Factor VII-activating protease

Unraveling the release and regulation of dead cell nuclear damps

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CHAPTER 8

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Summarizing discussion

SUMMARIZING DISCUSSION

Upon infection, vast numbers of host cells may die as a result of toxins released by an invading pathogen or of the collateral damage that accompanies the immune response against this pathogen[1–3]. Furthermore, damage associated molecular pattern (DAMP) molecules that are released from dying cells upon tissue damage may further drive inflammation, resulting in additional tissue damage (see review[4]). Therefore, in order to control inflammation, the efficient removal of dying cells is imperative. However, the clearance mechanisms responsible for the removal of dead cells from the body may become saturated in inflammatory conditions, resulting in insufficient dead cell clearance and the accumulation of dead cell debris[5–7]. When dead cells remain present in the circulation these are targeted by several plasma proteins such as C1q, FSAP, and DNase I[8–11]. Although the exact role of these plasma proteins in the clearance of dead cells is currently not entirely clear, deficiencies for DNase I, and for proteins of the classical pathway of the complement system are strongly linked with systemic lupus erythematosus (SLE). Notably, in the presence of serum, efferocytosis (phagocytosis of dead cells) of late apoptotic cells is enhanced[11]. Activation of FSAP also results in the release of chromatin from these cells[8]. It is clear that a range of DAMP molecules that are released from dying cells play an instrumental role in mediating the inflammatory response, but how this release is regulated, and which mechanisms are involved remains enigmatic.

The release of nuclear DAMPs from dying cells

The dynamics of DAMP exposure and release vary between different modes of cell death. Whilst apoptotic cells mostly confine DAMPs intracellularly by maintaining an intact plasma membrane, they have also been shown to present nucleosomes on their cell surface[12], and leak nucleosomes from apoptotic blebs[13]. However, the general confinement of DAMPs is lost when cells progress into a state of late apoptosis and plasma membrane integrity is lost. In contrast to apoptotic cells, necrotic cells have no mechanism in place to control DAMP release, and spontaneous leakage of DAMPs has been observed in other regulated forms of necrosis such as necroptosis (see review[14]), pyroptosis[15], and NETosis, whereby the timing of DAMP release depends on the type of cell death[16].

When investigating the immunogenicity of dying cells, one of the important

questions is whether the nature of DAMP release is passive or active; do DAMPs merely leak out of dying cells, or are there mechanisms that actively regulate their release? Some observations have been made which indicate that in addition to passive leakage[17], active regulation of DAMP release is present and plays an important role in determining the immunogenicity of dying cells. In the past, we have demonstrated that upon incubation of late apoptotic cells with healthy donor plasma, 70-80% of the chromatin content was released into the extracellular environment as measured by both flow cytometry and nucleosome-specific ELISA[18]. Plasma fractionation studies led to the identification of a plasma protease that exhibited chromatin-releasing activity: Factor VII-activating protease (FSAP), otherwise known as hyaluronic acid binding-protein 2 (HABP2)[8]. Importantly, inhibition of FSAP in plasma with a monoclonal antibody nearly entirely inhibited the release of chromatin from late apoptotic cells, underlining its importance for this mechanism.

Not just late apoptotic cells, but also necrotic cells are subject to FSAP mediated chromatin release, which we have shown in **Chapter 3**. However, in addition to FSAP, serum DNase I activity was also required. DNase I presumably fragments the chromatin into smaller parts that are more easily released from necrotic cells. Indeed, only the combined activity of DNase I and FSAP resulted in the formation of oligo-nucleosome fragments. In late apoptotic cells, chromatin fragmentation is mediated by caspase-activated DNase as part of the apoptotic process, and FSAP activity is sufficient for chromatin release from these cells. We also identified that histone H1 was cleaved by FSAP in necrotic cells treated with DNase I. Histone H1 is known to bind to the linker DNA region, where it regulates transcriptional access to the nucleosome[19]. In addition, histone H1 plays an indispensable role in chromatin compaction[20]. Therefore, it is tempting to speculate that histone H1 cleavage by FSAP allows for more efficient DNA digestion by DNase I in the linker DNA region, which facilitates efficient fragmentation of chromatin into oligo-nucleosomes[21]. This process is reminiscent of histone H1 cleavage by elastase during NETosis, where histone H1 is the first histone to be degraded in NET formation, and was identified as an inhibitor of NET formation[22]. Importantly, histone H1 is known to promote a condensed, closed state of chromatin[23], and histone H1 cleavage may therefore increase chromatin accessibility. Given that FSAP is sufficient for chromatin release from late apoptotic cells, we have attempted to investigate

