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A major reservoir for heparin-releasable TFPIα is extracellular matrix

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Abstract

Objective: Human endothelial cells produce two alternatively spliced tissue factor pathway inhibitor (TFPI) isoforms that maintain anticoagulant properties of the vasculature. TFPIβ is glycosylphosphatidyl inositol anchored on the cell surface. TFPIα has a basic C-terminus sharing homology with vascular endothelial growth factor (VEGF) and is a heparin-releasable protein, suggesting it binds glycosaminoglycans (GAGs) on the endothelium surface. However, this is unclear because TFPIα is not on the surface of cultured endothelial cells. This study identifies the source of heparin-releasable TFPIα.

Approach and Results: ELISA assays localized heparin-releasable TFPIα to the extracellular matrix (ECM) of Ea.hy926 cells and human umbilical vein endothelial cells. Immunofluorescence microscopy for TFPIα showed punctate intracytoplasmic staining and ECM staining beneath individual cells. Flow cytometry identified TFPIβ but not TFPIα on the cell surface. TFPIα localization to ECM was confirmed with ELISA and immunohistochemistry studies of umbilical cord veins. The TFPIα C-terminus interacted with Ea.hy926 ECM GAGs, and a homologous VEGF peptide competed for this binding, suggesting these interactions modulate VEGF responses. Immobilized TFPIα C-terminal peptide bound to several ECM proteoglycans in Ea.hy926 conditioned media. Immunofluorescence studies of human kidney colocalized TFPIα with four of these proteoglycans surrounding the microvasculature: glypican-1, syndecan-4, thrombospondin, and laminin-5. The absence of TFPIα on the surface of endothelial cells and its co-localization with specific ECM proteins suggests TFPIα binds to unique proteoglycan structures.

Conclusions: ECM contained the primary vascular pool of heparin-releasable TFPIα. By localizing to ECM, TFPIα is positioned to inhibit the procoagulant activity of tissue factor surrounding the vasculature.

Graphical Abstract

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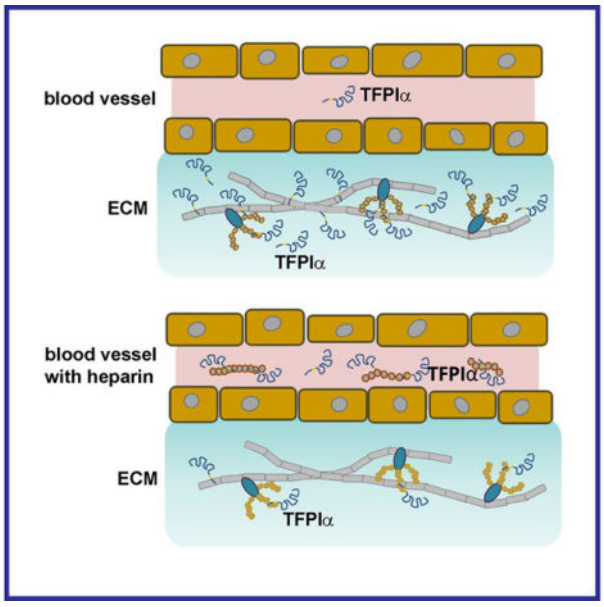

Keywords

TFPI; vascular endothelial cells; vascular endothelial growth factor; heparin-releasable; glycosaminoglycans on vascular endothelium

Subject terms:

Vascular Biology; Growth Factors/Cytokines; Endothelium/Vascular Type

Introduction

Tissue Factor Pathway Inhibitor (TFPI) is an anticoagulant glycoprotein that influences the outcome of bleeding[1] and thrombotic[2] disorders. Administration of a polyclonal antibody that blocks TFPI function to rabbits increases susceptibility to Tissue Factor (TF) induced disseminated intravascular coagulation.[3] Administration of a monoclonal antibody that blocks TFPI function to healthy subjects and patients with hemophilia increases plasma d-dimer and prothrombin fragment 1+2 concentrations.[4] Thus, TFPI dampens procoagulant potential within healthy vasculature.

TFPI mediates its anticoagulant activity by impeding early proteolytic events of the blood coagulation cascade, including those mediated by the TF-Factor VIIa (TF-FVIIa) catalytic complex,[5, 6] Factor Xa (FXa),[6] and early forms of the prothrombinase complex (FVa-FXa).[7] Human TFPI is an alternatively spliced protein expressed in two isoforms. TFPIα and TFPIβ have distinct structural features[8, 9] that produce differential anticoagulant activities and distinct expression patterns within endothelial and circulating blood cells.[10–13]

TFPIα has three Kunitz domains and a basic C-terminus.[14] The first and second Kunitz domains inhibit TF-FVIIa and FXa, respectively,[15] while the third Kunitz domain binds to Protein S.[16, 17] The basic C-terminus binds to heparin,[18–20] has homology with a region of

the FV B-domain,[7] and has a pattern of basic amino acids present in some isoforms of Vascular Endothelial Growth Factor (VEGF).[21] Human TFPIα is produced by endothelial cells and megakaryocytes. It is constitutively secreted by cultured human endothelial cells[22] and released from activated platelets.[12, 23] TFPIβ has two Kunitz domains and is GPI-anchored on the plasma membrane of endothelial cells and monocytes[8, 9, 13] but is not present on platelets.[12] Since it lacks the third Kunitz domain and basic C-terminal region present in TFPIα, TFPIβ does not bind to Protein S or heparin and does not inhibit prothrombinase.[24]

Plasma TFPI in humans is a complex mixture of full-length TFPIα (~25%) and variably C-terminally truncated forms of TFPI bound to lipoproteins (~75%).[25] In addition, TFPIα is a heparin-releasable protein. Plasma TFPIα levels promptly increase approximately 2- to 4-fold following infusion of unfractionated or low molecular weight heparin into adults[26, 27] or neonates.[28] Heparin-releasable TFPIα is also present in cultured human endothelial cells.[29, 30]

Heparin-releasable TFPIα and endothelial bound TFPIβ maintain anticoagulant properties of the vasculature.[4] Circumstantial evidence supports the notion that heparin-releasable TFPIα is bound to glycosaminoglycans (GAGs) on the surface of endothelial cells, placing TFPIα and TFPIβ at the same location on the vessel wall.[20, 29, 30] However, heparin-releasable TFPIα is not detectable on the surface of cultured endothelial cells with flow cytometry [31] implying heparin-releasable TFPIα is secreted from an internal store.[32, 33] The ECM is composed of two major classes of biomolecules including GAGs, which are most often covalently linked to protein forming proteoglycans, and fibrous proteins, which include collagen, elastin, fibronectin, and laminin. These components are secreted locally and assembled into the organized meshwork that forms the ECM. Here, we have used a variety of tissue culture, immunofluorescence, umbilical cord, and immunohistochemistry experiments to uncover extracellular matrix (ECM) as the primary source of heparin-releasable TFPIα.

Materials and Methods

The authors declare that all supporting data are available within the article and its online supplementary files.

