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Unraveling the release and regulation of dead cell nuclear dumps

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CHAPTER 3

Cooperation of Factor VII-activating protease and serum Deoxyribonuclease I in release of nucleosomes from necrotic cells

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ABSTRACT

Objectives: Removal of dead cells is essential in maintenance of tissue homeostasis and efficient removal prevents exposure of intracellular content to the immune system which could lead to autoimmunity. The plasma protease factor VII-activating protease (FSAP) can release nucleosomes from late apoptotic cells. FSAP circulates as an inactive single-chain protein, which is activated upon contact with either apoptotic or necrotic cells. In this study we investigated the role of FSAP in the release of nucleosomes from necrotic cells.

Methods: Necrotic Jurkat cells were incubated with serum, purified tcFSAP and/or DNase I. Nucleosome release was analyzed by flow cytometry and agarose gel electrophoresis was performed to detect DNA breakdown.

Results: We show that serum can release nucleosomes from necrotic cells. FSAP-deficient serum or serum in which FSAP is inhibited by an inhibiting antibody is unable to release nucleosomes from necrotic cells confirming that indeed FSAP is the essential serum factor in this process. Together with serum DNase I, FSAP induces release of DNA from the cells, the appearance of nucleosomes in the supernatant and fragmentation of the chromatin into eventually mononucleosomes.

Conclusions: FSAP and DNase I are the essential serum factors cooperating in DNA degradation and nucleosome release in necrotic cells. We propose that this mechanism may be important in the removal of potential autoantigens.

INTRODUCTION

In multicellular organisms, cell death is essential for the control of tissue homeostasis. Dying cells are efficiently removed by phagocytic cells in order to prevent release of potentially harmful cytotoxic and immunogenic cellular content into the microenvironment¹. Insufficient removal of dying cells or the inability to mask and digest nuclear material may lead to autoantibody formation, inflammation and development of autoimmune diseases, such as systemic lupus erythemathosus (SLE)²⁻⁴. SLE is a chronic inflammatory autoimmune disease, characterized by unpredictable exacerbations and remissions with variable clinical manifestations. One of the main characteristics is the formation of autoantibodies against ubiquitous intracellular antigens e.g. ds-DNA, histones and nucleosomes. During the course of SLE, nucleosomes are found in the blood of patients where they can bind antibodies to form immune complexes with the potency to deposit in the glomeruli⁵⁻⁸.

Napirei *et al.* have shown that incubation of necrotic cells with mouse serum leads to chromatin breakdown as well as to removal of nucleosomes and observed that serum DNase I plays a crucial role in this process⁹. Moreover the physiological importance of DNase I is demonstrated by the fact that decreased activity of serum DNase I has been shown in patients with SLE^{10,11}. However, Napirei *et al.* showed that serum DNase I was essential but insufficient by itself for chromatin breakdown and that the contribution of a serum serine protease was required. This protease was identified as plasmin, based on the observation that protease inhibitors specific for plasmin were able to inhibit the nucleosome generation in necrotic cells⁹.

We have previously shown that serum releases nucleosomes from late apoptotic cells¹². The serum factor responsible for this nucleosome release is Factor VII-activating protease (FSAP)¹³. FSAP, also known as plasma hyaluronic acid binding protein 2 (HABP2), is a serine protease circulating in plasma as an inactive single-chain molecule of 78 kDa. It is proteolytically converted into its active two-chain form consisting of a 50 kDa heavy and a 28 kDa light chain, connected by a disulfide bond¹⁴. FSAP has several plasma inhibitors including α_2 -antiplasmin¹⁵ and tissue factor pathway inhibitor (TFPI)¹⁶, which are inhibitors of plasmin as well. FSAP has been described to be activated upon contact with either late apoptotic or necrotic cells¹⁵ and circulating histones¹⁷. Based on the role of FSAP in the nucleosome release from late apoptotic cells,

we investigated in this study the role of FSAP in the release of nucleosomes from necrotic cells upon incubation with serum.

