Heparanase enhances the generation of activated factor X in the presence of tissue factor and activated factor VII

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ABSTRACT

Background

Heparanase is an endo-β-D-glucuronidase dominantly involved in tumor metastasis and angiogenesis. Recently, we demonstrated that heparanase is involved in the regulation of the hemostatic system. Our hypothesis was that heparanase is directly involved in activation of the coagulation cascade.

Design and Methods

Activated factor X and thrombin were studied using chromogenic assays, immunoblotting and thromboelastography. Heparanase levels were measured by enzyme-linked immunosorbent assay. A potential direct interaction between tissue factor and heparanase was studied by co-immunoprecipitation and far-western assays.

Results

Interestingly, addition of heparanase to tissue factor and activated factor VII resulted in a 3- to 4-fold increase in activation of the coagulation cascade as shown by increased activated factor X and thrombin production. Culture medium of human embryonic kidney 293 cells over-expressing heparanase and its derivatives increased activated factor X levels in a non-enzymatic manner. When heparanase was added to pooled normal plasma, a 7- to 8-fold increase in activated factor X level was observed. Subsequently, we searched for clinical data supporting this newly identified role of heparanase. Plasma samples from 35 patients with acute leukemia at presentation and 20 healthy donors were studied for heparanase and activated factor X levels. A strong positive correlation was found between plasma heparanase and activated factor X levels ($r=0.735$, $P=0.001$). Unfractionated heparin and an inhibitor of activated factor X abolished the effect of heparanase, while tissue factor pathway inhibitor and tissue factor pathway inhibitor-2 only attenuated the procoagulant effect. Using co-immunoprecipitation and far-western analyses it was shown that heparanase interacts directly with tissue factor.

Conclusions

Overall, our results support the notion that heparanase is a potential modulator of blood hemostasis, and suggest a novel mechanism by which heparanase increases the generation of activated factor X in the presence of tissue factor and activated factor VII.

Key words: heparanase, coagulation cascade, tissue factor, Xa level.

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Introduction

Heparanase is an endo-β-D-glucuronidase capable of cleaving heparan sulfate side chains at a limited number of sites, yielding heparan sulfate fragments of still appreciable size (~5–7 kDa).[1,2] Expression of heparanase is restricted primarily to the placenta, platelets, keratinocytes, and activated cells of the immune system, with little or no expression in connective tissue cells and most normal epithelia.[3,4] During embryogenesis, the enzyme is preferentially expressed in cells of the developing vascular and nervous systems.[5] Heparanase activity has been implicated in tumor growth, neovascularization, inflammation and autoimmunity.[3,4,6] A single human heparanase cDNA sequence was independently reported by several groups.[7-10] Thus, unlike the large number of proteases that can degrade polypeptides in the extracellular matrix, one enzyme appears to be used by cells to degrade the heparan sulfate side chains of heparan sulfate proteoglycans. Applying heparanase that lacks enzymatic activity, it was noted that heparanase also exerts non-enzymatic activities, independent of its involvement in extracellular matrix degradation and alterations in the extracellular microenvironment associated with tissue remodeling, angiogenesis, and cell invasion.[11-13]

The blood coagulation cascade is usually initiated as soon as the cell surface glycoprotein tissue factor (TF) comes into contact with circulating activated factor VII (factor VIIa), resulting in the formation of TF-factor VIIa complex. The TF-factor VIIa complex converts factor X to its activated form (factor Xa). Subsequently, factor Xa catalyzes the conversion of prothrombin to thrombin, thereby leading to fibrin formation, platelet activation, and, ultimately, generation of a thrombus. Tissue factor pathway inhibitor (TFPI) is a potent direct inhibitor of factor Xa and, in a factor Xa-dependent fashion, inhibits the factor VIIa-TF complex. Recently, we demonstrated that heparanase may also affect the hemostatic system. Heparanase was shown to up-regulate TF expression[14] and interact with TFPI on the cell surface, leading to dissociation of TFPI from the cell membrane, resulting in increased cell surface coagulation activity.[15] The present work further investigates the role of heparanase in activation of the coagulation system.

Design and Methods

Study group

The study was approved by the institutional Ethics Committee on human research at Rambam Medical Center. Thirty-five consecutive patients with newly diagnosed acute leukemia were enrolled over a 7-month period, between February 2007 and September 2007, at the Rambam Medical Center. The patients' ages ranged from 18-78 years (mean, 47 ± 20 years); there were 21 males and 14 females. Twenty-two patients had acute myeloid leukemia M1-2, nine had acute myeloid leukemia M4-5 and four had acute lymphocytic leukemia. The patients were diagnosed according to established morphological, immunophenotypic and molecular criteria. After obtaining informed consent, peripheral blood was taken at the time of diagnosis and prior to chemotherapy treatment. Patients were followed up prospectively for the occurrence of symptomatic thrombotic manifestations during the first month following the start of chemotherapy. Twenty samples were obtained from healthy donors (age 24-55 years, mean 36 ± 12 years, 14 males and 6 females). A total of 3 mL of peripheral blood was collected, with 3.2% (0.12 M) sodium citrate (1:10) as an anticoagulant. Plasma was obtained by centrifugation (1500 g for 15 min at 4°C) and all plasma samples were frozen and thawed once.