Endothelial cell culture and heparin treatment

Ea.hy926 cells were maintained with 10% fetal bovine serum (FBS) (BioWest, Riverside, MO) in Dublbecco's Modified Eagle Medium (DMEM) containing penicillin and streptomycin (Thermo Fisher Scientific, Waltham, MA). Human vein endothelial cells (HuVECs) were isolated from a fresh human umbilical cord with collagenase treatment and grown in EGM-2 Single Quots media (Lonza, Mapleton, IL) without addition of heparin. Adherent cells were washed with phosphate buffered saline (PBS), and incubated with 2% FBS in DMEM containing porcine heparin (1 U/ml) (Sigma Aldrich, St. Louis, MO) for 15 minutes at 37°C unless otherwise indicated. In some experiments the following agents also were incubated with adherent cells: Mouse monoclonal antibodies directed against the first, second, or third Kunitz domains of human TFPIα (10 nM), 40 ng/ml anti-TFPI aptamer, (all

kindly provided by Novo Nordisk, Copenhagen Denmark), 150 μM various peptides (generated at Versiti, Blood Research Institute), 0.01 mg/ml polyphosphate (Sigma Aldrich), or 0.1 mg/ml protamine sulfate (Sigma Aldrich). After incubation, media was removed from the cells and centrifuged at 1200 RPM to remove cell particles before TFPI measurements. All experiments were performed in triplicate unless otherwise noted.

ECM heparin treatment

ECM was exposed by manually dislodging cells in the absence of mild detergents to preserve the interaction of TFPIα with ECM proteoglycans as previously described.[34] Cultured endothelial cells were washed with PBS, incubated in PBS at room temperature for 15 minutes, and dissociated cells were further dislodged by gentle agitation with a Pasteur pipette leaving the remaining ECM attached to the plate surface. The surface adherent ECM was washed with PBS three times and then treated with heparin or other agents as described above for adherent cells.

Endothelial cell and ECM PIPLC treatment

Ea.hy926 cells or Ea.hy926 ECM were treated with 1U/ml phosphatidylinositol specific phospholipase C (PIPLC) (Thermofisher Scientific) in PBS containing 1% BSA at 37°C for one hour.

Human Subject Approval

Studies of human umbilical cords and kidney tissue were approved by the MCW Internal Review Board.

Umbilical cord heparin treatment

Human umbilical cords were obtained within 4 hours of birth from the MCW Tissue Bank. The umbilical cords were de-identified and sex information was not available. Umbilical cord veins were perfused profusely with PBS, cut in equal halves, and clamped on one end. DMEM containing 2% FBS with or without 1 U/ml heparin was perfused into the vein. After incubation at room temperature for 15 minutes the clamp was removed and flow through from the vein was collected for centrifugation and TFPI measurement. Treated umbilical cords were fixed for microscopic evaluation.

Measurement of TFPI

Total TFPI and TFPIα were measured by ELISA.[35] A 96 well plate was incubated overnight at 4°C with 2 μg/ml capture monoclonal anti-TFPI antibody against the second Kunitz domain in PBS, washed with PBS containing 0.05% Tween-20, and blocked with BLOK caesin buffer (Thermofisher Scientific) diluted 1:1 into 0.1% BSA in PBS. Samples were then added and incubated one hour at room temperature. After washing as above, a biotinylated monoclonal anti-TFPI antibody against the first Kunitz domain (total TFPI) or biotinylated monoclonal anti-TFPI antibody against the third Kunitz domain (TFPIα) was added at 1μg/ml and incubated at room temperature two hours. Washed wells were treated with HRP-streptavidin (Thermo Fisher Scientific) and Quanta Red Enhanced Chemifluorescent HRP (Thermo Fisher Scientific). The rate of substrate cleavage in each

well was determined by the slope and TFPI concentration calculated by comparison to a standard curve generated using purified TFPIα. All samples were tested in duplicate.

Flow cytometry

Ea.hy926 cells were harvested with PBS containing 1 mM EDTA and incubated with primary antibody for 1 hour followed by secondary antibody for 30 minutes, with washes performed in between. Isotype control antibody MOPC-21 was purchased from BD Biosciences (San Jose, CA). Secondary antibody AF488 Goat anti-Mouse was purchased from Jackson ImmunoResearch (Westgrove, PN). Data was analyzed using Flojo Software (Becton Dickinson, Franklin Lakes, NJ).

Pulldown experiments with immobilized GAGs or peptides

Heparan sulfate (HS, Sigma Aldrich) was biotinylated using Sulfo-NHS-SS-Biotin (Thermo Fisher Scientific) per manufacturer's protocol. Biotinylated HS or N-terminal-biotinylated TFPI C-terminal peptide KKGFIQRISKGGLIKTKRKRKKQRVKIAYEEIFVKN were incubated with streptavidin-coated magnetic beads (Thermo Fisher Scientific) overnight at 4°C. The beads were washed, blocked with 2% BSA in PBS, and added to Ea.hy926 conditioned media containing endogenously produced TFPIα, or to fresh media as a negative control, and incubated 2 hours at 4°C. Beads were then collected and washed with PBS containing 0.05% Tween-20. Beads were then boiled in 0.1% SDS-PAGE sample buffer to remove bound proteins. In competition studies, soluble GAGs in 10-fold or 100-fold molar excess of biotinylated HS were added to the conditioned media prior to addition of HS-coated beads.

SDS-PAGE and western blot analysis

SDS-PAGE was performed with 4–16% polyacrylamide gradient gels. Western blot analysis was performed as previously described.[36] Western blotting antibodies included a rabbit polyclonal anti-TFPI against the first and second Kunitz domains and a rabbit polyclonal anti-TFPIα against the C-terminal 11 amino acids of TFPIα. Images were developed on an Amersham Imager 680 (Cytiva, Marlborough, MA).

Protein identification by mass spectral analysis

Bands from a Coomassie-stained polyacrylamide gel were excised, washed, treated with tris(2-carboxyethyl)phosphine and iodoacetaminde to reduce and alkylate cysteines, respectively. Each band was digested overnight with trypsin and extracted peptides were injected onto a C18 column and analyzed on a Thermo Orbitrap Fusion Lumos (Thermo Scientific). Supplemental methods contain further details.

Histology

Healthy human kidney tissue was obtained from the MCW Tissue Bank, fixed with 10% formaldehyde buffered at pH 7.4, and processed to obtain four-micron sections. The tissue samples were de-identified, and sex and age information were not available. Tissues were stained for TFPI using a goat polyclonal anti-TFPI generated against full-length TFPIα or a rabbit polyclonal antibody specific for the C-terminus of TFPIα. Other antibodies included

sheep anti-thrombomodulin (R&D Systems, Minneapolis, MN), mouse anti-vWF (Versiti Blood Research Institute, Milwaukee, WI), goat anti-perlecan and mouse anti-collagen IV (LS Bio, Seattle, WA), mouse anti-agrin (Sigma Aldrich), mouse anti-glypican-1, mouse anti syndecan-4, mouse anti-thrombospondin and mouse anti-laminin-5 (Santa Cruz Biotechnology, Dallas, TX). Isotype-matched mouse, rabbit and goat IgG controls were from BD Biosciences, San Jose, CA. Secondary antibodies were from Thermo Fisher Scientific or Jacksons ImmunoResearch Laboratories. The Motic Easy scan imaging system (Motic 8 Inc., LTD, Hong Kong) was used to scan immunohistochemistry slides. Immunofluorescent images were obtained with a Nikon Eclipse Ti2 inverted microscope with a DS-Ri2 high speed color camera using either a 60X or 100x/1.45 numerical aperture oil objective (Nikon Instruments Inc., Melville, NY). Images were analyzed with the Nikon NIS-Elements software platform and processed with Imaris multi-channel microscopy software (Bitplane Inc., Concord, MA). Image formatting was performed in Photoshop CS-6 and Illustrator CS-6 (Adobe, San Jose, CA).

Statistics

Statistical analyses were performed using Prism GraphPad software version 7.05. P-values were calculated using a parametric (normality and variance were not tested), two-tailed unpaired Student's t-test or one-way ANOVA followed by post hoc analysis using Dunnett's multiple comparison test.

