



## Interaction of factor VII activating protease (FSAP) with neutrophil extracellular traps (NETs)



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### ABSTRACT

The circulating zymogen form of Factor VII activating protease (FSAP) can be activated by histones and nucleosomes *in vivo*. These cell-death-associated nuclear factors are also actively extruded into the extracellular space by neutrophils through a process called neutrophil extracellular trap (NET) formation (NETosis). NETs are thought to be involved in host defense, inflammation as well as thrombosis. We have investigated the bidirectional interactions of FSAP and NETs. Phorbol ester-mediated NET formation was marginally stimulated by FSAP. Plasma-derived FSAP as well as exogenous FSAP bound to NETs. There was co-localization of FSAP and NETs in coronary thrombi from patients with acute myocardial infarction. Contrary to our expectations no activation of pro-FSAP by NETs was evident. However, after disintegration of NETs with DNase, a robust activation of pro-FSAP, due to release of histones from nucleosomes, was detected. The released histones were in turn degraded by FSAP. Histone cytotoxicity towards endothelial cells was neutralized by FSAP more potently than by activated protein C (APC). One more consequence of histone degradation was a decrease in nucleosome release from apoptotic neutrophils. Taken together, NETs bind to FSAP, but do not activate pro-FSAP unless histones are released from NETs by DNase. This activation of FSAP is likely to be important in diminishing the cytotoxic effect of histones, thus limiting the damaging effect of NETosis.

### 1. Introduction

Neutrophil extracellular traps (NETs) were originally identified as structures secreted by neutrophils as part of their anti-microbial arsenal [1]. The main constituents of NETs are the decondensed DNA backbone and histones [2] and their primary function is to trap and kill bacteria in addition to classical neutrophil mechanisms such as phagocytosis, degranulation to release toxic mixtures of peptides and enzymes as well as secretion of oxygen radicals [3]. Bacteria possess defensive strategies in that they secrete nucleases that digest NETs [4], which frees them and allows their spreading in the host organism.

NET formation is a type of cell death accompanied by decondensation of chromatin, breakdown of granular and nuclear membrane and their fusion with the rest of the cytoplasm. The final step is the disruption of the plasma membrane and release of NETs by a process termed suicidal NETosis [5]. Another form of NET formation can occur,

without cell lysis, called vital NETosis [6]. Chromatin decondensation is a consequence of citrullination of histones through activation of peptidyl arginine deiminase 4 (PAD4) [7]. The generation of reactive oxygen species (ROS) by NADPH oxidase-dependent and independent pathways is also important in NETosis [8]. Different inducers of NETosis are likely to activate various pathways to differing degrees. While microorganisms and their products are physiological activators, phorbol esters, calcium ionophores, lipopolysaccharides and cytokines are often used as model activators. NETs are further coated with a variety of other antimicrobial proteins e.g., elastase, myeloperoxidase, cathepsin G, lactoferrin, pentraxin3, matrix metalloprotease, proteinase 3 and LL37 [9].

One of the main consequences of NETosis, is the promotion of coagulation by providing a secondary network, in addition to fibrin and von Willebrand factor, to promote platelet activation and aggregation [10]. NETs are associated with tissue factor [11] and also bind to

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various coagulation factors such as FXII, FX, APC, FVIIa and fibrinogen [12]. NET-bound elastase can also inactivate tissue factor pathway inhibitor (TFPI) [13], although NET formation per se is not dependent on elastase activity [14]. NETs promote hypercoagulability in patients with sepsis since DNA, histones and neutrophil proteases are all involved in regulating the pro-coagulant activity of NETs [15]. NETs can also influence coagulation and inflammation through their cytotoxic effects on cells [16]. Thus, NETs typify the emerging concept that an overactive inflammation promotes thrombosis.

Factor VII activating protease (FSAP) is a circulating serine protease that is involved at many levels in the coagulation and fibrinolysis cascade [17] and together with various cellular mechanisms it has been shown to be involved in thrombosis [17], stroke [18] and neointima formation [19]. FSAP binds to nucleic acids, histones and nucleosomes that are the major components of NETs [20]. Endotoxemia in mice can release histones which convert pro-FSAP into an active protease [20]. Addition of histones in plasma can activate pro-FSAP which, in turn, degrades histones [20]. Apoptotic cells can also activate pro-FSAP and, in turn, FSAP can release nucleosomes from apoptotic cells [21]. These findings led us to hypothesize that circulating FSAP would interact with NETs, thus influencing their function. We have tested the bidirectional interactions between FSAP and NETs at a physical and functional level and find that FSAP activity is increased by NETs if the histones, therein, are released. The cytotoxicity of histones is potentially neutralized by active FSAP through proteolytic degradation.

