Factor VII-activating protease

Unraveling the release and regulation of dead cell nuclear damps

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CHAPTER 6

Factor VII-activating protease mediates the degradation of HMGB1

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Gerben Marsman, Ingrid Bulder, Rowan F. van Golen, Francesco De Marchis, Marco E. Bianchi, Michal Heger, Sacha Zeerleder, Brenda M. Luken

ABSTRACT

During ischemia-reperfusion (I/R) injury, cells undergo necrotic cell death, which results in the release of pro-inflammatory damage-associated molecular patterns (DAMPs) including the DNA-binding protein high-mobility group box 1 (HMGB1). Factor VII-activating protease (FSAP) is a serine protease that is activated in serum upon incubation with necrotic cells. Activated FSAP degrades free histones which, like HMGB1, bind DNA and have potent pro-inflammatory extracellular effects. We therefore wondered whether FSAP may play a role in the regulation of extracellular HMGB1. We identified that HMGB1 released from necrotic cells is degraded by FSAP in healthy donor serum, but not in FSAP-depleted or in FSAP-deficient serum. Moreover, inhibition of FSAP activation with a monoclonal anti-FSAP antibody also prevented HMGB1 degradation. Notably, FSAP-mediated degradation of purified HMGB1 neutralized the chemotactic effects of HMGB1 on 3T3 fibroblasts, suggesting that FSAP activity indeed regulates HMGB1 functioning. We then investigated whether FSAP may play a role in the regulation of HMGB1 levels *in vivo*. In the plasma of hepatic liver surgery patients that were exposed to I/R injury, elevated HMGB1 levels were previously found at t=1 and thereafter rapidly declined between t=1 and t=6 h after surgery, whilst no such transient rise in HMGB1 was observed in liver surgery patients that had not been subject to I/R injury. In the plasma of these patients, we determined the levels of complexes formed by FSAP and alpha 2-antiplasmin (AP), a plasma inhibitor of FSAP, as a readout for FSAP activation. We found that patients that were exposed to I/R injury had significantly higher levels of FSAP activation at t=1 and t=6 h after surgery than control patients. Furthermore, we found a strong correlation between FSAP-AP levels at t=1, and the decline in HMGB1 levels between t=1 and t=6 h, which suggests that FSAP may play a role in HMGB1 proteolysis *in vivo*. Taken together, FSAP may be important in the regulation of HMGB1 which is released from necrotic cells in inflammation.

INTRODUCTION

During cell death, molecules that normally reside intracellularly may become exposed or released into the extracellular environment. Such exposure or release occurs in a controlled manner during programmed cell death (apoptosis, necroptosis, ferroptosis, pyroptosis) or uncontrolled, as is the case during primary necrosis. A range of molecules released from dead and dying cells have potent immunogenic effects and may initiate and support an inflammatory response[1], induce leukocyte migration[2], and facilitate wound healing[3]. Collectively, these molecules are referred to as damage-associated molecular patterns (DAMPs).

One of the most prominent DAMPs is high-mobility group protein B1 (HMGB1). HMGB1 contributes to disease severity and lethality in various murine models *in vivo*, including cecal ligation puncture-induced sepsis[4,5], LPS-induced and liver surgery-induced sterile inflammation[6–8], experimental arthritis[9], cancer[10], and thrombosis[11]. Inhibition of HMGB1 release from human macrophages with cholinergic agonists improved survival in the abovementioned experimental sepsis models, whilst neutralization with blocking anti-HMGB1 antibodies reduced lethality in LPS-induced inflammation[8]. The antagonistic A box of HMGB1 ameliorated collagen-induced arthritis[9]. In humans, increased HMGB1 levels were found during sepsis[12], severe sepsis[13,14], acute pancreatitis[15], severe infection[16], trauma[17], and cancer[18,19].

HMGB1 (29 kDa) contains two box domains and a C-terminal acidic tail. The first domain, Box A, is an N-terminally located ~11 kDa DNA-binding domain of HMGB1, and is flanked by the ~10 kDa Box B. HMGB1 normally resides in the nucleus, where it partially competes with histone H1 for binding to nucleosomes, and, like histone H1, is involved in higher order regulation of chromatin structure[20]. However, upon release into the extracellular environment, HMGB1 exerts potent immunogenic effects that are governed by the redox form in which HMGB1 is present. HMGB1 contains three cysteines; C23 and C45 which are located in Box A and may form an intramolecular disulfide bond, and C106 which is located in Box B. When all three cysteines are in the thiol state (reduced HMGB1), HMGB1 complexes with CXCL12 to signal via CXCR4 and mediates chemotactic effects on mouse fibroblasts, human monocytes[21], leukocytes[22,23], and macrophages[24]. However, when C23 and C45

form a disulfide bond and C106 is a thiol, HMGB1 lacks chemotactic activity. Instead, disulfide HMGB1 signals via the receptor for advanced glycation end-products (RAGE) and toll-like receptor (TLR) 4, which resulted in a pro-inflammatory cytokine response in human macrophages[21,25]. Importantly, recombinant HMGB1 Box A fragments have been found to inhibit mouse fibroblast chemotaxis induced by intact HMGB1[21].

Our group has previously shown that histone H1 is proteolytically cleaved upon incubation of necrotic cells with healthy donor serum. The protease responsible for the proteolytic degradation of histone H1 is Factor VII-activating protease (FSAP)[26]. FSAP is a serine protease that circulates as a single-chain zymogen in plasma at a concentration of ~12 µg/mL (187 nM)[27], and is activated upon contact with late apoptotic and necrotic cells[28] and histones[29], which results in the conversion of single-chain FSAP into a two-chain active protease. FSAP activation results in the release of chromatin from late apoptotic and, in cooperation with DNase I, necrotic cells[26,30], and it cleaves free histones[31].