Results

Heparin-releasable TFPIα was not on the surface of Ea.hy926 cells.

Adherent and suspended Ea.hy926 cells were treated with heparin. TFPIα was present in the heparin releasate from adherent cells but not detectable in suspended cells when measured by ELISA (Figure 1A). The absence of heparin-releasable TFPIα in the suspended cells was not caused by proteolytic activity because the suspended Ea.hy926 cells were lifted from the tissue culture plate with a non-proteolytic procedure. Ea.hy926 surface expression of TFPI detected with monoclonal antibodies directed against the first or second TFPI Kunitz domains was identical before and after heparin treatment, as judged by flow cytometry. By contrast, the TFPIα-specific monoclonal antibody directed against the third Kunitz domain did not detect TFPI by flow cytometry (Figure 1B), indicating the presence of TFPIβ but not TFPIα on the cell surface. These findings suggested that heparin-releasable TFPIα localized within the ECM laid down by the cultured Ea.hy926 cells.

Heparin-releasable TFPIα localized within Ea.hy926 cell ECM.

Heparin treatment of Ea.hy926 ECM following non-proteolytic removal of cells from the plate resulted in release of TFPIα in amounts similar to that released from plates containing adherent cells (Figure 1A). Microscopic examination of plates confirmed that all cells were lifted from the ECM in these experiments (Supplemental Figure I). We tested the possibility of heparin releasing TFPIα non-specifically bound to tissue culture plastic instead of ECM by incubating conditioned Ea.hy926 medium, which contains TFPIα, in empty plates followed by measurement of heparin-releasable TFPIα.The empty plate had one-third the amount of heparin-releasable TFPIα as ECM. These data indicated that a fraction of TFPIα

in conditioned media binds to tissue culture plastic (Figure 1A), but the majority of heparin-releasable TFPIα was within the ECM. Sequential heparin treatments of adherent Ea.hy926 cells produced a large release of TFPIα with the first treatment followed by diminished release of TFPIα with subsequent heparin treatments (Figure 1C). Following three heparin treatments, approximately 60% of the TFPIα was removed from ECM, while 40% was not released by heparin (Figure 1C). A variety of detergents were used to solubilize ECM for SDS-PAGE and western blot analysis. TFPIα was present in ECM solubilized with SDS, which yielded the largest amount of TFPIα, followed by NP-40 and sodium deoxycholate, while Chaps and Triton X-100 did not solubilize TFPIα from ECM (Figure 1D).

TFPIβ was present on the surface of Ea.hy926 cells but was not in ECM.

TFPIβ is a GPI-anchored protein that is removed from the surface of cultured endothelial cells by PIPLC.[9, 37] Adherent cells, suspended cells, and ECM were treated with PIPLC and released TFPI was measured using the total TFPI ELISA, because there is not an ELISA that specifically measures TFPIβ. PIPLC released TFPI from adherent and suspended cells but did not release it from ECM (Figure 1E). Only trace amounts of TFPIα were detected in the PIPLC releasates confirming that TFPIβ was the TFPI isoform released from the surface of the Ea.hy926 cells (Figure 1F).

TFPIa was present within the cytoplasm of Ea.hy926 cells and within ECM.

Immunofluorescence studies of Ea.hy926 cells were performed to identify the location of TFPIα. Ea.hy926 cells grown on collagen were fixed with 3% paraformaldehyde, permeabilized with methanol and stained with a polyclonal antibody that specifically recognizes the C-terminus of TFPIα (Supplemental Figure II). Punctate cytoplasmic staining of TFPIα was observed, which was consistent with previous reports by Lupu and colleagues.[38] In addition, a large amount of TFPIα was present in the ECM directly underneath each individual cell (Figure 2). The presence of TFPIα in ECM beneath individual cells strongly suggests basolateral secretion of TFPIα, while its presence in conditioned media indicates apical secretion as well.

Electrostatic forces at least partially mediated the interaction between TFPIα and ECM.

ECM is composed of an interlocking mesh of proteoglycans containing mostly heparan sulfate (HS) but also chondroitin sulfate (CS) and hyaluronic acid (HA) and fibrous proteins including collagens, elastins, fibronectin and laminins.[39] Heparin is a GAG consisting of a highly sulfated linear polysaccharide chain with repeating disaccharide units of uronic acid and glucosamine and is not present in ECM.[40] We investigated the nature of the interaction of TFPIα with ECM by examining the ability of several macromolecules to release of TFPIα from adherent Ea.hy926 cells (Figure 3A). Polyphosphate consists of multiple inorganic phosphate residues linked together by phosphoanhydride bonds[41] and is highly negatively charged. Like heparin, polyphosphate induced release of TFPIα. Protamine sulfate is a positively charged arginine rich nuclear protein.[42] It also induced TFPIα release. Since both positively and negatively charged polymers released TFPIα, the interaction of TFPIα with ECM is at least partially due to electrostatic forces. The opposing effects of negatively charged polyphosphate and positively charged protamine sulfate suggest that

release of the positively charged TFPIα C-terminus from ECM was mediated by protamine sulfate interacting with ECM GAGs and polyphosphate interacting with TFPIα.

TFPIα bound to GAGs with differential affinity.

The affinity of TFPIα for different GAGs was examined using HS immobilized on magnetic beads (Figure 3B, Supplemental Figure IIIA). HS-loaded beads removed TFPIα from the conditioned media, and soluble heparin added at 1U/ml totally prevented this binding interaction (Figure 3B). Soluble HS or HA added in ten-fold or 100-fold molar excess also prevented TFPIα from binding to immobilized HS, but to a lesser extent than heparin, while CS did not compete for binding (Figure 3B). These data suggest that within the ECM TFPIα has similar affinity for HA and HS with much less affinity for CS and that heparin removes TFPIα bound to any GAG. In control experiments, BSA loaded beads did not bind TFPIα (Supplemental Figure IIIB).

The first two Kunitz domains of TFPIα did not associate with ECM.

Monoclonal antibodies directed against the first and second TFPI Kunitz domains did not release TFPIα from adherent Ea.hy926 cells (Figure 3A). However, a monoclonal antibody directed against the third Kunitz domain, as well as an aptamer specific for full length TFPIα,[43] released TFPIα, but to a lesser extent than the charged polymers (Figure 3A).

The TFPIα C-terminus associated with ECM.

The electrostatic binding of TFPIα to ECM GAGs and monoclonal antibody results suggested TFPIα binds ECM via its basic C-terminus. Therefore, the ability of peptides mimicking the basic region of TFPIα to release TFPIα from adherent Ea.hy926 cells and isolated ECM was examined. A peptide corresponding exactly to the TFPIα basic domain (Figure 3C) released TFPIα from adherent cells and ECM (Figure 3D). Vascular endothelial growth factor (VEGF) isoforms 206, 189, 183 and 145 have stretches of basic amino acids similar to those present in the basic C-terminal region of TFPIα. Further, it has been reported that VEGF145 binds to endothelial ECM in a heparin-releasable manner.[44] A peptide mimicking this region of VEGF (Figure 3C) released TFPIα from cultured Ea.hy926 cells and ECM as effectively as the TFPIα C-terminal peptide (Figure 3D). The LIKT sequence of the TFPIα C-terminal peptide interacts with factor V and promotes TFPIα anticoagulant activity.[45] A peptide with these amino acids replaced with alanine (Figure 3C) was used to determine if they influence the interaction of TFPIα with ECM. This peptide released TFPIα from adherent cells and ECM. Interestingly, when compared to the unaltered TFPIα C-terminal peptide, TFPIα release from ECM was comparable, but release of TFPIα from adherent cells was decreased suggesting the LIKT amino acids enhanced access of the peptide to ECM shielded by adherent cells (Figure 3D). Control peptides with similar molecular weight or similar isoelectric point to the TFPIα C-terminal peptide (Figure 3C) did not release TFPIα from adherent cells or ECM (Figure 3D).