## 2. Material and methods

### 2.1. Induction of NET formation and immunofluorescence analysis

Blood was collected from healthy donors after informed consent and under approval of the local ethics committee. Neutrophils were isolated using the dextran sulphate method followed by centrifugation over a density gradient media as previously described [22]. Cells were seeded on glass slides in 24-well plates and stimulated with phorbol-12-myristate 13-acetate (PMA, 100 nM) and incubated for 3 h at 37 °C in serum-free RPMI medium. Purified plasma FSAP was added together with PMA to determine its influence on NET formation. FSAP was purified from human plasma using a monoclonal antibody column, as previously described [23] and is partially in the zymogen form and the active form. In some experiments 50% hirudin-plasma was added to the cells. Cells were washed and fixed with paraformaldehyde (PFA) (4% wt/vol) and NETs were visualized using an antibody against the DNA-histone complex (mouse monoclonal anti H2A-H2B-DNA complex; clone #PL2-6 [24]) as primary antibody and respective secondary antibody. Alternatively, cells were stained with 4',6-diamidino-2-phenylindole (DAPI). Finally, glass slides were embedded in ProlongGold® antifade with DAPI (Invitrogen, Olso, Norway) and analyzed by confocal fluorescence microscopy using a Leica TCS SP5 microscope with an HCX PL APO 40 × 0.75–1.25 oil immersion objective, and a Confocal microscope Zeiss LSM 510 (Carl Zeiss Germany) 63 × magnification, scale bar 20 μm. The number of NET-positive cells was determined using ImageJ software as previously described [25]. The criteria for NET-positive cells were (i) green staining, positive for histone-DNA complex, (ii) loss of nuclear lobulation, increased size of nucleus and less dense nuclei, and (iii) or an occurrence of a distinct extracellular off-shoot. The mean value from 6 images for each condition per experiment was used for statistical analysis to enable representative quantification per sample.

For the visualization of FSAP binding to NETs, fixed samples were blocked and permeabilized in 0.2% (wt/vol) Triton-X100/PBS. Then, samples were incubated over night with monoclonal mouse anti-FSAP antibody (clone 570; Sekisui, Pfungstadt, Germany) followed by rabbit anti mouse Alexa Fluor 488 (ThermoFischer Scientific). Histone H3 was localized using the rabbit monoclonal antibody D1H2 (Cell Signaling Technologies, Leiden, The Netherlands) followed by goat anti rabbit

Alexa Fluor 546 (ThermoFischer Scientific).

### 2.2. Pro-FSAP activation in plasma

For these experiments hirudin plasma was used in preference to heparin plasma or citrate plasma since heparin interacts with NETs and the use of citrate plasma would influence the viability of neutrophils. Hirudin plasma (25 μg/ml Lepirudin/ml fresh human blood) (Bayer, Wuppertal, Germany) was collected from healthy donors and centrifuged at 550 × g for 15 min to collect plasma. For the determination of the pro-FSAP activation, plasma was incubated with or without NETs in the absence or presence of DNaseI (Pulmozyme®, Genentech, San Francisco, CA) and RNase (Sigma Aldrich) for various time points. Thereafter, supernatants were collected and FSAP-inhibitor complexes were measured as previously described [26]. Histones were analyzed by Western blotting using an antibody to Histone H3 (rabbit monoclonal D1H2 antibody). Nucleosomes were measured by an ELISA kit (Roche Diagnostics, Mannheim, Germany). In this ELISA the capture antibody is directed against histone and the detecting antibody against DNA.

### 2.3. SDS-PAGE and Western blot analysis

Proteins were separated using SDS-PAGE and transferred to a nitrocellulose membrane by standard procedures. After blocking with Tris-buffered saline (50 mM Tris, pH 7.4, 100 mM NaCl, Tween 20 (0.1% wt/vol) (TBST) containing 5% (wt/vol) dry milk powder, membranes were incubated with the primary antibody in TBST with 2.5% (wt/vol) dry milk powder. Blots were washed thoroughly and stained with a horseradish peroxidase-conjugated secondary antibody and signals were developed using ECL prime Western Blotting Detection Reagent (Amersham Systems) as recommended by the manufacturer.