HMGB1 is released into the extracellular environment during necrotic cell death. During apoptosis, however, HMGB1 remains firmly attached to chromatin due to the hypoacetylation of histones in apoptotic cells[32]. It is currently unclear how necrotic cell-derived HMGB1 levels are regulated at sites of tissue injury. Since endogenous FSAP in serum proteolytically degrades DNA-free histones and histone H1 of necrotic cells, we investigated whether active FSAP also cleaves HMGB1 following its release during necrotic cell death.

MATERIALS & METHODS

Reagents

NuPAGE materials for SDS-PAGE and Western blotting were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Recombinant HMGB1 (disulfide and reduced form) was purchased from HMGBiotech (Milan, Italy). Purified, unfractionated calf thymus histones were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of laboratory grade and obtained from Sigma-Aldrich or Merck-Millipore (Billerica, MA, USA). Rat monoclonal antibody anti-mouse-kappa-light-chain conjugated to horse-radish-peroxidase and mouse monoclonal anti-FSAP antibody were prepared at our department[28,33]. Anti-HMGB1 antibody was obtained from R&D Systems (Minneapolis, MN, USA)

Study subjects

Blood samples were collected from 39 patients who underwent major liver surgery. The patient cohort has been described previously[34,35]. In brief, plasma samples were obtained from patients that underwent surgery either with or without the intraoperative use of liver ischemia, thereby creating an ischemia/reperfusion (I/R, n=25) and a control (n=13) group. The decision to use liver ischemia was left at the surgeon's discretion. Blood was drawn directly after induction of general anesthesia (baseline) and 1 hr and 6 hr after the start of reperfusion (I/R group) or on completion of liver resection (control group). The study protocols were approved by the Institutional Review Board of the Academic Medical Center (Amsterdam, the Netherlands) and were registered at https://clinicaltrials.gov under identifier #NCT01700660. All participants provided written informed consent.

Healthy donor serum

Serum was received from anonymous healthy donors at Sanquin Blood Supply (Amsterdam, the Netherlands). The samples were obtained and handled according to Dutch regulations for the use of patient material. The characterization of the FSAP-deficient donor has been described previously[26]. Briefly, a homozygous nonsense mutation (c.607C>T, p.R203X) led to replacement of an arginine with a premature stop codon.

Cell culture and induction of necrosis

Human T lymphocyte (Jurkat) cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, BioWhittaker) (Lonza, Basel, Switzerland) supplemented with 5% (v/v) fetal calf serum (FCS) (Bodinco, Alkmaar, the Netherlands), 100 U/mL penicillin (Invitrogen, Carlsbad, CA, USA), 100 µg/mL streptomycin (Invitrogen) and 50 µM β-mercaptoethanol (Sigma-Aldrich). Cells were maintained at standard culture conditions (humidified atmosphere composed of 5% CO_2 and 95% air, 37 °C). Mouse 3T3 fibroblasts (American Type Culture Collection), were cultured in Dublecco's Modified Eagle Medium (DMEM), supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. Necrosis was induced by freeze-thawing 4×10^6/mL Jurkat cells in FCS-free culture medium for three cycles at -80 °C and 37 °C.

Plasma-purified FSAP

Two-chain FSAP (tcFSAP) was purified from recalcified citrated plasma by affinity chromatography as described previously[30]. Briefly, plasma components were subjected to size exclusion chromatography using an Ultragel ACA34 column (Pall Corporation, Port Washington, NY). Subsequently, fractions containing FSAP were applied to an anti-FSAP-coupled Sepharose column. Column-bound FSAP was eluted and dialyzed to 10 mM phosphate buffer (pH = 6.0) containing 140 mM NaCl. As a result of the purification process, the FSAP zymogen is converted into active tcFSAP. The activity of the final product was determined in a chromogenic assay using substrate S2288 (Chromogenix), and the product was analyzed on SDS-PAGE. 100 U/mL of tcFSAP, as determined by ELISA[30], corresponds to a physiological FSAP concentration in 1 ml of healthy donor plasma.

FSAP depletion from serum

Anti-kappa-light-chain antibody (RM19) coupled to sepharose was incubated with anti-FSAP, followed by incubation with healthy donor serum. The FSAP-depleted serum was collected and stored at -20°C until further use. Mock-depleted serum was prepared through the same procedure using the isotype control antibody anti-IL6.8 (Sanquin). The depletion of FSAP from serum was confirmed by SDS-PAGE and Western blotting.

Activation of recombinant FSAP

The production and purification of the recombinant $FSAP^{R313Q}$ mutant (rFSAP) has been described in detail[36]. In short, rFSAP was freshly activated before each experiment, by incubation with 0.1 µg/mL thermolysin in 10 mM 2-(N-morpholino)ethanesulfonic acid (MES), 100 mM NaCl, 100 µM $CaCl_2$, 50 nM $ZnCl_2$, and 0.01% Tween-80, pH 5.0, for 2 h at 30 °C. The reaction was stopped by the addition of 30 µg/mL phosphoramidon disodium salt dissolved in MilliQ water. As a control, inactive rFSAP was subjected to the same conditions but in the absence of thermolysin. Activated rFSAP was stored on ice and used within 2 h.

HMGB1 proteolysis

Purified, recombinant disulfide- and fully reduced HMGB1 were obtained from

HMGbiotech. 1 µg of recombinant HMGB1 was incubated with 12.5-100 nM plasma-purified (tcFSAP) or rFSAP in 50 mM HEPES buffer containing 140 mM NaCl at 37 °C for 1 h. The cleavage products were separated on a 12% NuPAGE Bis-Tris polyacrylamide gel and visualized with the Silverquest kit (Invitrogen) according to the manufacturer's protocol.

