Extracellular histones promote fibrinolysis by single-chain urokinase-type plasminogen activator in a factor seven activating protease-dependent way

Fabrizio Semeraro[1,*], Concetta T. Ammollo[1], Nicola Semeraro, Mario Colucci

Dipartimento di Scienze Biomediche e Oncologia Umana, Università degli Studi di Bari Aldo Moro, Bari, Italy

[1]Contributed equally to the work

*Corresponding author at: Azienda Ospedaliero-Universitaria Policlinico, Ospedale Pediatrico "Giovanni XXIII", Via Amendola 207, 70123 Bari, Italy

E-mail address: brizioraro@hotmail.com (F. Semeraro)

Word count: 3842

Abstract

Introduction. Extracellular histones inhibit tissue plasminogen activator (t-PA)-mediated fibrinolysis by modifying fibrin structure and rheological properties. However, other plasminogen activators involved in intravascular and extravascular fibrinolysis have not been considered yet. **Objectives.** We investigated the effect of histones on fibrinolysis driven by different plasminogen activators. **Methods.** Clot lysis induced by t-PA, urokinase (u-PA) and its single chain precursor (scu-PA) was evaluated by turbidimetry. Conversion of scu-PA to u-PA and activation of factor seven activating protease (FSAP) were assessed by fluorogenic and chromogenic assays, respectively. **Results.** Histones delayed t-PA- and u-PA-mediated fibrinolysis but strongly accelerated scu-PA-driven clot lysis through the enhancement of scu-PA to u-PA conversion. This effect required a plasma factor identified as FSAP by the following findings: 1) histones enhanced neither scu-PA activation nor scu-PA-mediated clot lysis under purified conditions; 2) in plasma, the enhancement of fibrinolytic activity by histones was abolished by a neutralizing anti-FSAP antibody; 3) histones promoted the activation of plasma FSAP. The effect of the natural mixture of histones on scu-PA-driven fibrinolysis was differentially recapitulated by the individual recombinant histones, H4 displaying the strongest activity. When complexed to DNA, histones still accelerated scu-PA-mediated fibrinolysis but with a lesser efficiency due to a reduced FSAP activation. Finally, preincubation of histones with heparin or activated protein C, two known inhibitors of histones, further amplified histone-mediated boost of scu-PA-driven fibrinolysis. **Conclusions.** Enhancement of FSAP-mediated scu-PA activity by histones may play yet unforeseen roles in intravascular fibrinolysis and contribute to extravascular proteolysis and tissue damage.

Keywords: histones, plasminogen activators, fibrinolysis, proteolysis, FSAP

Abbreviations: ANOVA, analysis of variance; APC, activated protein C; FSAP, factor seven activating protease; HBS, hepes buffered saline; NETs, neutrophil extracellular traps; OD, optical density; p-EPR-MNA, Pyro-Glu-Pro-Arg-Methoxynitroanilide; scu-PA, single chain urokinase-type plasminogen activator; SEM, standard error of the mean; TF, tissue factor; t-PA, tissue plasminogen activator; UFH, unfractionated heparin; u-PA, urokinase-type plasminogen activator; Z-GGR-AMC, Z-Gly-Gly-Arg-7-Amino-4-MethylCoumarin

Introduction

In the last years, nuclear material extruded from dying or activated cells has been identified as a new player in inflammation and thrombosis. Among nuclear components, the basic proteins histones are pivotal in causing and amplifying vascular and tissue damage because of their cytotoxic, proinflammatory and prothrombotic activities [1]. With regard to the latter aspect, several in vivo data support the role of histones in thrombogenesis. When injected at high doses in mice, histones induce a septic-like disease while neutralization of histone H4 is protective in several sepsis models [2]. Sub-lethal doses of histones cause thrombocytopenia [3], stimulate thrombin generation [4] and foster deep vein thrombosis development in mice [5]. The importance of these new mediators is further supported by the presence of extracellular histones in animal [6] and human venous and arterial thrombi [7-9]. Multiple histone-mediated mechanisms act in concert to favor thrombin formation and fibrin deposition, including platelet activation and expression of procoagulant properties [10], phosphatidylserine flipping on erythrocyte membrane [11], tissue factor expression on monocytes and endothelial cells [12,13], prothrombin autoactivation [14], release of von Willebrand Factor [15], impairment of the protein C pathway [16] and interference with the anticoagulant activity of heparin-like substances [17].

In contrast with this rather abundant information on the procoagulant mechanisms of histones, only few data are available about the effect of histones on fibrinolysis. Varjù et al. [18] reported that incorporating histones into clots produces thicker fibrin fibers, which makes them more robust and resistant to shear forces; moreover, histones tightly bind to large fibrin(ogen) degradation products, which help stabilizing clots, and reduce clot permeability. Thus, the overall effect of histones on fibrinolysis appears to be inhibitory, especially when they are complexed with DNA. However, some concerns remain. First, most of these results have been obtained with fairly high concentrations of a lysine-rich histone preparation, which mainly consists of linker histone H1. Histone H1 is only a minor component of nuclear and extracellular histones, slightly lysine-rich

(H2A and H2B) and arginine-rich (H3 and H4) molecules representing the bulk of histone material. Second, most experiments were performed using fibrinolysis models where tissue plasminogen activator (t-PA) was applied externally to a preformed clot, thereby mimicking pharmacological rather than physiological fibrinolysis. Finally, the effect of histones was only evaluated using t-PA as fibrinolytic agent. It should be considered, however, that other plasminogen activators, such as urokinase-type PA (u-PA) and its single chain precursor (scu-PA), which are mainly regarded as key factors in extravascular proteolysis, have been shown to play an important role also in intravascular fibrin degradation [19]. In addition, there is evidence that factor seven-activating protease (FSAP), a plasma pro-enzyme endowed with multiple biological functions [20], is an efficient activator of scu-PA [21] and can be activated by histones [22], suggesting an intriguing liaison with potential biological consequences on the fibrinolytic system. Therefore, in this study we sought to shed more light on the influence of histones on fibrinolysis by investigating in more detail the effect of histones on plasma clot lysability driven by several plasminogen activators under conditions of pathophysiological relevance.

