Res Pract Thromb Haemost. 2023 Aug; 7(6): 102179.
Published online 2023 Aug 30. doi: 10.1016/j.rpth.2023.102179
PMCID: PMC10520574
PMID: 37767062

Tissue factor-heparanase complex: intracellular nonhemostatic effects

Shorook Ghanem,[1] Anat Keren-Politansky,[2] Victoria Kaplan,[1] Yonatan Crispel,[1] and Yona Nadir[1,*]

Abstract

Background

Heparanase, known to be involved in angiogenesis, cancer progression, and inflammation, was shown to form a complex with tissue factor (TF) via its procoagulant domain and to enhance the hemostatic system.

Objectives

To reveal a potential role of heparanase procoagulant domain in nonhemostatic effects.

Methods

Effects of peptides 16 and 16AC derived from the heparanase procoagulant domain, discovered by our group, were studied using the XTT proliferation assay, western blot analysis, and immunostaining *in vitro* and a mouse wound-healing model.

Results

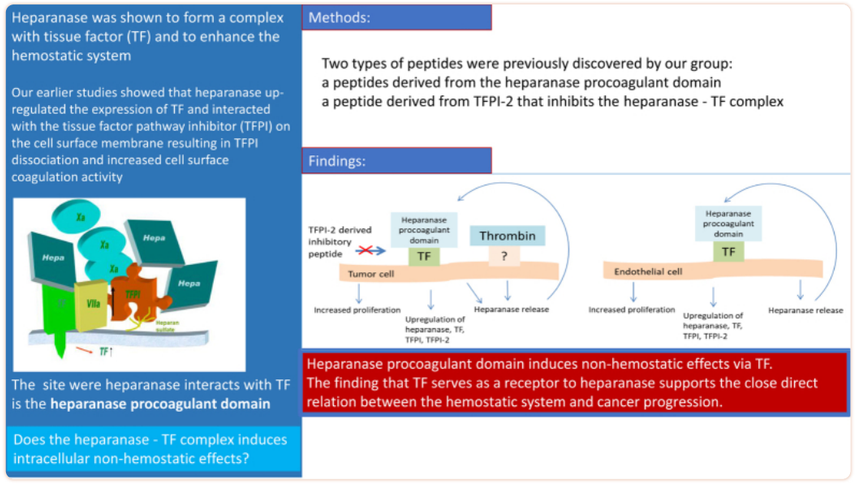
Procoagulant peptides induced increased proliferation, release of heparanase, and upregulation of heparanase, TF, tissue factor pathway inhibitor (TFPI), and TFPI-2 in U87, T47D, and MCF-7 tumor cell lines and in endothelial cells. These results were reversed by a peptide derived from TFPI-2 that inhibited the heparanse procoagulant domain-TF complex. Thrombin had a similar effect on tumor cell proliferation and heparanase release, although the impact of thrombin on cell proliferation was mediated by the heparanase procoagulant domain. A mouse model of full-thickness skin incision exhibited higher levels of heparanase, TF, TFPI, and TFPI-2 in the healing skin, mainly in the blood vessel wall and lumen in animals injected with the procoagulant peptides compared to controls. The cells transfected to overexpress full-length TF or TF devoid of the cytoplasmic domain demonstrated that the procoagulant domain conveyed intracellular signaling via TF.

Conclusion

Heparanase procoagulant domain induces nonhemostatic effects via TF. The finding that TF serves as a receptor to heparanase supports the close direct relation between the hemostatic system and cancer progression.

Keywords: heparanase, neoplastic processes, receptor, tissue factor, tissue factor pathway inhibitor

Graphical abstract



Essentials

- Heparanase was shown to form a complex with tissue factor and to enhance the hemostatic system.
- Effects of peptides 16 and 16AC derived from the heparanase procoagulant domain were studied.
- Peptides increased proliferation, release, and upregulation of heparanase, tissue factor, tissue factor pathway inhibitor, and tissue factor pathway inhibitor-2.
- Peptide effects were not conveyed in cells overexpressing tissue factor devoid of the cytoplasmic domain.

1. Introduction

Heparanase, a β-D-endoglucuronidase, was discovered as an enzyme that cleaved heparan sulfate side chains on the cell surface and in the extracellular matrix [1,2]. It was later shown to be an enhancer of inflammation, angiogenesis, and metastasis [3,4]. We previously demonstrated that heparanase might also affect the hemostatic system in a nonenzymatic manner [5, 6, 7]. Our findings demonstrated that heparanase directly enhanced the tissue factor (TF) activity, leading to increased factor Xa production and subsequent activation of the coagulation system [7]. Our earlier studies showed that heparanase upregulates the expression of TF [5] and interacts with the tissue factor pathway inhibitor (TFPI) on the cell surface membrane of endothelial and tumor cells, resulting in TFPI dissociation and increased cell surface coagulation activity [6]. We recently detected the procoagulant domain in the heparanase protein and generated peptides derived from this site (peptides 16 and 16AC). Additionally, we revealed the domain in TFPI-2 that is capable of inhibiting the interaction between heparanase and TF complex and generated a peptide derived from that site (peptide 7) [8,9]. Interestingly, the inhibition of the latter complex reduced tumor growth and vascularization [10], implying that the interaction between heparanase and TF also exerted nonhemostatic effects. The present study was aimed at exploring a potential role of TF as a heparanase receptor promoting induction of intracellular effects.