MATERIALS AND METHODS

Reagents

3

Mouse monoclonal antibodies antiFSAP-4¹³, AAP-20, AAP-11, AP-1¹⁸ and ANA58 and ANA60¹⁹ were prepared at our department. IMDM medium was obtained from BioWhittaker Europe (Verviers, Belgium). Fetal calf serum was obtained from Bodinco BV (Alkmaar, The Netherlands). Penicillin and streptomycin were obtained from Gibco/Invitrogen (Groningen, the Netherlands). RNase A, DNase I and β -mercaptoethanol were obtained from Sigma (Zwijndrecht, 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). QIAamp[®] DNA Blood Mini kit was obtained from Qiagen (Venlo, The Netherlands). NuPage 12% polyacrylamide gels, SDS sample buffer, SilverQuest kit and Dynal[®] CD4⁺ Isolation kit were obtained from Invitrogen (Groningen, The Netherlands). Lymphoprep[™] was obtained from Axis-shield (Oslo, Norway)

Plasma-derived two-chain FSAP has been obtained by purification as described¹³. The construction, production, purification and activation of thermolysin-activatable FSAP^{R313Q} mutant has been described previously²⁰. Blood was collected from donors and allowed to clot for 30 min at room temperature. After centrifugation at 1300 x *g* for 10 min, serum was removed and stored at -20°C. All healthy donors were homozygous for the wild-type form of FSAP. All sera and buffycoats from healthy donors have been obtained as anonymized samples from the diagnostic laboratory, according to the Dutch rules and regulation for the use of patient material.

FSAP-deficient donor

The sera received from anonymous healthy donors were tested for nucleosome releasing activity, FSAP activation and FSAP antigen levels¹³. Serum of one donor was not functional in releasing nucleosomes from late apoptotic cells. In addition, no FSAP-inhibitor-complexes could be detected upon activation of FSAP and the serum was negative in the FSAP antigen ELISA. Subsequently

DNA was isolated from serum of the FSAP-negative donor as well as from a healthy donor using the QIAamp® DNA Mini Kit (Qiagen). Sequence analysis was performed as described by Kuijpers *et al.*²¹ A homozygous nonsense mutation substituting an arginine with a stopcodon (c.607C>T p.R203X) was found, leading to a truncated protein lacking activity.

Cell culture, isolation of human CD4⁺ T lymphocytes and induction of necrosis

Human PBMCs were isolated by density gradient centrifugation on Lymphoprep™ from buffycoats obtained from healthy donors. From the PBMCs, CD4⁺ cells were purified with a Dynal® CD4⁺ Isolation kit. Jurkat cells were cultured in culture medium (Iscove's Modified Dulbecco's Medium (IMDM) containing 5% (v/v) fetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and 50 µM β-mercaptoethanol. Necrosis was induced by incubation of Jurkat cells or isolated T-cells (2*10⁶ cells/ml) at 56°C for 30 min. After induction of necrosis, cells (1*10⁶ cells/ml) were cultured in FCS-free medium at an atmosphere of 5% CO₂ at 37°C for 48 h in the presence of human serum, purified tcFSAP and/or DNase.

Analysis of nucleosome release by flow cytometry

Necrotic cells were washed with HN buffer (50 mM Hepes, 100 mM NaCl at pH 7.4). To examine the release of nucleosomes, cells were stained with propidium iodide (PI) diluted in HN buffer (1 µg/ml). Samples were analyzed by flow cytometry. The median fluorescence intensity of PI was measured and quantified using the FACS Diva Software (Becton Dickinson, Mountain view, CA, USA). In our assay the FSAP content of serum was set on 100 arbitrary units per ml (AU/ml). Dilutions of serum (expressed as % serum) were compared with the same amount of AU/ml purified FSAP.

Nucleosome ELISA

Nucleosome levels were determined by an ELISA as recently described^{12,19}. Briefly, monoclonal antibody CLB-ANA/60, which recognizes histone H3 was used as a catching antibody. Biotinylated F(ab')₂ fragments of CLB-ANA/58, which recognizes an epitope exposed on complexes of histone H2A, histone H2B and dsDNA, in combination with poly-HRP was used for detection.