The TFPIα C-terminal region bound to GAGs associated with several ECM proteins.

ECM is generated by ordered aggregation of secreted proteins and is constantly remodeled by association of newly secreted proteins and enzymatic removal of existing proteins.[46]

Thus, soluble ECM proteins are abundant in conditioned media. A peptide corresponding to the human TFPIα C-terminal amino acids from lysine 240 to asparagine 275 immobilized on beads was used to pull down proteins from Ea.hy926 cell conditioned medium (Figure 4). Following SDS-PAGE, three bands were pulled down from conditioned media but not fresh media (Figure 4). These three bands and a fourth band at 50 kDa that was also pulled down from fresh media were subjected to mass-spectral analysis. The proteins detected in each band are shown in Table 1. Several of those identified were ECM proteoglycans, including agrin, perlecan, collagen XVIII, syndecan-4 and glypican-1. The 50 kDa band pulled down from conditioned media was factor X, which has previously been shown to interact with the TFPIα C-terminus.[19, 47, 48] The corresponding band pulled down from fresh media was not analyzed but likely was bovine factor X present in the fetal calf serum added to the media.

Heparin released TFPIα from HuVEC ECM.

Studies were performed with cultured HuVECs to confirm findings from Ea.hy926 cells in non-transformed endothelial cells. As observed in Ea.hy926 cells, heparin released TFPIα from adherent but not suspended HuVECs (Figure 5A). Following removal of the cells, heparin released TFPIα from HuVEC ECM in amounts similar to adherent cells (Figure 5A).

Heparin releases TFPIα from umbilical cord veins.

Ex vivo studies of umbilical cords were performed to experimentally mimic heparin infusion. Perfusion of umbilical cord veins with fresh media containing heparin resulted in release of TFPIα when compared to perfusion with fresh media lacking heparin (Figure 5B). Perfused cords were subsequently fixed and imaged using immunohistochemistry. There was no difference between heparin and PBS perfused umbilical veins when examined with trichrome staining (Figure 5C and F). However, the ECM surrounding the heparin perfused umbilical vein had substantially less immunohistochemical TFPI staining than the non-heparin perfused umbilical vein (Figure 5 D, E, G, H). Experiments with umbilical arteries were not performed, because they were contracted and could not be reproducibly perfused.

TFPIα was present in ECM surrounding human kidney microvasculature

Immunofluorescence studies of kidney were performed to characterize the location of TFPIα and TFPIβ in an adult human tissue. An antibody that specifically recognizes TFPIβ is not available, so studies were performed with a polyclonal antibody that recognizes total TFPI and a polyclonal antibody that specifically recognizes the C-terminus of TFPIα (Supplemental Figure II). The total TFPI antibody prominently stained vascular endothelial cells in a pattern similar to the endothelial protein thrombomodulin reflecting the expression of TFPIβ on the endothelium surface (Figure 6A–D). In contrast, the TFPIα antibody stained within the subendothelial matrix and not on the surface of the endothelial cells (Figure 6E–H). The presence of TFPIα in ECM was well demonstrated by its staining outside the thrombomodulin stained vascular endothelial layer (Figure 6H). It is important to note that the antibody recognizing total TFPI antibody stained TFPIβ more brightly than TFPIα. Thus, the total TFPI antibody-stained endothelium brightly and the ECM weakly (Supplemental Figure IV). This may reflect a lower amount of TFPIα in the ECM relative to TFPIβ on the endothelium. However, it may also reflect a difference in affinity of the total

TFPI antibody for TFPIα and TFPIβ and no conclusions about the relative amounts of TFPI isoform expression can be made based on the differences in staining intensity.

TFPIα colocalized with glypican-1, syndecan-4, thrombospondin, and laminin-5 in human kidney ECM.

Immunofluorescence studies were used to determine if TFPIα colocalized with the individual proteins within the ECM that pulled down with the C-terminal peptide of TFPIα (Table 1). In these studies, TFPIα colocalized with glypican-1 (Figure 7A), syndecan-4 (Figure 7B), thrombospondin (Figure 7C) and laminin-5 (Figure 7D). In contrast, TFPIα was in close proximity to VWF (Figure 7E), perlecan (Figure 7F), agrin (Figure 7G) and collagen IV (Figure 7H) but did not colocalize with them.

Discussion

Heparin-releasable TFPIα accounts for a substantial portion of the TFPI accessible to circulating plasma in humans and is widely reported to originate from a pool bound to GAGs on the surface of endothelial cells.[20, 26, 27] The data presented here challenge this notion and implicate proteoglycans within ECM as the major source of heparin-releasable TFPIα. Our findings indicate that TFPIα and TFPIβ reside within distinct locations in the vessel wall to inhibit the procoagulant activity of different TF pools. TFPIα is in the ECM where it can modulate the procoagulant activity of TF that is constitutively expressed in adventitia surrounding blood vessels and initiates coagulation following vascular injury,[49] while TFPIβ is on the endothelial surface where it can inhibit TF expressed within the vasculature following inflammatory stimuli.[37]

Early studies identified that TFPIα is promptly released into the circulation upon heparin infusion.[26, 27] Since the plasma TFPIα concentration increases too rapidly to be attributed to synthesis and secretion of new protein, it was proposed that heparin displaced TFPIα from the endothelium glycocalyx.[26, 27] Eloquent studies performed by Lupu and colleagues subsequently found that TFPI localized to granules within endothelial cells that are distinct from Weibel Pilade bodies,[38] identified a heparin mediated decrease of intracellular TFPI,[32] and found that heparin does not alter cell surface TFPI even under flow conditions,[50] suggesting that heparin effects are more complex than simple displacement of TFPI from the endothelial glycocalyx. Consistent with this previous work, we observed punctate immunofluorescent TFPIα staining within Ea.hy926 cells and did not detect TFPIα on the surface of Ea.hy926 cells with flow cytometry or immunofluorescence. Thus, the current study complements and expands earlier work by finding that ECM is a major reservoir for heparin-releasable TFPIα.

Vascular ECM is composed of proteoglycans, collagens and nonproteoglycans typically thought to lie beneath a layer of endothelial cells that separates it from flowing blood. Thus, the mechanistic underpinning for how heparin accesses ECM and rapidly redistributes TFPIα from ECM into circulating blood remains unclear. Vascular beds containing fenestrated endothelium, such as liver and bone marrow, are a possible source of heparin-releasable TFPIα. However, TFPIα localized to ECM surrounding the umbilical vein was

released by heparin perfusion suggesting the release also occurs in vascular beds that do not have fenestrated endothelium.

Heparin-released TFPIα redistributes out of the circulation upon protamine administration to reverse the anticoagulant activity of heparin administered to patients undergoing heart surgery requiring cardiopulmonary bypass.[28, 51] Therefore, the flux of heparin-releasable TFPIα occurs in both directions into and out of plasma. Additional *in vivo* evidence that heparin releases TFPIα from ECM comes from studies performed by White and colleagues, who examined transgenic mice over expressing extravascular TFPIα under control of a vascular smooth muscle cell promoter. These mice had normal plasma TFPI levels indicating the transgenic TFPIα did not have direct access to plasma. However, upon heparin infusion they had almost double the normal amount of heparin-releasable plasma TFPIα.[52] These studies clearly indicate that heparin redistributes TFPIα from the extravascular space into plasma and provide strong additional support for our findings that the primary reservoir of heparin-releasable TFPIα is the ECM and not the endothelium.