Materials and Methods

Materials

The following reagents were purchased: single-chain recombinant t-PA (Boehringer Ingelheim GmbH, Florence, Italy); human thromboplastin (Recombiplastin, Instrumentation Laboratory, Milan, Italy); type II-A calf thymus histones, recombinant human histones (H1, H2A, H2B, H3.3, H4), calf thymus DNA, bovine fibrinogen, De-N-sulfated heparin sodium salt (Sigma, Milan); hirudin (Abbott GmbH, Ludwigshafen, Germany); reptilase from Bothrops atrox venom (American Diagnostica, Pfungstadt, Germany); fluorogenic substrate Z-Gly-Gly-Arg-7-Amino-4-MethylCoumarin (Z-GGR-AMC) (Diagnostica Stago, Asnieres, France); chromogenic substrate Pyro-Glu-Pro-Arg-Methoxynitroanilide (p-EPR-MNA, Berichrom® Protein C, Dade Behring,

Marburg, Germany); sodium heparin (Heparin BMS, Bristol-Myers Squibb, Anagni, Italy); u-PA (Urokinase Crinos, Crinos SpA, Milan). Phospholipid vesicles (20% phosphatidylserine, 60% phosphatidylcholine, 20% phosphatidylethanolamine, Avanti Polar Lipids, Alabaster, AL, USA) were prepared by sonication. scu-PA was kindly provided by Prof. R.H. Lijnen (Center for Molecular and Vascular Biology, Department of Cardiovascular Sciences, KU Leuven, Leuven). Neutralizing anti-FSAP antibody was a kind gift of Prof. S.M. Kanse (Oslo University Hospital and University of Oslo, Oslo, Norway). DNA was fragmented by sonication to 200-400 bp size, as assessed by agarose gel electrophoresis. Where indicated, DNA was preincubated with histones at 1:1 (w:w) ratio for 10 minutes at room temperature to form complexes. The same procedure was used to form heparin-histone complexes at the indicated heparin concentrations. In some experiments, histones were digested with 6 μg/mL activated protein C (APC, Xigris, Eli Lilly, Indianapolis, IN, USA) for 1 h at 37 °C, as described elsewhere [2].

Plasma preparation

Peripheral venous blood was collected into 3.2% trisodium citrate from healthy volunteers who gave informed consent, and platelet-poor plasma was prepared by double centrifugation at 1000xg and 12000xg for 10 minutes each. Plasma from at least three different subjects was pooled and used for each experiment.

Clot lysis assay

Clot lysis was studied by a turbidimetric assay as already described [23] with minor modifications. The wells of a microtiter plate were loaded with 100 μL plasma, procoagulant liposomes (10 μg/mL), thromboplastin as source of tissue factor (TF, 6 pM) and 8.3 mM calcium (100 μL). Histones and plasminogen activators (at the indicated concentrations) were added to the mixture (final volume 240 μL) right before starting the test. In some cases, clotting was induced by reptilase (1:24 final dilution, f.d.) and calcium in the presence of 200 U/mL hirudin to inhibit endogenous

thrombin. Changes in the optical density at 405 nm were monitored at 37°C in a Multiskan GO spectrophotometer (Thermo Fisher Scientific, Dreieich, Germany) and lysis times calculated as the interval between the midpoint of the clear to maximum turbidity transition and the midpoint of the maximum turbidity to clear transition. For the evaluation of clot lysis under purified conditions, a plasminogen-containing bovine fibrinogen preparation (1 mg/mL) was clotted with reptilase (1:24 f.d.) in the presence of 2.5 mM calcium and lysis calculated as described above.

Evaluation of scu-PA conversion to u-PA

The generation of active u-PA from scu-PA was evaluated either in plasma or in a purified system with the fluorogenic substrate Z-GGR-AMC [24]. In the former setting, hirudin-inhibited plasma was recalcified (8.3 mM calcium) in the presence of histones, 300 µM Z-GGR-AMC and 1 µg/mL scu-PA. In the purified system, 10 µg/mL scu-PA were tested in the presence of histones, 300 µM Z-GGR-AMC and 2.5 mM calcium. The activity of u-PA was followed at 390 nm excitation and 460 nm emission at 30 seconds intervals in a Fluoroskan Ascent fluorometer (Thermo Scientific) and measured as the slope (OD/min) of the fluorescence curve (GraphPad Prism 8 software, GraphPad, San Diego, CA, USA).

Plasma FSAP activation

The activation of plasma FSAP was analyzed by the cleavage of the chromogenic substrate p-EPR-MNA [25]. In order to make the assay specific for active FSAP, histones and chromogenic substrate (600 µM) were added to plasma containing the thrombin quencher hirudin (200 U/mL) and, after recalcification, color development was followed at 405 nm at 37°C over time.

Statistical analysis

Results shown are mean ± standard error of the mean (SEM). Comparisons between two means were performed by Student's t-test for paired data. Multiple comparisons were performed by ANOVA for repeated measures followed by post-test for linear trend (dose-response experiments),

Dunnett's post hoc test (comparison between each sample and the control) or Newman-Keuls post hoc test (comparison of the samples to each other). P values <0.05 were considered statistically significant.

Results

Histones differentially modulate clot lysis depending on the plasminogen activator

We investigated the effect of a natural mixture of histones on the fibrinolytic activity of t-PA, u-PA or scu-PA by a turbidimetric clot lysis assay. As shown in Fig. 1 (panels A, B, D and E), histones inhibited t-PA- and u-PA-mediated fibrinolysis at concentrations ≥80 μg/mL. Moreover, prolongation of t-PA- and u-PA-induced clot lysis was accompanied by a concentration-dependent increase in maximal optical density of clots, suggesting a change in the thickness of fibrin fibers in the presence of histones (Fig. 1A and B). At variance with these findings, despite similar changes in clot optical density, the fibrinolytic activity of scu-PA was strongly and dose-dependently enhanced by histones (Fig. 1C), a significant effect being observed with as low as 2.5 μg/mL histone (Fig. 1F). Maximal stimulation of fibrinolysis, amounting to >5-fold reduction of lysis time, was seen with 40 μg/mL and was maintained at concentrations exceeding 500 μg/mL histones (not shown).
The effect of the natural mixture of histones on scu-PA-driven fibrinolysis was differentially recapitulated by the individual recombinant histones, H4 displaying the strongest profibrinolytic activity, followed by H3, H2A and H2B. H1 had virtually no effect on clot lysis (Supplementary Fig. 1).