histone H1 cleavage by FSAP in late apoptotic cells, but found that the bulk of histone H1 in these cells was already degraded during apoptosis (unpublished observations). Since we did not detect histone H1 in late apoptotic cells, another target of FSAP may be responsible for retaining chromatin in late apoptotic cells before it is proteolysed by FSAP. Whether histone H1 cleavage is a key event in the process of chromatin release from necrotic cells by promoting chromatin decondensation remains speculative.

Chromatin release in SLE

Although FSAP antigen levels are relatively stable, serum DNase I activity is known to vary[24,25]. Given that, in contrast to late apoptotic cells, chromatin release from necrotic cells depends on both FSAP and DNase I activity, this may imply that the kinetics of chromatin release from necrotic cells may also vary between individuals. Moreover, a decrease in DNase I activity has been demonstrated in patients that suffer from systemic lupus erythematosus (SLE) compared to healthy donors, rheumatoid arthritis, or scleroderma patients[24,26,27]. Interestingly, C1q-deficiency has also been linked to SLE[28], and C1q has been demonstrated to enhance plasma DNase I activity[10]. In **Chapter 4**, we investigated FSAP-mediated chromatin release from late apoptotic cells in the serum of SLE patients. We did not detect differences in the activation of FSAP in serum between healthy donors and SLE patients upon incubation with late apoptotic cells. However, anti-nuclear antibodies inhibited FSAP-mediated chromatin release from late apoptotic cells in these patients, likely by cross-linking nuclear antigens and forming large immune complexes that are difficult to release by FSAP. These immune complexes are potent inducers of complement activation and may further drive inflammation upon deposition, and thereby propagate disease. Although it is therefore conceivable that the inhibition of chromatin release from late apoptotic cells may result in increased inflammation during high disease activity, no inhibition of chromatin release from late apoptotic cells was observed during low disease activity, when anti-nuclear antibody levels are low.

Since chromatin release from necrotic cells also requires DNase I, and decreased DNase I activity is implicated in SLE, chromatin release from necrotic cells may be impaired in SLE sera. A decrease in DNase I activity may slow down FSAP-mediated chromatin release from necrotic cells, resulting in the increased

exposure of the immune system to putative nuclear auto-antigens, which might trigger SLE. Notably, the defective clearance of neutrophil extracellular traps in SLE has been shown to result in complement-mediated inflammation[29]. Therefore, as FSAP-mediated chromatin release from necrotic cells is DNase dependent, studies on the release of chromatin from necrotic cells by the combined actions of FSAP and DNase I may prove valuable to gain more insight in SLE disease pathogenesis. Subsequent continued exposure of the immune system to uncleared nuclear antigen may facilitate the development of an uncontrolled auto-immune response.

Histone and HMGB1 cleavage by FSAP

Histone H1 is cleaved by FSAP, and exposure of serum to unfractionated calf thymus histones has previously been shown to result in FSAP activation, and the subsequent degradation of histone H3[30]. We therefore wondered whether the different/other histone subtypes served as a substrate for FSAP. In **Chapter 5** we demonstrated that FSAP in serum is activated upon incubation with unfractionated histones, and that all different histone subtypes were efficiently degraded by activated FSAP. Histones are cytotoxic as a result of their highly positive charge, and FSAP-mediated degradation of histones turned out to protect against histone cytotoxicity. In contrast, histones as part of a nucleosome complex were not cytotoxic, and did not induce FSAP activation as strongly as free histones.