2. Methods

2.1. Reagents and antibodies

A single-chain GS3 heparanase gene construct, comprising the 8 and 50 kDa heparanase subunits (8 + 50), was purified from the conditioned medium of baculovirus-infected cells. GS3 heparanase was assayed for the presence of bacterial endotoxin (Biological Industries) using the gel-clot technique (limulus amebocyte lysate test) and was found to contain <10 pg/mL of endotoxin [5]. The 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 glutathione-S-transferase fusion protein [11]. Antibody 733 was raised in rabbits against a 15 amino acid peptide that maps at the N-terminus of the 50 kDa heparanase subunit [11]. Polyclonal anti-TF, polyclonal anti-TFPI, and polyclonal anti-p38 antibodies were purchased from Santa Cruz Biotechnology. Polyclonal anti-TFPI-2 was purchased from Bioss Inc, and the polyclonal antibody to phospho-p38 was acquired from Cell Signaling. Adenosine monophosphate (ATP) was obtained from Helena Biosciences. Thrombin was purchased from SIEMENS. PAR-1 antagonist (SCH 530348, vorapaxar), PAR-1 antagonist (FR) (Fr 171113), PAR-2 antagonist (FSLLRY-NH2), and PAR-1 agonist TRAP-6 were acquired from Tocris Bioscience.

Amino acids sequences were as follows: peptide 16, GSKRRKLRVYLHCT; peptide 16AC, amid GSKRRKLRVYLHCT acetyl (amid and acetyl residues prevent peptide degradation and enhance activity); and peptide 7, NNAEICLLPLDYGP. Peptides 16 and 16AC derive from the procoagulant domain of heparanase, and peptide 7 derives from TFPI-2 and inhibits the interaction between TF and heparanase.

2.2. Cell culture and transfection

Human umbilical vein endothelial cells (HUVECs), U87 human glioma, T47D human breast carcinoma, MCF-7 human adenocarcinoma, and HEK-293 human embryonic kidney cells were cultured at 37 ºC, 5% CO_2. Tumor cell lines were maintained in Dulbecco's modified Eagle's medium (Biological Industries) and supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin-amphotericin (rich medium). The HUVEC cell line was grown in Medium 199, Earle's Salts (Biological Industries) and supplemented with 20% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin-amphotericin in addition to endothelial growth factor (100 μg/mL; Biomedical Tech). U87, T47D, and MCF-7 cell lines were stably transfected with a vector containing the human heparanase cDNA to generate high levels of heparanase. Full-length human TF cDNA was inserted into the mammalian expression vector pSecTag2 (Invitrogen). A cDNA encoding the TF mutant lacking amino acid residues (248-263) (ΔTF) was prepared as described earlier [12]. An expression plasmid for this mutant was prepared by inserting the cDNA into the expression plasmid pSecTag2. Cells were stably transfected to HEK-293 cells using the FuGENE 6 Reagent (Roche Applied Science), according to the manufacturer's instructions. Transfection proceeded for 48 hours, followed by selection with Zeocin for 2 weeks. Stable transfectant pools were further expanded and analyzed.

2.3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblot analysis

Proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis under reducing conditions using 10% gradient polyacrylamide gels. After electrophoresis, the gels were transferred to a polyvinylidene fluoride membrane (BioRad). The membrane was probed with the heparanase antibody (63 immunoglobulin G) followed by an horseradish peroxidase-conjugated secondary antibody (Jackson Immuno Research) and chemiluminescence substrate (Pierce).

2.4. Immunostaining

Cells were seeded on slides in 10 cc plates; the following day, peptides or thrombin were added to the cells. The cells were further fixed using 4% formaldehyde for 20 minutes and then washed twice with phosphate-buffered saline (PBS). Blocking steps included successive incubation in 3% H_2O_2 in methanol for 20 minutes, washing with PBS 3 times for 3 minutes each, and incubation with 10% normal goat serum in PBS for 30 minutes to block nonspecific binding. The cells were incubated (90 minutes, 25 °C) with the anti-heparanase polyclonal antibody (733; diluted 1:250), anti-TF polyclonal antibody (1:100), anti-TFPI-1 polyclonal antibody (1:100), or anti-TFPI-2 polyclonal antibody (1:100). Slides were extensively washed with PBS and incubated with a secondary reagent (Envision Kit) according to the manufacturer's instructions (Dako). Following additional washes, the color was developed using the AEC Reagent (Dako); sections were counterstained with hematoxylin and mounted, as previously described [7]. Paraffin-embedded 5-μm sections of the wound area were used for staining formalin-fixed tissue. Slides were deparaffinized with xylene, rehydrated, and endogenous peroxidase activity was quenched for 30 minutes with 3% hydrogen peroxide in methanol. Slides were then subjected to antigen recovery by boiling (20 minutes) in 10 mM citrate buffer (pH 6). They were further incubated with 10% normal goat serum in PBS for 60 minutes to block nonspecific binding, followed by incubation (20 hours, 4 ºC) with relevant antibodies. The rest of the procedure was performed as described above. Analyses of tissue immunostaining results were performed by 2 of the authors, unaware of the slide allocation. Discrepancies in the analyses were reconciled following the assessment by a third reviewer. Five high-power fields were evaluated in each stained slide. Staining intensity was scored as follows: 0, no staining; 1, weak intensity; 2, moderate intensity; and 3, marked intensity.

