Factor VII-activating protease: Mechanism and regulation of nucleosome release from dead cells
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Differential activation of factor VII-activating protease (FSAP) by various blood cells

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Abstract

**Introduction:** Factor VII-activating protease (FSAP) has been reported as plasma protease responsible for nucleosome release from late apoptotic and necrotic cells. FSAP circulates as an inactive single-chain molecule and is activated upon contact with dead cells. FSAP activation has been observed in several inflammatory conditions. Neutrophils are the main effector cells seen early in the response to pathogens. They have a short half-life and high turnover rate. Moreover, they have the ability to release their nucleosomes in the form of neutrophil extracellular traps (NETs). The aim of this project is to investigate the activation of FSAP by neutrophils and to identify the structure(s) responsible for FSAP activation.

**Methods:** Formation of complexes of FSAP with its inhibitor α₂-antiplasmin is used as a measure for FSAP activation. Plasma was incubated with necrotic cells and FSAP-α₂-antiplasmin complexes were measured by ELISA. To get more insight in which organelle of the cell is involved in FSAP activation, binding of FSAP to cells was analyzed by confocal microscopy.

**Results and Conclusions:** PBMCs were able to activate FSAP in serum. Interestingly, necrotic neutrophils fail to activate FSAP in the serum. The absence of activation was not due to proteolysis of FSAP-α₂-antiplasmin complexes or the activator of FSAP present in the cells. FSAP displayed a strong nucleolar staining and diffuse staining pattern in the cytoplasm. Several forms of purified RNA failed to activate FSAP in serum. In conclusion, we show that necrotic neutrophils are not capable of activating FSAP. Neutrophils lack a factor that is involved in FSAP activation. The nucleolar binding of FSAP and the lack of activation by RNA might suggest that a ribosomal protein is involved in FSAP activation.
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Introduction

FSAP, also known as plasma hyaluronic acid binding protein 2 (HABP2), has been initially described as a protease which was able to activate factor VII (FVII) in vitro in the absence of tissue factor (TF) (1). We recently have reported a novel role for FSAP, being the plasma protease responsible for nucleosome release from late apoptotic cells (2). In addition, in cooperation with serum deoxyribonuclease I (DNase I), FSAP can release nucleosomes from necrotic cells (3). In purified systems, several inhibitors such as C1-inhibitor (C1inh), α2-antiplasmin (AP), antithrombin III (AT-III), tissue factor pathway inhibitor (TFPI) and PAI-1 have been reported to inhibit the amidolytic activity of two-chain FSAP (tcFSAP). In plasma, C1inh and AP have been demonstrated to form complexes with activated tcFSAP (4).

FSAP is a serine protease circulating as an inactive single-chain molecule of 78 kDa in plasma. In purified systems, single-chain FSAP (scFSAP) has been reported to be susceptible to autoproteolysis. Upon activation scFSAP is converted to its active two-chain form consisting of a 50 kDa heavy and a 28 kDa light chain, connected by a disulfide bond (5). Kannemeier et al. have shown that this autoactivation was enhanced in the presence of heparin, whereas Ca2+ ions stabilized scFSAP (6). Negatively charged polyanions, such as heparin, polyphosphates, DNA and RNA have been demonstrated to accelerate autoproteolysis of purified scFSAP (1;5;7-9). Also polycations, such as polyamine and histones have been demonstrated to activate scFSAP (10;11).

However, FSAP in plasma is quite resistant to autoactivation (4). Upon purification FSAP becomes very susceptible to autoactivation (2). For a molecule described to be involved in coagulation and fibrinolysis is seems unusual that even total coagulation of blood does not lead to FSAP activation. Moreover in serum no activation of FSAP was observed. We have shown that FSAP in plasma becomes activated upon contact with either late apoptotic or necrotic cells (4).

We have observed FSAP activation in post-surgery patients, and patients suffering from severe sepsis, septic shock, and meningococcal sepsis (4). The inflammatory response during sepsis leads to the induction of widespread cell death and levels of FSAP activation correlated with nucleosome levels, disease severity and mortality in these patients (4). Neutralization of nucleosomes and DNA-binding proteins such as histones has been shown to attenuate the pro-inflammatory response and rescue animals in lethal sepsis models (12).