Analysis of DNA degradation by agarose gel electrophoresis

After induction of necrosis, cells were cultured in an atmosphere of 5% CO₂ at 37°C for 24 h in presence of purified FSAP and/or DNase I. Samples were treated with RNase (10 U/ml) for 30 min at 37°C. DNA was isolated using a QIAamp® DNA Blood Mini kit and samples were loaded on a 0.9% agarose gel containing 0.5 µg/ml ethidium bromide. The gel was run in TAE (Tris-acetate-EDTA) buffer.

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Plasmin-α₂-antiplasmin complex ELISA

Plasmin-α₂-antiplasmin (PAP) complexes were measured using ELISA. Monoclonal antibody AAP-11, directed against inactivated and complexed α₂-antiplasmin was used as a catching antibody and monoclonal antibody AP-1 against plasmin in combination with poly-HRP was used for detection. Plasma incubated with urokinase was used as a standard with a concentration of 2100 nmol/l PAP-complexes. Results were expressed in nmol/l¹⁸.

FSAP-α₂-antiplasmin complex ELISA

FSAP-α₂-antiplasmin (FSAP-AP) complex levels were determined by an ELISA as recently described¹⁵. Briefly, a monoclonal antibody (AAP-20) 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.

SDS-PAGE and silverstaining

After induction of necrosis, cells (5*10⁶ cells/ml) were cultured in an atmosphere of 5% CO₂ at 37°C for 48 h in presence or absence of plasma-purified FSAP or thermolysin-activated recombinant FSAP^{R313Q}²⁰. Cells were washed with HN buffer, taken up in SDS-PAGE sample buffer and samples were applied to NuPage 12% polyacrylamide gel. After electrophoresis, silverstaining was performed using the SilverQuest silverstaining kit.

Statistics

Results are presented as mean ± SEM. In case of normal distribution, comparison between groups is performed by using parametric testing by means of t-test. A p-value <0.05 was considered to be statistically significant.

RESULTS

To test whether human serum is able to release nucleosomes from necrotic cells, necrotic Jurkat cells or isolated T-cells were incubated with serum and the remaining cellular DNA content was measured by staining the cell remnants with propidium iodide (PI) followed by flow cytometry analysis. Untreated cells retained all DNA in the cells as shown by the positive grey shaded PI-peak in **Figure 1A**. When cells were incubated with serum of a healthy donor, a shift of the PI-peak was observed indicating that indeed part of the cells lose their DNA (**Figure 1A**). When FSAP in the serum was blocked with a neutralizing antibody, no decrease in PI-staining was observed indicating that FSAP in fact is involved in nucleosome release from necrotic cells (**Figure 1B**). The role of FSAP in nucleosome release was confirmed by using serum of an FSAP-deficient donor. Again no nucleosome release could be detected (**Figure 1C**). Results of the quantitative analysis of the nucleosome release are shown in **Figure 1D**.

To test whether FSAP is sufficient for nucleosome release from necrotic cells or that, as indicated by Napirei *et al.*⁹, also DNase I is required for this process, we incubated necrotic cells with purified FSAP alone or in combination with DNase I. When cells were incubated with plasma-derived purified FSAP, the cells retained the DNA in the cell comparable to the untreated cells (**Figure 2A**). This indicates that there is no nucleosome releasing activity of purified FSAP in necrotic cells. Incubation of DNase I alone does not lead to a decrease in PI signal either (**Figure 2B**). When cells were incubated with DNase I in combination with purified FSAP a shift in the PI peak was observed, indicating that FSAP cooperates with DNase I in releasing nucleosomes from necrotic cells (**Figure 2C**). Results of the nucleosome release are summarized in **Figure 2D**.

To confirm that the cells release their DNA in the form of nucleosomes, an ELISA was performed to measure nucleosomes in the supernatant. Indeed, high levels of nucleosomes were detected in the supernatant of the cells that were incubated with serum or with purified FSAP and DNase I, whereas no or low nucleosome levels could be measured in the supernatant of untreated cells or cells incubated with either purified FSAP or DNase I alone (**Figure 3**).