To assess HMGB1 degradation in dead cells, 4×10^6/mL necrotic Jurkat cells were incubated with 20 nM tcFSAP, 20 nM rFSAP, 10% healthy donor serum in the absence or presence of 20 µg/mL anti-FSAP antibody, or 10% FSAP-deficient serum at 37 °C for 1 h. Samples were separated on a 12% NuPAGE Bis-Tris polyacrylamide gel and transferred to a nitrocellulose membrane using the iBlot system (Invitrogen). Membranes were blocked with 1% Western blocking reagent (Roche) in PBS. HMGB1 was detected using 1 µg/mL anti-HMGB1 followed by incubation with 0.5 µg/mL rat anti-mouse kappa light chain conjugated to horseradish peroxidase. Blots were developed using Pierce ECL 2 Western blotting substrate (Thermo Scientific) and visualized with the Chemidoc MP system (Bio-Rad, Hercules, CA, USA) or by exposure to high performance chemoluminescence film (GE Healthcare, Little Chalfont, UK).

Chemotaxis assays

Chemotaxis assays were performed as previously described[21]. In short, 50,000 murine (3T3) fibroblasts in 200 µl serum-free DMEM were added to the upper chamber of 8-µm modified Boyden chambers (Neuro Probe) coated with 50 µg/mL fibronectin (Roche). HMGB1 or HMGB1 that had been pre-incubated with active rFSAP was added to the lower chamber, and cells were allowed to migrate for 3 h at 37°C. To assess a potential inhibitory effect of FSAP-generated HMGB1 fragments on HMGB1-mediated chemotaxis, a fixed concentration of intact HMGB1 was mixed with FSAP-generated HMGB1 fragments, and migration was determined. Non-migrating cells were removed with a cotton swab. After fixation in ethanol, the migrated cells were stained with Giemsa and counted in 10 random fields/filter. All assays were performed in triplicate.

Nucleosome, FSAP-AP, and HMGB1 ELISA

Nucleosome levels were determined as previously described[33]. Briefly, a monoclonal anti-histone H3 capture antibody and biotinylated $F(ab')_2$ fragments of an antibody recognizing an epitope formed by histone H2A,

H2B, and DNA were used in combination with streptavidin-polyHRP for detection. FSAP-AP levels were determined as recently described[28]. In short, a monoclonal antibody that recognizes α2-antiplasmin was used as a capture antibody. Biotinylated anti-FSAP4, which recognizes the light chain of FSAP, was used for detection in combination with streptavidin-polyHRP. HMGB1 levels were determined using an HMGB1 ELISA kit (IBL International) following the manufacturers' instructions (regular sensitivity mode). To correct for hemodilution, we measured total plasma protein levels with the Pierce BCA Protein Assay Kit (Thermo-Fischer Scientific) and expressed the values as U/mg plasma protein.

Statistical analysis

Statistical analyses for comparisons between two time-points within one group were performed using paired, two-tailed Student's t-tests (Wilcoxon matched-paires signed rank test), whilst for comparisons between two groups at one time-point an unpaired (Mann-Whitney) rank test was used in GraphPad Prism (GraphPad Software, San Diego, CA). For correlation analysis, Spearman's rank correlation coefficient was determined. Results are expressed as mean ± SEM for $n \geq 3$. For data sets comprising $n < 3$, values are expressed as mean ± SD. A p-value of ≤ 0.05 was considered statistically significant.

RESULTS

Plasma-derived FSAP and recombinant FSAP cleave oxidized and reduced HMGB1

To investigate whether HMGB1 is susceptible to proteolytic cleavage by FSAP, we incubated recombinant HMGB1 in the disulfide form and the reduced form with increasing concentrations of active plasma-purified FSAP for 30 min at 37 °C. Subsequently, samples were separated by SDS-PAGE and cleavage products were visualized by silver staining. As shown in **Figure 1A**, both the disulfide form and the reduced form of HMGB1 were efficiently cleaved by FSAP. At 12.5 nM FSAP, HMGB1 (29 kDa) was cleaved into multiple smaller bands. At a 50 nM FSAP concentration, no native HMGB1 remained after 30 min incubation. The cleavage pattern generated by FSAP was not dependent on HMGB1 redox status, as the observed cleavage products of disulfide and reduced HMGB1 were of similar size.

To rule out that the HMGB1 proteolysis was caused by an impurity in the purified FSAP preparation, we also incubated HMGB1 with recombinant $FSAP^{R313Q}$ (rFSAP). $FSAP^{R313Q}$ is an enzymatically inactive form of FSAP, as the activation site is mutated to prevent auto-activation. The mutation introduces a cleavage site for thermolysin that mediates *in vitro* activation. Active, but not inactive, rFSAP efficiently proteolyzed both the reduced-HMGB1 (**Figure 1B**) and disulfide-HMGB1 (data not shown).

Plasma-derived FSAP cleaves HMGB1 released from necrotic cells

It was previously reported that FSAP is activated upon contact with late apoptotic or necrotic cells[28]. Therefore, we investigated whether necrotic cell-derived HMGB1 is degraded by activated FSAP. Accordingly, necrotic Jurkat cells were incubated with plasma-purified active FSAP or 10% serum of a healthy donor. Additional experiments were conducted with serum in which FSAP was inhibited with a monoclonal anti-FSAP antibody and with FSAP-deficient serum. After 1 h incubation at 37 °C, the proteins were separated by SDS-PAGE, followed by detection of HMGB1 on immunoblot. As shown in **Figure 2A**, HMGB1 derived from necrotic cells was efficiently degraded upon incubation with plasma-purified FSAP and healthy donor serum, but not when FSAP activation was inhibited or when FSAP was absent. To exclude FSAP-unrelated causes of HMGB1 degradation in healthy donor serum, we employed

FSAP-deficient serum and also depleted FSAP from healthy donor serum by affinity chromatography using Sepharose-conjugated anti-FSAP antibodies. We found no degradation of necrotic cell-derived HMGB1 in FSAP-deficient and FSAP-depleted serum, whilst HMGB1 in control serum was degraded (**Figure 2B**). In addition, active but not inactive rFSAP was able to degrade HMGB1, confirming that FSAP directly proteolyzes HMGB1 upon its release from necrotic cells (**Figure 2C**).