2.5. Proliferation assay

The XTT Cell Proliferation Kit (Biological Industries) was used according to the manufacturer's instructions. The proliferation kit is designed to assess the mitochondrial activity as an indicator of the cell division rate. Briefly, 1×10^5 cells were cultivated in a 96-well plate; 100 μL of growth media was added to each well and incubated for 24 hours. A blank containing complete medium without cells was used as negative control. The reaction solution included 0.1 mL of the activation solution combined with 5 mL of the XTT reagent. After the addition of 50 μL of the reaction solution to each well, the plate was incubated for 4 hours, and the absorbance was measured using an ELISA reader at a wavelength of 450 nm. Nonspecific readings were measured at a wavelength of 630 nm and subtracted from the 450 nm measurement.

2.6. The wound-healing model

The study was approved by the Technion Ethics Committee for Animal Research, and the procedures were carried out in accordance with institutional guidelines. All the experiments were performed in 7-8-week-old male mice in order to avoid hormonal effects. Institute of Cancer Research (ICR) mice (without specific genetic background) were anesthetized with isoflurane systemically and lidocaine locally throughout all the procedures. The hair on the dorsal side was removed, and a 10-mm full-thickness skin incision

was done. Following the surgery, either peptide 16 or 16AC was injected opposite the wound (not in the incision site) and was compared to the injection of the vehicle. On day 7, mice were sacrificed, and the wound skin was analyzed using immunostaining.

2.7. Statistical analysis

Data were evaluated using the SPSS software for Windows version 13.0 (SPSS Inc). Statistics were calculated using the nonparametric Mann–Whitney U-test. Values were reported as median and range. The significance level was set at $P < .05$.

3. Results

3.1. Procoagulant peptides stimulate a significant release of heparanase from tumor cells and upregulate the expression of heparanase, tissue factor, tissue factor pathway inhibitor, and tissue factor pathway inhibitor-2

Peptides 16 or 16AC were added to the tumor cells at various concentrations, and the effect on heparanase levels was studied. The procoagulant peptides were found to induce significant heparanase secretion from U87 heparanase overexpressing cells (Figure 1A). Western blot analysis revealed a strong band at 65 kDa, representing the heparanase protein released from the cells in response to the addition of the procoagulant peptides. In contrast, cell exposure to the anticoagulant peptide 7 failed to increase the release of heparanase from the cells compared to their basal level (Figure 1A). This result was confirmed using immunostaining analysis with U87 heparanase overexpressing cells (Figure 1B). Peptide 16 or 16AC (5 μg/mL) was added to U87 cells and compared to control cells. After overnight incubation at 37 ºC, the cells were stained using the immunostaining assay. A significant increase in heparanase, TF, TFPI, and TFPI-2 levels was observed in the treated cells (Figure 1C). In addition, nuclear staining was observed in heparanase.

Figure 1

Procoagulant peptides stimulate a significant release of heparanase from tumor cells and upregulate the expression of heparanase, tissue factor (TF), tissue factor pathway inhibitor (TFPI), and tissue factor pathway inhibitor-2 (TFPI-2). (A) Western blot analysis. U87 heparanase overexpressing cells were seeded in tissue culture dishes. Peptide 16 (5 μg/mL), peptide 16AC (5 μg/mL), and heparanase inhibitory peptide 7 (50 μg/mL) were added to the cells. Cells were incubated for 30 minutes at 37 ºC, following collection of the medium. A strong band at 65 kDa was identified in the peptides 16 and 16AC lanes (right panel). A mild reduction in heparanase levels was observed with peptide 7 compared with control (left panel). Levels of heparanase were evaluated using densitometry analysis (upper panel). The assay was performed in triplicate. The results are presented as the mean and range. $*P < .05$. (B) Immunostaining assay. Peptide 16 or 16AC (5 μg/ml) was added to U87 heparanase overexpressing cells at 37 ºC for 30 minutes, and cells were fixed using 4% formaldehyde as described in the Methods section. A significant reduction in the heparanase level was observed in the cells treated with the procoagulant peptides. (C) Peptide 16 or 16AC (5 μg/mL) was added to U87 cells and compared with control cells after overnight incubation at 37 ºC. Immunostaining revealed a significant increase in heparanase, TF, TFPI, and TFPI-2 levels in the treated cells compared with controls. Nuclear staining was observed in heparanase. Representative images were visualized at × 50 magnification, with a 0.82 MDC objective lens, captured with a Nikon E995 digital camera (Nikon), and processed with Adobe Photoshop software (Adobe Systems).

3.2. Procoagulant peptides induce tumor cell proliferation

According to our previously published data, the peptides derived from TFPI-2 that inhibit the heparanase-TF complex not only impede the heparanase procoagulant activity but are also associated with a significant reduction in tumor growth [10]. To support our hypothesis that the procoagulant domain is involved in cancer progression, the current study evaluated the effect of the procoagulant peptides on the proliferation of tumor cells. A series of experiments were performed on 2 types of human breast cancer cell lines, T47D and MCF-7, and the human glioma cell line U87, using the XTT assay. All 3 cell lines demonstrated a significant increase in cell proliferation ($P < .05$) after incubation for 48 hours with the procoagulant peptide (16 or 16AC) in a dose-dependent manner (Figure 2A–C). Notably, results of the XTT proliferation assay showed that incubation of the cells with peptide 7, inhibiting interaction of the heparanase-TF complex prior to the addition of the procoagulant peptides (16 or 16AC), significantly reduced their proliferative effect (Figure 2D–F).