### 2.4. Immunofluorescence analysis of acute coronary thrombi

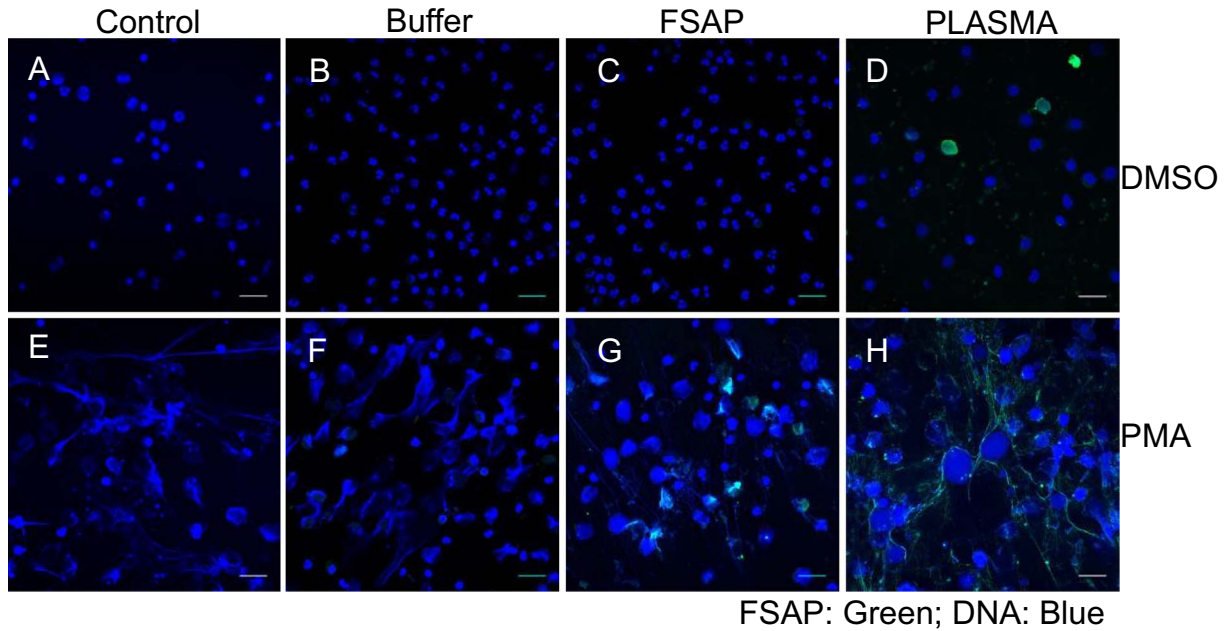
Fresh coronary thrombus was aspirated from the culprit coronary artery in the catheter laboratory, immediately fixed in formalin and embedded in paraffin for immunohistochemistry. Study participants gave written informed consent under an approval of the Ethics Committee of the Medical University of Vienna, Austria (approval reference numbers 114/2011, 303/2005, and 581/2006). For NET assessment, paraffin sections of the thrombus material ( $n = 5$ ) were stained for FSAP (rabbit polyclonal antibody), anti-human mouse DNA-Histone-Ab (Merck, MAB3864 concentration 1:500), rabbit anti citrullinated Histone H3 (Abcam), donkey anti-mouse secondary antibody (Alexa Fluor 555) Cell Signaling Technology, 4409S, concentration 1:500, goat anti-rabbit Alexafluor 647, and cell nuclei (DAPI, Sigma-Aldrich, D9542, concentration 1:500) as described [27]. Images were taken with the TissueFAXS system (TissueGnostics, Vienna, Austria) and a representative example is shown in Fig. 3.

### 2.5. Histone cytotoxicity on endothelial cells

Human umbilical vein endothelial cells (HUVEC) were isolated using collagenase and cultivated on collagen-coated dishes (5 μg/ml) with endothelial basal medium (PromoCell, Heidelberg, Germany), containing hydrocortisone (1 μg/ml), EGF (10 ng/ml), bFGF (10 ng/ml) and FCS (5% vol/vol). Cells were treated with histones (mixed histone preparation from Calf Thymus, Roche) at the indicated concentrations in the presence or absence of other test substances. After incubation of 4 h cells were washed extensively, stained with propidium iodine and analyzed by flow cytometry on FACS Calibur (Becton Dickinson, San Jose, CA).

### 2.6. Statistical analysis

All experiments were replicated in 3–5 independent experiments.



**Fig. 1.** Binding of FSAP to NETs: Neutrophils were treated for 3 h in absence (A–D) or presence (E–H) of PMA (100 nM) in serum-free medium and incubated for further 2 h with either control buffer (B, F), purified FSAP (5  $\mu\text{g}/\text{ml}$ ) (C, G) or 50% autologous hirudin plasma (D,H). Cells have been stained for FSAP (green) with anti-FSAP monoclonal antibody and for DNA with DAPI (blue). Results are representative confocal microscope pictures of three independent experiments, scale bar 20  $\mu\text{m}$ .

The data were analyzed using GraphPad Prism 7.02 (GraphPad Software). Differences between the two groups were analyzed by using a one-way or two-way ANOVA followed by Bonferroni post-test. The results are shown as mean  $\pm$  SEM and the significance is indicated as  $*p < 0.05$ .

### 3. Results

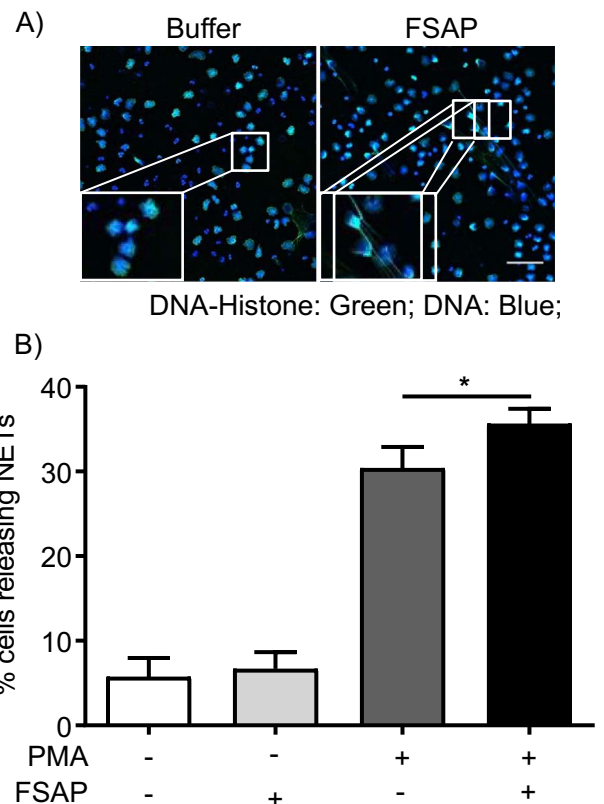
#### 3.1. Binding of FSAP to NETs *in vitro*