Since FSAP is constitutively present in the circulation, becomes activated upon incubation with histones, and subsequently degrades these histones, we wondered whether free histones exist in the circulation. In our study in **Chapter 5** we did not detect free histones in the circulation of *E. coli* challenged baboons or derived from patients suffering from meningococcal sepsis. Instead, histones were only detected in the form of nucleosomes. Important to note, is that we determined that the sensitivity of our assay to detect free histones is ~10 µg/ml. However, we cannot exclude that low levels of circulating free histones go unnoticed in the assay. The apparent absence of free histones in the circulation is a novel finding that raises several questions regarding the role of free histones in inflammation. In a number of studies, the cytotoxic effects of histones have been investigated when added to cultured endothelial cells at concentrations ranging from 50-100 µg/ml, or by

intravenously injecting purified DNA-free histones in high concentrations (>50 mg/kg body weight) into mice or baboons[31–33]. Lower concentrations were not found to induce any cytotoxicity. We only found histones in the form of a nucleosome complex in the circulation during inflammation, and free histones did not circulate in concentrations that are cytotoxic to cultured endothelial cells or induce inflammation *in vivo*.

Furthermore, histones are normally present in the nucleus in the form of core protein components of a nucleosome. The affinity of histones for DNA is high, ranging from 2-30 nM for the different histone subtypes[34]. Furthermore, histones are produced at levels matching the requirements for chromatin assembly, as the production of the core histones is tightly coupled to DNA replication (see review[35]). Only several histone H1 subtypes have been found to follow replication-independent expression patterns[36,37], and histone H1 exchange on chromatin is known to regulate chromatin condensation[38]. These observations raise the question whether free histones exist both inside and outside of the cell. Therefore, it is likely that upon cell death, nucleosomes, and not free histones, are the major chromatin component that is released from these cells. Nonetheless, the activity of serum DNases and nucleases produced by a pathogen may result in the liberation of histones from nucleosomes. Thus, we cannot exclude that free histones released from nucleosomes or dead cells in tissues confer their cytotoxic and immunostimulatory effects locally. We found that, in contrast to free histones, nucleosomes were not cytotoxic to cultured HEK293 cells. Our results are supported by the results of an *in vivo* nucleosome clearance study, in which injection of up to 1 mg of purified nucleosomes into mice was not lethal, and no cytotoxic effects were reported up to 1 h after infusion[39]. For comparison, injection of 1.5 mg of purified histones was lethal within 30 min[31]. Since histones circulate as nucleosomes, the focus on the cytotoxic and inflammatory effects of histones may have to be broadened to include the role of nucleosomes.

Interestingly, histone H3 was found to be cleaved in nucleosomes that circulated in *E. coli* challenged baboons and in meningococcal disease patients. Furthermore, we demonstrated that the cleavage of H3 in purified Jurkat-derived nucleosomes in serum occurred in an FSAP-dependent manner. In more detail, the ~16 kDa histone H3 was clipped into a ~13 kDa fragment. This cleavage resulted in a nucleosome complex containing the

~13 kDa fragment that was still detectable in our nucleosome ELISA. These results suggest that the cytotoxicity of histones is regulated by FSAP upon their release from nucleosomes. The consequences of histone H3 cleavage in nucleosomes require further investigation. The histone H3 tail extruding from the nucleosome complex spans ~40 amino acids[40]. Although we have not N-terminally sequenced the H3 fragment that remains in nucleosomes after FSAP incubation, the size of this fragment matches the expected size of histone H3 minus its N-terminal tail. Furthermore, removal of the N-terminal tail of histone H3 has been shown not to result in nucleosome desintegration[41], which may explain why we detect these modified nucleosomes in a nucleosome ELISA. If the H3 fragments produced by FSAP in a nucleosome have lost their cytotoxic potential, then this histone H3 cleavage may prevent histone H3 cytotoxicity prior to its release from a nucleosome. What remains to be determined is whether histone H3 in the chromatin of late apoptotic and necrotic cells is cleaved by FSAP. It is important to note that the tail of histone H3 has been demonstrated to play an important role in the condensation and folding of chromatin[41,42]. Perhaps not only histone H1 cleavage, but also H3 cleavage by FSAP may serve to decondense chromatin and facilitate chromatin release from dead cells. Additional studies are required to elucidate the effects that histone H3 cleavage in nucleosomes may have on the extracellular effects of nucleosomes.