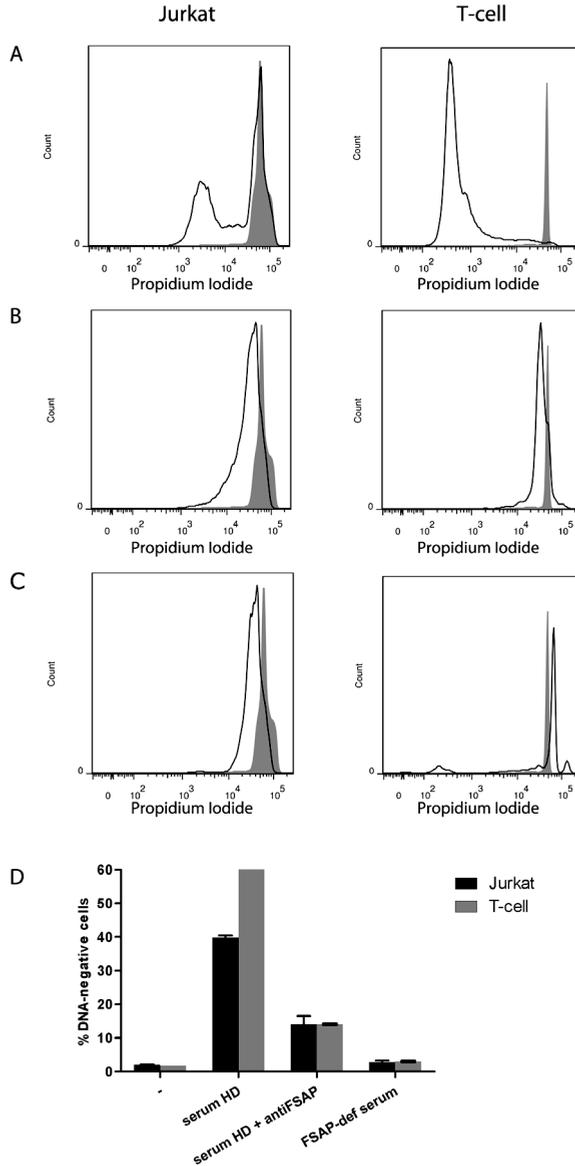


Figure 1: Analysis of nucleosome release from necrotic cells by serum in which FSAP is inhibited or absent.

After induction of necrosis, Jurkat cells or isolated T-cells were cultured with (A) 10% serum of a healthy donor, (B) 10% serum of a healthy donor in presence of a monoclonal antibody against FSAP (20 $\mu\text{g}/\text{ml}$) or (C) 10% serum of a FSAP-deficient donor at 37°C for 48 h. Next cells were stained with propidium iodide (1 $\mu\text{g}/\text{ml}$) and analyzed by flow cytometry. The untreated cells are indicated by the grey shaded area. (D) The percentage of DNA-positive cells is shown as mean \pm SEM, $n=3$. An antibody with the same isotype and of an irrelevant specificity did not inhibit nucleosome release (not shown).

