Factor VII-activating protease: Mechanism and regulation of nucleosome release from dead cells
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General Introduction

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In multicellular organisms, cell death is essential to control tissue homeostasis. Plasma proteins, such as components of the classical pathway of complement (e.g. C2, C4) play an important role as they facilitate phagocytosis of dying cells. Insufficient removal of dying cells or the inability to mask and digest cellular material may lead to the release of potentially harmful cytotoxic and immunogenic cellular content into the environment (1). A number of these immunogenic endogenous molecules are damage-associated molecular pattern molecules (DAMPs). Several DAMPs such as DNA, RNA, histones and the DNA-binding protein HMGB1 are known to stimulate Toll-like receptors (TLRs). Hence, circulating nucleosomes might function as endogenous danger signals. Recently, plasma was shown to release DNA in the form of nucleosomes from late apoptotic cells (2). Factor VII-activating protease (FSAP) was identified as the plasma serine protease responsible for this nucleosome release (3).

FSAP has initially been described as a plasma hyaluronan acid binding protein 2 (HABP2), purified from plasma by affinity chromatography using hyaluronan-conjugated sepharose (4). At later time point, when purifying vitamin-K dependent coagulation factors from cryoplasma, Hunfeld et al. have found a protease-activity towards a thrombin-sensitive chromogenic substrate, which could not be inhibited by hirudin, and identified this protein as HABP2 (5). At the same time another group identified a protease which was able to activate factor VII (FVII) in vitro in absence of tissue factor (TF) (6). In view of this activity the protease was termed as “factor VII-activating protease (FSAP)" which turned out to be identical to HABP2.

**Structure of FSAP**

FSAP consists of 560 amino acids, including a 23 amino acid pro-peptide at the amino-terminal part of the molecule (4). It is synthesized in the liver and has a plasma concentration of ~12 μg/ml. FSAP consists of three epidermal growth factor (EGF) domains, a kringle domain and a serine protease domain at its C-terminus. It has a high homology to urokinase, plasminogen, FXII and hepatocyte growth factor-activator (HGF-A) (4;7). In plasma, FSAP circulates as an inactive single-chain molecule of 78 kDa that can be converted into its active two-chain form which consists of a 50 kDa heavy and a 28 kDa light chain, connected by a disulfide bond. Sequence analysis has revealed that the first cleavage step of FSAP occurs at Arg290-Ile291 (8), resulting in the active two-chain form. The FSAP light chain harbors the proteolytic domain consisting of the catalytic triad formed by His239, Asp388 and Ser486 (4). The N-terminal heavy chain contains two possible N-linked glycosylation sites (Asn31 and Asn284) (2). The isoelectric point of FSAP was determined at 4.9–5.5 (9). Two single nucleotide polymorphisms (SNPs) were found in the FSAP gene (10), named Marburg I (G511E) and Marburg II (G380Q). The presence of the Marburg I polymorphism gives rise to diminished proteolytic activity towards pro-urokinase (pro-UK) whereas the activity towards FVII is unaffected (10). The Marburg II (E393Q) variant is not associated with an altered FSAP function.
FSAP activation and regulation

Single-chain FSAP (scFSAP) in purified systems is susceptible to autoproteolysis. Kannemeier et al. have shown that this autoactivation was enhanced in the presence of heparin, whereas Ca\(^{2+}\) ions were shown to stabilize scFSAP (8). Furthermore, an acidic environment has been shown to reduce the activation and degradation of scFSAP (8). Negatively charged polyanions with a high charge-to-size ratio enhance autoactivation of FSAP resulting in the formation of its active two-chain form (11). Besides heparin, also polyphosphates, DNA and RNA have been demonstrated to accelerate autoproteolysis of purified scFSAP (6;8;11-13). The EGF3 domain, containing a positively charged cluster, has been shown to be essential for this polyanion binding and heparin-induced acceleration of autoproteolysis (11;12). Polycations, such as polyamine, and positively charged histones have been demonstrated to activate scFSAP as well (14;15). Based on these findings, Yamamichi et al. suggested a model on how polyanions and -cations might promote autoactivation of purified FSAP (Figure 1). They suggested that an intramolecular interaction between the N-terminus containing acidic amino acids and positively charged basic clusters within the EGF3 domain prevent autoproteolysis.