Cell culture and transfection

HEK-293 cells were purchased from the American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics (Biological Industries, Beit Haemek, Israel). Cells were stably transfected with full-length human heparanase (65 kDa) cDNA cloned into the pSecTag2 vector (Invitrogen, Carlsbad, CA, USA), using the FuGENE 6 reagent, according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IL, USA).[16-18] Transfection proceeded for 48 h, followed by selection with Zeocin (Invitrogen, Carlsbad, CA, USA) for 2 weeks. Stable transfectant pools were further expanded and analyzed. Modified species of heparanase were used: a single chain GS3 active heparanase gene construct comprising the 8 and 50 kDa heparanase subunits (8+50) (kindly provided by Dr. Christian Steinkuhler, IRMB/Merck Research Laboratories, Pomezia, Italy),[19] and a heparanase gene construct mutated at Glu^{225} and Glu^{343}, critical for the enzyme's catalytic activity.[13,20]

Reagents and antibodies

The single chain GS3 active heparanase gene construct, comprising the 8 and 50 kDa heparanase subunits (8+50), was purified from the conditioned medium of baculovirus-infected cells.[14] GS3 active heparanase was assayed for the presence of bacterial endotoxin by Biological Industries (Beit Haemek, Israel), using the gel-clot technique (*Limulus* amebocyte lysate – LAL test) and was found to contain less than 10 pg/mL endotoxin. Polyclonal antibody 1453 was raised in rabbits against the entire 65 kDa heparanase precursor isolated from the conditioned medium of heparanase-transfected HEK-293 cells. The antibody was affinity-purified on immobilized bacterially expressed 50 kDa heparanase GST fusion protein.[18] Anti-heparanase monoclonal antibody 130 was kindly provided by InSight Pharmaceuticals (Rehovot, Israel). Lipidated recombinant human TF, monoclonal and polyclonal anti-human TF antibodies, and monoclonal anti-human factor X and Xa heavy chain (I.D. 5010) were purchased from American Diagnostica (Stanford, CT, USA). Recombinant human factor VIIa, recombinant human inactivated factor VIIa, recombinant TFPI, plasma-derived human factor X, plasma-derived human prothrombin, chromogenic substrate for factor Xa (I.D. 222, Formula: MeO-CO-D- CHG-Gly-Arg-pNA.AcOH solubility: Tris buffer, pH – 8.4) and chromogenic substrate for thrombin (I.D. 238, Formula: H-D-HHT-Ala-Arg-pNA.2AcOH solubility: Tris buffer, pH – 8.4) were purchased from American Diagnostica (Stanford, CT, USA). Recombinant TFPI-2 was purchased from R&D (Minneapolis, MN, USA) and unfractionated heparin was purchased from Sigma (St. Louis, MO, USA). Rivaroxaban was purchased from Bayer (Leverkusen, Germany). All coagulation factors were dissolved in double-distilled water.

Activated factor X generation assay

Following preliminary experiments to quantify the appropriate protein doses, we performed a basic experiment of factor Xa generation in the following manner: the concentrations mentioned are the final concentrations. Lipidated recombinant human TF (0.004 μM, 200 pg/mL), recombinant human factor

VIIa (0.04 μM) and plasma-derived human factor X (1.4 μM) were incubated in 50 μL assay buffer (0.06 M Tris, 0.04 M NaCl, 2 mM $CaCl_2$, 0.04 % bovine serum albumin, pH 8.4) to a total volume of 125 μL in a 96-well sterile plate. After 15 min at 37°C, chromogenic substrate for factor Xa was added (1 mM). After 20 min, the reaction was stopped with 50 μL of glacial acetic acid and the level of factor Xa generation was determined using an enzyme-linked immunosorbent assay (ELISA) plate reader (Power Wave XS, BIO-TEK, VT, USA). In order to visualize the increased production of factor Xa by western blotting, the experiment was repeated with 7 μM factor X. After 15 min at 37°C, half of the reaction volume was subjected to blotting using monoclonal anti-human factor X/Xa heavy chain antibody.

Sodium dodecylsulfate polyacrylamide gel electrophoresis and immunoblot analysis

Proteins were subjected to 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (BioRad, Maylands, CA, USA). The membrane was probed with the appropriate antibody followed by horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) and a chemiluminescence substrate (Pierce, Rockford, IL, USA), as described elsewhere.[14]

Thromboelastography

The assay was performed according to the manufacturer's recommendations. Briefly, 3 mL of healthy donor's whole blood were collected in a bottle containing 3.2% (0.12M) sodium citrate and stored at room temperature. Recalcification and TEG® measurements at 37°C were performed in disposable cups of the Thrombelastograph® coagulation analyzer (Haemoscope Corporation, Skokie, IL, USA).