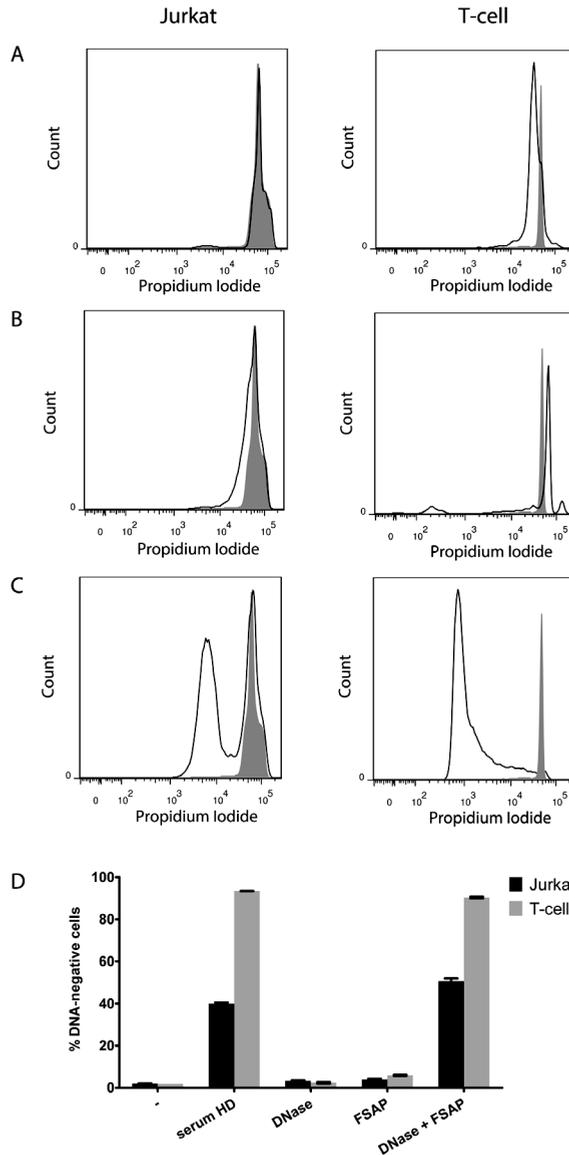


Figure 2: Analysis of nucleosome release from necrotic cells by plasma-purified FSAP in combination with DNase I.

After induction of necrosis, Jurkat cells or isolated T-cells were cultured with (A) plasma-purified FSAP (10 U/ml), (B) DNase I (50 mU/ml) or (C) a combination of DNase I (50 mU/ml) and FSAP (10 U/ml) at 37°C for 48 h. Next cells were stained with propidium iodide (1 µg/ml) and analyzed by flow cytometry. The untreated cells are indicated by the grey shaded area. (D) The percentage of DNA-positive cells is shown as mean±SEM, n=3.

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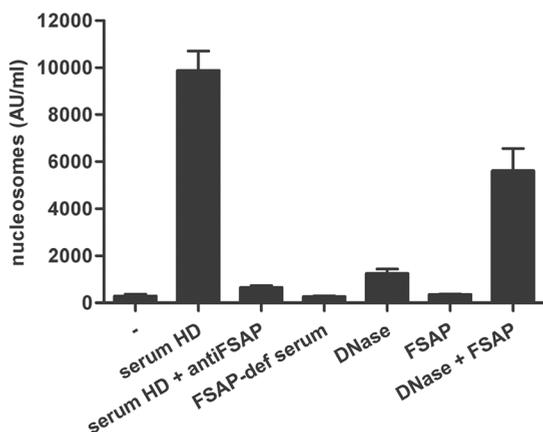


Figure 3: Nucleosomes in supernatant of necrotic cells after incubation with serum in which FSAP is inhibited or absent and plasma-purified FSAP in combination with DNase I. After induction of necrosis, Jurkat cells were cultured with 10% serum of a healthy donor (HD), 10% serum of a healthy donor in presence of a monoclonal antibody against FSAP (20 $\mu\text{g}/\text{ml}$), 10% serum of a FSAP-deficient donor, plasma-purified FSAP (10 U/ml), DNase I (50 mU/ml) or a combination of DNase I (50 mU/ml) and FSAP (10 U/ml) at 37°C for 48 h. Next nucleosomes were measured in supernatant by ELISA. Nucleosomes were expressed as AU/ml. Results are given as mean \pm SEM, n=3.

DNA fragmentation by FSAP and DNase I

To analyze DNA breakdown of necrotic cells, DNA was isolated and agarose gel electrophoresis was performed. Cells were incubated with purified FSAP and/or DNase I. Necrotic cells cultured in absence of both components showed no DNA-laddering as shown in **Figure 4**. Necrotic cells incubated with FSAP alone did not show DNA fragmentation either whereas cells incubated with DNase I alone demonstrated a DNA smear of high molecular weight (HMW) DNA indicating random cleavage of the DNA (**Figure 4**). Notably, necrotic cells incubated with FSAP in combination with DNase I showed DNA fragmentation of HMW DNA leading to a banding pattern characteristic for nucleosomes.