Figure 1: Autoactivation of FSAP by polyanions and polycations

Model on how polyanions and -cations might promote autoactivation of purified FSAP. An intramolecular interaction between the N-terminus containing acidophilic amino acids and positively charged basophilic clusters within the EGF3 domain prevent autoproteolysis. Polycations interfere with the respective intramolecular interaction thereby allowing intermolecular binding of the N-terminus to the EGF3 domain of an adjacent FSAP molecule to form an autoactivation complex. Polyanions offer a scaffold to which scFSAP molecules can bind via their EGF3 domain and when located in juxtaposition may lead to autoactivation. Figure adapted from Yamamichi et al. (14). Abbreviations: EGF: epidermal growth factor; scFSAP: single-chain FSAP
Polycations were suggested to interfere with that intra-molecular interaction, thereby allowing intermolecular binding of the N-terminus to the EGF3 domain of an adjacent FSAP molecule to form an autoactivation complex. In contrast, polyanions offer a scaffold to which scFSAP molecules can bind via their EGF3 domain and when located in juxtaposition may lead to autoactivation (14).

In purified systems, several serpins such as C1-inhibitor (C1inh), α₂-antiplasmin (AP), antithrombin III (AT-III) and the kunitz-type inhibitor inter-α-trypsin-inhibitor have been reported to inhibit the amidolytic activity of activated two-chain FSAP (tcFSAP) (5;8;16-18). In plasma, C1inh has been reported to be the main inhibitor of tcFSAP (16). In bronchoalveolar fluid (BALF) of patients suffering from adult respiratory distress syndrome (ARDS) complexes of FSAP with plasminogen activator inhibitor-1 (PAI-1) can be detected suggesting that PAI-1 might be involved in the regulation of FSAP activity as well (19).

**Role in coagulation and fibrinolysis**

Römisch et al. reported that FVII could be activated by tcFSAP in a tissue factor independent manner in a purified system and the same effect of tcFSAP on FVII could be observed in plasma (6). However, FSAP concentrations above the physiological concentration were used in plasma and FVIIa generation was lower than in the purified system (6). Moreover, Stavenuiter et al. have reported that FVII appears remarkably resistant to activation by recombinant FSAP (20). Therefore it remains doubtful whether FVII activation in plasma by FSAP is of physiological importance. In addition, FSAP has been reported to contribute to fibrinolysis. Pro-urokinase has been demonstrated to act as a substrate for purified tcFSAP in vitro (18). Because the Marburg I polymorphism results in diminished proteolytic activity towards pro-urokinase while the activity towards FVII remains preserved, the Marburg I polymorphism was implicated as a risk factor for thrombosis (10). The effect of the FSAP Marburg I polymorphism in deep venous thrombosis is not clear yet (21-24). However, there appears to be an association between the presence of FSAP Marburg I polymorphism and cardiovascular risk and the risk for late complications of carotic stenosis suggesting a role in the pathogenesis of atherosclerosis (25;26). These effects are unlikely to be due to the procoagulant and profibrinolytic properties, as FSAP was demonstrated to inactivate platelet derived growth factor BB (PDGFBB), thereby inhibiting proliferation and migration of smooth muscle cells (27;28). Moreover, FSAP has been identified in atherosclerotic plaques (29). In a mouse model, Sedding and colleagues have demonstrated that local application of wild-type plasma-derived FSAP reduced neointima formation after vascular damage, whereas local application of the Marburg I variant did not have any effect on this process (30). These findings suggest a role of FSAP in the pathogenesis of neointima formation and hence a possible role in the pathogenesis of atherosclerosis.
General Introduction

Role in inflammation
During inflammation, plasma cascade systems and immune cells are activated. Among others, the activation of endothelial cells plays a crucial role in the pathogenesis of local and systemic inflammation. Production of pro-inflammatory mediators results in an upregulation of procoagulant as well as adhesion molecules and to a fundamental change of the composition and contents of glycosaminoglycans on endothelial cells (31;32). Moreover, these stimuli may also increase vascular permeability leading to vascular leakage, which is a crucial event in the pathogenesis of sepsis (33). FSAP was demonstrated to regulate endothelial cell function. Proteolytic release of basic fibroblast growth factor (bFGF) by FSAP was shown to activate ERK1/2 kinases via stimulation of the FGF receptor 1 in endothelial cells finally resulting in phosphorylation of the transcription factor c-Myc (34). Etscheid and colleagues have demonstrated that FSAP proteolytically cleaves high molecular weight kininogen (HMWK), resulting in the release of the highly vasoactive bradykinin (BK) (17). BK induces an intracellular calcium flux via stimulation of the bradykinin receptor 2 (B2R) resulting in an increase of vascular permeability (35;36). BK release by FSAP might contribute to the development of potentially fatal hypotension in severe sepsis and septic shock (37). Indeed, the fact that FSAP activation could be demonstrated, by the detection of FSAP-PAI-1 complexes, in the BALF in patients suffering from ARDS, an acute long pathology of various etiology characterized by increased permeability of the pulmonary vasculature may point to the possible physiological relevance of this pathway (19). Histones have been shown to induce FSAP activation in plasma resulting in the liberation of BK (15).