Neutrophils are the main effector cells to recognize and destroy foreign bacteria at sites of infection. Neutrophils belong to the class of polymorphonuclear cells (PMNs), which contain nuclei with a multilobulated shape. They constitutively undergo apoptosis both in vivo and in vitro. Upon maturation, neutrophils are released into the bloodstream where they circulate for ~10–24 hours before migrating into tissue where they may function for an additional 1–2 days before undergoing apoptosis and being cleared by macrophages (13;14). Recently,
it has been reported that activation of neutrophils can lead to the formation of neutrophil extracellular traps (NETs). These NETs consist of DNA, histones and antimicrobial enzymes and can be secreted by neutrophils to trap and kill pathogens (15). Neutrophils have been shown to contribute to the initiation and progression of severe sepsis and may induce organ damage through the extracellular release of proteolytic enzymes (16-18), oxygen radicals (19;20) and neutrophil extracellular traps (NETs) (21).

Since there is a high turnover of neutrophils and they have the ability to release their nucleosomes in the form of NETs, the aim of this project is to investigate the activation of FSAP by neutrophils and to identify the structure(s) responsible for FSAP activation.

**Materials and methods**

**Reagents**

Mouse monoclonal antibodies anti-FSAP4 (2) and AAP20 to α\(_2\)-antiplasmin were prepared at our department. Iscove’s modified Dulbecco’s medium was obtained from BioWhittaker Europe (Verviers, Belgium). Fetal calf serum was obtained from Bodinco BV (Alkmaar, The Netherlands). High performance ELISA buffer (HPE) and Poly-HRP labelled streptavidin were obtained from Sanquin (Amsterdam, The Netherlands). (3,5,3′,5′)-tetramethylbenzidine (TMB) was obtained from Merck (Darmstadt, Germany). 4′,6-diamidino-2-phenylindole (DAPI) was obtained from Sigma (Zwijndrecht, The Netherlands). HEp2 ANA slides were obtained from INOVA Diagnostics (San Diego, USA). Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody (GaM-FITC) was from DAKO (Glostrup, Denmark). All sera from healthy donors (HD) have been obtained as anonymized samples from the diagnostic laboratory, according to the Dutch rules and regulation for the use of patient material. All donors were homozygous for the wild-type form of FSAP (22).

**Cell culture, isolation of PMNs and PBMCs and induction of necrosis**

Human PBMCs and neutrophils were isolated by density gradient centrifugation on Lymphoprep™ from citrated blood from healthy donors. PBMCs were washed and resuspended with HN-buffer (10 mM Hepes, 140 mM NaCl, pH 7.2). Following erythrocyte lysis, neutrophils were washed and resuspended with HN-buffer. Necrosis was induced by 3 cycles of freezing and thawing the cells (5x10\(^6\) cells/ml).

**FSAP activation**

FSAP was activated by incubating serum for 30 minutes at 37°C with necrotic cells (2.5x10\(^6\) cells/ml) in HN-buffer. Samples were centrifuged at 1300 x g for 10 minutes and FSAP activation was measured in the supernatant by detection of FSAP- α\(_2\)-antiplasmin (FSAP-AP) complexes.
Determination of FSAP-α₂-antiplasmin complexes by ELISA

FSAP-AP complex levels were determined by an ELISA as recently described (4). Briefly, a monoclonal antibody (AAP20) which recognizes α₂-antiplasmin was used as a catching antibody. Biotinylated anti-FSAP4 which recognizes the light chain of FSAP in combination with poly-HRP was used for detection.