In addition to histones, another DNA-binding protein that is known to play an important role in inflammation is HMGB1. HMGB1 is an ambiguous molecule, since its extracellular effects are modulated by the redox state of three cysteines in the molecule. Two of the cysteines, C23 and C45, may form an intramolecular disulfide bond. Disulfide HMGB1 has been demonstrated to activate immune cells via RAGE signaling, whilst the fully reduced form of HMGB1 binds to CXCL12 and has chemoattractive properties through signalling via CXCR4[43,44]. Since FSAP activation is induced by histones, which are subsequently degraded by FSAP, and HMGB1 is also a DNA-binding protein, we aimed to investigate HMGB1 degradation by FSAP in **Chapter 6**. Indeed, we found that although FSAP was not activated in serum upon incubation with HMGB1, activated FSAP did degrade HMGB1, which in turn neutralized the chemoattractive effects of HMGB1 on fibroblasts. Furthermore, FSAP activation induced by necrotic cells resulted in the degradation of the HMGB1

that was released from these cells. We therefore hypothesized that FSAP may modulate extracellular HMGB1 functioning. Indeed, in patients that had undergone major liver surgery, and that therefore had suffered major tissue damage, we observed that FSAP activation at 1hr post-surgery correlated with the decrease in HMGB1 levels between t=1 hr and t=6 hr.

Interestingly, given that HMGB1 alone does not induce FSAP activation, FSAP mediated HMGB1 proteolysis may only occur in situations where FSAP activation is induced by another molecule. Since FSAP is activated upon incubation with late apoptotic or necrotic cells, and HMGB1 is degraded in necrotic cells by FSAP, this suggests that FSAP may modulate the extracellular release of HMGB1 in cell death. In contrast to the release of HMGB1 from dying cells, HMGB1 may also be released from activated NK cells or macrophages[45,46]. Thus, supposedly, when HMGB1 release occurs in the absence of FSAP activation by histones, , the HMGB1 released by these cells will not be subject to proteolysis by FSAP. This suggests that FSAP only modulates HMGB1 extracellular functioning upon specific conditions that also induce FSAP activation.

FSAP activity: good or bad?

We described that FSAP mediates the release of chromatin from late apoptotic and necrotic cells whilst it efficiently cleaves free histones and HMGB1. Although it is unclear whether the cleavage activity of FSAP towards histones and HMGB1 is in fact required for the mechanism of chromatin release from dying cells, these processes might be linked. Importantly, another question arises from this observation: what purpose does FSAP-mediated release and proteolysis of these DAMPs have in inflammation?

Free histones are known to be cytotoxic, and both free histones and HMGB1 have well established immune-stimulatory effects. Their degradation by FSAP may therefore serve to modulate the inflammatory response. However, FSAP-mediated chromatin release from late apoptotic or necrotic cells may prove to have both beneficial and detrimental effects on the inflammatory response. Chromatin release by FSAP may facilitate more efficient removal of dead cells by liberating them of potent nuclear DAMPs. Indeed, it has been found that cells of which the chromatin had been removed upon incubation with serum were more efficiently efferocytosed by monocytes[11]. The decrease

in DNA content coincided with increased C1q binding, but chromatin removal was unaffected in C1q-depleted serum. We have verified that chromatin removal from late apoptotic cells by FSAP is normal in C1q-deficient plasma (unpublished observations). Whether chromatin removal from late apoptotic cells really potentiates efferocytosis is unclear, but it may simply lighten the phagocytic burden and make efferocytosis more manageable.

Although efferocytosis may be more efficient once the chromatin of the dead cells has been removed, the released chromatin may have effects extracellularly. Although nucleosomes lack cytotoxic effects, they have been demonstrated to activate neutrophils and DCs. However, nucleosomes infused in mice have been shown to be efficiently cleared by the liver, and no inflammation was reported. It is conceivable that the release of chromatin from a limited number of uncleared dying cells may enhance phagocytosis and has little inflammatory effects. In contrast, chromatin release from large numbers of dying cells during an inflammatory response may drive further inflammation. It is interesting to note that HMGB1, which binds nucleosomes, is degraded by FSAP that is activated upon contact with necrotic cells. HMGB1 has been shown to facilitate endocytosis of endogenous DNA and subsequent TLR-9 stimulation[47]. The immunostimulatory properties of nucleosomes have been linked to the presence of HMGB1 in one study[48]. The specific removal of HMGB1 by FSAP may thus render nucleosomes less inflammatory. Alongside the degradation of free histones, these results further support a regulatory role for FSAP in nuclear-DAMP driven inflammation. Nonetheless, the release of large quantities of chromatin may have additional extracellular effects, although *in vivo* studies are required to further delineate the role of circulating chromatin in inflammation. We speculate that FSAP-mediated chromatin release from dead cells serves to assist in the clearance of these cells and the maintenance of homeostasis, but may have pro-inflammatory effects upon extensive cell death.