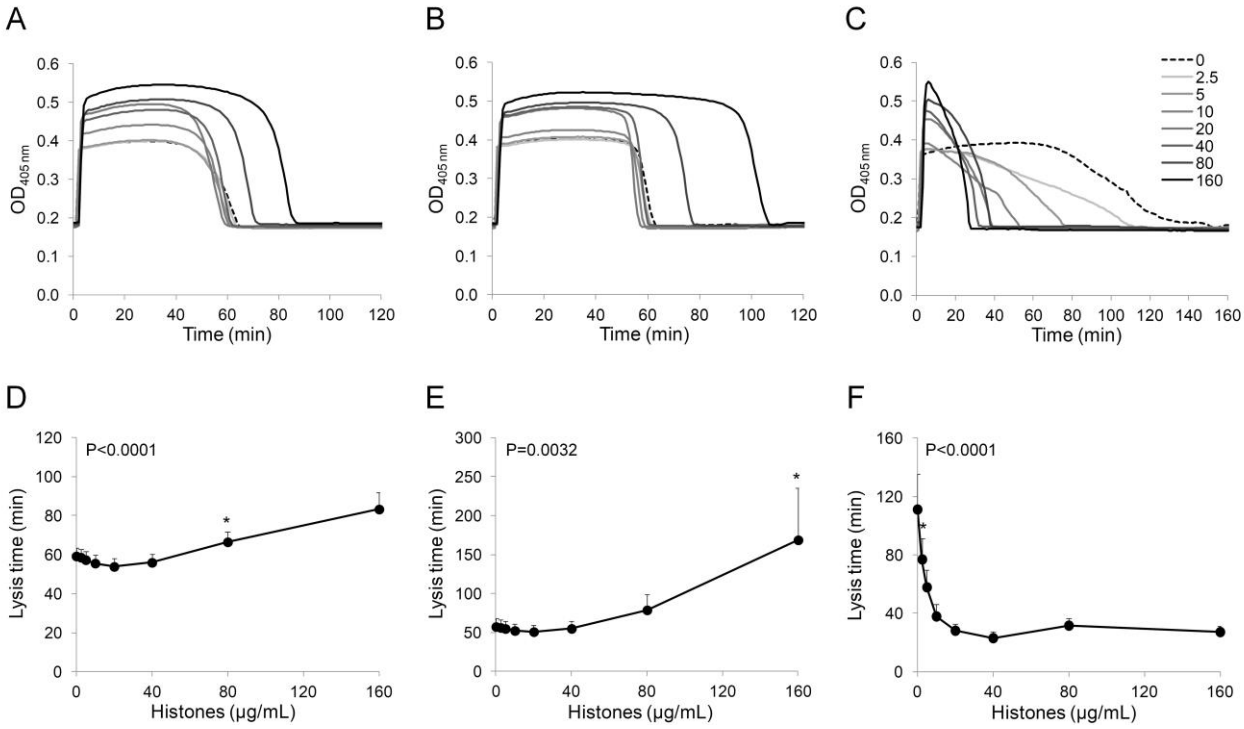

Fig. 1. *Histones differentially influence plasma fibrinolysis induced by various plasminogen activators.* Plasma was supplemented with increasing concentrations of calf thymus histones (0-160 µg/mL), plasminogen activators, TF (6 pM), procoagulant liposomes (10 µg/mL) and calcium (8.3 mM). Coagulation and fibrinolysis were analyzed by turbidimetry as detailed in Material and Methods. In A, B and C representative optical density (OD) tracings are shown. In D, E and F lysis time changes as a function of histone concentration are reported as mean ± SEM (5-7 independent experiments). (A, D) t-PA (40 ng/mL); (B, E) u-PA (100 U/mL); (C, F) scu-PA (10 µg/mL). Differences were assessed by ANOVA for repeated measures followed by post test for linear trend (p value in the graph) and Dunnett's post-hoc test (*, lowest concentration significantly different from control, $P<0.05$).

Histones induce scu-PA to u-PA conversion in plasma

To elucidate the mechanism behind the enhancement of scu-PA fibrinolytic activity, we evaluated the influence of histones on the conversion of scu-PA to u-PA by means of a fluorogenic substrate sensitive to u-PA. In hirudin-inhibited recalcified plasma, the appearance of u-PA activity was dose-dependently enhanced by histones, reaching a maximum stimulation at 20 µg/mL and then gradually declining in the presence of higher concentrations (Fig. 2A). On the contrary, when scu-PA was challenged with histones in buffer, no increase in u-PA activity was observed (Fig. 2B). To further prove that histones do not activate scu-PA in the absence of plasma, we performed a clot

lysis assay using purified reagents. Under this condition, histones did not hasten but rather delayed fibrinolysis (Fig. 2C), suggesting that stimulation of scu-PA activity by histones is mediated by one or more plasma-derived factor(s).