Confocal microscopy

HEp2 ANA slides were used containing a human epithelial cell line (HEp2) optimally-fixed on a 12 wells slide. All incubation steps were performed in a humid plastic container. HEp2 ANA slides were incubated with plasma of a healthy donor (1:5 dilution in PBS) for 30 min at RT. After washing with PBS, the cells were incubated with anti-FSAP4 (50 µg/ml) or a control monoclonal antibody of the same isotype with an irrelevant specificity (anti-IL6.8, 50 µg/ml), for 30 min at RT. Antibodies were diluted in PBS with 0.5% bovine serum albumin (BSA). The cells were washed with PBS and incubated with FITC labeled GaM (1:5) and DAPI (1:1.000.000). After 30 min incubation in the dark, de cells were washed again with PBS and analyzed by confocal microscopy (Zeiss LSM 510 META/TIRF microscope) and Zeiss micro imaging software (Carl Zeiss 2009 Zen).

Statistics

Results are expressed as mean ± SEM. The Mann-Whitney rank-sum test was used to assess differences between groups at a given time. A P-value < 0.05 was considered to be statistically significant.

Results

Lack of FSAP activation by dead neutrophils

Recently we have shown that FSAP in serum is activated upon contact with late apoptotic as well as necrotic cells. A Jurkat cell line was used for these experiments. To investigate the activation of FSAP in serum by different types of primary cells, peripheral blood mononuclear cells (PBMCs) and polymorphonuclear cells (PMNs) were isolated from blood of a healthy donor (HD), necrosis was induced and cells were incubated with HD serum. After incubation, FSAP-α₂-antiplasmin (FSAP-AP) complex formation was detected by ELISA as a measure for FSAP activation. PBMCs were able to activate FSAP in serum. Interestingly, necrotic PMNs fail to activate FSAP in the serum (Figure 1). To exclude that FSAP is a property of cell lines exclusively we also tested lymphocytes and monocytes isolated from peripheral blood. Both necrotic lymphocytes and monocytes were able to activate FSAP in serum (data not shown).
Proteolysis by neutrophil proteases
The next step was to investigate whether there was a lack of activation by the PMNs or inhibition of the activation by a factor in the PMNs. Since PMNs are known to release proteases, the absence of FSAP-AP complexes upon incubation with PMNs could be due to proteolysis of FSAP-AP complexes or the activator of FSAP present in the cells could still be the target of proteolysis. Because necrosis was induced in the absence of serum, the natural inhibitors of neutrophil proteases are absent. Therefore, to directly inhibit the proteases with their serum inhibitors, necrosis was induced in presence of serum of a FSAP-deficient donor. Necrosis induction in PMNs in presence of serum confirmed that PMNs are not able to activate FSAP (Figure 2). To check whether there was still an inhibiting factor present, PMNs were added to PBMCs and necrosis was induced in presence of FSAP-deficient serum. Further incubation with FSAP-sufficient serum leads to the formation of FSAP-AP complexes indicating that PMNs do not inhibit the activation by PBMCs (Figure 2). These results suggest that the activator of FSAP is not present in PMNs.

Structure of neutrophils
Neutrophils contain nuclei with a multilobulated shape and mature neutrophils contain few mitochondria and ribosomes in their cytoplasm (23). To confirm that one of these
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Figure 3: FSAP activation by necrotic human and chicken erythrocytes

Isolated human and chicken erythrocytes (RBC) (12.5x10^6 cells/ml) were sonicated and incubated with serum of a healthy donor for 30 minutes at 37°C. After incubation, FSAP activation was measured in the supernatant by detection of FSAP-AP complexes. FSAP-AP complexes were expressed in AU/ml. Results are given as mean ± SEM, (n = 3).

organelles that are different in PMNs compared to PBMCs are involved in the activation of FSAP we used sonicated human erythrocytes which lack a nucleus and cytoplasmic organelles such as mitochondria, endoplasmic reticulum, Golgi apparatus and ribosomes. We compared these human erythrocytes with avian erythrocytes, which contain a nucleus and its cytoplasmic organelles. Serum was incubated with sonicated human erythrocytes and chicken erythrocytes. Sonicated human erythrocytes were unable to activate FSAP while sonicated chicken erythrocytes can activate FSAP in plasma as shown by the FSAP-AP complex formation (Figure 3). This indeed suggests that the nucleus or one of the missing organelles in neutrophils and erythrocytes is involved in FSAP activation by dead cells.