For these studies, we have used a model of NETosis based on the activation of neutrophils with PMA which mimics the ROS-dependent activation of NETs by activating NADPH oxidases similar to several microorganisms [28]. Neutrophils were stimulated with 100 nM PMA for 3 h to induce formation of NETs. Then, purified FSAP or 50% autologous hirudin-plasma was added to the wells for further 2 h of incubation. Stimulation of cells with PMA led to cells releasing extracellular strands of DNA, i.e., NETosis (Fig. 1A, E). Addition of FSAP (Fig. 1C, G) or plasma (Fig. 1D, H) to PMA-stimulated cells lead to co-staining of FSAP and extracellular DNA. No FSAP staining was observed in cells without FSAP or plasma (Fig. 1A, E and B, F).

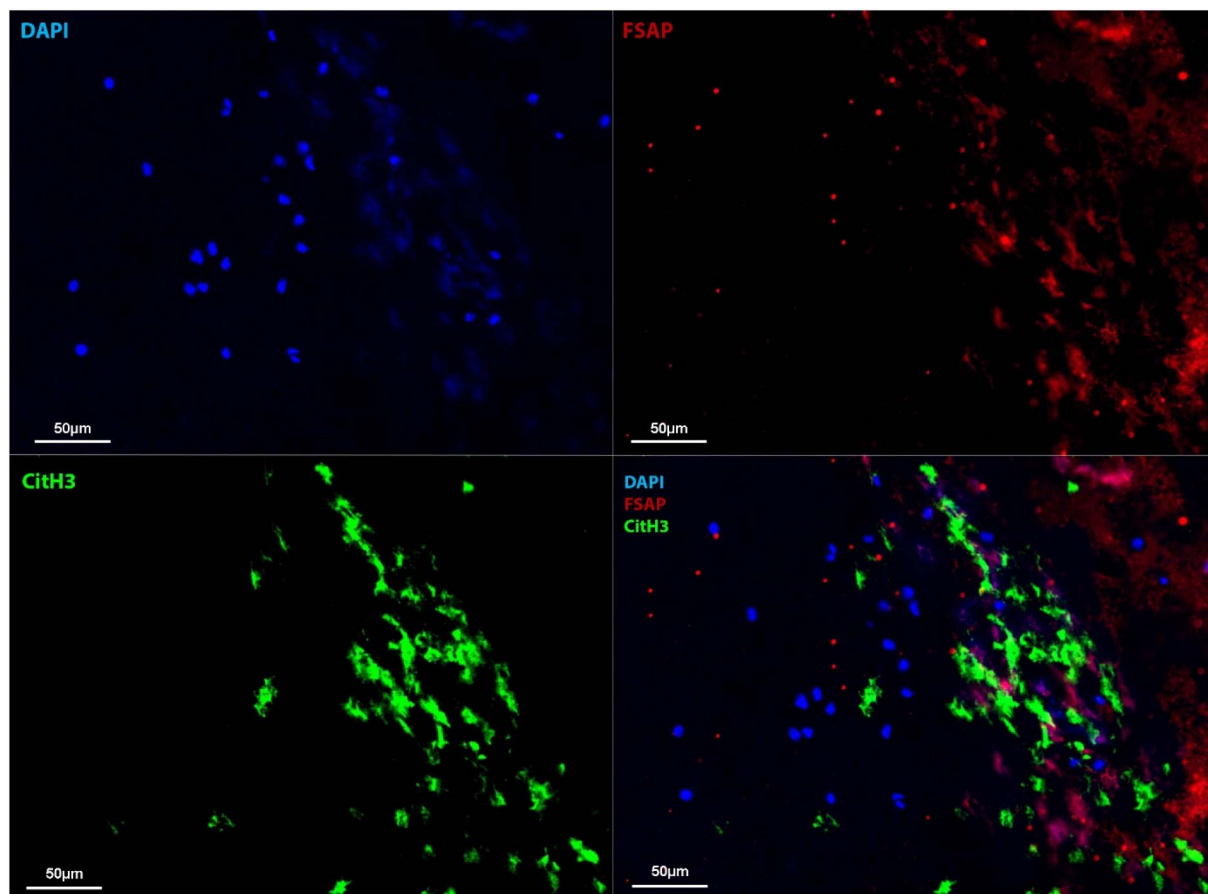
To test whether FSAP influences formation of NETs, purified FSAP (10  $\mu\text{g}/\text{ml}$ ) was added together with or without 100 nM PMA from the beginning of the 4 h incubation. FSAP itself did not induce formation of NETs, but slightly increased formation of NETs by PMA (Fig. 2A, B). Thus, in line with its known properties, FSAP binding to NETs was observed.