Others and our group have demonstrated that, in addition to FSAP activation upon contact with dead cells, free histones can induce FSAP activation in plasma or serum, which results in FSAP-mediated histone proteolysis. Similar to histones, HMGB1 is proteolytically degraded by FSAP and is a DNA-binding protein. It was tested whether HMGB1 also induces FSAP activation in serum. To that end, we incubated HMGB1 (50 µg/mL; both

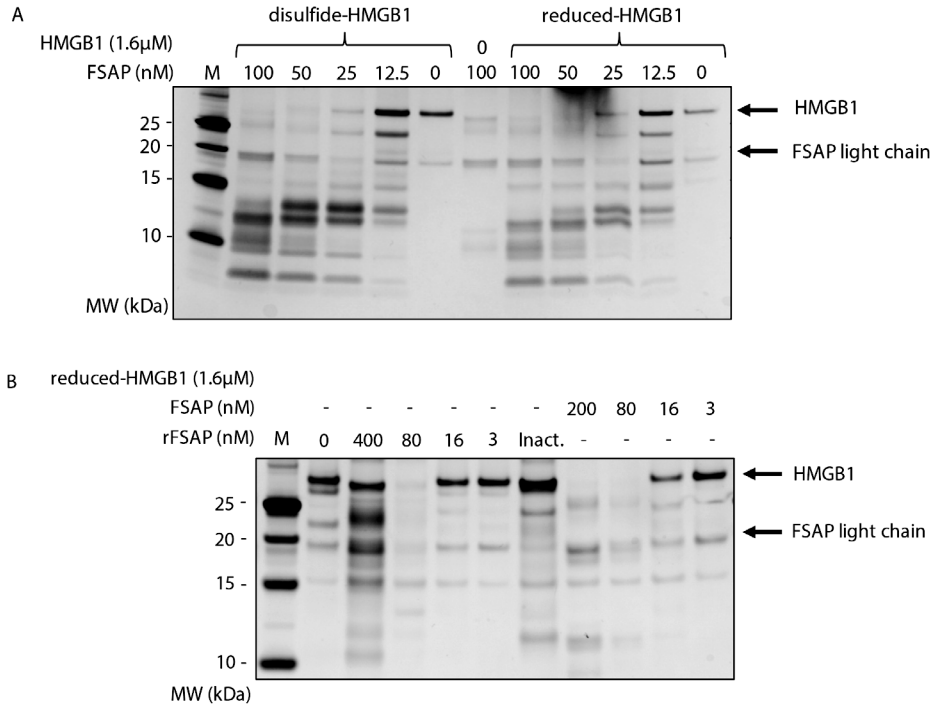

Figure 1. FSAP proteolyzes reduced and oxidized HMGB1
Both disulfide and reduced HMGB1 were incubated with plasma purified active FSAP (A) or recombinant FSAP (B) at 37 °C for 1 h. The cleavage products were separated on SDS-PAGE and visualized by silver staining. Arrows indicate intact HMGB1 and the light-chain of active FSAP. Inact.: inactive rFSAP. The depicted gels are representative of three independent experiments.

disulfide- and reduced HMGB1) or histones (50 µg/mL) with 50% healthy donor serum at 37 °C for 1 h. Complexes of FSAP with one of its serpins, alpha-2-antiplasmin (AP), were used as a measure of FSAP activation. FSAP-AP complex formation indicates that FSAP activation and subsequent inactivation by AP had occurred. In contrast to FSAP activation induced by histones, no activation of FSAP in serum following incubation with HMGB1 occurred (**Figure 2D**), which indicates that the degradation of necrotic cell-derived HMGB1 by FSAP in serum requires prior activation of FSAP by a molecule other than HMGB1.