Heparanase enzyme-linked immunosorbent assay

Wells of microtiter plates were coated (18 h, 4°C) with 2 μg/mL of anti-heparanase monoclonal antibody in 50 μL coating buffer (0.05 M Na_2CO_3, 0.05 M $NaHCO_3$, pH 9.6) and were then blocked with 2% bovine serum albumin in phosphate-buffered saline for 1 h at 37 °C. Samples (200 μL) were loaded in duplicate and incubated for 2 h at room temperature before the addition of 100 μL antibody 1453 (1 μL/mL) for an additional period of 2 h at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit IgG (1:20,000) in blocking buffer was added (1 h, room temperature) and the reaction was visualized by the addition of 50 μL chromogenic substrate (TMB; MP Biomedicals, Germany) for 30 min. The reaction was stopped with 100 μL H_2SO_4 and absorbance at 450 nm was measured using an ELISA plate reader (Power Wave XS, BIO-TEK, VT, USA). Plates were washed five times with washing buffer (PBS, pH 7.4 containing 0.1% (v/v) Tween 20) after each step. As a reference for quantification, a standard curve was established by serial dilutions of recombinant 8+50 GS3 active heparanase (390 pg/mL - 25 ng/mL).[21] The assay detects almost exclusively the active heparanase and only poorly the full-length heparanase (65 kDa).

Co-immunoprecipitation

The interaction between TF and heparanase was analyzed by co-immunoprecipitation. The ProFound™ Co-Immunoprecipitation Kit, in which the antibody is coupled to gel support, was employed according to the manufacturer's (Pierce, Rockford, IL, USA) instructions. Briefly, coupling gel was washed with coupling buffer. Polyclonal anti-TF or polyclonal anti-heparanase (1453) antibodies (100 μg) and 5 M sodium cyanoborohydride were added to the gel support and incubated at 4°C for 4 h. The gel was then washed with quenching buffer and incubated with the quenching buffer and sodium cyanoborohydride at 20°C for 30 min. Next, the gel was washed four times with wash solution, once with elution buffer, and twice with coupling buffer. Purified proteins (0.5 μg) were added to the gel and incubated for 2 h at 4°C. The gel was washed four times with coupling buffer and bound proteins were eluted with elution buffer (pH 2.5), neutralized by 1 M Tris-HCl, pH 9.5, and subjected to immunoblot analysis. Monoclonal anti-TF and monoclonal anti-heparanase antibodies were used to detect the respective coupled protein. Irrelevant anti-GST polyclonal antibody (100 μg) and uncoupled beads were used as controls.

Statistical analysis

Data were evaluated by SPSS software for Windows version 13.0 (SPSS Inc., Chicago, IL, USA). Pearson's correlation (r) was used to assess the link between plasma heparanase and factor Xa levels. A t-test for independent samples was used to asses the difference between patients and controls regarding heparanase levels and factor Xa levels. Values are reported as mean ± standard deviation. *P* values less than 0.05 were considered statistically significant.

Results

Heparanase increases activated factor X levels

The factor Xa generation assay was performed as described in the *Design and Methods* section with increasing doses of recombinant human heparanase (GS3) added prior to the addition of the coagulation factors. Interestingly, heparanase increased factor Xa production in a dose-dependent manner visualized by a chromogenic substrate (Figure 1A) and by western blot (Figure 1B). It should be noted that factor X is composed of two polypeptide chains, a heavy chain with a molecular weight of 38 kDa and a light chain with a molecular weight of 18 kDa joined by a dipeptide Arg-Arg. The factor VIIa-TF complex activates the conversion of factor X to factor Xa by cleaving the heavy chain to a 33 kDa peptide. The factor Xa produced in the presence of heparanase has the same molecular weight as factor Xa produced without heparanase, indicating that the site of cleavage in factor X heavy chain is most likely the same. In order to exclude contamination of the heparanase preparation during the routine procedure of purification described in the *Design and Methods* section, we tested the effect of overnight serum-free without phenol medium of 293 HEK cells over-expressing heparanase and its derivatives compared to mock. The proteins were further purified from the culture medium and analyzed. Similar quantities of proteins were obtained from the culture media. A 2-fold increase in factor Xa levels was observed with the medium of full-length heparanase and active heparanase (Figure 1C). The double mutant inactive heparanase also increased factor Xa levels, indicating that the effect is non-enzymatic (Figure 1C).

Heparanase increases thrombin levels

In order to verify that factor Xa produced in the presence of heparanase is active, thrombin generation, the next step in the coagulation cascade, was examined. The

experiment described above (Figure 1A) was repeated and plasma-derived human prothrombin (222 μM) was added following the addition of factor X. After 15 min at 37°C, chromogenic substrate for thrombin was added (1 mM). In the presence of heparanase, thrombin generation was increased (Figure 1D).

Heparanase increases coagulation without substituting one of the coagulation factors

In order to verify that the heparanase molecule is a cofactor for the factor VIIa-TF complex, heparanase was added to the conditions described in Figure 1A. Factor Xa was not generated in the absence of each of the essential factors (i.e., TF, factor VIIa, and factor X) or in the presence of inactivated VIIa, as shown in Figure 1E, indicating that the heparanase preparation is neither contaminated with, nor does it replace, any of these coagulation factors. Moreover, no factor Xa was generated when heparanase was added to factor X for 15 min at 37°C, followed by the addition of a chromogenic substrate for Xa. Similarly, when heparanase was incubated (15 min, 37°C) with prothrombin followed by the addition of a chromogenic substrate for thrombin, no thrombin was generated (*data not shown*). These experiments confirm that heparanase does not activate factor X or prothrombin directly, but rather serves as a cofactor for the TF-factor VIIa complex.