Heparin-releasable TFPIα was identified in ECM produced by Ea.hy926 cells, HuVECs, and human umbilical cord veins. Although the proteoglycan structures within ECM of these cell lines and umbilical cord has not been well characterized, the presence of heparin-releasable TFPIα in ECM from several different sources indicates that our findings are not limited to specific ECM produced by an individual cell line, but rather may be a general property of vascular ECM.

Treatment of Ea.hy926 cells with negatively or positively charged polymers dissociated TFPIα from ECM. This suggested the basic C-terminal region of TFPIα mediated binding to GAGs in ECM which was confirmed by studies with domain specific anti-TFPI monoclonal antibodies. TFPIα bound to immobilized HS, and heparin, HS or HA competed for this binding to different degrees. Interestingly, CS did not compete for TFPIα binding to HS. The differential ability of HS, HA and CS to compete for TFPIα binding to immobilized HS suggests specificity of the binding interactions between TFPIα and GAGs and may partially explain why TFPIα associated with proteoglycans within ECM, but not with proteoglycans on the surface of endothelial cells.

VEGF and several other growth factors, including platelet derived growth factor, fibroblast growth factor, and epidermal growth factor, have a basic domain with homology to the TFPIα C-terminus.[21] There is increasing evidence that this domain targets VEGF to the ECM providing a physiologically important growth factor reservoir during morphogenesis and wound healing.[34, 44, 53] Similarly, TFPIα has been implicated in wound healing and angiogenic responses to injury.[21] We found that a peptide corresponding to this basic region in VEGF effectively competed with TFPIα for binding to ECM. Thus, another biological role for heparin-releasable TFPIα may be to modulate VEGF activity within ECM.

Pull-down experiments using a peptide corresponding to the TFPIα C-terminal region immobilized on magnetic beads removed multiple ECM proteins from Ea.hy926 conditioned media. In human kidney immunofluorescence studies, four of these proteins, glypican-1, syndecan-4, thrombospondin, and laminin-5, colocalized with TFPIα, while four others,

VWF, perlecan, agrin, and collagen IV, did not. Associations between ECM proteins that bind TFPIα and ECM proteins that do not bind TFPIα likely caused proteins that did not colocalize with TFPIα to be pulled down by the TFPIα C-terminal peptide. The proteins that colocalized with TFPIα are consistent with previous studies that have identified thrombospondin,[54] glypican-3,[55, 56] and syndecan-3[57] as TFPI-binding proteins. Proteoglycans consist of a core protein covalently attached to HS or CS chains. Often a proteoglycan is decorated with only one type of GAG chain[58] but glypican-1, syndecan-4 and perlecan are examples of hybrid proteoglycans that contain primarily HS chains but also may contain CS chains.[58] The co-localization of TFPIα with several GAG-containing ECM proteins further implicates a specific interaction between TFPIα and GAGs within ECM, as opposed to a specific protein-protein interaction. The nature of this interaction remains to be defined and may be important as proteoglycan glycosylation is a dynamic process that varies between tissues and in different diseases associated with blood coagulation and vascular wall pathologies.[59, 60]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Disclosures

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Abbreviations

TFPI	Tissue Factor Pathway Inhibitor
VEGF	Vascular Endothelial Growth Factor
GAG	Glycosaminoglycans
ECM	Extracellular Matrix
TF	Tissue Factor
FVIIa	Factor VIIa
FXa	Factor Xa
FVa-FXa	Factor Va-Factor Xa, prothrombinase
FBS	Fetal Bovine Serum
DMEM	Dublbecco’s Modified Eagle Medium
HuVEC	Human vein endothelial cells
PIPLC	phosphatidylinositol specific phospholipase C

HS	Heparan Sulfate
vWF	von Willebrand Factor
CS	Chondroitin Sulfate
HA	Hyaluronic Acid

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Highlights

- ECM contained the primary vascular pool of heparin-releasable TFPIα.
- The TFPIα C-terminal region bound to GAGs within the ECM, at least partially through electrostatic interactions.
- TFPIα is optimally positioned to inhibit the procoagulant activity of tissue factor surrounding the vasculature.

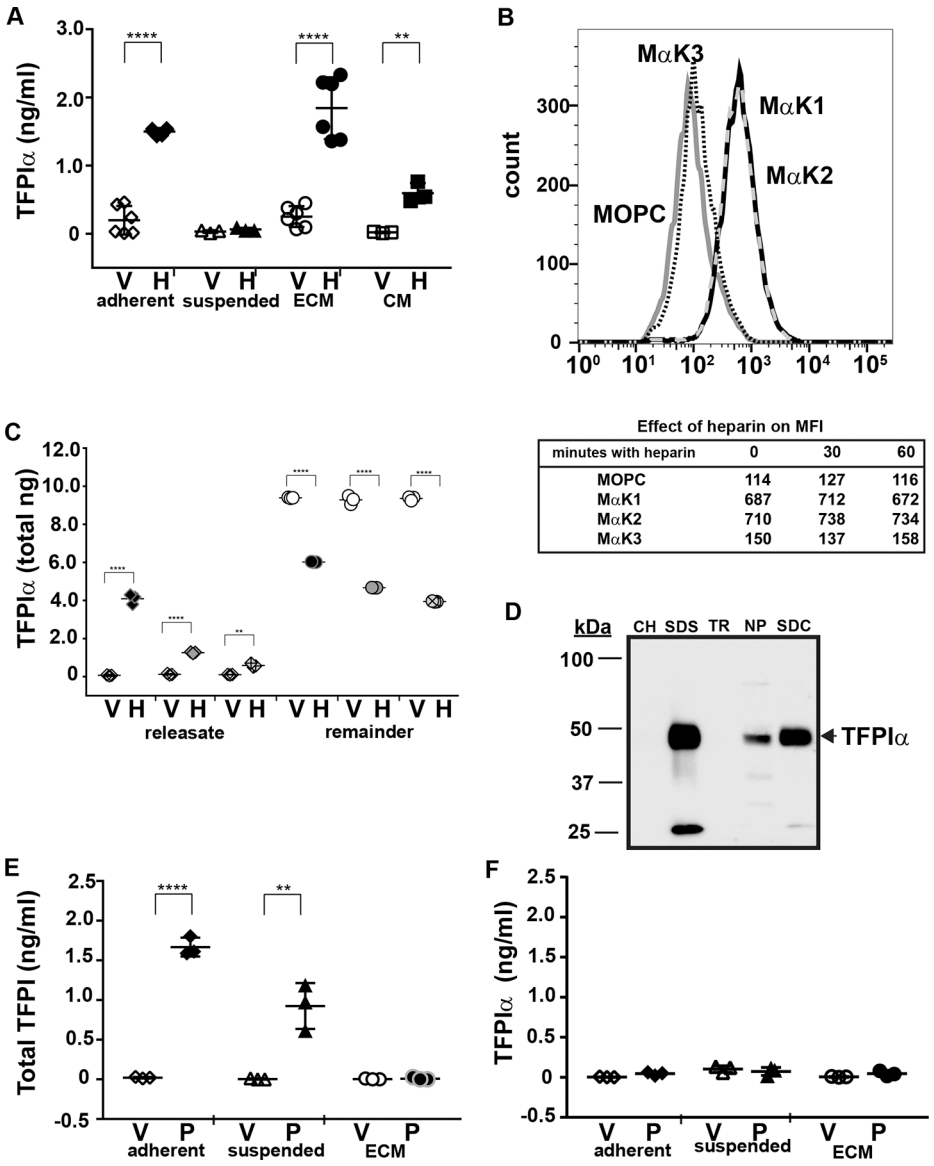

Figure 1. Heparin-releasable TFPIα in Ea.hy926 cells was in the ECM.