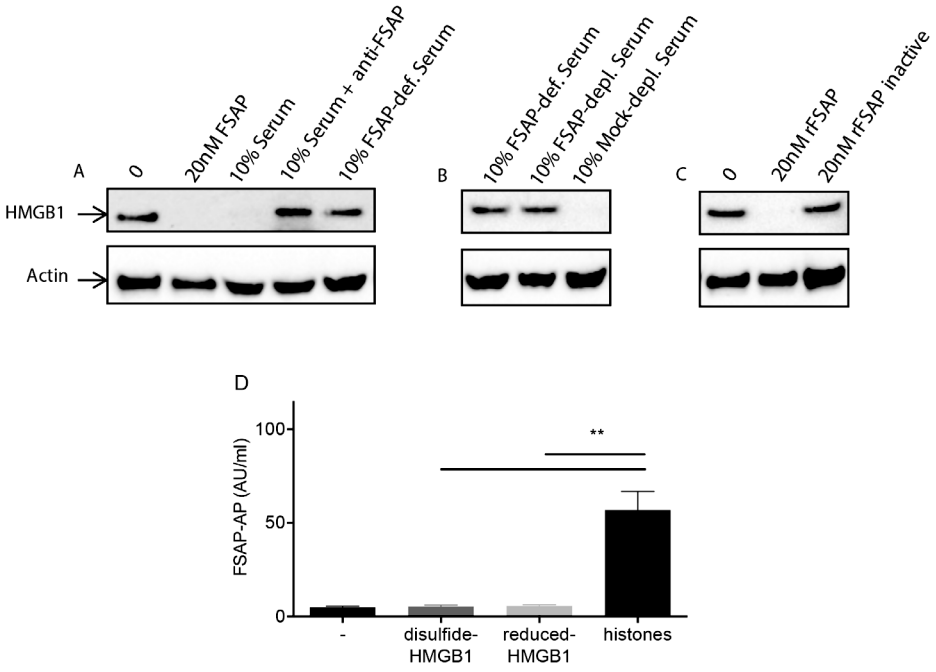

Figure 2. FSAP in serum proteolyzes HMGB1 derived from necrotic cells

Necrotic Jurkat cells were incubated for 1 h at 37 °C with 20 nM plasma-purified FSAP or 10% serum in the presence or absence of an inhibitory monoclonal antibody directed against FSAP, or 10% FSAP-deficient serum (A). The cleavage products were separated by SDS-PAGE and HMGB1 was detected by immunoblotting. Actin was also visualized on the same blot as a loading control. As a control, necrotic Jurkat cells were incubated with 10% FSAP-deficient serum, 10% FSAP-depleted or 10% mock-depleted serum (B), or with 20 nM active or inactive rFSAP (C) and HMGB1 was detected on immunoblot. The depicted blots are representative of three independent experiments. The ability of HMGB1 to induce the activation of FSAP in serum was determined by incubating disulfide or reduced HMGB1 (50 µg/mL) with 50% healthy donor serum for 1 h at 37 °C. Subsequently FSAP-AP complexes were detected by ELISA (D). As a control, purified calf thymus histones (50 µg/mL) were used to induce FSAP activation in serum. n = 6 Data are expressed as mean ±SEM. **p<0.01 was determined by Mann-Whitney Rank Sum test.

FSAP-mediated proteolysis of HMGB1 abrogates fibroblast chemotaxis

HMGB1 is released during cell death, and results in neutrophil migration to the site of injury[7]. However, it is unclear how HMGB1 levels at the site of injury are regulated. In this set of experiments we therefore determined whether FSAP affects HMGB1-mediated chemotaxis through HMGB1 proteolysis. A fixed amount of HMGB1 was incubated overnight with increasing concentrations of activated FSAP at 37 °C. Chemotaxis of 3T3 murine fibroblasts towards the cleaved HMGB1 fragments was analyzed in relation to the size of the cleavage fragments, determined by SDS-PAGE. As can be observed in **Figure 3A**, the chemotactic movement of fibroblasts towards HMGB1 was reduced with increasing concentrations of activated rFSAP. A pronounced ~20 kDa cleavage product was detected at 5 and 16 nM FSAP, but HMGB1 was fully degraded when using higher concentrations of FSAP (**Figure 3B**). A similar ~20 kDa HMGB1 cleavage product had been observed in **Figure 1B**.

We hypothesized that one of the generated HMGB1 cleavage fragments may contain the ~11 kDa Box A of HMGB1, which is known to inhibit HMGB1-mediated chemotaxis[21]. To test this hypothesis, we incubated a fixed amount of intact HMGB1 with an equal amount of HMGB1 that had been proteolyzed by increasing concentrations of active rFSAP and analyzed the capacity of the HMGB1 cleavage products to inhibit HMGB1-mediated fibroblast migration. Compared to the migration induced by intact HMGB1, fibroblast migration was not inhibited by the FSAP-cleaved HMGB1 fragments, suggesting that they did not contain an intact Box A (**Figure 3C**).

In conclusion, FSAP neutralizes HMGB1-mediated 3T3 fibroblast migration through degradation of HMGB1. HMGB1 levels and its chemotactic properties are therefore likely modulated by FSAP at the site of tissue injury.

Plasma HMGB1 levels are negatively correlated with plasma FSAP-AP complex levels in liver surgery patients

During inflammatory disease, FSAP is activated upon contact with dead cells. FSAP has been demonstrated to release nucleosomes from these cells *in vitro*. In line with these findings, elevated FSAP-AP levels have been detected in patients suffering from inflammatory conditions such as sepsis and FSAP-AP levels strongly correlate with circulating nucleosome levels in these patients[28]. In addition to nucleosomes, elevated HMGB1 levels have been found in