In addition to the proteolysis of HMGB1 and free histones by FSAP, other substrates of FSAP have previously been identified. These include scuPA[49], fibrinogen[50], high molecular weight kininogen (HMWK)[51], platelet-derived growth factor[52] (PDGF-BB), and TFPI[53]. These substrates were identified with purified proteins *in vitro*, and, with the exception of HMWK, their degradation by FSAP in plasma remains to be verified. Notably, the Marburg I (G511E)

single-nucleotide polymorphism (SNP) of FSAP shows a decreased proteolytic activity towards all known substrates[54], although we have not investigated its activity towards histones and HMGB1. The Marburg I SNP was also identified as a general risk factor for vascular disease[55], and is associated with stroke[56], carotid stenosis[57], cancer[58], and liver fibrosis[59]. In line with the association between the Marburg I SNP and stroke, thrombin-induced stroke displayed an increased volume and neurological deficit in FSAP$^{-/-}$ mice when compared to wildtype mice[60]. Furthermore, the formation of a stable, occlusive thrombus was impaired in FSAP$^{-/-}$ mice[61]. However, it is unclear whether the association with these diseases may be ascribed to the decreased proteolytic activity of FSAP towards one of its known substrates. For example, FSAP has been shown to degrade PDGF-BB *in vitro*, and thereby inhibits vascular smooth muscle cell (VSMC) proliferation. Upon vascular damage, excessive VSMC proliferation may result in neointima formation, and indeed, increased neointima formation was linked to the Marburg I polymorphism. However, given that free histones and HMGB1 are implicated in inflammation, wound healing[62,63], and platelet activation and coagulation[64,65], it would be interesting to investigate the relevance of FSAP-mediated histone and HMGB1 proteolysis in the context of thrombotic and vascular disease.

On the regulation of FSAP activation

We have established that free histones, and to a greatly reduced extent histones as part of a nucleosome complex, induce the activation of FSAP in serum. In contrast to the core histones, of which the potential to activate FSAP is likely inhibited by the DNA that wraps these histones, histone H1 is not part of the nucleosome core, but resides on the outside of the core and regulates the wrapping of DNA around the nucleosome core. We speculate that, as a result of its location, histone H1 may be the main histone available to induce FSAP activation. Unfortunately, human histone H1 knock-out cell lines are not viable, which renders this hypothesis hard to test. However, we have tested the activation of endogenous FSAP in serum upon contact with sonicated cells from various species, including yeast. Yeast belongs to the eukaryote domain, and contains histones and organizes its DNA in nucleosomes. Remarkably, yeast does not have a linker histone H1[66]. A H1-like protein has been identified, but it does not seem to have the same role in regulating gene expression as

linker histone H1 in other eukaryotes, as a knockout of the gene only had small effects in a genome-wide epxression analysis[67]. Strikingly, we found that sonicated yeast cells did not induce FSAP activation in serum (unpublished observations). Hence histone H1 might be an important histone for FSAP activation by dead cells, but this hypothesis requires further investigation.

We have demonstrated in **Chapter 7** that inactive FSAP binds strongly to late apoptotic Jurkat cells, and that this binding is mediated by RNA, as cellular RNA digestion significantly decreased the binding of FSAP to the cells. Interestingly, the activation of FSAP in serum upon incubation with late apoptotic cells was similarly reduced by RNA digestion. However, various forms of purified RNA were unable to induce FSAP activation in serum, although they have been described to potentiate FSAP auto-activation[68]. In contrast to RNA, purified histones strongly induce FSAP activation in serum. In line with that observation, DNA digestion of late apoptotic cells significantly increased cellular FSAP binding, as well as FSAP activation, likely because histones become more available upon DNA digestion. We then demonstrated that the activation of FSAP induced by a suboptimal concentration of free histones was potentiated by the addition of purified RNA. Thus it appears that RNA potentiates the activation induced by histones derived from dead cells. Histones and RNA therefore appear to be important cellular constituents that mediate FSAP activation by dead cells.