#### 3.2. Immunostaining of FSAP in NETs in acute coronary thrombi

We next examined coronary thrombi to understand the *in vivo* distribution of FSAP. For this, the staining pattern of FSAP in a panel of acute coronary thrombi, that are particularly rich in NETs [27], was characterized. As reported [27], NETs were found in association with cell nuclei of neutrophils as seen by citrullinated histone H3 staining (Fig. 3). The merged picture shows colocalization of FSAP, citrullinated H3 and cell nuclei (red, green, blue). There are also regions where nuclei are intact and lack citrullinated H3 DNA or FSAP



**Fig. 2.** Effect of FSAP on NET formation: (A) Neutrophils were stimulated with PMA (100 nM) in serum-free medium in the absence or presence of FSAP (10  $\mu\text{g}/\text{ml}$ ) for 3 h and stained with monoclonal anti H2A-H2B-DNA complex to visualize the NETs. Representative figure displaying neutrophils stimulated with PMA in the presence or absence of FSAP, scale bar 80  $\mu\text{m}$ . (B) Percentages of NET-forming cells in relation to the total number of cells were counted and data are shown as mean  $\pm$  SD of five independent experiments;  $*p < 0.05$ .



**Fig. 3.** Immunofluorescence staining of FSAP in coronary thrombi: 3  $\mu\text{m}$  Tissue sections were stained for DAPI (top-left), FSAP (top right) (rabbit polyclonal) and citrullinated Histone H3 (CitH3) (bottom left) (rabbit polyclonal). Virtual merge of immunofluorescence staining of DAPI, CitH3 and FSAP in coronary thrombus (bottom right). Two parallel sections were stained with CitH3 (rabbit anti-CitH3) and DAPI or with FSAP (rabbit anti-FSAP) and DAPI. Since host species of both primary antibodies is rabbit, these stainings could not be performed on the same slide. For both primary antibodies a secondary goat anti-rabbit AlexaFluor 647 antibody was used. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

immunoreactivity, or regions where FSAP immunoreactivity is localized outside of NETs. We also performed immunohistochemical staining of these thrombi with the same antibodies as used for fluorescence-based analysis above. A similar pattern of staining was observed confirming that the immunofluorescence-staining pattern was reproducible using different methods (data not shown). We conclude that endogenous FSAP co-localizes with NETs in human coronary thrombi *in vivo*.

### 3.3. Activation of plasma pro-FSAP by NETs

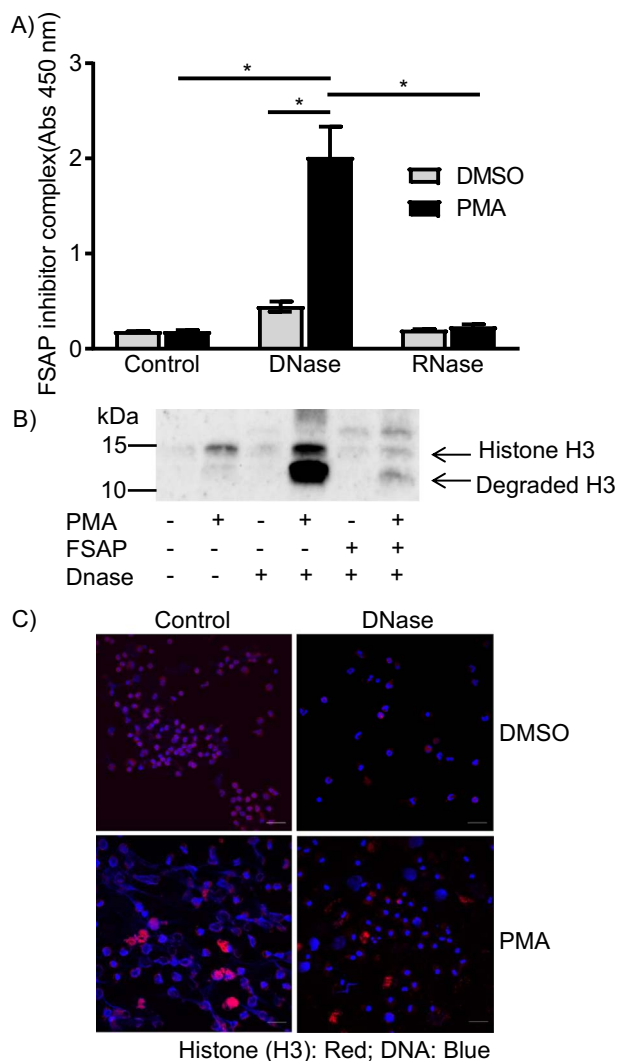
Since pro-FSAP can be activated by histones and nucleosomes [20] we expected an activation of plasma derived pro-FSAP in the presence of NETs. This was investigated using an ELISA assay for FSAP- $\alpha 2$  anti-plasmin complexes that are formed once pro-FSAP is activated in plasma. Contrary to our expectations, the extent of pro-FSAP activation in plasma showed no difference between control and PMA-stimulated neutrophils (Fig. 4A). Addition of RNase did not alter this pattern of pro-FSAP activation, but the addition of DNaseI, instead, induced a robust increase in pro-FSAP activation in PMA-treated cells but not in control cells (Fig. 4A). We hypothesized that this was due to the release of histones from nucleosomes, thus, we performed Western blot analysis of cell supernatants, and observed more histone release in DNaseI treated and PMA-induced cells (Fig. 4B). A lower MW band indicating degradation of histone H3 in the supernatants was observed and histone H3 bands disappeared in the presence of exogenous FSAP (Fig. 4B). Immunofluorescence analysis of histone H3 showed that the normal