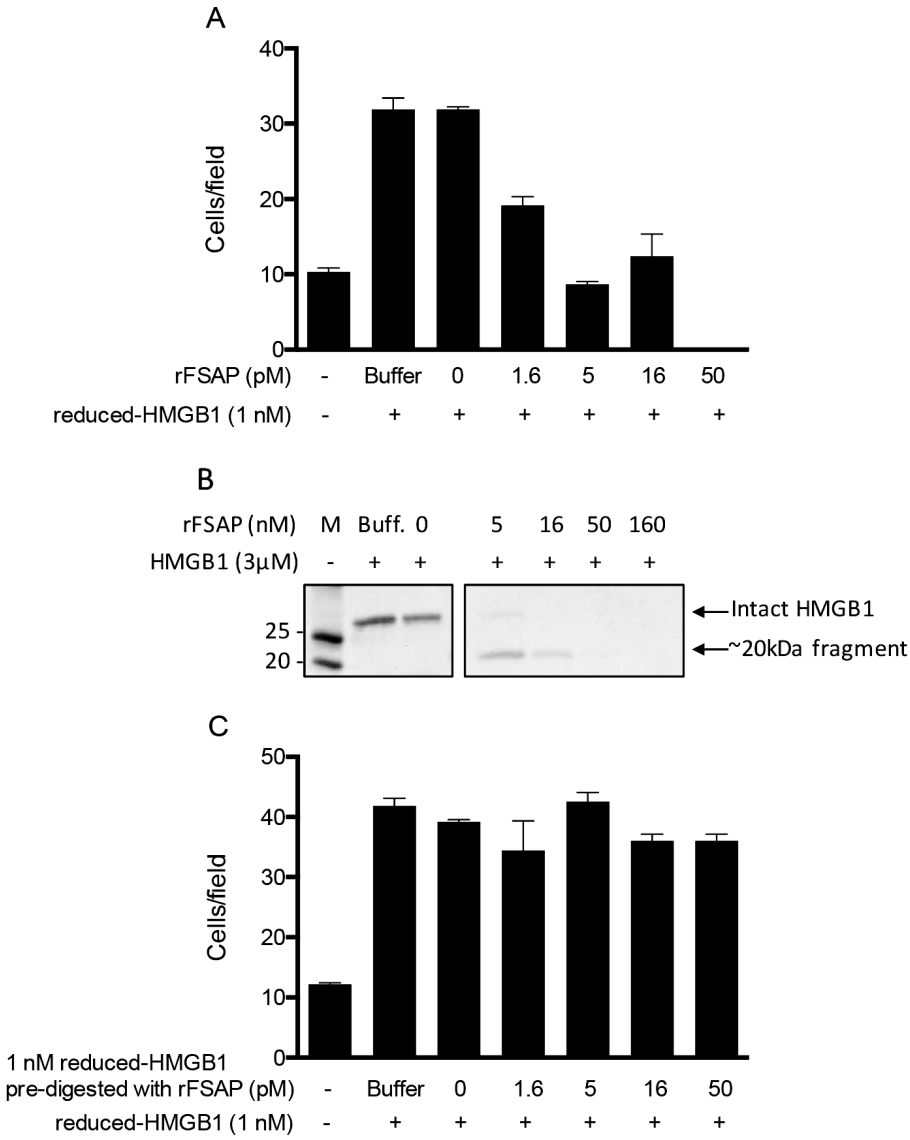

Figure 3. FSAP-mediated proteolysis of HMGB1 reduces fibroblast chemotaxis
3T3 fibroblasts were added to the upper compartment of a modified Boyden chamber and intact HMGB1 (30 ng/mL), or HMGB1 that had been pre-incubated with active rFSAP (0.1-30 ng/mL), was added to the lower compartment. Cells were left to migrate for 3 h at 37°C and migrated cells were quantified (A). HMGB1 cleavage products were separated on SDS-PAGE and visualized by Coomassie stain (B). Both insets are from the same gel. To test the inhibitory capacity of the ~20 kDa HMGB1 cleavage product, intact HMGB1 (30ng/mL) was incubated with rFSAP-processed HMGB1 cleavage products and its chemotactic act ivity was subsequently determined (C). Data are expressed as mean ±SEM obtained from two independent experiments performed in triplicate.

patients suffering from inflammatory disease[12,13,17]. However, it is unclear how the systemic HMGB1 and nucleosome levels are interrelated. Moreover, since FSAP degrades HMGB1 *in vitro* (**Figure 1**), we investigated a possible role for FSAP in regulating HMGB1 levels in patients with an inflammatory condition. In a previous study, plasma HMGB1 and nucleosome levels were determined in patients that had undergone major liver surgery with ischemia/reperfusion (I/R) injury – a condition that predisposes patients to sterile inflammation[34,37]. Data were compared to a control patient cohort that underwent major liver surgery without I/R. Plasma was assayed at t = 0 h (prior to surgery) and at t = 1 h and t = 6 h postoperatively. HMGB1 levels were elevated in the I/R group at t = 1 h but had declined at t = 6 h, suggesting rapid HMGB1 clearance or breakdown (**Supplementary Figure 1A**). Nucleosomes were elevated in the I/R group at t = 1 h and t = 6 h but the concentration did not differ between these time points (**Supplementary Figure 1B**). Nucleosomes were surgery-induced inasmuch as their post-ischemia levels were comparable between the I/R and control group. Thus the rise in HMGB1 and not nucleosomes specifically correlated with I/R injury.

In order to follow up on the *in vitro* finding that FSAP degrades HMGB1, we determined FSAP-AP levels in plasma from these patients and investigated a possible relation between FSAP-AP levels and HMGB1. FSAP-AP levels were elevated at t = 1 h and remained elevated at t = 6 h ($p < 0.0001$ for both time points) **(Figure 4A**). Importantly, FSAP-AP levels were significantly higher at t = 1 h and t = 6 h in patients that had been subjected to I/R injury compared to the control group ($p = 0.01$).

As previously found in severe sepsis, septic shock, and meningococcal sepsis[28], nucleosome and FSAP-AP levels correlated strongly in both the I/R group ($r = 0.80$, $p < 0.0001$) (**Figure 4B**) and the control group ($r = 0.71$, $p < 0.0001$). Nucleosome and HMGB1 levels correlated in the I/R group ($r = 0.51$, $p < 0.0001$) and the control group ($r = 0.47$, $p < 0.0063$), albeit the correlation was weaker in the latter group. These findings are in line with the observation that HMGB1 is a more specific marker for I/R injury. Furthermore, HMGB1 and FSAP-AP levels correlated well in the I/R group ($r = 0.63$, $p < 0.0001$) but less profoundly in the control group ($r = 0.49$, $p < 0.0194$) (**Figure 4C**).

To investigate a possible relation between FSAP activation and the decrease in HMGB1 levels observed between t = 1 h and t = 6 h, we calculated