No plasminogen activation upon incubation with necrotic cells

Since plasmin is described to function together with DNase I in the degradation of the chromatin and release of nucleosomes from necrotic cells as well⁹, we tested whether plasminogen is activated after incubation with necrotic cells. Serum was incubated with or without necrotic cells and plasmin- α_2 -antiplasmin (PAP) complexes were measured by ELISA. No PAP-complexes could be detected upon incubation of serum with necrotic cells, indicating

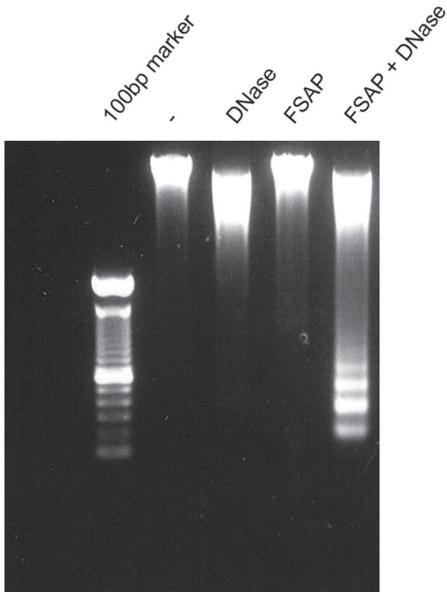


Figure 4: Analysis of DNA fragmentation by FSAP and DNase I

After induction of necrosis, Jurkat cells were cultured in an atmosphere of 5% CO₂ at 37°C for 24 h in presence of purified FSAP (10 U/ml) and/or DNase I (50 mU/ml). DNA was isolated and samples were subjected to agarose gel electrophoresis. A 100 bp DNA marker served as molecular weight marker.

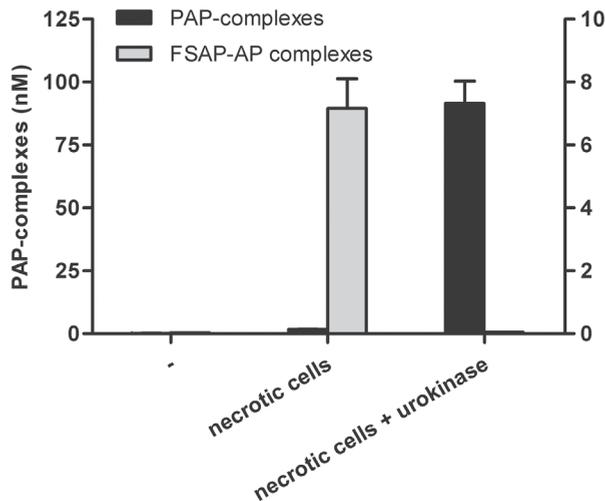


Figure 5: Analysis of plasminogen and FSAP activation upon incubation with necrotic cells

After induction of necrosis, Jurkat cells were cultured in an atmosphere of 5% CO₂ at 37°C for 48 h in presence of serum of a healthy donor. Next plasmin-alpha₂-antiplasmin (PAP) and FSAP-alpha₂-antiplasmin (FSAP-AP) complexes were measured in supernatant by ELISA. Serum incubated without necrotic cells and serum incubated with necrotic cells and urokinase (10 µg/ml) was used as a negative and positive control, respectively. Results are given as mean ± SEM, n=3.

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that plasminogen is not activated into plasmin (**Figure 5**). However, FSAP is activated when serum is incubated with necrotic cells as shown by the formation of FSAP- α_2 -antiplasmin complexes in the serum (**Figure 5**).

Histone H1 degradation by FSAP

Napirei *et al.* have shown that there is a loss of histone H1 upon incubation of necrotic cells with DNase1-/- serum and that this effect is inhibited by the addition of aprotinin or PAI-1⁹. To test whether histone H1 was cleaved by FSAP, we incubated necrotic cells with purified plasma-derived FSAP. Indeed, histone H1 was degraded after FSAP incubation as shown by **Figure 6A**. Western blotting with an antibody against histone H1 and mass spectrometry analysis confirmed that indeed the band that disappeared on gel is histone H1 (**Figure 6B**). To exclude the possibility that a contamination in the plasma-purified FSAP is responsible for the histone H1 degradation, necrotic cells were also incubated with activated recombinant FSAP^{R313Q}²⁰. Again, the cleavage of histone H1 was seen upon incubation with recombinant FSAP^{R313Q}, confirming the observation that FSAP indeed cleaves histone H1.