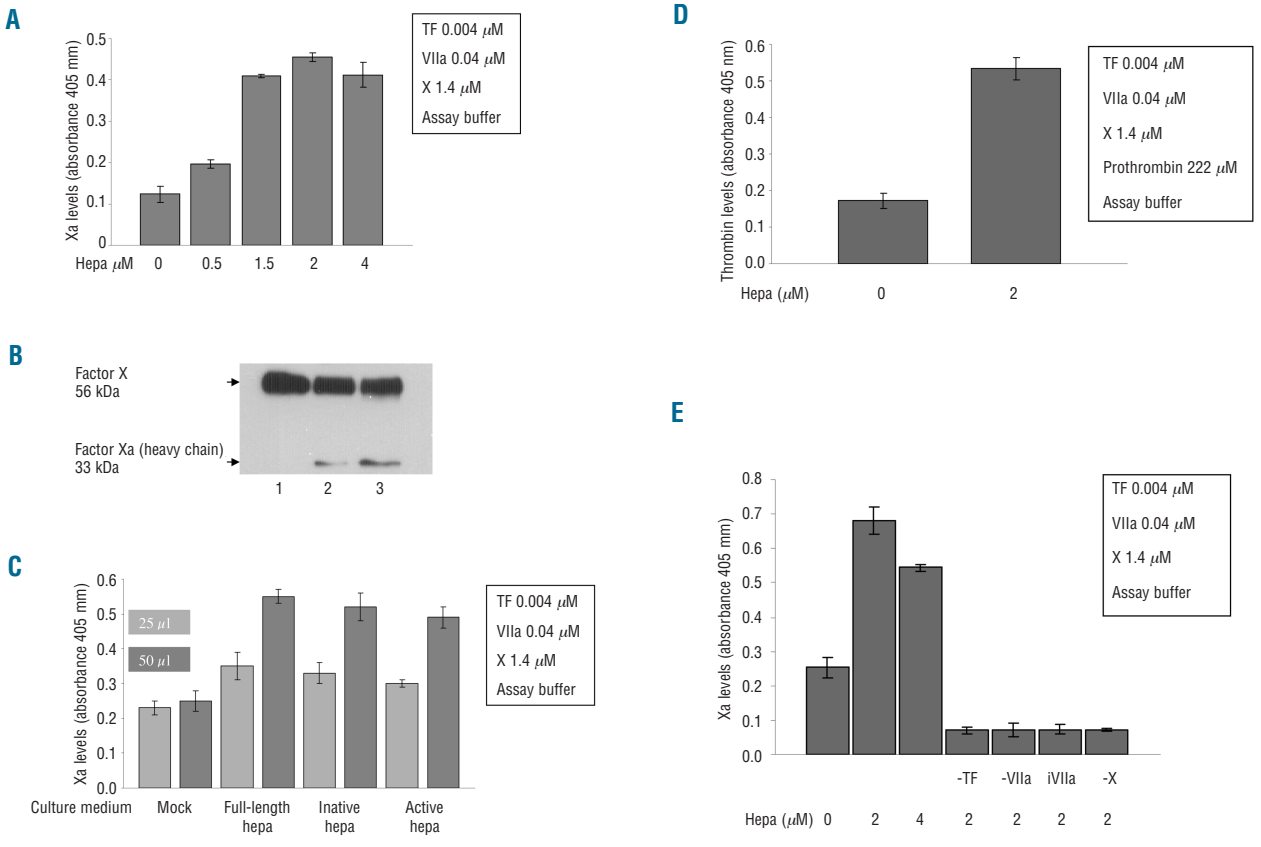

Figure 1. Heparanase increases factor Xa levels. (A) Recombinant human TF, recombinant human factor VIIa, and plasma-derived human factor X were incubated (15 min, 37°C) in a total assay volume of 125 μL. Chromogenic substrate for the detection of factor Xa was then added (final concentration 1 mM). Recombinant active heparanase GS3 (Hepa) was added prior to the addition of the coagulation factors. The mean value ± SD of three independent experiments in shown. A 3- to 4-fold increase in coagulation activity was obtained in the presence of heparanase. (B) Western blot analysis. Lane 1: 50 ng of human factor X served as a control on SDS/PAGE followed by immunoblotting in which anti-human monoclonal antibody directed against the heavy chain of factor X and Xa was employed. Lane 2: sample, incubated as in A, in a reaction mixture containing 50 ng factor X (7 μM), was subjected to immunoblotting. Lane 3: Same as lane 2, except that heparanase (2 μM) was added prior to the addition of the coagulation factors. Note an increased generation of Xa in the presence of heparanase. (C) 25 μL or 50 μL of overnight serum-free medium without phenol of 293 HEK cells over-expressing full-length heparanase (65 kDa), enzymatically inactive heparanase (double mutant), active heparanase (GS3 8+50) and mock were incubated with TF, factor VIIa and X. After 15 min at 37°C, chromogenic substrate for Xa detection was added. The mean value of two experiments is shown. Note a 2-fold increase in coagulation activity when the medium contained heparanase or its derivatives. (D) Heparanase increases thrombin levels. Recombinant human TF, recombinant human factor VIIa, plasma-derived human factor X, and plasma-derived human prothrombin were incubated (15 min, 37°C) in a total volume of 125 μL. Chromogenic substrate for the detection of thrombin was then added (1 mM). Human recombinant heparanase (Hepa) was added prior to the addition of the coagulation factors. The mean value ± SD of three independent experiments is shown. Note a 3-fold increase in coagulation activity in the presence of heparanase. (E) Each of the coagulation factors is needed for the heparanase-mediated increase in coagulation. Recombinant heparanase was incubated as described in Figure 1A with either complete reaction mixture, reaction mixture lacking TF, factor VIIa, or factor X, or reaction mixture containing inactivated factor VIIa instead of factor VIIa. The mean value ± SD of three independent experiments is shown. The results imply that the heparanase preparation is neither contaminated with, nor does it replace, any of the coagulation factors.

Heparanase in human plasma

Next, the procoagulant effect of heparanase in human plasma was tested. Using an ELISA method,[21] it was found that the level of active heparanase (GS3 8+50 kDa) in plasma drawn in sodium citrate was 500±29 pg/mL (0.0085 μM).[22] When heparanase, factor VIIa and factor X were incubated (15 min, 37°C) with pooled normal human plasma from five healthy donors, followed by the addition of a chromogenic substrate for factor Xa, a 7- to 8-fold increase in factor Xa levels was obtained compared to levels in plasma incubated in the absence of heparanase (Figure 2). In this condition the coagulation was mainly dependent on the concentration of endogenous TF, as described previously.