(A) Vehicle treatment with DMEM containing 2% FBS (open symbols, V) and Heparin (closed symbols, H) treatment of adherent Ea.hy926 cells (diamonds), suspended Ea.hy926 cells (triangles), Ea.hy926 ECM (circles), and an empty plate incubated with Ea.hy926 conditioned media (CM, squares). Heparin released TFPIα in approximately equal amounts from adherent cells and ECM but did not release TFPIα from suspended cells. Heparin released smaller amounts of TFPIα from an empty plate incubated with conditioned media than from adherent cells or ECM. (B) Flow cytometry was used to identify TFPIα and TFPIβ on the surface of suspended Ea.hy926 cells. Before heparin treatment, monoclonal antibodies directed against the first (MαK1) and second (MαK2) Kunitz domains, which recognize both TFPIα and TFPIβ, detected surface TFPI, while an antibody directed against the third Kunitz domain (MαK3), which recognizes only TFPIα, did not stain differently than MOPC, the isotype control antibody. After heparin treatment, the mean fluorescence

intensity (MFI) values for each antibody were unchanged (lower box) indicating an absence of heparin-releasable TFPIα.(C) Sequential treatment of adherent Ea.hy926 cells with vehicle (open diamonds, V) and Heparin (filled diamonds, H). The first Heparin exposure (black) released substantially more TFPIα than the second (grey) and third treatments (grey with crosshairs). Solubilization of ECM after each vehicle (V, open circle) or Heparin (H, closed circle) treatment of adherent cells revealed that each heparin treatment reduced the amount of TFPIα remaining in ECM but about 40% of TFPIα remained in the ECM following 3 heparin treatments. (D) ECM was solubilized with PBS containing 10 mM EDTA and 30 mM Chaps (CH), 0.1% sodium dodecyl sulfate (SDS), 0.1% Triton X-100 (TR), 1% Nonidet P40 (NP) or 0.5% deoxycholate (SDC) and subjected to SDS-PAGE and western blot with a TFPIα specific antibody. Small amounts of proteolyzed TFPIα were present in SDS and SDC treated samples. (E and F) Adherent Ea.hy926 cells (diamonds), suspended Ea.hy926 cells (triangles), and Ea.hy926 ECM (circles) were treated for 1 hour at 37°C with 1 U/mL PIPLC (filled, P) or vechicle consisting of PBS containing 1% BSA (open, V). PIPLC removed total TFPI from the surface of adherent and suspended cells but not from ECM (E) but did not remove TFPIα from cells or ECM (F) indicating that GPI-anchored TFPIβ was on the surface of adherent and suspended cells but was not in the ECM. (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$; (****) $p<0.0001$.

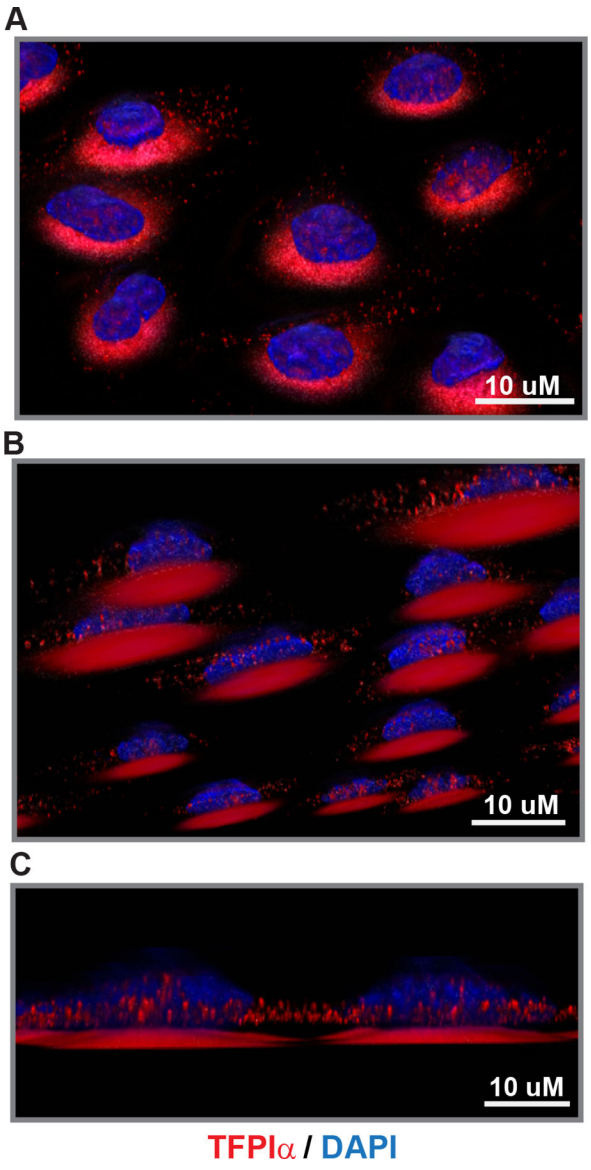

Figure 2. TFPIα in Ea.hy926 cells localized to punctate cytoplasmic granules and to ECM directly beneath individual cells.

Serial volume immunofluorescence images (41stacked z-step images at 0.25uM intervals) of Ea.ahy926 cells stained with polyclonal antibody against the TFPIα C-terminus (red) and dapi (blue) are shown. (A) Top view. (B) Bottom view. (C) Side view.

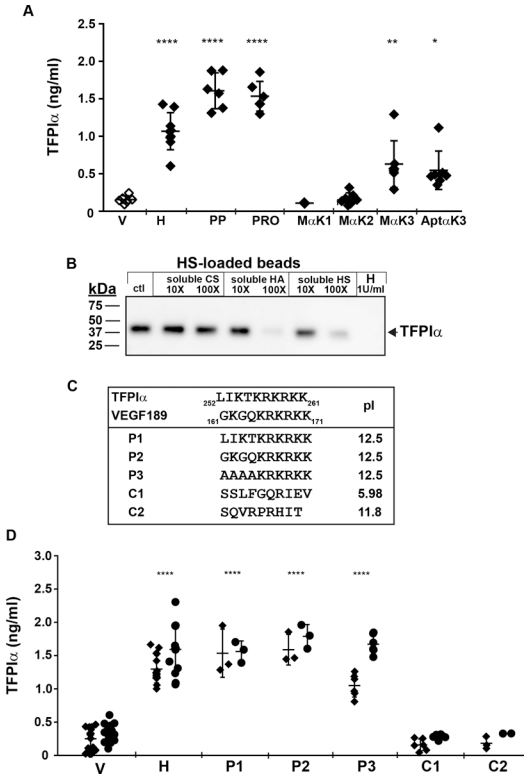

Figure 3. TFPIα interacts with ECM through electrostatic forces.