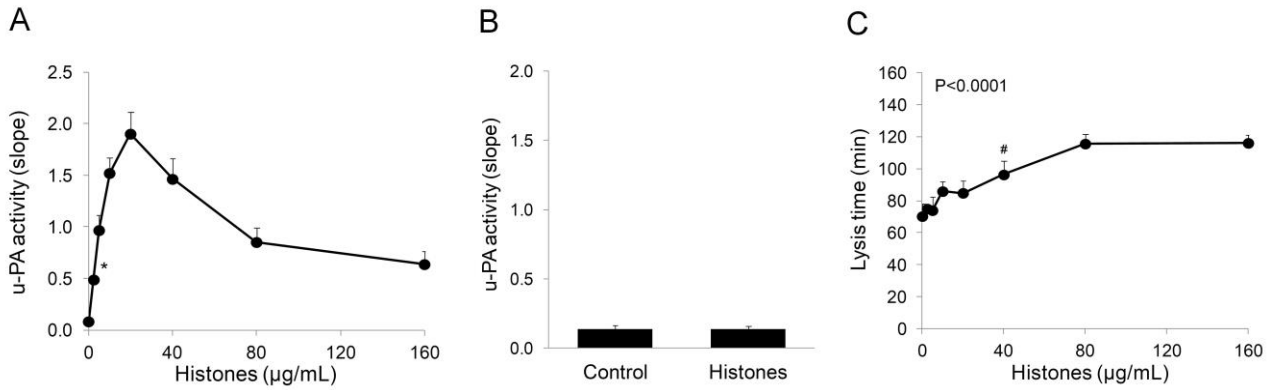

Fig. 2. *Histones stimulate scu-PA activation in plasma but not under purified conditions.* The conversion of scu-PA into u-PA was evaluated by means of the fluorogenic substrate Z-GGR-AMC in recalcified and hirudin-treated plasma (A) or in a purified system (B) in the presence of increasing concentrations (0-160 µg/mL) or 20 µg/mL of histones, respectively. Substrate cleavage was followed at 390 nm excitation and 460 nm emission at 37°C over time. Results are expressed as the slope (OD/min) of the fluorescence curve. Means ± SEM from 3 independent experiments are shown. In C, a mixture of plasminogen-containing bovine fibrinogen (1 mg/mL), histones (0-160 µg/mL) and scu-PA (30 ng/mL) was clotted by reptilase and fibrinolysis evaluated by turbidimetry. Means ± SEM (n=5) are shown. Differences were assessed by ANOVA for repeated measures followed by post test for linear trend (p value in the graph) and Dunnett's post-hoc test (lowest concentration significantly different from control: *, $P<0.05$; #, $P<0.01$).

Histones promote plasma FSAP activation

As histones have been shown to promote the autoactivation of FSAP [22], and the latter, in turn, reportedly may activate scu-PA [21], we investigated the role of FSAP in histone-induced enhancement of scu-PA-driven fibrinolysis. When added to hirudin-inhibited plasma, histones induced the cleavage of a FSAP-sensitive chromogenic substrate in a dose-dependent manner, with a peak stimulation at 20-40 µg/mL (Fig. 3A). The enzymatic activity generated in the presence of histones was completely quenched by a specific anti-FSAP antibody (Fig. 3B), indicating that histones indeed induced FSAP activation.

To prove that histones stimulated scu-PA fibrinolytic activity in a FSAP-dependent manner, plasma clot lysis was performed in the presence of the anti-FSAP antibody. Under this condition no enhancement of fibrinolysis by histones was observed, thus confirming that histones favor FSAP autoactivation, which in turn enables scu-PA to express its full plasminogen activator potential (Fig. 3C).

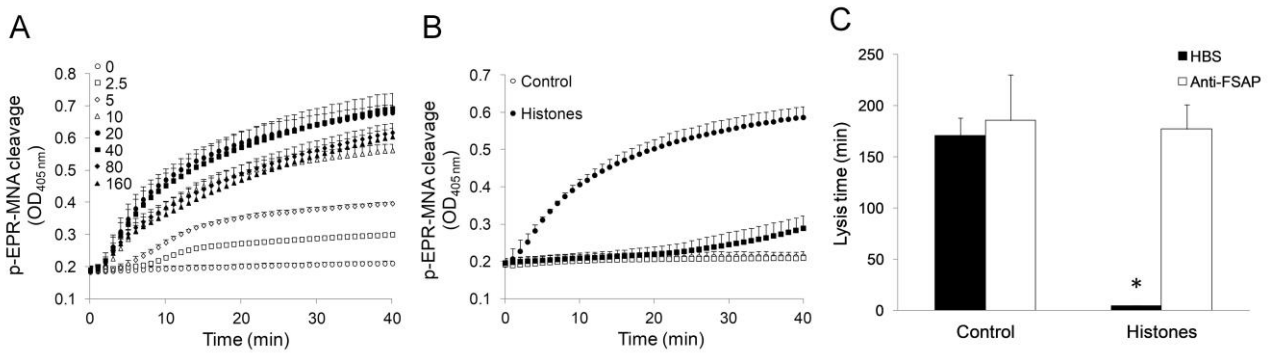

Fig. 3. *Histones stimulate FSAP activation.* Recalcified, hirudin-treated normal plasma was challenged with histones alone (0-160 μg/mL) (A) or histones (40 μg/mL) in the absence or presence of anti-FSAP antibody (50 μg/mL) (B). Cleavage of p-EPR-MNA (600 μM) was followed at 405 nm at 37°C at 1 min intervals over time. Panel C shows the effect of anti-FSAP antibody (50 μg/mL) on lysis time induced by scu-PA in control plasma and in plasma containing histones (40 μg/ml). Experimental conditions as in legend to Fig. 1. HBS, hepes buffered saline. Means and SEM from 3 experiments are shown. *, $P<0.01$ vs control by t-test for paired data.

Effect of DNA on the activity of histones

Histones are usually released from cells in tight complex with DNA. We therefore evaluated whether DNA influenced the biological activity of histones. Histones were preincubated with an equal weight amount of calf thymus DNA and then added to plasma. In the standard clot lysis assay, histone-DNA complex caused a clear-cut hastening of scu-PA-mediated fibrinolysis, but the magnitude of the effect was significantly lower compared to histones only (Fig. 4A). DNA alone, instead, had a minor, though significant, effect on clot lysis time (Fig. 4A). Concerning FSAP activation, the histone-DNA complex induced a delayed and reduced cleavage of the FSAP-sensitive substrate, compared to histones alone, whereas DNA was totally inactive (Fig. 4B).