regular nuclear pattern of staining was disrupted in PMA-treated cells and clusters of extracellular histones were evident. DNase treatment led to a reduction of histone staining on the cells/NETs (Fig. 4C). Only histones, but not pure DNA, dose-dependently increased FSAP-inhibitor complex formation in plasma as has been shown before [26]. Thus, the release of histones from NETs is required for the activation of pro-FSAP.

### 3.4. Interactions of FSAP with nucleosomes

In an earlier study it was reported that FSAP can release nucleosomes from late apoptotic cells [21] thus, we also measured nucleosome-release from neutrophils in the presence of FSAP. PMA treatment of neutrophils led to an increase in the concentration of nucleosomes release from the cells into the supernatants under serum-free conditions (Fig. 5A). In the additional presence of purified FSAP there was about a 50% decrease in nucleosome release. This pattern was identical in the presence of RNase. In DNaseI-treated cells no nucleosomes were measurable, because the digestion of DNA leads to a loss of DNA-histone complex-epitope that is detected by this ELISA. The pattern of nucleosomes was also mirrored exactly by the measurements of cell-free DNA (data not shown).

One possible explanation for the FSAP-mediated decrease in immunoreactive nucleosomes may be that the activated FSAP digests histones, as observed above (Fig. 4B) and as reported previously [20]. To test this hypothesis, purified nucleosomes isolated from the nucleosome ELISA kit standard were incubated with and without FSAP in the further presence or absence of the protease inhibitor aprotinin and

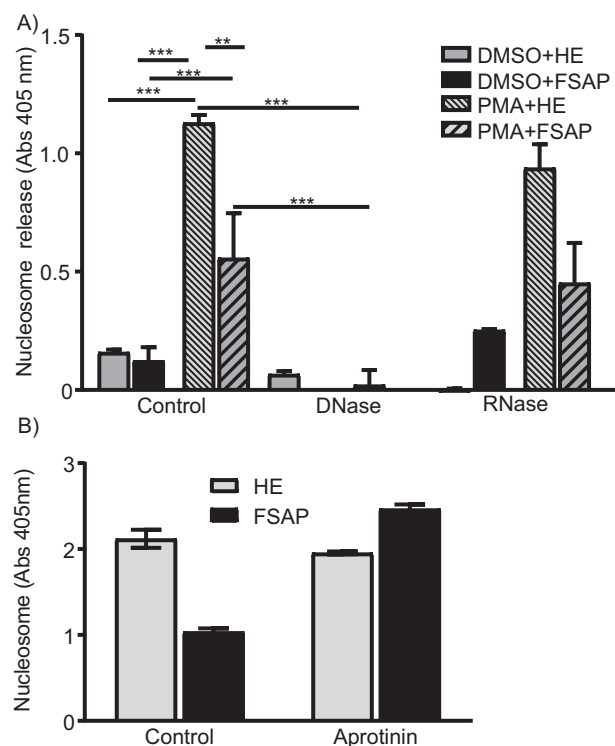


**Fig. 4.** Pro-FSAP activation by NETs: Neutrophils were treated for 3 h in absence or presence of PMA (100 nM) in serum-free medium and incubated for further 2 h with 50% autologous hirudin plasma. DNaseI (10 µg/ml) or RNase (50 µg/ml) was added for the last 30 min prior to plasma incubation. (A) Levels of FSAP-α2 antiplasmin (inhibitor) complexes in supernatants were measured by ELISA and results are given as absorbance values; mean ± SEM; \**p* < 0.05. (B) From a similar experiment as above in serum-free conditions, histone H3 was measured in cell supernatant by Western Blotting under reducing conditions. In some wells FSAP (10 µg/ml) was added for the last 2 h of the incubation. (C) Cells treated as above, were stained for histone H3 (red) and DNA (blue). Results are representative confocal pictures of three independent experiments; scale bar 20 µm.

intact nucleosomes were detected by ELISA. FSAP clearly reduced the levels of nucleosomes and this was not the case in the presence of aprotinin (Fig. 5B). Thus, FSAP promotes the loss of nucleosomes from NETotic cells by degrading histones and does not release nucleosomes from late apoptotic cells as has been previously suggested [21,29].