(A) TFPIα released from adherent Ea.hy926 cells following treatment with vehicle consisting of DMEM media containing 2% FBS (V) heparin (H), polyphosphate (PP), protamine sulfate (PRO), monoclonal antibodies directed against the three TFPIα Kunitz domains (MαK1, MαK2, MαK3), or anti-TFPI aptamer (AptαK3). (B) Western blot analysis of TFPIα removed from Ea.hy926 cell conditioned media with immobilized heparan sulfate (HS, ctl) and in the presence of soluble chondroitin sulfate (CS), hyaluronic acid (HA), HS or heparin. (C) Sequences and isoelectric points of TFPIα, VEGF, and control peptides used. (D) TFPIα released from adherent Ea.hy926 cells (diamonds) cells or ECM (circles) following treatment with peptides shown in (C). Experiments were performed with DMEM containing 2% FBS (V) and heparin (H) are shown for comparison. (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$; (****) $p<0.0001$.

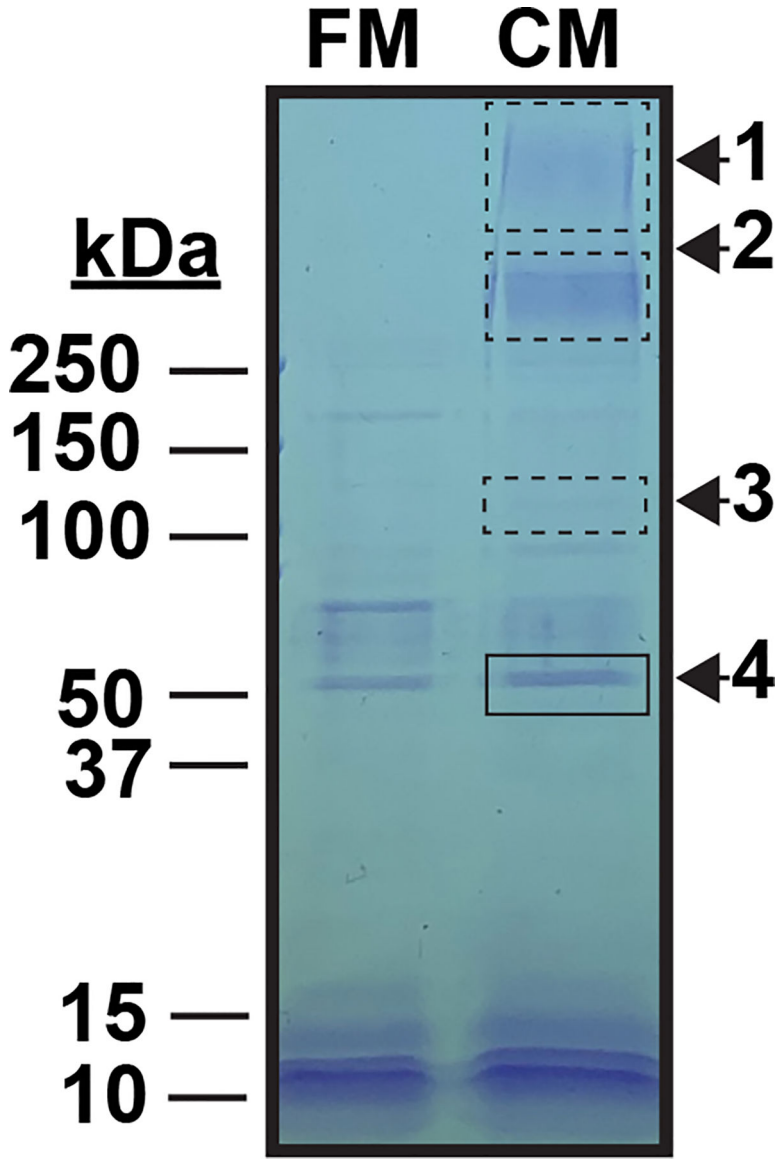

Figure 4. Proteins removed from Ea.hy926 conditioned media by the TFPIα C-terminus.
SDS-PAGE with coomassie blue stain of proteins removed from Ea.hy926 cell conditioned media (CM) or fresh media (FM) by a peptide corresponding to the 36 C-terminal amino acids of TFPIα immobilized on magnetic beads. Bands 1–4 from CM were subjected to mass spectral analysis. Proteins identified in each band are presented in Table 1.

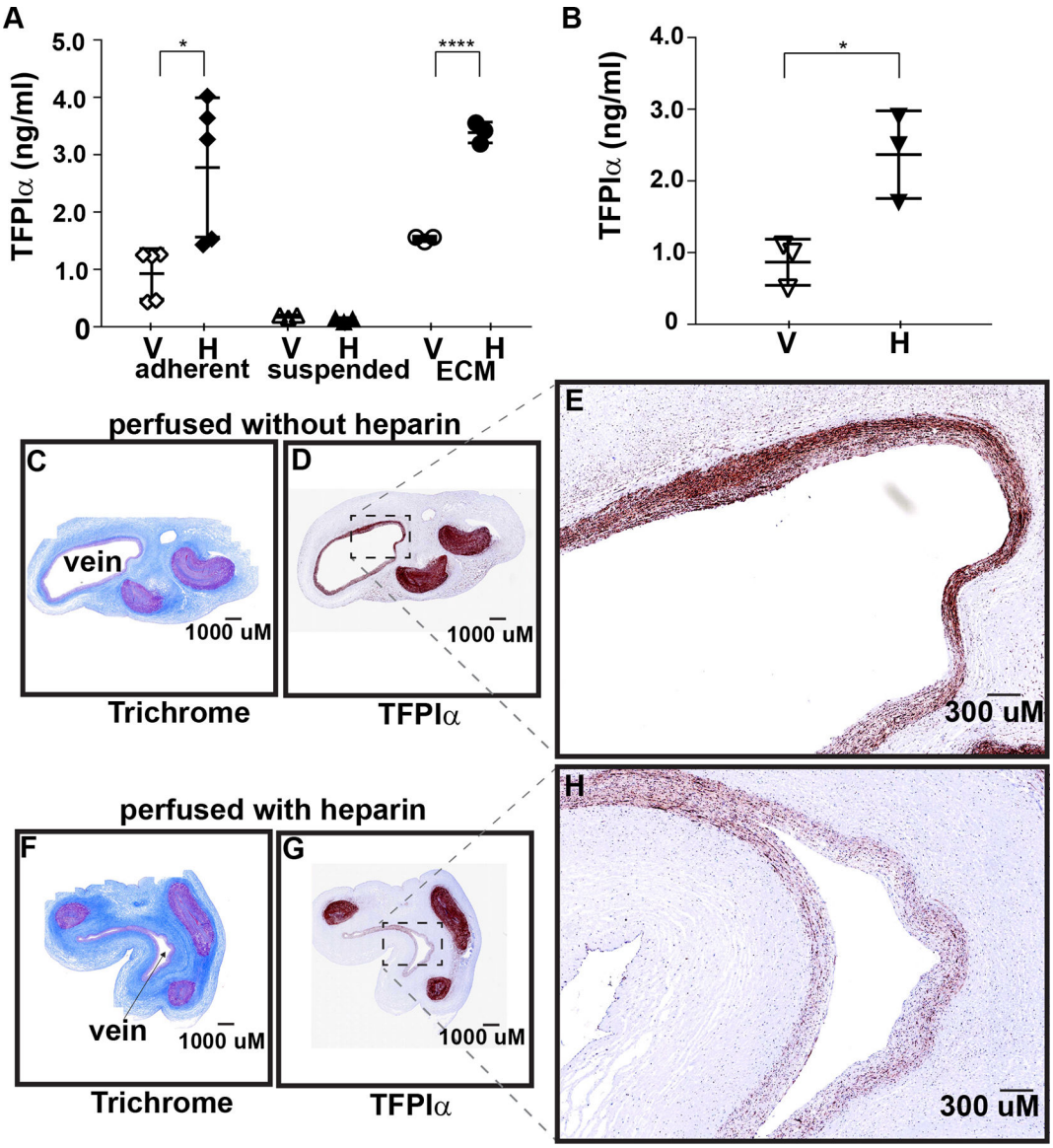

Figure 5. Heparin releases TFPIα from HuVEC and human umbilical cord vein ECM.