Figure 2

Procoagulant peptides induce tumor cell proliferation. (A) T47D cells (5×10^3 cells/well) were seeded in a 96-well plate. Peptides 16 (P16) and 16AC (P16AC) (5 μg/mL, 10 μg/mL) were added to the cells. Following incubation at 37 ºC for 48 hours, the XTT assay was performed as described in the Methods section. A significant increase in cell proliferation was observed in the cells exposed to P16 or P16AC. Similar results were obtained when the study was performed in 2 additional tumor cell lines: U87 (B) and MCF-7 (C). Subsequently, T47D (D), U87 (E), and MCF-7 (F) cells were seeded in a 96-well plate (5×10^3 cells/well). Peptide 7 (P7) inhibiting the heparanase-TF complex (50 μg/mL) was added to the cells for 1 hour, followed by the addition of P16 or P16AC (5 μg/mL). After incubation at 37 °C for 48 hours, the XTT assay was performed. A significant reduction in cell proliferation was observed when P7 was added to P16 or P16AC ($P < .05$). All assays were performed in triplicate. The results are presented as the mean and range. $*P < .05$.

3.3. Heparanase procoagulant peptides increase the level of heparanase, tissue factor, tissue factor pathway inhibitor, and tissue factor pathway inhibitor-2 and induce cell proliferation in human umbilical vein endothelial cells

Peptide 16 or 16AC was added to HUVECs for overnight incubation. Staining for heparanase, TF, TFPI, and TFPI-2 was significantly stronger in HUVECs cocultured with the peptides compared to controls (Figure 3A). The XTT proliferation assay demonstrated a major increase in the proliferation of HUVECs upon the addition of either peptide 16 or 16AC (Figure 3B). Furthermore, incubation with peptide 7, inhibiting the heparanase-TF complex, significantly reversed the proliferative effect (Figure 3C).

Figure 3

Heparanase procoagulant peptides increase the level of heparanase, tissue factor (TF), tissue factor pathway inhibitor (TFPI), and tissue factor pathway inhibitor-2 (TFPI-2) and induce cell proliferation in human umbilical vein endothelial cells (HUVECs). (A) Immunostaining assay. Peptide 16AC (P16AC; 5 μg/mL) was added to HUVECs and compared with control cells. After overnight incubation at 37 ºC, the cells were fixed using 4% formaldehyde and immunostained. Levels of heparanase, TF, TFPI, and TFPI-2 were significantly higher in the treated cells. Representative images were visualized at × 50 magnification, with a 0.82 MDC objective lens, captured with a Nikon E995 digital camera (Nikon), and processed with Adobe Photoshop software (Adobe Systems). The contingency table (lower panel) shows the staining intensity in the HUVECs. Significance was determined by the Mann–Whitney U-test. (B) Heparanase procoagulant peptides induce HUVEC proliferation. HUVECs were seeded in a 96-well plate (5×10^3 cells/well). Heparanase procoagulant peptides 16 (P16) and P16AC (2.5 μg/mL, 5 μg/mL) were added at increasing concentrations to the cells. After 48 hours, a significant increase in cell proliferation was observed using the XTT assay. $*P < .05$. (C) Inhibition of heparanase-TF complex impedes proliferation in HUVECs. A similar experiment is described in B, but 50 ng/mL of heparanase inhibitory peptide 7 (P7) was added to the cells for 1 hour prior to the addition of P16 (5 μg/mL) or P16AC (5 μg/mL). P7 significantly inhibited the proliferative effect of P16 and P16AC.

3.4. Procoagulant peptides upregulate the expression and enhance secretion of heparanase, tissue factor, tissue factor pathway inhibitor-1, and tissue factor pathway inhibitor-2 in the wound-healing model

In order to verify the *in vitro* results in endothelial cells, we studied the effect of the procoagulant peptides in an animal model of wound healing that involves angiogenesis. In this experimental model, a full-thickness skin incision was made on the backs of ICR mice, as described in the Methods section. Following the incision, either peptide 16 or 16AC was injected subcutaneously into the mice opposite the wound site for 1 week on alternate days. At the end of the wound-healing experiment, the mice were sacrificed, and the wound skin was analyzed using immunostaining. The results showed that the procoagulant peptides not only significantly enhanced wound healing and vascularization, as we previously reported [9], but also significantly upregulated the expression and intraluminal secretion of heparanase, TF, TFPI, and TFPI-2, mainly in the small blood vessel wall and lumen of the tissue (Figure 4).

Figure 4

Procoagulant peptides upregulate the expression and enhance heparanase secretion, tissue factor (TF), tissue factor pathway inhibitor-1 (TFPI-1), and tissue factor pathway inhibitor-2 (TFPI-2) in the wound-healing model. Our previous study showed that the procoagulant peptides accelerated wound healing [9]. In the current model, a full-thickness skin incision of 10 mm was made in the back skin of ICR mice (with no specific genetic background). On days 1, 3, and 5, peptide 16, peptide 16AC, or the vehicle (phosphate-buffered saline) were injected subcutaneously opposite the wound at a dose of 225 μg/kg. On day 7, mice were sacrificed, and the wound skin was analyzed by immunostaining. A significant increase in the expression of heparanase, TF, TFPI-1, and TFPI-2 was observed, either directly induced by the peptides or as part of the wound-healing process. Increased staining was most prominent in the small blood vessel wall and lumen of the subcutis tissue. Representative images were visualized at × 50 magnification, with a 0.82 MDC objective lens, captured with a Nikon E995 digital camera (Nikon), and processed with Adobe Photoshop software (Adobe Systems).