### 3.5. FSAP inhibits the cytotoxic effect of histones on endothelial cells

Extracellular histones are toxic for cells, which can be neutralized by proteases such as activated protein C (APC) [30]; thus we compared the effects of FSAP to APC. FSAP by itself did not induce apoptosis in endothelial cells, however histones treatment led to a strong cytotoxic effect in cells, determined by propidium iodide (PI) staining as a marker of cell death (Fig. 6A). Preincubation of histones with FSAP reduced this toxicity and this effect was neutralized by aprotinin (Fig. 6A). FSAP was more potent compared to APC in reducing cytotoxicity of histones

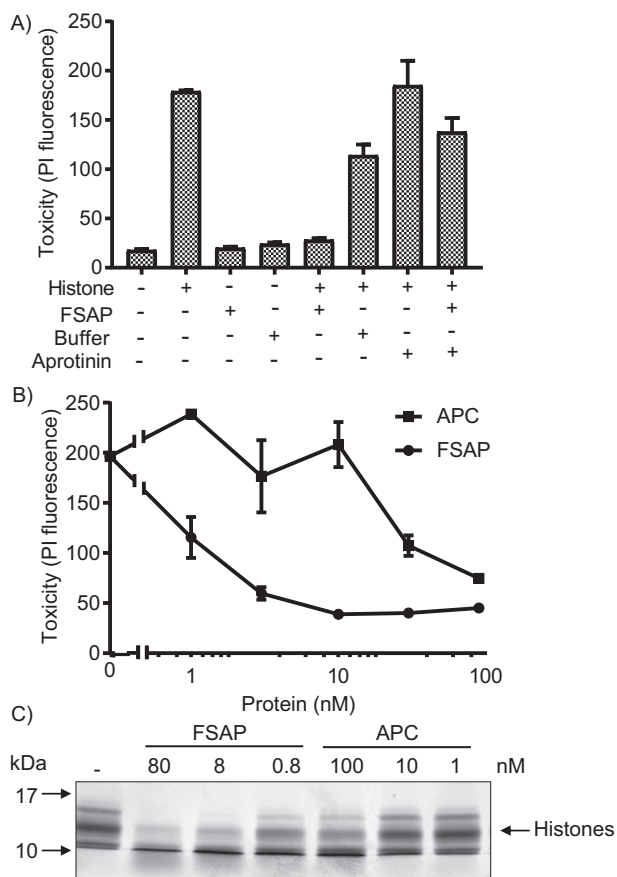


**Fig. 5.** Nucleosome release during NETosis: Neutrophils were treated for 3 h in absence or presence of PMA (100 nM) in serum-free medium and incubated for further 1 h with FSAP (10 µg/ml) or control buffer (HE). DNaseI (10 µg/ml) or RNase (50 µg/ml) was added for the last 30 min of incubation. (A) Supernatants were used to measure nucleosome release using an ELISA and results are given as absorbance values; mean ± SEM; \**p* < 0.05. (B) In a cell-free system nucleosomes were incubated 1 h at 37 °C in presence or absence of FSAP (5 µg/ml) and Aprotinin (25 µg/ml) and then nucleosome were quantified by ELISA; mean ± SEM; \**p* < 0.05. Similar results were obtained in three independent experiments.

(Fig. 6B). This difference was also seen at the level of degradation of purified histones by FSAP and APC (Fig. 6C). Incubation of histones with FSAP led to a complete degradation of histones. Thus, FSAP could reduce the cytotoxicity of histones towards endothelial cells much more effectively than APC.

## 4. Discussion

Pro-FSAP zymogen has a unique mechanism of activation into the active enzyme. Extracellular histones [20] and nucleosomes released from apoptotic cells were shown to activate pro-FSAP [21]. Thus, FSAP may serve as a sensor of cell death or it can be considered to be a circulating damage-associated molecular pattern recognition molecule (DAMP). Although DNA has been shown to activate FSAP under purified conditions [31], this is not the case in the presence of plasma [26]. Our finding that NETs, which comprise of chromatin DNA-histone complexes, bind to FSAP in vitro and in vivo to coronary thrombi was not surprising. But, the failure of NETs to activate FSAP was unexpected. However, treatment of NETs with DNaseI released histones and enabled a strong activation of pro-FSAP. A similar result has been shown in a recent report, showing that NETs did not have any pro-coagulant effects per se, but when separated into the individual components, both histones and DNA from NETs were active [32]. Thus, histones and DNA neutralize each other with respect to coagulation as well as FSAP activation. The observations in earlier studies, concluding that nucleosomes activate FSAP [21], may have been due to the presence of free histones in the nucleosome preparations. A caveat in this study is that PMA was used as an inducer of NETosis. Although widely used as a model inducer it may activate NETosis in a different way