Binding of FSAP to dead cells

FSAP binds to late apoptotic as well as necrotic Jurkat cells as shown by flow cytometry analysis (4). To get more insight in which organelle of the cell is involved in FSAP activation, we investigated the binding of FSAP to cells by confocal microscopy. Since cellular structures in Jurkat cells are hard to distinguish by microscopy, we made use of HEp2 cells with a clear cellular morphology. First necrotic HEp2 cells were tested in FSAP activation and were shown to be able to activate FSAP in serum (data not shown). To visualize the binding of FSAP to cells, fixed HEp2 cells were incubated with plasma followed by incubation with a monoclonal anti-FSAP antibody. Cells were stained with a FITC-labeled goat anti-mouse antibody. FSAP yields a strong nucleolar staining and diffuse staining pattern in the cytoplasm (Figure 4). Cells incubated with FSAP-deficient serum or an isotype control antibody were negative for FSAP staining (data not shown). These findings suggest that FSAP binds to a target present in the nucleoli and cytoplasm of the cells. The nucleoli and also the ribosomes are made up of ribosomal proteins and RNA. RNA is described to activate FSAP in a purified system (9). We tested total RNA, transfer RNA and poly I:C, the synthetic form of double-stranded RNA, in FSAP activation in serum. All tested forms of RNA fail to activate FSAP in serum (data not shown).
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Discussion

Single-chain FSAP (scFSAP) in purified systems has been reported to be susceptible to autoproteolysis and several activators of purified scFSAP have been described. However, FSAP in plasma is quite resistant to autoactivation (4). We have previously shown that FSAP in plasma becomes activated upon contact with either late apoptotic or necrotic cells (4). In this study we show that necrotic neutrophils are not capable of activating FSAP. Neutrophils lack a factor that is involved in FSAP activation.

FSAP is a serine protease present in serum that is responsible for nucleosome release from late apoptotic and necrotic cells. Upon contact with dead cells, FSAP becomes activated and can release nucleosomes from the cells (2;4). In absence of serum, nucleosomes remain bound to dead cells (24). Circulating phagocytes may not be very efficient in taking up these large fragments of cellular material. The removal of nucleosomes from dead cells may therefore help in phagocytosis of the cell remnants and prevent exposition of the immunogenic intracellular

![Figure 4: FSAP binding to HEp2 cells](image)

HEp2 cells fixed on ANA slides were incubated with 20% plasma for 30 minutes at RT. Cells were then incubated with anti-FSAP4 (50 µg/ml) and stained with a secondary antibody FITC labeled GαM (1:5) to visualize FSAP binding to the cells. DNA was counterstained with DAPI (1:1,000,000). Images were acquired with confocal microscopy. Scale bars = (A) 50 µm or (B) 20 µm.
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content and thereby the development of an autoimmune response. Dead neutrophils however do not activate FSAP and consequently FSAP is not able to remove nucleosomes from the neutrophils. Neutrophil turnover is rapid and they are the most abundant but also very short-lived human white blood cells. Upon maturation, neutrophils are released into the bloodstream where they circulate for 10–24 hours before undergoing apoptosis and being cleared by macrophages (25;26). When dead neutrophils would be able to activate FSAP, there would be continuous FSAP activation, which is not the case since FSAP activation cannot be detected in plasma of healthy subjects (4). Furthermore netosis is an important neutrophil function leading to the release of neutrophil extracellular traps (NETs) in response to various stimuli. NETs are networks of extracellular fibers, primarily composed of DNA, histones and proteases from the neutrophil, which binds pathogens (15). Immediate breakdown of these NETs by FSAP due to nucleosome release would abrogate the antimicrobial role of the NET forming neutrophil. Recently it was suggested that NETs also participate in the pathogenesis of a variety of inflammatory and autoimmune diseases, including systemic lupus erythematosus (SLE). An imbalance between NET formation and degradation in SLE patients may play a role in the perpetuation of autoimmunity and the exacerbation of disease (27). To our knowledge DNase is the only factor described to play a role in this NET degradation. However, FSAP might also have a function in the degradation of NETs. We described that in the chromatin breakdown and release of nucleosomes from necrotic cells FSAP cooperates with serum DNase I (3). A similar mechanism might function in the degradation of NETs.