3.5. Tissue factor conveys intracellular signaling induced by heparanase-derived peptides

HEK-293 cells were stably transfected to overexpress full-length TF or TF devoid of the cytoplasmic domain (ΔTF) and were compared to empty plasmid transfected cells (vector only, control). The cells were washed twice with PBS, and either peptide 16 or 16AC was added to the serum-free media for overnight incubation. P38 kinase signaling was implicated in cellular responses to virtually all types of stresses, from environmental and intracellular insults to pathologies such as infection and tumorigenesis [13]. We previously demonstrated that heparanase upregulated the expression of TF via p38 signaling [5]. Peptide 16AC, derived from heparanase, increased the p-p38 intracellular signaling in the cells overexpressing TF, as demonstrated in our previous study [5], compared with the cells overexpressing the TF devoid of the intracellular part (ΔTF), indicating that heparanase activates intracellular signaling via TF (Figure 5A). Peptides 16 and 16AC induced a more effective release of heparanase from the cells overexpressing TF than those overexpressing ΔTF, which points to the fact that the release is mediated by TF (Figure 5B).

Figure 5

Tissue factor (TF) conveys intracellular signaling induced by heparinase-derived peptides. HEK-293 cells stably transfected to overexpress full-length TF or TF devoid of the cytoplasmic domain (ΔTF) were transiently transfected to overexpress heparinase and compared with empty plasmid transfected cells (Vo, vector only; control). Twenty-four hours posttransfection, cells were washed twice with phosphate-buffered saline, and either peptide 16 or peptide 16AC (2.5 μg/mL) was added to the serum-free media for overnight incubation. (A) Peptide 16AC derived from the heparinase procoagulant domain increased p-p38 intracellular signaling in cells overexpressing TF compared with cells overexpressing TF devoid of the intracellular part (ΔTF), indicating that heparinase activates intracellular signaling via TF. (B) The heparinase release, induced by peptides 16 and 16AC, was more prominent in the cells overexpressing TF than in those overexpressing ΔTF, indicating that the release is mediated by TF. Levels of p-p38 or heparinase in the Western blot were evaluated using densitometry analysis (upper panel). Assays were performed in triplicate. The results are presented as the mean and range. $*P < .05$.

3.6. Thrombin induces heparanase release from tumor cells

The results previously obtained at our laboratory demonstrated that thrombin induced release of heparanase from platelets and granulocytes [14]. In addition, Shafat et al. [15] reported that ATP triggered release of heparanase from tumor cell lines. We hypothesized that thrombin would induce a similar effect in tumor cells and used ATP as a control. In addition, as peptides 16 and 16AC derived from heparinase induced release and upregulation of heparanase (Figure 1), we speculated if the effect of thrombin will partially involve the interaction of heparanase with TF. U87 cells overexpressing heparanase were incubated with thrombin, ATP, and peptides 16 and 16AC at 37 ºC for 30 minutes. Then, the medium was collected and analyzed with Western blot. A similar experiment was performed using U87 and MCF-7 cells overexpressing heparanase, and the results were evaluated by immunostaining. The findings demonstrated that the thrombin-induced release of heparanase in the medium was similar to the effect of ATP (Figure 6A, B), while the effect of peptides 16 and 16AC on heparanase release was less pronounced (Figure 6A).

Figure 6

Thrombin induces heparinase release from tumor cells. (A) Western blot analysis. U87 heparanase overexpressing cells were seeded in tissue culture dishes and incubated with 1 unit/mL of thrombin, 5.5 μg/mL (1 μM) of ATP, 5 μg/mL of peptide 16, and 5 μg/mL of peptide 16AC at 37 ºC for 30 minutes. Forty μl of sample medium were loaded on the gel. Compared with the control lane, a strong band at 65 kDa was observed in the thrombin, ATP, 16, and 16AC lanes. Levels of heparinase were evaluated using densitometry analysis (upper panel). The assay was performed in triplicate. The results are presented as the mean and range. $*P < .05$. (B) Immunostaining assay. One unit/mL of thrombin or 5.5 μg/mL (1 μM) of ATP was added to U87 heparanase overexpressing cells and compared with the control. The cells were incubated at 37 ºC for 30 minutes and then fixed using 4% formaldehyde, as described in the Methods section. A significant reduction in the cell heparanase content was observed in treated cells. Similar results were obtained using MCF-7 heparanase overexpressing cells. Representative images were visualized at × 50 magnification, with a 0.82 MDC objective lens, captured with a Nikon E995 digital camera (Nikon), and processed with Adobe Photoshop software (Adobe Systems).

3.7. PAR-1 and PAR-2 are not involved in thrombin-mediated release of heparinase

Our group demonstrated that PAR-1 was involved in heparanase release from neutrophils and platelets [14]. Based on these data, the current study intended to test whether PAR-1 was involved in heparanase release from tumor cells. PAR-1 was inhibited with 2 specific inhibitors (SCH and FR) and activated with TRAP-6. A specific inhibitor to PAR-2, also present on the tumor cell surface membrane, was investigated. Western blot analysis revealed a strong band at 65 kDa, implying that inhibition of PAR-1 or PAR-2 did not affect the heparanase release from U87 cells (Figure 7A). The experiments were repeated in T47D cells to verify the results (Figure 7B). PAR-1 and PAR-2 signaling has not been shown to be involved in heparanase release from these tumor cells, which is not in line with previously published data on their contribution to heparanase release from neutrophils and platelets [14].