The inflammatory response during sepsis leads to the induction of widespread cell death of parenchymal as well as immune cells. Circulating nucleosomes, a measure for cell death, were demonstrated to correlate with disease severity and fatality in sepsis patients (38;39). Circulating nucleosomes and DNA-binding proteins such as histones, released either by dead or activated cells were reported to induce a potential fatal inflammatory response in sepsis (40;41). Histone release contributes to death induced by inflammatory injury or chemical-induced cellular injury in mouse models, mediated in part through TLRs (42). Interestingly, histones are able to induce FSAP activation in plasma resulting in the liberation of BK (15). In contrast, FSAP was shown to negatively regulate vascular permeability via activation of protease-activated receptor signaling/RhoA/Rho kinase signaling. In murine LPS- and acute lung injury models, FSAP has been demonstrated to reduce the permeability of the pulmonary vasculature (43). Whether this observation is a general phenomenon or is restricted to the lung vasculature, is not known yet. Therefore, the net effect of FSAP on the vascular permeability during inflammation remains to be established.
Role in autoimmunity
In 2008 a novel role for FSAP was reported by Zeerleder et al. FSAP was identified as the plasma serine protease responsible for nucleosome release from late apoptotic cells (2;3). Cell death is a fundamental biological process to control development and tissue homeostasis of multicellular organisms. Dying cells are efficiently removed by phagocytic cells in order to prevent release of potentially harmful cytotoxic and immunogenic cellular content into the environment (1). Impaired removal of dying cells results in circulating apoptotic waste, which may serve as immunogen for the induction of autoantibody formation, inflammation and development of autoimmune diseases, such as systemic lupus erythematosus (SLE) (44-46). One of the main characteristics of SLE is the formation of autoantibodies against ubiquitous intracellular antigens present in the cell nucleus and cytoplasm e.g. ds-DNA, histones and nucleosomes. During the course of the disease, nucleosomes are found in the blood of SLE patients, which can trigger the formation of antinuclear antibodies (ANAs). These ANAs form immune complexes, which in turn contribute to the pathogenesis of SLE upon deposition. Serum DNase I activity has been shown to be decreased in SLE patients compared to that of healthy controls (47;48). Furthermore Macanovic et al. have demonstrated that lupus-prone NZB/NZW mice had significantly lower serum and urine concentrations of DNase I than normal mice, and this reduction did not correlate with the presence of the DNase inhibitor actin or anti-DNA antibodies (49). Napirei et al. have shown that mouse serum DNase I functions together with the serine protease plasmin in the chromatin breakdown of necrotic cells (50). Since nucleosomes play a central role in the antinuclear antibody response in SLE (51-53) and FSAP has been shown to release nucleosomes from late apoptotic cells (3), it would be of great interest to investigate whether the nucleosome releasing activity of FSAP is disturbed in patients with SLE.

Scope of this thesis
Since its discovery, several functions for FSAP have been proposed, including its role in coagulation and fibrinolysis. Recently however, FSAP was identified as the plasma protease that releases nucleosomes from late apoptotic cells. Cell death and the release of circulating nucleosomes and histones forms a critical step in the pathogenesis of sepsis and contributes to lethality. Furthermore, nucleosomes represent a set of major autoantigens in the induction of SLE and appear to be essential for the development of lupus nephritis. In this thesis we explored the role of FSAP in cell death, inflammation and autoimmunity. In chapter 2 the activation of FSAP in plasma by dead cells is described as well as its inhibition by the plasma inhibitors α2-antiplasmin and C1-inhibitor. Furthermore FSAP activation was investigated in several inflammatory conditions (e.g. sepsis and meningococcal sepsis) where cell death is a central event in the pathogenesis. We demonstrate FSAP activation during inflammation
and levels of FSAP-inhibitor complexes correlate with nucleosome levels and correlate with severity and mortality in these patients. In Chapter 3 the presence of complexes of FSAP with plasminogen activator inhibitor-1 in the plasma of severe sepsis, septic shock or meningococcal sepsis patients was demonstrated. In Chapter 4 we demonstrate that besides the serine protease inhibitors (serpins) AP, C1inh and PAI-1, also tissue factor pathway inhibitor (TFPI), a Kunitz-type inhibitor, is an efficient inhibitor of FSAP. In addition to the regulation of FSAP, we have studied the activation of FSAP by several primary cells in Chapter 5. We show that dead neutrophils fail to activate FSAP. In Chapter 6 FSAP was identified as the factor that cooperates with serum DNase I in the release of nucleosomes from necrotic cells. The fact that FSAP releases nucleosomes from dead cells is a remarkable finding in view of the fact that nucleosomes play a central role in the antinuclear antibody response in SLE. Therefore we have investigated in Chapter 7 whether the nucleosome releasing activity of FSAP is disturbed in patients with SLE. SLE patients in exacerbation show decreased nucleosome release which is mainly due to antinuclear antibodies.
References