[23,24] The effect of heparanase was also tested in whole blood using thromboelastography. Citrated blood samples from healthy donors to which factor VIIa (0.04 μM) and factor X (1.4 μM) were added were recalcified according to the manufacturer's recommendations (final concentration of $CaCl_2$ 8 mM) and arranged in the disposable cups of the thromboelastogram. With increasing doses of heparanase there was a significant reduction in time to clot formation and an increase in thrombus strength (Figure 3). At the highest dose (4 μM) the effect on time to clot formation was abolished (Figure 3).

In order to attribute clinical relevance to the newly identified function of heparanase, 55 plasma samples were tested for their heparanase content using ELISA and the heparanase content correlated to factor Xa levels using a chromogenic substrate for Xa. Thirty-five samples were from patients with acute leukemia and 20 samples were from healthy donors. Using Pearson's analysis, a strong positive correlation was found between the two parameters (r=0.735, *P*=0.001), indicating a potential *in vivo* effect of heparanase on factor Xa levels in plasma (Figure 4). Using a t-test for independent samples, a significant difference was found between patients and controls in heparanase levels (1091±352 *versus* 499±129, repectively; *P*=0.0001) and factor Xa levels (0.83±0.32 *versus* 0.51±0.17, *P*=0.004), respectively. Four thrombotic manifestations (two pulmonary emboli, one catheter-related deep vein thrombosis, and one myocardial infarction) occurred in the group of patients in the first month during which chemotherapy was initiated. The levels of heparanase in these patients were exceedingly high (1353, 1276, 1220, and 1276 pg/mL) as were the levels of factor Xa (1.23, 0.61, 1.12, and 0.87; optical density of 405-490 nm). The subgroup with thrombotic manifestations is, however, too small for statistical analysis.

Inhibition of heparanase procoagulant activity

Four inhibitors were tested: unfractionated heparin, TFPI, TFPI-2, and anti-Xa derivative (rivaroxaban). Whereas unfractionated heparin and the anti-Xa derivative abolished the procoagulant effect of heparanase (Figure 5A, 5C), TFPI and TFPI-2 attenuated the effect

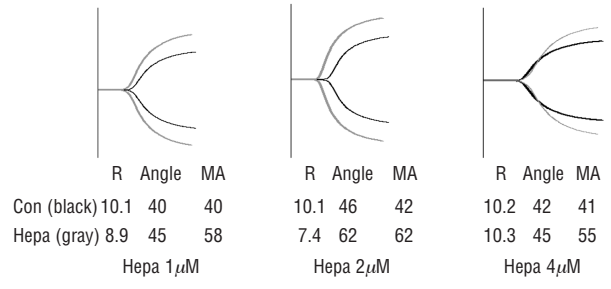

Figure 3. Heparanase reduces time to clot formation and increases thrombus strength. Thromboelastogram studies. The assay includes addition of factor VIIa, X and calcium to blood drawn into citrate in the presence or absence of heparanase. Each time, a sample with addition of heparanase (Hepa) and a sample without (Con) - were analyzed in parallel. R - The period of latency from the time that the blood was placed in the analyzer until the initial fibrin formation: this represents the coagulation factor activation. Angle - measures the rapidity of fibrin build-up and cross-linking: this represents the coagulation factor amplification. MA – A direct function of the maximum dynamic properties of fibrin and platelet bonding: signifies the ultimate strength of the clot. Note that in the presence of heparanase the clot is formed significantly faster and is stronger. At the highest dose, no effect on R is detected. The assays were done in pooled whole blood from three healthy donors and were repeated three times at each concentration revealing consistency. Representative results are shown.