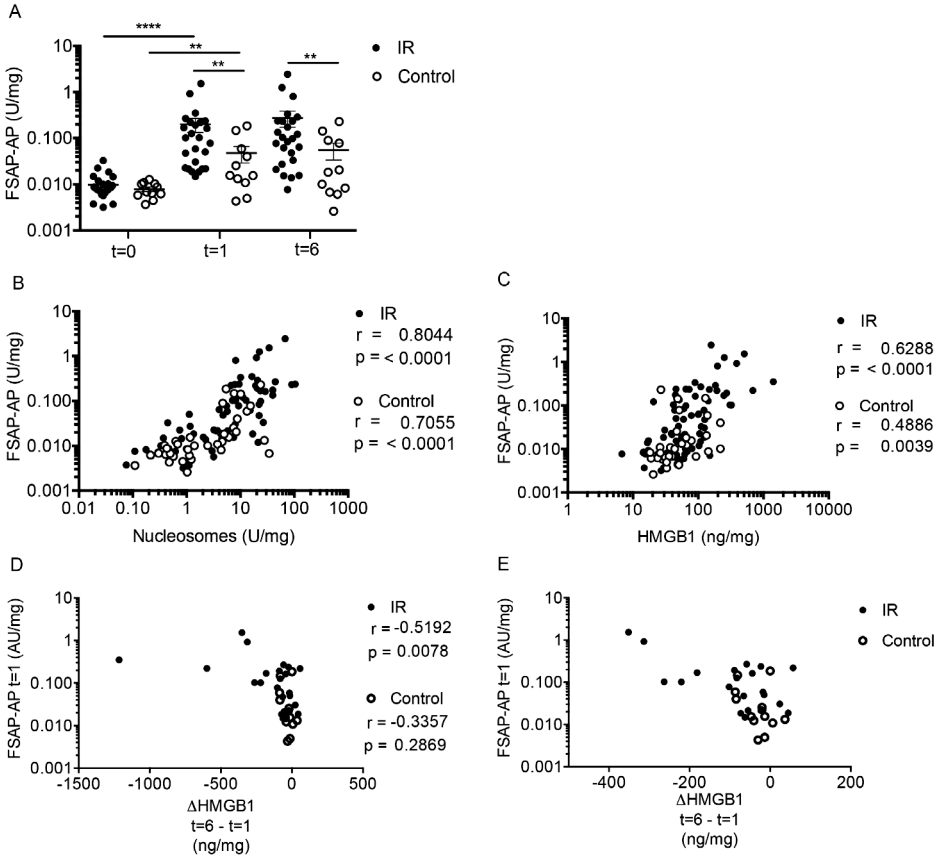

Figure 4. FSAP-AP complexes are elevated in hepatic surgery patients

Levels of FSAP-AP complexes (A) in the plasma of patients that had undergone hepatic surgery followed by I/R were determined by ELISA. As a control group, hepatic surgery patients that had not been exposed to I/R were included. Blood was drawn prior to surgery (T = 0), 1 h postoperatively (T = 1) and 6 h postoperatively (T = 6). Nucleosome levels (B) and HMGB1 levels (C) were correlated to FSAP-AP levels, and FSAP-AP levels at T = 1 were correlated to delta HMGB1 levels between T = 1 and T = 6 (D). (E) is a zoom-in of (D) with x-axis limits set between -500 and 200 ng/mg. Nucleosomes and FSAP-AP levels are expressed as AU/mg plasma protein. HMGB1 is expressed as ng/mg plasma protein. Data are expressed as individual values and the mean ±SEM are indicated. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001 were calculated using a Wilcoxon matched-pairs signed rank test. For correlation analysis, Spearman's rank correlation coefficient was determined.

the correlation between FSAP-AP levels at t = 1 h and the difference in HMGB1 levels between t = 1 h and t = 6 h (DHMGB1). As expected, HMGB1 exhibited a negative correlation with FSAP-AP (r = -0.52, p = 0.008) in the IR group. No correlation was found in the control group (r = -0.34, p = 0.29) (**Figure 4D and E**). These findings suggest that the degree of FSAP activation at t = 1 h is associated with a more substantial decrease in HMGB1 levels. The data also yield credence to our *in vitro* results demonstrating that activated FSAP in plasma degrades HMGB1.

DISCUSSION

HMGB1 is a molecule with different functions depending on the redox form of the molecule and its cellular location. Although the biological effects of extracellular HMGB1 are gradually becoming clearer, the regulation of HMGB1 levels in the circulation has received little attention. In this study we show that HMGB1 is degraded by FSAP under controlled in vitro conditions and in plasma samples from a clinical patient cohort. Furthermore, FSAP-mediated proteolysis of HMGB1 was not restricted to a specific redox form, as both the reduced and free thiol forms of HMGB1 were degraded with similar efficiency. The degradation of HMGB1 by FSAP abrogated HMGB1-mediated fibroblast chemotaxis, indicating that FSAP neutralized the pro-inflammatory properties of HMGB1.

From previous studies, it has become clear that the chemotactic and immunostimulatory effects of HMGB1 are not only dependent on the redox state of HMGB1, but also on its concentration. Low levels of HMGB1 (50-100 ng/mL) were found to inhibit fMLF- and IL-8-mediated neutrophil migration, whereas high levels of HMGB1 (5,000 ng/mL) had a direct chemoattractant effect on neutrophils[22]. Moreover, a recombinant HMGB1 Box A fragment antagonized the chemotactic effect of intact HMGB1[21]. Since the function of HMGB1 depends so critically on its concentration, and also on the specific exosite interactions of its different functional domains, HMGB1 proteolysis by FSAP may constitute an important regulatory mechanism of HMGB1 function. HMGB1 proteolysis by FSAP produced several cleavage products. We hypothesized that one of these cleavage products may contain the intact Box A domain. However, the inhibitory capacity of the HMGB1 cleavage products generated by a broad titration of FSAP was absent in terms of fibroblast

chemotaxis. This suggests that FSAP may cleave in the Box A domain, but further biochemical studies investigating the interactions between FSAP and HMGB1 and the identification of the exact FSAP cleavage sites in HMGB1 are required to test this hypothesis.

FSAP is activated upon incubation of serum with late apoptotic and necrotic cells[28], but seems otherwise relatively stable in its zymogen form. For instance, the initiation of coagulation during serum preparation does not lead to FSAP activation[28]. We showed that HMGB1 itself does not induce FSAP activation, in contrast to histones. Since activation of FSAP is required for the enzymatic proteolysis of HMGB1, HMGB1 release in the absence of cell death, for example by activated NK cells or macrophages, may not coincide with FSAP activation and may be less susceptible to FSAP-mediated regulation and degradation.

The role of HMGB1 as a DAMP has been thoroughly characterized following hepatic I/R injury[6,37,38] and circulating HMGB1 may potentially serve as a marker for ischemic hepatocyte injury after clinical liver I/R. Although nucleosome levels were equally elevated in the liver I/R and control group, both FSAP-AP levels and HMGB1 levels were significantly higher in the I/R group one hour after surgery. Notably, the type of cell death that is induced under ischemic conditions is different from that induced under normoxic conditions, which may account for the varying HMGB1 levels. Ischemic conditions result in ATP depletion, which is one of the main reasons that necrosis and not apoptosis is the predominant mode of cell death during liver I/R injury[39–41]. Necrotic cells release HMGB1 into the extracellular environment, in contrast to apoptotic cells[32]. This may explain the different HMGB1 levels found in the I/R group versus control.