Figure 7

PAR-1 and PAR-2 are not involved in the thrombin-mediated release of heparanase. (A) Western blot analysis. U87 heparanase overexpressing cells (4×10^5/plate) were seeded in dishes. When cells reached 80% confluence, PAR-1 antagonist (SCH 530348, vorapaxar; 0.5 μM and 1 μM) was added for 10 minutes, followed by the addition of thrombin (1 unit/mL) for 20 minutes. TRAP-6, a direct PAR-1 activator (1 μM), was also tested. The medium was collected, and 40 μL of samples were loaded on the gel. A strong band at 65 kDa was observed in all the lanes compared with the baseline level observed in the control. (B) Similar to the experiment described in A, T47D heparanase overexpressing cells were incubated with PAR-1 antagonist (FR) (FR 171113, 0.05 μM) and/or PAR-2 antagonist (FS) (FSLLRY-NH2, 0.05μM) for 10 minutes, followed by the addition of thrombin (1 unit/mL) for 20 minutes. TRAP-6, a direct PAR-1 activator (2 μM), was also tested. The medium was collected, and 40 μL of samples were loaded on the gel. A strong band at 65 kDa was observed in all the lanes compared with the control, indicating that neither PAR-1 nor PAR-2 is involved in heparanase release in this cell line. Levels of heparanase in the Western blot were evaluated using densitometry analysis (upper panel). The assay was performed in triplicate. The results are presented as the mean and range. $*P < .05$.

3.8. Thrombin-associated increase in tumor cell proliferation is mediated by heparinase

T47D, U87, and MCF-7 cells were incubated with thrombin (0.5 unit/mL, 1 unit/mL) at 37 °C for 48 hours. The XTT test was performed after incubation. A significant increase in cell proliferation was observed in the cells exposed to thrombin compared with control ($P < .05$, Figure 8A–C), as previously shown [16]. Subsequently, in a similar experiment, peptide 7, inhibiting the heparanase-TF complex, was added to the cells for 1 hour, which was followed by the addition of thrombin (1 unit/mL). After incubation at 37 °C for 48 hours, the XTT assay was performed. A significant reduction in cell proliferation was observed following peptide 7 addition to thrombin ($P < .05$, Figure 8D–F), implying that the proliferative effect of thrombin was mediated by the release of heparanase.

Figure 8

Thrombin-related increase in tumor cell proliferation is mediated by heparanase. T47D (A), U87 (B), and MCF-7 (C) cells were seeded in a 96-well plate (5×10^3 cells/well) with or without thrombin (T) (0.5 unit/mL, 1 unit/mL). After incubation at 37 °C for 48 hours, the XTT test was performed. A significant increase in cell proliferation was observed in the cells exposed to T compared to control ($*P < .05$). All assays were done in triplicates. The result represents mean and range. Subsequently, T47D (D), U87 (E), and MCF-7 (F) cells were seeded in a 96-well plate (5×10^3 cells/well). Fifty μg/mL of the peptide inhibiting heparanase-TF complex (P7) were added to the cells for 1 hour, followed by the addition of T (1 unit/mL). After incubation at 37 °C for 48 hours, the XTT assay was performed. A significant reduction in cell proliferation was observed when P7 was added to T ($*P < .05$). All assays were performed in triplicate. The results are presented as the mean and range.

4. Discussion

Preliminary results reported by our group suggest the existence of 2 novel peptides originating from the third heparin-binding domain of heparanase. These peptides have a procoagulant effect and may be defined as a putative procoagulant domain of heparanase [9].

The present study investigated the involvement of the heparanase procoagulant domain in nonhemostatic effects. The findings demonstrate that the procoagulant domain of heparanase induces the release and upregulation of this protein and that of TF, TFPI, and TFPI-2. Moreover, this domain is found to be capable of inducing tumor and endothelial cell proliferation. These data have been further strengthened by the evidence obtained using the wound-healing mouse model. Inhibition of the interaction between the procoagulant domain of heparanase and TF has resulted in the reversal of the effects. Furthermore, the use of a construct of TF, devoid of the intracellular part, showed that the procoagulant domain of heparanase conveys intracellular signaling via TF. Essentially, these data imply that heparanase is a ligand, and TF serves as its receptor. These data are summarized in the schematic abstract.

In our study, peptides 16 and 16AC induced nuclear staining of heparanase in U87 cells (Figure 1C). This finding was not detected in HUVECs (Figure 3A). Similar results using western blot and staining were previously demonstrated in U87 cells. Schubert et al. [17] showed that exogenous addition or overexpression of heparanase in U87 cells induced translocation of heparanase into the cell nucleus, where it may degrade the nuclear heparan sulfate and thereby affect nuclear functions that are thought to be regulated by heparan sulfate. This finding emphasizes that heparanase interaction with TF is responsible for important nonhemostatic activities of heparanase.

According to our previous publication on the procoagulant peptide 16, we found that the increase in coagulation system activation was induced at concentrations of 2.5-10 μg/mL [9]. Accordingly, in the current study we used similar concentrations to induce heparanase nonhemostatic effects. In the wound-healing mouse model, 225 μ/kg of peptide 16 or 16AC was injected opposite the wound (not in the incision site). The mice weighed about 35 grams, with a blood volume of approximately 2 mL. Thus, 8 μg of peptide 16 or 16AC were subcutis injected into a blood volume of 2 mL (4 μg/mL). In the current study, we investigated several human cell lines (breast cancer, glioma, and embryonic kidney) and primary human endothelial cells in order to show that the effect is not cell-line or tissue origin-specific.