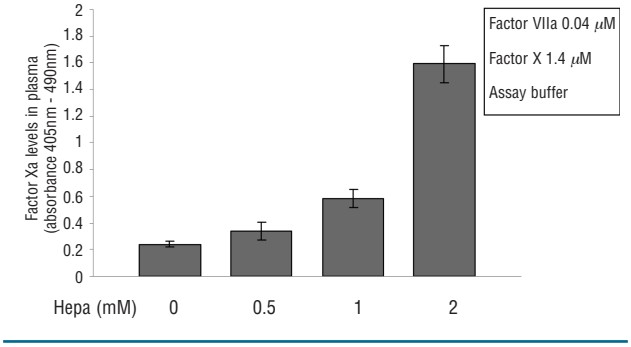

Figure 2. Heparanase increases coagulation in normal human plasma. Increasing doses of heparanase were added to pooled normal human plasma (25 μL plasma). After 15 min at 37°C, chromogenic substrate for the detection of factor Xa was added (1 mM). The mean value ± SD of three independent experiments is shown. Note a 7- to 8-fold increase in coagulation activity in the presence of heparanase.

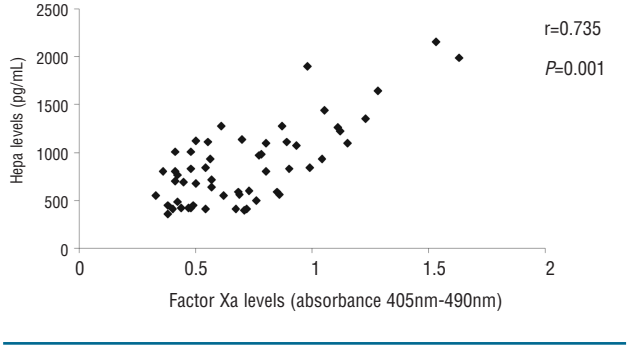

Figure 4. Correlation between plasma heparanase and factor Xa levels. Plasma samples drawn from 35 patients with acute leukemia at presentation and from 20 healthy donors were evaluated for heparanase levels using ELISA and for factor Xa levels present in plasma (50 μL plasma, 50 μL assay buffer) applying a chromogenic substrate (1 mM, 30 min incubation). A strong positive correlation was found between the two parameters, suggesting an effect of heparanase on factor Xa levels.

(Figure 5B). As far as we know, rivaroxaban does not inhibit the TF-factor VIIa complex but it succeeded in abolishing the factor Xa effect generated in the presence of heparanase. Interestingly, when heparanase, was added, TFPI-2 was a more effective inhibitor than TFPI (Figure 5B).

Co-interaction between tissue factor and heparanase

Using co-immunoprecipitation and far-western analyses, we demonstrated that heparanase actually interacts with TF (Figure 6). Unfractionated heparin abrogated this interaction completely (Figure 6A), in accordance with the abolishment of the heparanase procoagulant effect (Figure 5A).

Discussion

TF is the main initiator of blood coagulation. In the present study it was demonstrated that heparanase may serve as a cofactor for TF, suggesting that heparanase is directly involved in the activation of the coagulation cascade. Our findings are supported by results of experiments indicating that heparanase increases the level of factor Xa in the presence of the TF-factor VIIa complex and that the effect is enzymatically independent. The newly generated factor Xa has the same molecular weight as factor Xa cleaved by TF-factor VIIa and is active, as shown by increased conversion of prothrombin to thrombin (Figure 1). The recombinant heparanase preparation used in our study was free of the relevant coagulation factors (Figure 1E) and failed to activate factor X or prothrombin, directly. The newly identified direct involvement of heparanase in the coagulation system was demonstrated by three different methods: a chromogenic assay, western blot and thromboelastography (Figures 1 and 3). Factor Xa generation in plasma was more robust (Figure 2) compared to that in the assay involving isolated proteins (Figure 1) possibly because of the optimal concentrations of coagulation factors VII and X that are also present in the plasma. Increased factor Xa generation in the presence of heparanase was shown to be relevant in the clinical setting. Thus, apart from the ability of heparanase to increase factor Xa levels in normal human plasma (Figure 2), a statistically significant positive correlation was found between the plasma levels of heparanase and factor Xa in both patients with acute leukemia and healthy donors (Figure 4).

To elucidate the mode of action of heparanase we demonstrated a direct interaction between TF and heparanase, as illustrated by co-immunoprecipitation and far-western blot analyses (Figure 6), although possible addition interaction with factor VIIa was not excluded. Unfractionated heparin was found to abolish heparanase procoagulant activity completely (Figure 5A), most probably through disruption of the TF-heparanase interaction, as demonstrated by co-immunoprecipitation experiments (Figure 6A). This result widens our understanding of heparin anticoagulant activity, indicating that unfractionated heparin not only enhances anti-thrombin activity, but also inhibits the heparanase's procoagulant effect. Interestingly, a direct anti-factor Xa derivative (rivaroxaban) was also found to abolish the procoagulant effect of heparanase (Figure 5C).