In vivo, increased levels of HMGB1 have been found in various inflammatory conditions. Similarly, activated FSAP has been found in post-surgery patients, major trauma patients, patients suffering from severe sepsis, septic shock, meningococcal sepsis, and systemic lupus erythematosus[28,42,43]. In this study we showed that FSAP activation had occurred in patients that had undergone major liver surgery, and was significantly more increased in patients that had been subjected to I/R injury when compared to the control group. The FSAP activation coincided with a decrease in HMGB1 levels. We hypothesize that activated FSAP is responsible for the decline in HMGB1 in

these patient sera. Although activated FSAP in serum degrades HMGB1, and FSAP activation in major liver surgery correlated with the decrease in HMGB1, we cannot exclude that other HMGB1 clearance or degradation mechanisms, including HMGB1 degradation by thrombin-thrombomodulin complexes[44], may play a role in the observed decline in HMGB1 levels.

Both FSAP and HMGB1 levels were increased one hour after surgery compared to baseline. However, we did not determine what the levels of HMGB1 and FSAP-AP were at earlier time points after surgery. Elevated HMGB1 levels have been previously described in patients at t = 2-6 h after trauma, and have been found to decline steadily afterwards. To our knowledge no studies have thus far assessed HMGB1 levels at earlier time points[45]. Nevertheless, upon incubation of late apoptotic or necrotic cells with healthy donor plasma, FSAP-AP complexes were already strongly elevated after 30 min, indicating that FSAP activation occurs rapidly[28]. These results support the hypothesis that FSAP regulates HMGB1 levels released from dying cells in the circulation and suggest an important role for FSAP in the regulation of HMGB1 functioning *in vivo*. Furthermore, the type of cell death involved may be important. Apoptotic cell death is generally accepted to occur in an immunologically silent manner. This is in line with the idea that HMGB1 is not released by apoptotic cells. However, necrotic cell death is immunostimulatory, partially due to the release of HMGB1, and HMGB1 degradation by FSAP may serve to reduce the inflammatory potential of necrotic cells.

In conclusion, we describe for the first time that FSAP proteolyzes the proinflammatory DAMP HMGB1 during its release from necrotic cells. We suggest that proteolysis of HMGB1 by FSAP plays a role in the regulation of HMGB1 levels and its immunological effects during (sterile) inflammation. We additionally show that FSAP activation correlates with decreasing HMGB1 levels in liver I/R patients, supporting an *in vivo* role for FSAP in the regulation of HMGB1. These results shed light on the regulation of HMGB1 released from dying cells into the circulation. Furthermore, the administration of recombinant FSAP may provide new opportunities to therapeutically target HMGB1.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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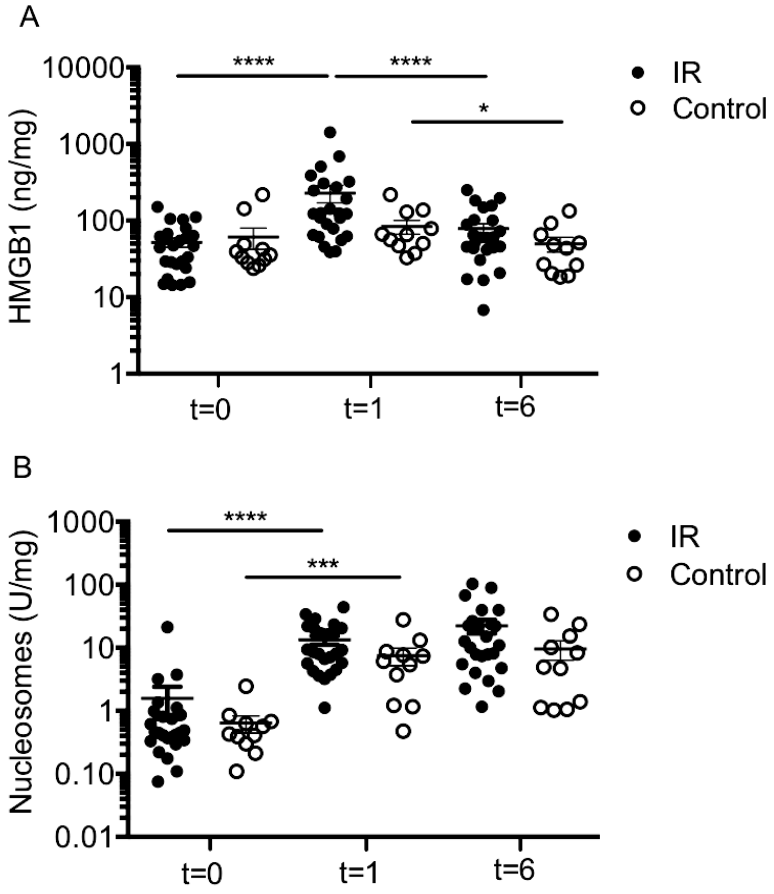
Supplementary Figure 1



Supplementary Figure 1. HMGB1 and nucleosome levels in hepatic surgery patients (adapted from van Golen et al., manuscript in preparation)

Circulating HMGB1 (A) and nucleosome (B) levels were determined by ELISA in the plasma of patients that underwent hepatic surgery followed by I/R (IR). As a control group, hepatic surgery patients that had not been exposed to I/R were included. Blood was drawn prior to surgery (t=0), 1h postoperatively (t=1) and 6h postoperatively (t=6). Data are expressed as individual values and the mean ±SEM are indicated. *p<0.05 ***p<0.001 ****p<0.0001 were calculated using a Wilcoxon matched-pairs signed rank test.