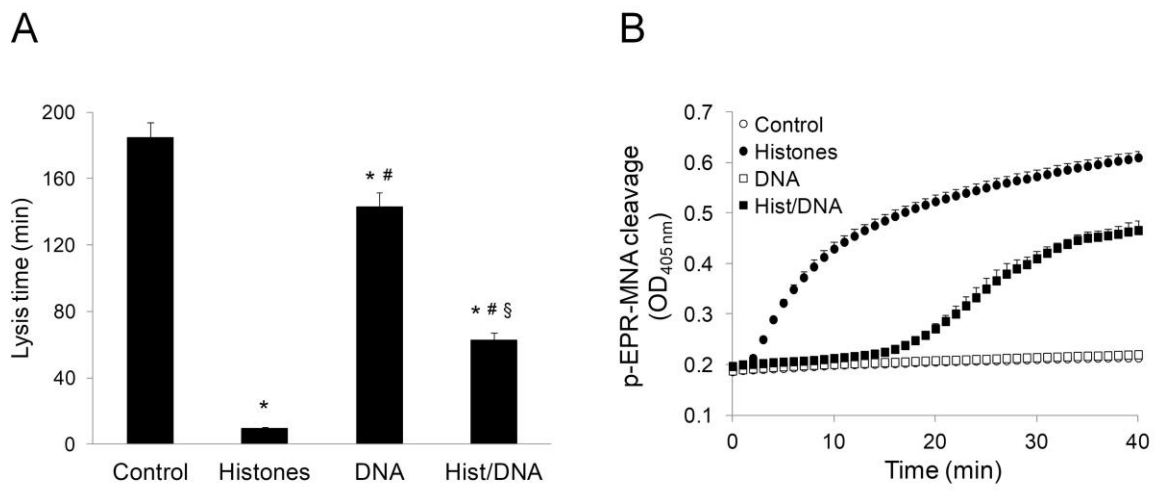

Fig. 4. *DNA influences histone activity on scu-PA-mediated clot fibrinolysis.* (A) Plasma was supplemented with histones (40 µg/mL), DNA (40 µg/mL) or histone-DNA complex (Hist/DNA, 40 µg/mL each), scu-PA (10 µg/mL), TF (6 pM), liposomes (10 µg/mL) and calcium (8.3 mM). Fibrinolysis was analyzed by turbidimetry. Mean lysis times ± SEM are showed (n=6). Differences were assessed by ANOVA for repeated measures followed by Newman-Keuls post-hoc test (*, $P<0.001$ vs control; $^{\#}$, $P<0.001$ vs histones; §, $P<0.001$ vs DNA). (B) Hirudin-containing plasma was supplemented with histones (40 µg/mL), DNA (40 µg/mL) or histone-DNA complex (Hist/DNA, 40 µg/mL each) in the presence of p-EPR-MNA (600 µM); after recalcification substrate cleavage was followed at 405 nm at 37°C over time. Means and SEM from 4 experiments are shown.

Heparin and APC promote histone-mediated enhancement of scu-PA fibrinolytic activity

Heparin neutralizes the cationic charges of histones and is therefore considered a histone inhibitor [26,27]. Therefore, we evaluated the ability of unfractionated heparin (UFH) to prevent the histone-induced stimulation of scu-PA. To avoid any interference on fibrinolysis due to the anticoagulant activity of UFH [28], we performed the lysis assay in the presence of hirudin and induced clot formation with reptilase. Under these conditions, UFH alone had no effect on scu-PA-induced clot lysis (Fig. 5A). When tested in combination with histones, UFH did not attenuate but rather boosted histone-mediated enhancement of scu-PA-driven fibrinolysis over an ample range of UFH concentrations (0.04-1 U/mL) (Fig. 5A). In accordance with these data, the generation of active FSAP was more intense in the presence of the UFH-histone complex (Fig. 5B). Enhancement of

both FSAP activation and scu-PA fibrinolytic activity was also observed with a de-sulfated form of heparin characterized by impaired anticoagulant activity (Supplementary Fig. 2).

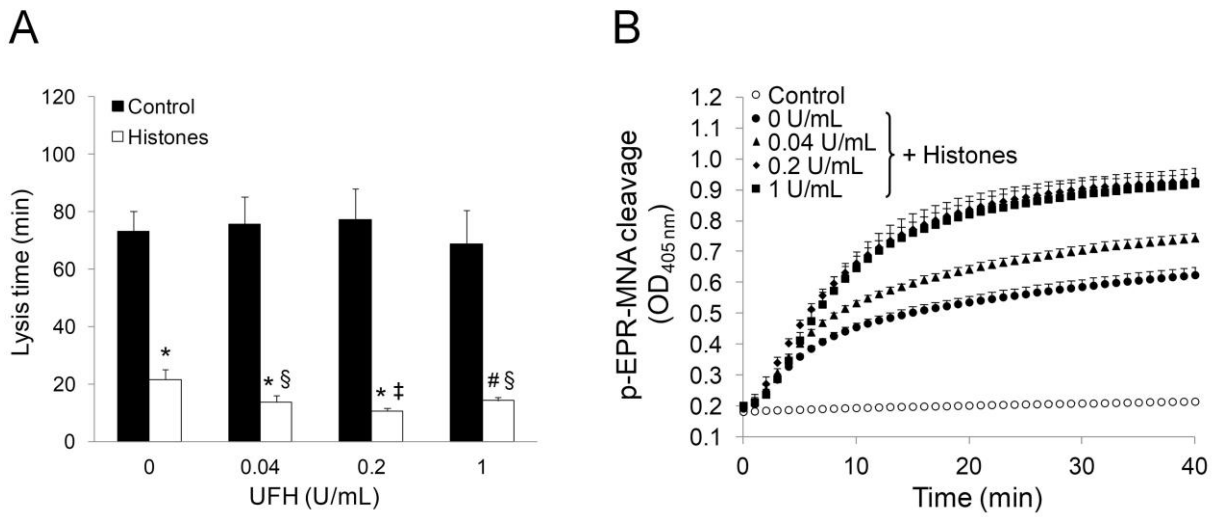

Fig. 5. *Heparin impacts on histone-mediated enhancement of scu-PA fibrinolytic activity and FSAP activation.* (A) Recalcified, hirudin-inhibited plasma was supplemented with histones (0-40 μg/mL) and UFH at different concentrations (0.04-0.2-1 U/mL), clotted by reptilase and lysed by scu-PA (1 μg/mL) as described above. Differences were assessed by t-test for paired data (*, $P<0.01$; #, $P<0.05$ vs control) and by ANOVA for repeated measures followed by Dunnett's post-hoc test (§, $P<0.05$; ‡, $P<0.01$ vs no UFH). (B) Recalcified, hirudin-inhibited plasma was supplemented with histones (0-40 μg/mL) and UFH at different concentrations (0.04-0.2-1 U/mL), in the presence of p-EPR-MNA (600 μM); substrate cleavage was followed at 405 nm at 37°C over time. For clarity, all the curves in the presence of sole UFH were omitted as they were not different from the control (absence of histones). Means and SEM from 4 experiments are shown.

A similar enhancement of scu-PA-induced fibrinolysis was observed when histones were preincubated with APC (Supplementary Fig. 3), which has been reported to degrade and inhibit histones [2]. In this case, however, we could not evaluate the effect of APC-treated histones on FSAP activation, because of the high sensitivity to APC of the chromogenic substrate employed in the assay.