Given the multiple pathological clinical situations in which heparanase is involved, the ability to inhibit heparanase's hemostatic response completely positions direct anti-factor Xa compounds as potential strong inhibitors of pro-angiogenic and pro-metastatic processes involving heparanase and elevated coagulation. TFPI and TFPI-2 attenuated the effect of heparanase, but even at high doses failed to abolish the procoagulant effect of heparanase fully (Figure 5B). Notably, TFPI-2 was a stronger inhibitor than TFPI in the presence of heparanase. Taking into account the high levels of heparanase and TFPI-2 in the placenta,[3,4,25] a potential regulatory role in placental hemostasis is suggested.

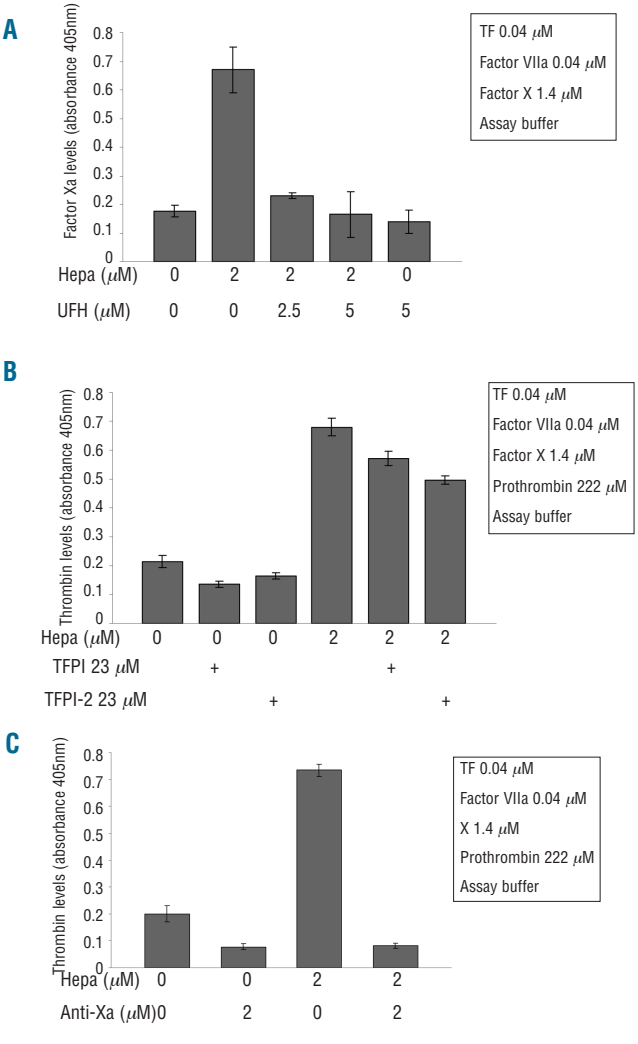

Figure 5. Inhibition of heparanase procoagulant activity. (A) Heparanase procoagulant activity is abrogated by heparin. Same as Figure 1A, except that unfractionated heparin (UFH) was added (2.5 μM = 7.5 IU/mL) prior to heparanase. Note that UFH abolished heparanase activity. (B) Heparanase procoagulant activity is attenuated by TFPI and TFPI-2. Same as Figure 1D, except that recombinant human TFPI and TFPI-2 were added prior to heparanase addition. The mean value ± SD of three independent experiments is shown. Note that TFPI and TFPI-2 mildly attenuated thrombin generation but failed to abolish the procoagulant effect of heparanase. TFPI-2 was more potent than TFPI in the presence of heparanase. (C) Heparanase procoagulant activity is abrogated by an anti-Xa derivative. Same as Figure 1D, except that an anti-Xa derivative (Rivaroxaban) was added after factor X. Note that the anti-Xa derivative abolished the effect of heparanase, resembling the effect of UFH.