Neutrophils differ from PBMCs in their morphology in the way that they have multilobed nuclei. During maturation the number of mitochondria and ribosomes decreases and the Golgi apparatus is small and the rough endoplasmic reticulum is absent. Glycogen granules, the main source of energy, fill the cytoplasm of mature neutrophils. Like neutrophils, human erythrocytes also lack cytoplasmic organelles such as mitochondria, endoplasmic reticulum, Golgi apparatus and ribosomes. In addition, erythrocytes also lose their nucleus during maturation. Similar to neutrophils also dead erythrocytes fail to activate FSAP whereas avian erythrocytes containing a nucleus and cytoplasmic organelles do activate FSAP. These results suggest that the nucleus or one of the missing organelles in neutrophils and erythrocytes is involved in FSAP activation by dead cells.

To get an indication which organelle of the cell is involved in FSAP activation, we investigated the binding of FSAP to cells. The binding structure of FSAP in the cells does not necessarily have to be the activator of FSAP, however this may give an indication of the activator of FSAP. Confocal microscopy analysis showed a nucleolar and cytoplasmic binding pattern. This binding pattern showed similarities with an antinuclear antibody (ANA) nucleolar staining pattern observed in patients with scleroderma or CREST syndrome (28). The nucleolar staining
pattern is associated with many antibodies including anti-Scl-70 (anti-topoisomerase 1), anti-PM-Scl (anti-exosome), anti-fibrillarin, anti-Th/To ribonucleoprotein and anti-RNAPI/III (anti-RNA polymerase I/III) (29-33). Since most of the autoantibodies showing this distinct staining pattern are directed against structures containing- or are composed of ribosomal proteins and RNA these structures seem plausible candidates for FSAP binding and/or activation.

The nucleolus is the site of rRNA transcription and processing, and of ribosome assembly. The nucleoli are made up of both, ribosomal proteins and RNA. Nakazawa et al. identified cell-derived RNA species as potent cofactors for the autoactivation of FSAP. They have shown that several forms of RNA were able to autoactivate the isolated proenzyme of FSAP. Indeed, the FSAP binding pattern to HEp2 cells showed a similar pattern as the RNA staining of the cells with acridine orange. However, we do not see activation of FSAP in plasma by total RNA, transfer RNA or synthetic RNA. This discrepancy in results may be explained by the fact that we use a plasma system. Purified FSAP may be more susceptible for activation by RNA compared to scFSAP present in plasma, or factors plasma may bind and neutralize the RNA. Another difference could be the purity of the several forms of RNA. In addition, we routinely use RNase-treated cells to induce FSAP activation, since RNase treatment improves the specificity of the propidium iodide staining used in the nucleosome releasing factor assay (24). No differences in FSAP activation could be found between RNase treated and untreated cells suggesting that FSAP activation by RNA in our system is rather unlikely. The nucleolar binding of FSAP and the fact that FSAP activation is unaffected by RNase treatment might suggest that a ribosomal protein is involved in FSAP activation.

Furthermore DNA and histones are described to activate FSAP in a purified system (11;34). However, in our system that is unlikely, since the nucleus present in the neutrophils consists of DNA and histones. Furthermore the binding analysis of FSAP to the HEp2 cells did not show a nuclear staining. Nakazawa et al. showed that DNA was not capable of mediating the autoactivation of FSAP (9). Moreover, bacterial circular double-stranded DNA, did not activate FSAP in our plasma system (data not shown). Further research is required to elucidate what the exact activator of FSAP is. Furthermore, it is of interest to investigate whether activated FSAP is also not able to release nucleosomes of dying neutrophils or cooperate in the degradation of neutrophil extracellular traps. In summary, we show that necrotic neutrophils are not capable of activating FSAP. Neutrophils lack a factor that is involved in FSAP activation.
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