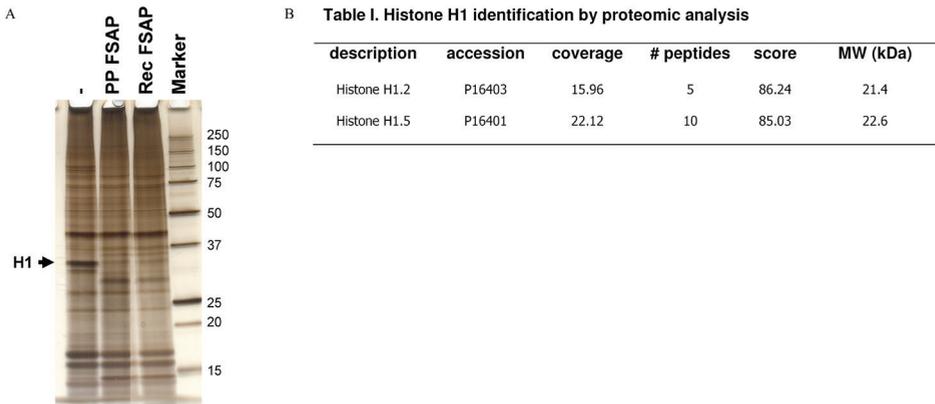


Figure 6: Histone 1 degradation upon incubation of necrotic cells with plasma-purified FSAP

After induction of necrosis, Jurkat cells were cultured in an atmosphere of 5% CO₂ at 37°C for 48 h in presence of plasma-purified FSAP (10 U/ml) or recombinant thermolysin-activatable FSAP^{R313Q} (10 U/ml). Cells were washed and samples were subjected to SDS-PAGE 12% gel and silverstaining was performed (A). The corresponding cleaved protein was excised from an untreated sample and subjected to analysis by mass spectrometry. A description of the histone H1 subtypes, the uniprot accession number, sequence coverage (in % of total number of amino acids), number of peptides identified, protein score as calculated using Proteome Discoverer 1.1 and molecular weight (MW) are provided (B). PP FSAP, plasma-purified FSAP; rec FSAP, thermolysin-activated FSAP^{R313Q}; H1, histone H1

DISCUSSION

We previously showed that the plasma serine protease FSAP is the factor in serum that can release nucleosomes from late apoptotic cells¹³. DNA release from necrotic cells seems to be a multistep process. In this study we showed that the plasma serine protease FSAP cooperates with DNase I in the degradation and removal of DNA from necrotic cells.

Impaired clearance of dead cells can lead to the induction of inflammation, autoantibody formation and development of autoimmune diseases, such as SLE²⁻⁴. In absence of serum, nucleosomes remain bound to late apoptotic cells¹². 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 content. Moreover, nucleosomes that are released into the circulation are rapidly cleared by hepatocytes²². When nucleosomes are not removed from the cells and stay attached to the cells, they might persist longer and can play an immunogenic and pathogenic role in the development of autoimmune diseases, such as SLE.

In contrast to apoptosis, upon necrosis the integrity of the cell membrane is distorted without cleavage of DNA by intracellular nucleases. Degradation of the DNA of necrotic cells by serum DNase I followed by removal of nucleosomes might be an important event in recognition by phagocytes and thereby preventing the development of SLE. The physiological importance of DNase I is demonstrated by the fact that decreased activity of serum DNase I has been shown in patients with SLE^{10,11}. Furthermore Macanovic *et al.* demonstrated that lupus-prone NZB/NZW mice had significantly lower serum and urine concentrations of DNase I than normal mice, and this reduction was not related to the presence of the DNase inhibitor actin or anti-DNA antibodies²³. Napirei *et al.* showed that mouse serum DNase I functions together with the serine protease plasmin in the generation of nucleosomes in necrotic cells⁹. This identification was based on the observation that protease inhibitors specific for plasmin were able to inhibit the nucleosome generation in necrotic cells. FSAP is a plasma serine protease with structural and functional similarities to plasmin. Both proteases are serine proteases which are inhibited by aprotinin. FSAP has several plasma inhibitors including α_2 -antiplasmin¹⁵ and tissue factor pathway inhibitor¹⁶ which are inhibitors of

plasmin as well. Moreover FSAP is described to be inhibited by plasminogen activator inhibitor²⁴. This is a serine protease inhibitor that functions as the principal inhibitor of tissue plasminogen activator (tPA) and urokinase (uPA), activators of plasminogen.