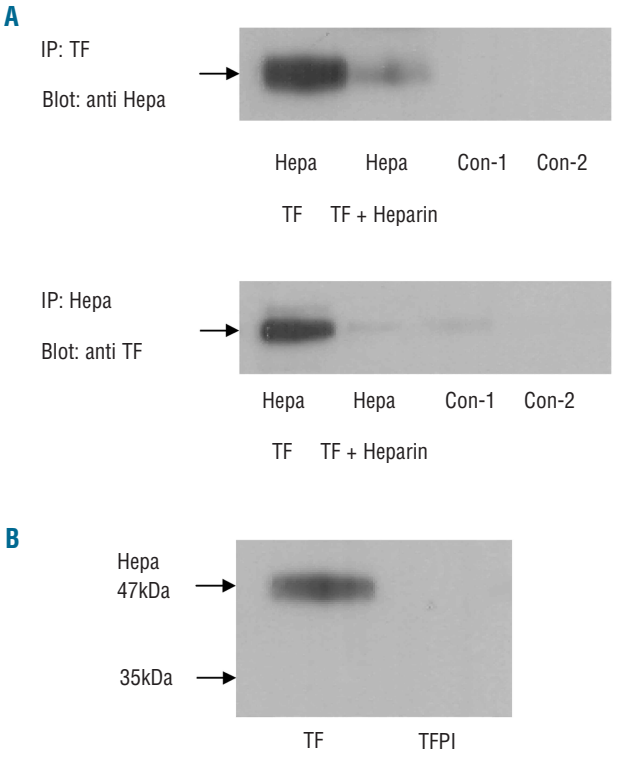

Figure 6. Direct interaction between heparanase and TF. (A) Co-immunoprecipitation. Purified TF (1 μg/mL) and GS3 active heparanase (1 μg/mL) proteins were incubated with beads coupled to polyclonal anti-TF (upper panel), or anti-heparanase (lower panel) antibodies. Bound proteins were analyzed by immunoblotting for the presence of heparanase (upper panel) or TF (lower panel), using the respective monoclonal antibody. Note the interaction between TF and heparanase, reduced or lack of interaction in the presence of unfractionated heparin (1 μg/mL=0.2 IU/mL), and lack of interaction with beads coupled to irrelevant anti-GST polyclonal antibody (Con-1), or to uncoupled beads (Con-2). (B) Far-western analysis. Recombinant TF (100 ng) and TFPI (100 ng) were resolved by SDS-PAGE under reducing conditions using a 10% gradient polyacrylamide gel. After electrophoresis, proteins were transferred to a polyvinylidene fluoride membrane. The membrane was incubated for 2 h with GS3 active heparanase protein (1 μg/mL, 4°C). Next, the membrane was probed with anti-heparanase antibody followed by horseradish peroxidase-conjugated secondary antibody and chemiluminescence substrate, as described in the *Design and Methods* section. A clear band was revealed in the TF-heparanase lane but not in the TFPI-heparanase lane.

Most TF is found in intracellular compartments, predominantly in the Golgi body. TF at the cell surface is localized in cholesterol-rich lipid rafts and is extensively co-localized with caveolin-1.[26] Recently, it was found that heparanase induces Akt phosphorylation via a lipid raft receptor, suggesting that such receptors mediate heparanase signaling.[27] It is possible that TF, which is a transmembrane receptor concentrated in lipid rafts, interacts with heparanase at the cell surface, enhancing both coagulation and intracellular signaling.

The extent of TF protein induction in vascular cells does not always correlate with TF activity.[28,29] One possible explanation is the concomitant secretion of TFPI, the endogenous inhibitor of TF. Another possible explanation is the distribution of TF in several cellular compartments. While biologically active TF is located at the cell surface, intracellular TF constitutes a pool that is only released upon cell damage.[29,30] Discrepancies between TF protein expression and activity can further be accounted for by the induction of a functionally inactive form of TF at the cell surface, termed latent or encrypted TF. Expression of encrypted TF enables a cell to rapidly increase TF activity in response to certain stimuli without a need for *de novo* protein synthesis. De-encryption of TF has been observed secondary to changes in intracellular calcium levels, alterations in membrane phosphatidylserine expression, or modifications in the quaternary structure of TF.[31] Hence, the relative contributions of TF protein induction, cellular localization and structural modification appear to determine the net procoagulant effect elicited by a given mediator. It is conceivable that the procoagulant effect of TF also depends on the presence and level of its potential, newly identified co-factor, heparanase.

According to our data, heparanase has an optimal effect at a concentration that is about 250-fold higher than active heparanase in plasma as measured by ELISA. Although the total level of heparanase (active and non-active forms) is still unknown, in view of the abundance of heparanase in platelets, the levels of heparanase released locally upon degranulation of platelets[32] may be high and further facilitate blood coagulation. It was previously demonstrated that secretion of active heparanase from malignant cells is brought about by cell stimulation with physiological concentrations of adenosine, ADP and ATP.[33] Activated platelets secret ADP and ATP from their δ-granules and may, therefore, contribute to heparanase secretion from tumor cells.

The main limitation of our findings is the lack of data from an animal model supporting a direct role of heparanase in the coagulation cascade. Nevertheless, the strong positive correlation between plasma heparanase and plasma factor Xa levels in patients with acute leukemia and in normal donors strongly supports our findings in the clinical setting. Overall, our results support the notion that heparanase is a potential modulator of blood hemostasis, and suggest a novel mechanism by which heparanase is involved in direct activation of the coagulation cascade. Our previous findings on the regulation of TF[14] and TFPI[15] by heparanase indicate a more fundamental role of heparanase in coagulation. The elevation of heparanase levels in human tumors, together with the pro-thrombotic state of most neoplasms, suggests possible clinical relevance of the procoagulant function of heparanase, as demonstrated for leukemia patients (Figure 4). Targeting domains in the heparanase protein that mediate its enzymatic-dependent and -independent functions may prove beneficial for patients with cancer and pro-thrombotic conditions.

Authorship and Disclosures

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