Although the clearance of dead cells is important to maintain tissue homeostasis, neutrophils that die and form neutrophil extracellular traps (NETs) may be (temporarily) beneficial in combatting invading pathogens. Indeed, NETs have been shown to capture and kill various pathogens[69]. Premature clearance of the expelled neutrophil chromatin may therefore negatively impact pathogen clearance. Indeed pretreatment of *E. coli* challenged mice with DNase to degrade NETs markedly increased bacteremia[70]. Since activated FSAP degrades free histones, and to a lesser extent histones in nucleosomes[71], NETs may be sensitive to degradation by FSAP. However, given that necrotic neutrophils induce marginal FSAP activation[72], NET-induced activation may also be limited. Therefore, the mechanism of NET-induced FSAP activation and subsequent NET-degradation requires further investigation. Future studies that investigate NET degradation by FSAP are needed. In addition, given that HMGB1 is degraded upon the incubation of necrotic cells with FSAP, and

HMGB1 has been identified on NETs[73,74], it would be interesting to investigate whether NET-associated HMGB1 is protected from degradation by FSAP. Therefore, further studies that investigate FSAP-mediated clearance of NETs are needed. One of the reasons that necrotic neutrophils, and perhaps NETs, do not induce high levels of FSAP activation may be found in the relatively low levels of cellular RNA that are present in neutrophils. As discussed above and in Chapter 7, RNA appears to play an important role in FSAP activation by dead cells. Mature neutrophils are known to contain ~10x lower levels of mRNA compared to PBMCs[75]. Although the evidence is only suggestive, it would be interesting to investigate whether the observed reduction in FSAP activation induced by necrotic neutrophils when compared to PBMCs of the same donor, is indeed due to these low RNA levels.

So far, the studies on FSAP have focused on its role in the circulation. However, although FSAP is produced in the liver, and is present in relatively high amounts in the circulation, the presence of high levels of FSAP has been detected in various tissues. These include glandular cells present along the gastro-intestinal tract covering the stomach until the rectum, with high levels found in the small intestines and the colon[76]. Additionally, high FSAP levels have been found in Leydig cells in the testis, and in respiratory epithelial cells in the bronchus[76]. Important to note is that the cellular turnover in all these tissues is very high. The epithelium covering the intestines may renew itself every few days, whilst 75% of all male germ cells are discarded during spermatogenesis[77]. It would be interesting to investigate whether the presence of FSAP serves as an additional safeguard that mediates the inflammatory effects of nuclear DAMPs when apoptotic cells in these tissues are untimely cleared.

Future recommendations

The functionality and activation of FSAP in the regulation and release of dead cell nuclear DAMPs has increasingly been unraveled in *in vitro* studies. Our results describing the degradation of HMGB1 and free histones by FSAP suggest that FSAP activity may be beneficial in resolving nuclear DAMP-mediated inflammation. To further address this functionality, and to study the relevance of FSAP-mediated chromatin release from inadequately removed dead cells in inflammation, *in vivo* studies are needed. By introducing FSAP knockout mice in mouse models of sterile inflammation, or pathogen-induced

sepsis, more insight in the *in vivo* regulation of dead cell nuclear DAMPs may be obtained. Furthermore, i*n vivo* studies are also needed to investigate the potential of therapeutic FSAP administration in highly inflammatory disease. Moreover, the role of chromatin clearance from dead cells by FSAP in the context of SLE etiology may be assessed in lupus-prone mice.

In conclusion, we show that FSAP regulates the release and degradation of important nuclear DAMPs from dead cells. The pro-inflammatory role of nuclear DAMPs, and specifically the immunity of chromatin, is increasingly being recognized. With this recognition, the regulation of DAMPS and chromatin immunity has also become more apparent, and studies investigating DAMP regulation may result in new opportunities to treat inflammatory diseases. Therefore, our results support an important role for FSAP in the regulation of inflammation in situations where the homeostatic clearance of dying cells has been compromised.

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