Discussion

In this work we show that histones influence fibrinolysis in a complex manner that strictly depends on the plasminogen activator involved. In the presence of either t-PA or u-PA, both of which express full plasminogen-activating potential, histones hamper fibrinolysis at high concentrations (≥80 μg/mL). As to the mechanism of fibrinolysis inhibition, Varjù et al. [18] provided evidence suggesting that histones modify the fibrin structure by increasing the fiber thickness and stabilize the clot by binding to large fibrin degradation products. This view harmonizes with our observation that histones increased plasma clot turbidity (Fig. 1A and B), which is an indirect sign of larger fibrin strand formation [23,29].

The new finding of our work is that, at variance with t-PA and u-PA-induced fibrinolysis, histones strongly stimulate scu-PA-mediated fibrinolysis. The main difference between these activators is that scu-PA holds very little plasminogen activator activity and needs to be converted to the two-chain form u-PA (also referred to as tcu-PA) to activate plasminogen into plasmin with full efficiency [30]. We suggest that the profibrinolytic effect of histones results from stimulation of scu-PA conversion into u-PA via activation of FSAP. Several findings support this hypothesis: 1) histones failed to stimulate u-PA activity; 2) histones enhanced scu-PA conversion to u-PA in plasma but not under purified conditions; 3) histones induced plasma FSAP activation, and 4) FSAP blockade prevented histone-induced stimulation of scu-PA-induced clot lysis. However, there was an apparent inconsistency between the results of clot lysis and scu-PA activation concerning the concentration-response curve. Indeed, while in the fibrinolytic assay the effect of histones increased dose-dependently up to a maximum (Fig. 1F), in the u-PA generation assay the effect of histones reached a maximum at 20-40 μg/ml and then declined (Fig. 2A). The reason for this discrepancy is unknown, but the different assay conditions, particularly the presence of the thrombin inhibitor hirudin in the scu-PA activation test, might have accounted for the different behavior of histones.

296 The relationship between histones and FSAP is intriguing. Similarly to other cationic proteins,
297 binding of histones to FSAP promotes the formation of intermolecular interactions which allow
298 FSAP autoactivation [22]. Active FSAP, in turn, induces fast histone cleavage [31] and catalyzes
299 the release of nucleosomes from secondary necrotic cells [32,33]. FSAP activation, therefore,
300 represents a novel histone detoxification mechanism. However, this "beneficial" effect comes at a
301 price. Indeed, histone-mediated activation of FSAP has been associated with bradykinin formation
302 [22,34] and anaphylatoxins generation, particularly C5a [35], which exacerbate inflammation.
303 Activation of fibrinolysis through scu-PA and generation of the potent, non-specific enzyme
304 plasmin can be another detrimental consequence of histone release and FSAP activation.
305 An interesting point pertains to the influence of interacting charged molecules on the biological
306 activities of histones. Histones are generally released as a complex with their natural ligand DNA in
307 the form of nucleosomes, chromatin or neutrophil extracellular traps (NETs), so evaluation of their
308 activity in the presence of DNA cannot be overlooked. We found that DNA attenuates to a
309 significant extent the stimulatory effect of histones on FSAP activation and scu-PA-mediated
310 fibrinolysis. However, this notwithstanding, the magnitude of the effect induced by histone/DNA
311 complex remained remarkable, suggesting that both free and complexed histones are capable of
312 promoting scu-PA-mediated plasmin generation.
313 Besides DNA, other negatively-charged molecules such as heparin-like substances can bind to
314 histones in such a tight manner that they are regarded as effective histone neutralizers [26,27]. In
315 this study we observed that preincubation of histones with UFH or de-sulfated heparin does not
316 hamper but rather increase the profibrinolytic activity of histones trough the enhancement of FSAP
317 activation. In vivo, heparin-like substances (sulfated glycosaminoglycans) are constitutively
318 expressed by endothelial cells and are an important component of the extracellular matrix, implying
319 that the improvement of histone effect may take place both inside and outside the bloodstream.
320 Interestingly, APC, another well-known histone inhibitor [2], was also able to heighten the capacity
321 of histones to enhance scu-PA fibrinolytic activity. This observation may have clinical implications

as APC, thanks to its ability to degrade histones, is considered a potential remedy against histone toxicity. Supposedly, the enhancement of scu-PA-driven plasmin generation brought about by histone fragments might exacerbate the bleeding side effects of APC.

From a pathophysiological standpoint, histones are thus able to either impair or promote fibrinolysis depending on their concentration, the nature of the plasminogen activator and the local conditions. Inhibition of t-PA-induced fibrinolysis by high histone concentrations adds to prothrombotic activity of histones and may be a further mechanism of harm in disease, especially in severe systemic conditions such as septic shock and trauma where high histone levels may be found in the circulation [2,4]. On the other side, FSAP-mediated enhancement of scu-PA-driven fibrinolysis has been observed over an ample range of histone concentrations, including the lower ones, and should conversely expose the patient to the risk of bleeding. It is generally assumed that t-PA represents the main player in intravascular fibrinolysis, while (sc)u-PA is considered more important in extravascular fibrin degradation [36]. However, this assumption has been revisited and it is now supposed that the two PAs play a complementary role in the resolution of intravascular thrombi [37-39], which suggests that the net effect of histones on fibrinolysis likely depends on the balance between the two PA systems. Outside the bloodstream, u-PA-dependent plasmin formation has been implicated in several processes characterized by extracellular matrix (ECM) degradation and remodeling, and cell migration, e.g. angiogenesis, inflammatory diseases, cancer invasion and metastasis formation [40-42]. Among these, atherosclerosis is, perhaps, the condition that best illustrates the possible role of FSAP-mediated activation of the u-PA system. Atherosclerotic plaques contain (sc)u-PA and the content is generally proportional to the severity of the lesions, with high amounts typically detected in unstable or vulnerable plaques which are more prone to rupture or to induce vessel dilation [43]. Both plasmin-mediated degradation of ECM and intracellular signaling triggered by u-PA bound to its receptor (u-PAR) are involved in atherosclerosis progression and the ensuing complications [43]. More recently, extracellular histones in the form of NETs released by infiltrating neutrophils and macrophages have been

identified in atherosclerotic lesions, especially in the lipid-rich core [44]. Moreover, FSAP has been found to abundantly decorate atherosclerotic plaques [45]. It is therefore tempting to speculate that in the atherosclerotic environment the release of histones can drive a chain reaction through FSAP autoactivation, u-PA formation, and, in the end, excessive plasmin generation which may favor aneurysm formation, plaque rupture and development of arterial thrombi. The abundance of heparin-like molecules in the ECM may further exalt the detrimental consequences of histone release.

