes around 130 kDa which most likely represents FSAP-α2-antiplasmin complex. Different exposure times were required to visualize FSAP as compared to FSAP-inhibitor complex. The presence of FSAP inhibitor complexes mirrors quite well the FSAP-α2-antiplasmin complex, FSAP activity and the FSAP antigen levels measured by ELISA. Subject No. 2 (●) and subject No. 11 (○).