Heparanase, TF, TFPI, and TFPI-2 are coagulation proteins locally synthesized in the tissue microcirculation. In contrast, other coagulation proteins are synthesized in the liver, and their levels are expected to be similar in most tissues. We have previously shown that heparanase and the levels of its related proteins are not the same in different normal tissues. Our earlier studies have demonstrated that in normal ICR mice (without any genetic manipulation), levels of these proteins are low in the microcirculation of the liver, lung, brain cortex, and bone and high in the microcirculation of the subcutis, skeletal muscle, brain subcortex, and bone marrow [18]. The current study showed a positive feedback regulation of heparanase that occurs via TF, enabling local regulation of the tetrad: heparanase, TF, and their inhibitors. What determines the normal level of heparanase in the microcirculation of various tissues is an intriguing question that should be further investigated. Although staining of TF and TFPI in Figure 3A was less prominent compared with heparanase and TFPI-2, staining of these 4 parameters was similar in U87 cells (Figure 1C) and mice wounds (Figure 4). Comparable results were obtained in our previous works [18,19]. The mechanism in which TFPI and TFPI-2 exert an inhibitory effect on the heparinase-TF complex in the presence of similar levels should be further investigated.

Coagulation, cancer, inflammation, and angiogenesis are interconnected processes. Our data demonstrate that the interaction between heparanase and TF not only enhances the activation of the coagulation system but also has intracellular effects on tumor cells and endothelial cells, pointing to a dominant mechanism that may underlie the interconnections between these processes. In light of these findings, inhibition of the heparanase-TF complex is expected to attenuate not only coagulopathy but also tumor growth, blood vessel formation, and inflammation, as shown in our earlier studies [8,10].

Previous results obtained at our laboratory have demonstrated that thrombin induces release of heparanase from platelets and granulocytes via PAR-1 [14]. The present study has revealed a similar effect of thrombin on tumor cells, although neither PAR-1 nor PAR-2 has been found to be involved. Possibly, the thrombin-mediated release of heparanase in tumor cells could be attributed to thrombin interaction with other receptors. Further investigation of this issue is crucial to enable the development of specific inhibitors. In a similar way, ATP that was shown to release heparanase from tumor cells in the present study and by another group [15] had no effect on heparanase release from platelets and granulocytes [14]. Notably, this study has shown that peptide 7, inhibiting the heparanase-TF complex, has also impeded the proliferation effect of thrombin on tumor cells. This result may imply a new mechanism of thrombin action, ie, thrombin induces immediate release of heparanase from tumor cells that in a vicious cycle triggers tumor cell proliferation. Other interrelations between thrombin receptors and TF have been previously demonstrated. The FVIIa-TF complex has been shown to mediate PAR-2 activation on tumor cells, thereby enhancing tumor growth. Furthermore, tumor cells have been reported to use additional signaling networks of the coagulation cascade by activating PAR-1 through the ternary FVIIa-TF-FXa complex in the vascular and potentially lymphatic system [20]. TF forms multiple complexes and interacts with coagulation factors and other entities, and it is possible that heparanase affects these interactions indirectly. Parallel pathways of tumor growth induced by TF may support the strategy of using several modalities to block TF intracellular signaling in cancer therapy.

While receptors that capture secreted heparanase precursor and traffic it to the intracellular site of processing/activation, such as low-density lipoprotein receptor, mannose 6-phosphate receptors, and heparan sulfate, have been described [21,22], a specific heparanase receptor that induces intracellular signaling as presented in the current study has not been previously defined. Heparanase was shown to enhance inflammation, angiogenesis, and metastasis. We assume that part of these effects is exerted by the interaction with TF, although interactions with other receptors and mechanisms may be involved. Further research is needed to evaluate the actual effect of the heparanase-TF complex inhibition as a therapeutic modality to attenuate angiogenesis, cancer progression, and inflammation.

Funding

The authors received no funding for this study.

Author contributions

S.G. performed most of the research and analyzed data. A.K.P., V.K., and Y.C. performed research. Y.N. designed and performed research, contributed vital new reagents or analytical tools, analyzed data, and wrote the paper.

Relationship disclosure

The authors have no conflicts of interest to declare.

Footnotes

Handling Editor: Prof Yotis Senis.

References

1. Freeman C., Parish C.R. Human platelet heparanase: purification, characterization and catalytic activity. *Biochem J.* 1998;330:1341–1350. [PMC free article] [PubMed] [Google Scholar]

2. Pikas D.S., Li J.P., Vlodavsky I., Lindahl U. Substrate specificity of heparanases from human hepatoma and platelets. *J Biol Chem.* 1998;273:18770–18777. [PubMed] [Google Scholar]

3. Parish C.R., Freeman C., Hulett M.D. Heparanase: a key enzyme involved in cell invasion. *Biochim Biophys Acta.* 2001;1471:M99–M108. [PubMed] [Google Scholar]

4. Vlodavsky I., Friedmann Y. Molecular properties and involvement of heparanase in cancer metastasis and angiogenesis. *J Clin Invest.* 2001;108:341–347. [PMC free article] [PubMed] [Google Scholar]