**Fig. 6.** Cleavage of histones by FSAP and inhibition of its cytotoxic effect: (A) Histones (50  $\mu\text{g/ml}$ ) were incubated with FSAP (2  $\mu\text{g/ml}$ ) or Aprotinin (25  $\mu\text{g/ml}$ ) for 1 h at 37  $^{\circ}\text{C}$  and mixtures were then added onto endothelial cells. Toxicity was determined by measuring propidium iodide-associated fluorescence by flow cytometry. (B) Histones (50  $\mu\text{g/ml}$ ) were incubated with different concentration of FSAP or APC for 1 h at 37  $^{\circ}\text{C}$  and toxicity determined as described above. Experiments have been performed in duplicate and PI fluorescence is given in arbitrary units; mean  $\pm$  SD. Similar results were obtained in five independent experiments. (C) Histones (50  $\mu\text{g/ml}$ ) were incubated with different proteases in a volume of 100  $\mu\text{l}$  for 1 h at 37  $^{\circ}\text{C}$  and the samples were used for SDS-PAGE followed by Coomassie staining. Similar results were obtained in three independent experiments.

compared to microorganisms and bacterial products or cytokines [28].

DNases are present in blood [33] and, to a certain extent, any NETs produced in blood would therefore be digested by them [34]. The fact that NETs and NET-associated FSAP is found in thrombi indicates that endogenous DNases are not completely effective in degrading NETs, and that there is probably equilibrium between the formation and dissolution of NETs. DNase treatment of NETs neutralizes some of the negative effects of NETs in the context of vascular biology; e.g., it reverses the pro-thrombotic effects of NETs [35], decreases platelet aggregation and increases microvascular perfusion [36]. However, DNase treatment would also release histones, which would have strong toxic effects [37–39] as well as being prothrombotic [32]. Activated protein C (APC) was shown to have cytoprotective effects in a baboon model of sepsis through degradation of histones [39]. Comparison of FSAP and APC, showed that FSAP was by far more potent than APC in reducing the cytotoxic effects of histones on endothelial cells. Although degradation of histones by FSAP has been previously reported [20], the context of this in NETosis, neutralization of histone toxicity and comparison with APC are novel findings. Neutrophil elastase is also released during NETosis, and these can also contribute to degradation of histones in order to reduce their cytotoxicity [40]. The observation that after lipopolysaccharide (LPS) challenge of mice there is a 2.5-fold increase in plasma and 6-fold in lung FSAP mRNA [41], further

strengthens the case for a cytoprotective function of FSAP. These findings provide a basis for considering FSAP as an endogenous neutralizer of histone toxicity in a situation such as sepsis and activation of endogenous FSAP or supplementation with active FSAP could be a therapeutic concept in this condition.

One of the reported functions of FSAP has been its ability to release nucleosomes from late apoptotic cells [29,42,43]. This was demonstrated by staining of cells/nucleosomes with propidium iodide and annexin V as well as by ELISA for nucleosomes. Although the exact significance of this is not known, it was hypothesized that this would be important in regulating inflammation and autoimmunity [42]. We found that NETosis was related to the release of nucleosomes and thus could test the effect of FSAP on nucleosome release in our model system. Our findings show that FSAP generally decreased nucleosomes released by cells, and that this was most likely due to degradation of histones and disruption of the nucleosomes as could be demonstrated in the cellular and cell-free systems. Thus, at least in the context of NETosis, FSAP does not show any nucleosome release activity. It is notable that although intact NETs cannot activate pro-FSAP, once active FSAP can degrade histones within a nucleosome complex. Thus, FSAP binding to NETs and degradation of histones within NETs may have different requirements than the activation of pro-FSAP by NETs.

The prima facie function of NETs is to protect the host against microorganisms. In this context we did preliminary experiments to determine if FSAP could influence the viability of bacteria but this was not the case (data not shown). The other function of NETs seems to be to modulate the haemostasis process in microbial infections. The presence of FSAP on NETs in human thrombi raises the possibility that NETs may serve to concentrate FSAP activity at sites of clot formation where FSAP may have other functions over and above histone degradation. Active FSAP might regulate coagulation and fibrinolysis through cleavage of pro-urokinase, FVII and tissue factor pathway inhibitor [44,45]. It may also regulate inflammation through cleavage of kininogen as well as generation of anaphylatoxins C5a and C3a [26,45] and activate protease-activated receptors [46]. Because the balance between NET burden and endogenous DNase activity is one factor determining outcome in vascular thrombosis [27], FSAP-NET colocalization may be illustrating the NETs-DNase interaction, and mirror-image the degree of histone neutralization. Carriers of the Marburg I single nucleotide polymorphism of FSAP, which have very low enzymatic activity [47], would fail to neutralize histones resulting in increased disease susceptibility.

#### Conflict of interest

None.

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#### Author contributions

SMK and MKB designed the study, analyzed the data and edited the manuscript. SG performed most of the experiments and analyzed the data and co-wrote the manuscript with SMK. AN performed some of the experiments analyzed the data and edited the manuscript. IJL performed and analyzed the immunofluorescence analysis of thrombi and edited the manuscript. ME provided reagents and edited the manuscript.

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