In conclusion, extracellular histones exert differential activities on plasminogen activators that translate into opposite effects on the fibrinolytic system, whose pathophysiological relevance is complex to predict and probably depends on the site and timing of histone release.

Addendum

F.S. and C.T.A. designed the study, performed research and wrote the manuscript. M.C. and N.S. supervised the study and critically revised the manuscript.

Acknowledgements

We are very grateful to Prof. H.R. Lijnen (Center for Molecular and Vascular Biology, Department of Cardiovascular Sciences, KU Leuven, Leuven) and Prof. S.M. Kanse (Oslo University Hospital and University of Oslo, Oslo, Norway) for kindly providing scu-PA and anti-FSAP antibody, respectively.

Funding

The study was partially supported by a grant from the University of Bari “Aldo Moro” (M.C.).

Declaration of competing interest

All the authors state that they have no conflict of interest.

Appendix. Supplementary data

References

1. R. Chen, R. Kang, X. Fan, D. Tang, Release and activity of histone in diseases, Cell Death Dis 5 (2014) e1370.
2. J. Xu, X. Zhang, R. Pelayo, M. Monestier, C.T. Ammollo, F. Semeraro, et al., Extracellular histones are major mediators of death in sepsis, Nat. Med. 15 (2009) 1318-1321.
3. T.A. Fuchs, A.A. Bhandari, D.D. Wagner, Histones induce rapid and profound thrombocytopenia in mice, Blood 118 (2011) 3708-3714.
4. S.T. Abrams, N. Zhang, J. Manson, T. Liu, C. Dart, F. Baluwa, et al., Circulating histones are mediators of trauma-associated lung injury, Am. J. Respir. Crit. Care Med. 187 (2013) 160-169.
5. A. Brill, T.A. Fuchs, A.S. Savchenko, G.M. Thomas, K. Martinod, S.F. De Meyer, et al., Neutrophil extracellular traps promote deep vein thrombosis in mice, J. Thromb. Haemost. 10 (2012) 136-144.
6. T.A. Fuchs, A. Brill, D. Duerschmied, D. Schatzberg, M. Monestier, D.D. Jr. Myers, et al., Extracellular DNA traps promote thrombosis, Proc. Natl. Acad. Sci. USA. 107 (2010) 15880-15885.
7. O.J. de Boer, X. Li, P. Teeling, C. Mackaay, H.J. Ploegmakers, C.M. van der Loos, et al., Neutrophils, neutrophil extracellular traps and interleukin-17 associate with the organisation of thrombi in acute myocardial infarction, Thromb. Haemost. 109 (2013) 290-297.
8. E. Laridan, F. Denorme, L. Desender, O. François, T. Andersson, H. Deckmyn, et al., Neutrophil extracellular traps in ischemic stroke thrombi. Ann. Neurol. 82 (2017) 223-232.

9. A.S. Savchenko, K. Martinod, M.A. Seidman, S.L. Wong, J.I. Borissoff, G. Piazza, et al., Neutrophil extracellular traps form predominantly during the organizing stage of human venous thromboembolism development, J. Thromb. Haemost. 12 (2014) 860-870.
10. F. Semeraro, C.T. Ammollo, J.H. Morrissey, G.L. Dale, P. Friese, N.L. Esmon, et al., Extracellular histones promote thrombin generation through platelet-dependent mechanisms: involvement of platelet TLR2 and TLR4, Blood 118 (2011) 1952-1961.
11. F. Semeraro, C.T. Ammollo, N.L. Esmon, C.T. Esmon, Histones induce phosphatidylserine exposure and a procoagulant phenotype in human red blood cells, J. Thromb. Haemost. 12 (2014) 1697-1702.
12. T.J. Gould, Z. Lysov, L.L. Swystun, D.J. Dwivedi, R. Zarychanski, A.E. Fox-Robichaud, et al., Extracellular histones increase tissue factor activity and enhance thrombin generation by human blood monocytes, Shock 46 (2016) 655-662.
13. X. Yang, L. Li, J. Liu, B. Lv, F. Chen, Extracellular histones induce tissue factor expression in vascular endothelial cells via TLR and activation of NF-κB and AP-1, Thromb. Res. 137 (2016) 211-218.
14. S. Barranco-Medina, N. Pozzi, A.D. Vogt, E. Di Cera. Histone H4 Promotes Prothrombin Autoactivation, J. Biol. Chem. 288 (2013) 35749-35757.
15. F.W. Lam, M.A. Cruz, K. Parikh, R.E. Rumbaut, Histones stimulate von Willebrand factor release in vitro and in vivo, Haematologica 101 (2016) e277-279.
16. C.T. Ammollo, F. Semeraro, J. Xu, N,L. Esmon, C.T. Esmon, Extracellular histones increase plasma thrombin generation by impairing thrombomodulin-dependent protein C activation, J. Thromb. Haemost. 9 (2011) 1795-1803.
17. C.T. Ammollo, N. Semeraro, M.R. Carratù, M. Colucci, F. Semeraro, Histones Differentially Modulate the Anticoagulant and Profibrinolytic Activities of Heparin, Heparin Derivatives, and Dabigatran, J. Pharmacol. Exp. Ther. 356 (2016) 305-313.