Plasminogen, the proenzyme of plasmin, is present in the circulation. Activation of plasminogen into plasmin occurs when plasminogen activators cleave a unique bond in the serine protease domain resulting in two chains, linked by two disulfide bonds. Similar to plasminogen, FSAP circulates in its inactive single-chain form and has to be activated by proteolytic cleavage resulting in a heavy and a light chain connected by a disulfide bond. We recently showed that FSAP is activated upon contact with late apoptotic cells as well as necrotic cells¹⁵. In contrast, plasminogen activation requires the presence of plasminogen activators, which need to be released to the site of action (e.g. tPA from the endothelium) and is not activated by contact with necrotic cells. Since no additional factor besides the contact with necrotic cells is required for FSAP activation, the role of FSAP in the release of nucleosomes from necrotic cells might be physiologically relevant. This is supported by the finding that FSAP-deficient serum or serum in which FSAP is inhibited by an inhibiting antibody is not able to release nucleosomes from necrotic cells.

C1q has also been demonstrated to release nucleosomes from necrotic cells in cooperation with serum DNase²⁵. However C1q deficient plasma showed identical results as normal plasma in our system (data not shown).

Whereas FSAP is essential to induce removal of nucleosomes from necrotic cells, purified FSAP is not able to induce nucleosome release. We now show that FSAP needs DNase I to release nucleosomes from the necrotic cells, in contrast to late apoptotic cells where FSAP alone is sufficient to release nucleosomes. We previously reported that serum was not able to induce nucleosome release from necrotic cells. However, we observed this using a system in which the cells were incubated for 30 minutes with serum¹². Since Napirei *et al.* demonstrated that mouse serum was able to generate nucleosomes in necrotic cells after a longer incubation period⁹ we changed our system to a 48 hour incubation period. With this longer incubation period we showed that serum indeed can release nucleosomes from necrotic cells. The increased incubation time may be required for efficient DNA cleavage by serum DNase I after necrosis, whereas intracellular endonucleases already

confer DNA cleavage during the process of apoptosis.

The mechanism by which DNase I promotes FSAP-induced nucleosome release is not entirely clear yet. DNase I is able to cleave the DNA of necrotic cells in a random manner as illustrated by a HMW DNA smear after DNA isolation and agarose gel electrophoresis. However, this DNA is not released from the cells in the form of nucleosomes. One interpretation could be that the DNA fragments are too large to be released from the cells or that the DNA is still linked to the cell by a DNA-binding protein. When the isolated DNA from necrotic cells treated with a combination of FSAP and DNase I was analyzed on agarose gel LMW bands were visible, which is a typical feature of nucleosomes. This indicates that in presence of FSAP, internucleosomal cleavage occurs by DNase I. Various explanations for this specific cleavage pattern can be hypothesized. One could be that the FSAP cleaves a DNA-binding protein, thereby changing the conformation of the chromatin resulting in increased accessibility for DNase I. This hypothesis is supported by the fact that histone H1 is degraded upon incubation of necrotic cells with FSAP. The other explanation could be that inhibitors of DNase I are cleaved by FSAP, resulting in an increased activity of DNase I. Further investigation is needed to elucidate the mechanism by which FSAP and DNase I function together in the degradation and release of DNA from necrotic cells. In view of the fact that nucleosomes play a central role in the antinuclear antibody response in SLE⁵⁻⁸ and the role of FSAP together with DNase I in the release of nucleosomes from necrotic cells, it would be of interest to investigate whether the nucleosome releasing activity of FSAP is disturbed in patients with SLE.

In conclusion, this study showed that the plasma protease FSAP cooperates with DNase I in the degradation and removal of DNA from necrotic cells. This mechanism may be important in the clearance of potential autoantigens, thereby preventing the induction of an autoantigen-driven autoimmune response in SLE.

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CONFLICT OF INTEREST

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