5. Nadir Y., Brenner B., Zetser A., Ilan N., Shafat I., Zcharia E., Goldshmidt O., Vlodavsky I. Heparanase induces tissue factor expression in vascular endothelial and cancer cells. *J Thromb Haemost.* 2006;4:2443–2451. [PubMed] [Google Scholar]

6. Nadir Y., Brenner B., Gingis-Velitski S., Levy-Adam F., Ilan N., Zcharia E., Nadir E., Vlodavsky I. Heparanase induces tissue factor pathway inhibitor expression and extracellular accumulation in endothelial and tumor cells. *Thromb Haemost.* 2008;99:133–141. [PubMed] [Google Scholar]

7. Nadir Y., Brenner B., Fux L., Shafat I., Attias J., Vlodavsky I. Heparanase enhances the generation of activated factor × in the presence of tissue factor and activated factor VII. *Haematologica.* 2010;95:1927–1934. [PMC free article] [PubMed] [Google Scholar]

8. Axelman E., Henig I., Crispel Y., Attias J., Li J.P., Brenner B., Vlodavsky I., Nadir Y. Novel peptides that inhibit heparanase activation of the coagulation system. *Thromb Haemost.* 2014;112:466–477. [PubMed] [Google Scholar]

9. Crispel Y., Ghanem S., Attias J., Kogan I., Brenner B., Nadir Y. Involvement of the heparanase procoagulant domain in bleeding and wound healing. *J Thromb Haemost.* 2017;15:1463–1472. [PubMed] [Google Scholar]

10. Crispel Y., Axelman E., Tatour M., Kogan I., Nevo N., Brenner B., Nadir Y. Peptides inhibiting heparanase procoagulant activity significantly reduce tumour growth and vascularisation in a mouse model. *Thromb Haemost.* 2016;116:669–678. [PubMed] [Google Scholar]

11. Zetser A., Levy-Adam F., Kaplan V., Gingis-Velitski S., Bashenko Y., Schubert S., Flugelman M.Y., Vlodavsky I., Ilan N. Processing and activation of latent heparanase occurs in lysosomes. *J Cell Sci.* 2004;117:2249–2258. [PubMed] [Google Scholar]

12. Sorensen B.B., Freskgard P.O., Nielsen L.S., Rao L.V., Ezban M., Petersen L.C. Factor VIIa-induced p44/42 mitogen-activated protein kinase activation requires the proteolytic activity of factor VIIa and is independent of the tissue factor cytoplasmic domain. *J Biol Chem.* 1999;274:21349–21354. [PubMed] [Google Scholar]

13. Canovas B., Nebreda A.R. Diversity and versatility of p38 kinase signalling in health and disease. *Nat Rev Mol Cell Biol.* 2021;22:346–366. [PMC free article] [PubMed] [Google Scholar]

14. Tatour M., Shapira M., Axelman E., Ghanem S., Keren-Politansky A., Bonstein L., Brenner B., Nadir Y. Thrombin is a selective inducer of heparanase release from platelets and granulocytes via protease-activated receptor-1. *Thromb Haemost.* 2017;117:1391–1401. [PubMed] [Google Scholar]

15. Shafat I., Vlodavsky I., Ilan N. Characterization of mechanisms involved in secretion of active heparanase. *J Biol Chem.* 2006;281:23804–23811. [PubMed] [Google Scholar]

16. Darmoul D., Gratio V., Devaud H., Lehy T., Laburthe M. Aberrant expression and activation of the thrombin receptor protease-activated receptor-1 induces cell proliferation and motility in human colon cancer cells. *Am J Pathol.* 2003;162:1503–1513. [PMC free article] [PubMed] [Google Scholar]

17. Schubert S.Y., Ilan N., Shushy M., Ben-Izhak O., Vlodavsky I., Goldshmidt O. Human heparanase nuclear localization and enzymatic activity. *Lab Invest.* 2004;84:535–544. [PubMed] [Google Scholar]

18. Nevo N., Ghanem S., Crispel Y., Tatour M., Cohen H., Kogan I., Ben-Arush M., Nadir Y. Heparanase level in the microcirculation as a possible modulator of the metastatic process. *Am J Pathol.* 2019;189:1654–1663. [PubMed] [Google Scholar]

19. Hardak E., Peled E., Crispel Y., Ghanem S., Attias J., Asayag K., Kogan I., Nadir Y. Heparan sulfate chains contribute to the anticoagulant milieu in malignant pleural effusion. *Thorax.* 2020;75:143–152. [PubMed] [Google Scholar]

20. Schaffner F., Ruf W. Tissue factor and PAR2 signaling in the tumor microenvironment. *Arterioscler Thromb Vasc Biol.* 2009;29:1999–2004. [PMC free article] [PubMed] [Google Scholar]

21. Vreys V., Delande N., Zhang Z., Coomans C., Roebroek A., Durr J., David G. Cellular uptake of mammalian heparanase precursor involves low density lipoprotein receptor-related proteins, mannose 6-phosphate receptors, and heparan sulfate proteoglycans. *J Biol Chem.* 2005;280:33141–33148. [PubMed] [Google Scholar]

22. Nadav L., Eldor A., Yacoby-Zeevi O., Zamir E., Pecker I., Ilan N., Geiger B., Vlodavsky I., Katz B.Z. Activation, processing and trafficking of extracellular heparanase by primary human fibroblasts. *J Cell Sci.* 2002;115:2179–2187. [PubMed] [Google Scholar]