18. I. Varjú, C. Longstaff, L. Szabó, Á.Z. Farkas, V.J. Varga-Szabó, A. Tanka-Salamon, et al., DNA, histones and neutrophil extracellular traps exert anti-fibrinolytic effects in a plasma environment, Thromb. Haemost. 113 (2015) 1289-1298.
19. T.H. Bugge, M.J. Flick, M.J. Danton, C.C. Daugherty, J. Romer, K. Dano, et al., Urokinase-type plasminogen activator is effective in fibrin clearance in the absence of its receptor or tissue-type plasminogen activator, Proc. Natl. Acad. Sci. USA 93 (1996) 5899-5904.
20. F. Stephan, L.A. Aarden, S. Zeerleder, FSAP, a new player in inflammation?, Hämostaseologie 32 (2012) 51-55.
21. J. Romisch, S. Vermohlen, A. Feussner, H.A. Stohr, The FVII activating protease cleaves single-chain plasminogen activators, Haemostasis 29 (1999) 292-299.
22. S. Yamamichi, Y. Fujiwara, T. Kikuchi, M. Nishitani, Y. Matsushita, K. Hasumi, Extracellular histone induces plasma hyaluronan-binding protein (factor VII activating protease) activation in vivo, Biochem. Biophys. Res. Commun. 409 (2011) 483-488.
23. C.T. Ammollo, F. Semeraro, F. Incampo, N. Semeraro, M. Colucci, Dabigatran enhances clot susceptibility to fibrinolysis by mechanisms dependent on and independent of thrombin-activatable fibrinolysis inhibitor, J. Thromb. Haemost. 8 (2010) 790-798.
24. M. Zimmerman, J.P. Quigley, B. Ashe, C. Dorn, R. Goldfarb, W. Troll, Direct fluorescent assay of urokinase and plasminogen activators of normal and malignant cells: kinetics and inhibitor profiles, Proc. Natl. Acad. Sci. USA 75 (1978) 750-753.
25. A. Hunfeld, M. Etscheid, H. König, R. Seitz, J. Dodt, Detection of a novel plasma serine protease during purification of vitamin K-dependent coagulation factors, FEBS Lett. 456 (1999) 290-294.
26. K.C. Wildhagen, P. García de Frutos, C.P. Reutelingsperger, R. Schrijver, C. Aresté, A. Ortega-Gómez, et al., Nonanticoagulant heparin prevents histone-mediated cytotoxicity in vitro and improves survival in sepsis, Blood 123 (2014) 1098-1101.

448 27. Y. Zhang, Z. Wen, L. Guan, P. Jiang, T. Gu, J. Zhao, et al., Extracellular histones play an
449 inflammatory role in acid aspiration-induced acute respiratory distress syndrome,
450 Anesthesiology 122 (2015) 127-139.
451 28. C.T. Ammollo, F. Semeraro, N. Semeraro, M. Colucci, The contribution of anti-Xa and anti-
452 IIa activities to the profibrinolytic activity of low-molecular-weight heparins, Thromb.
453 Haemost. 101 (2009) 782-785.
454 29. M.E. Carr, D.A. Gabriel, Dextran-induced changes in fibrin fiber size and density based on
455 wavelength dependence of gel turbidity, Macromolecules 13 (1980) 1473-1477.
456 30. H.R. Lijnen, B. Van Hoef, L. Nelles, D. Collen, Plasminogen activation with single-chain
457 urokinase-type plasminogen activator (scu-PA). Studies with active site mutagenized
458 plasminogen (Ser740----Ala) and plasmin-resistant scu-PA (Lys158----Glu), J. Biol. Chem.
459 265 (1990) 5232-5236.
460 31. G. Marsman, H. von Richthofen, I. Bulder, F. Lupu, J. Hazelzet, B.M. Luken, et al., DNA
461 and factor VII-activating protease protect against the cytotoxicity of histones, Blood Adv. 1
462 (2017) 2491-2502.
463 32. S. Zeerleder, B. Zwart, H. te Velthuis, R. Manoe, I. Bulder, I. Rensink, et al., A plasma
464 nucleosome releasing factor (NRF) with serine protease activity is instrumental in removal
465 of nucleosomes from secondary necrotic cells, FEBS Lett. 581 (2007) 5382-5388.
466 33. S. Zeerleder, B. Zwart, H. te Velthuis, F. Stephan, R. Manoe, I. Rensink, et al., Nucleosome-
467 releasing factor: a new role for factor VII-activating protease (FSAP), FASEB J. 22 (2008)
468 4077-4084.
469 34. M. Etscheid, N. Beer, E. Fink, R. Seitz, D. Johannes, The hyaluronan-binding serine
470 protease from human plasma cleaves HMW and LMW kininogen and releases bradykinin.
471 Biol. Chem. 383 (2002) 1633-1643.

35. S.M. Kanse, A. Gallenmueller, S. Zeerleder, F. Stephan, O. Rannou, S. Denk et al., Factor VII-activating protease is activated in multiple trauma patients and generates anaphylatoxin C5a, J. Immunol. 188 (6) (2012) 2858-2865.
36. H. Matsuno, O. Kozawa, M. Niwa, S. Ueshima, O. Matsuo, D. Collen, et al., Differential role of components of the fibrinolytic system in the formation and removal of thrombus induced by endothelial injury, Thromb. Haemost. 81 (1999) 601-604.
37. K. Yamamoto, D.J. Loskutoff, Fibrin deposition in tissues from endotoxin-treated mice correlates with decreases in the expression of urokinase-type but not tissue-type plasminogen activator, J. Clin. Invest. 97 (1996) 2440-2451.
38. K. Bdeir, J.C. Murciano, J. Tomaszewski, L. Koniaris, J. Martinez, D.B. Cines, et al., Urokinase mediates fibrinolysis in the pulmonary microvasculature, Blood 96 (2000) 1820-1826.
39. K. Schafer, S. Konstantinides, C. Riedel, T. Thinnes, K. Muller, C. Dellas, et al., Different mechanisms of increased luminal stenosis after arterial injury in mice deficient for urokinase- or tissue-type plasminogen activator, Circulation 106 (2002) 1847-1852.
40. B.R. Binder, J. Mihaly, G.W. Prager, uPAR-uPA-PAI-1 interactions and signaling: a vascular biologist's view, Thromb. Haemost. 97 (2007) 336-342.
41. C.A. Reichel, S.M. Kanse, F. Krombach, At the interface of fibrinolysis and inflammation: the role of urokinase-type plasminogen activator in the leukocyte extravasation cascade, Trends Cardiovasc. Med. 22 (2012) 192-196.
42. J. Madunić, The Urokinase Plasminogen Activator System in Human Cancers: An Overview of Its Prognostic and Predictive Role, Thromb. Haemost. 118 (2018) 2020-2036.
43. B. Fuhrman, The urokinase system in the pathogenesis of atherosclerosis, Atherosclerosis 222 (2012) 8-14.
44. Y. Döring, O. Soehnlein, C. Weber, Neutrophil Extracellular Traps in Atherosclerosis and Atherothrombosis, Circ. Res. 120 (2017) 736-743.

45. M.S. Parahuleva, S.M. Kanse, B. Parviz, A. Barth, H. Tillmanns, R.M. Bohle, et al., Factor Seven Activating Protease (FSAP) expression in human monocytes and accumulation in unstable coronary atherosclerotic plaques, Atherosclerosis 196 (2008) 164-171.