(A) TFPIα released from DMEM media containing 2% FBS treated (V) or heparin (H) treated adherent or suspended HuVECs and HuVEC ECM. (B) TFPIα released from human umbilical cord veins perfused with DMEM media containing 2% FBS (V) or heparin (H). (C, D, E) Umbilical cord following perfusion of the vein with DMEM containing 2% FBS. (F, G, H) Umbilical cord following perfusion of the vein with heparin. C and F were stained with trichrome to display tissue histology. D, E, G, and H were stained with a polyclonal antibody specific for the C-terminus of TFPIα. E and H are enlarged regions from D and G, respectively, that highlight the reduced staining for TFPIα in the ECM surrounding the heparin treated umbilical vein. (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$; (****) $p<0.0001$.

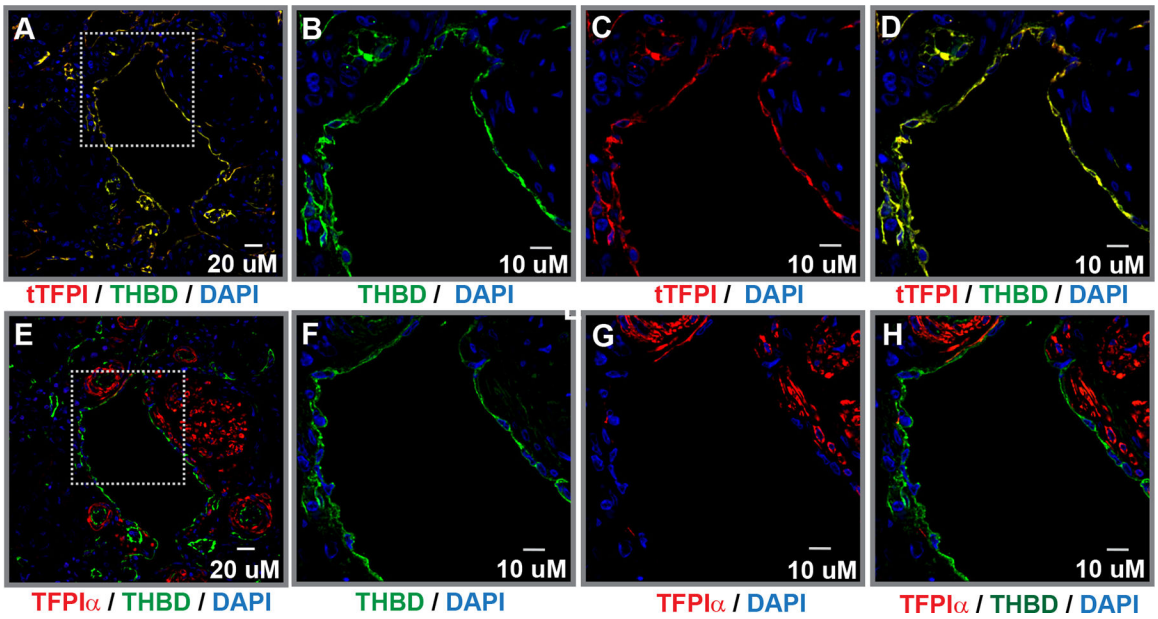

Figure 6. TFPIα was located in ECM surrounding human kidney vessels.

(A) Serial sections (5 microns) from healthy human kidney were stained with polyclonal antibodies against thrombomodulin (THBD, green) and total TFPI (tTFPI, red) and with dapi stained nuclei (blue). The region in the dotted box is enlarged in panels B-D. (B) THBD and dapi only. (C) tTFPI and dapi only. (D) Overlay of THBD and tTFPI showing co-localization of THBD and tTFPI (yellow) within the vascular endothelial cell layer. (E) Serial sections (5 microns) from healthy human kidney were stained with polyclonal antibodies against thrombomodulin (THBD, green) and the TFPIα C-terminus (TFPIα, red) and with dapi stained nuclei (blue). The region in the dotted box is enlarged in panels F-H. (F) THBD and dapi only. (G) TFPIα and dapi only. (H) Overlay of THBD and TFPIα staining showing THBD and TFPIα did not co-localize, as TFPIα was in the ECM surrounding the endothelial layer stained with THBD.

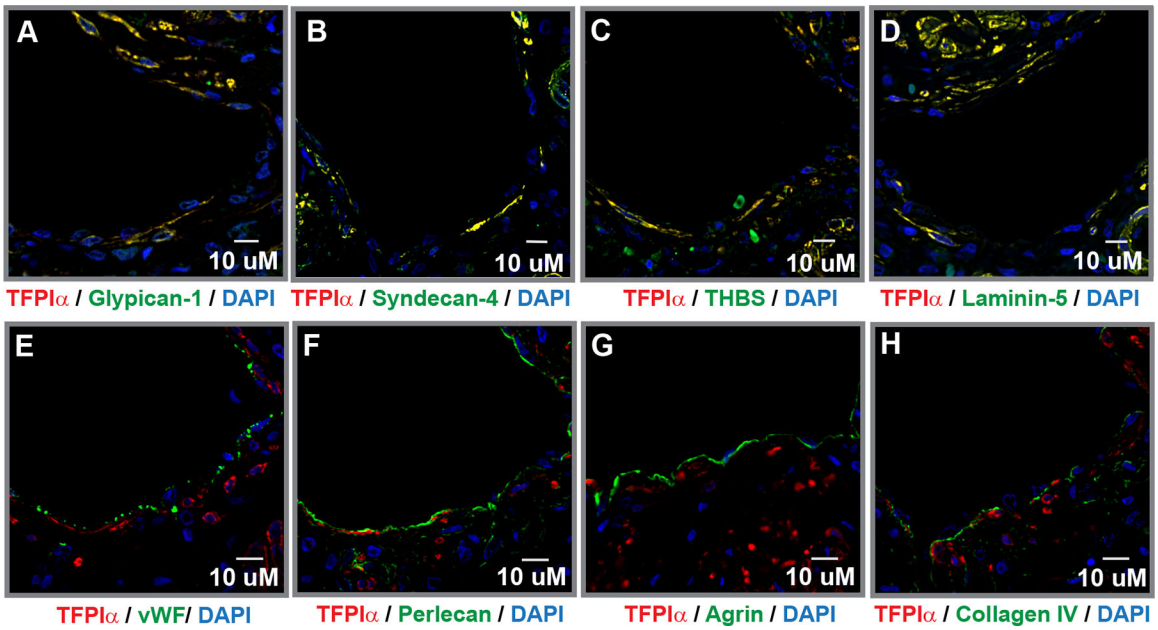

Figure 7. TFPIα colocalized with specific ECM proteins in human kidney.

In all panels 5 micron serial sections of healthy human kidney were stained with a polyclonal antibody directed against the TFPIα C-terminus (red). Sections were costained with antibodies specific for eight of the ECM proteins removed from Ea.hy926 conditioned media by immobilized TFPIα C-terminal peptide (Table 1). TFPIα colocalized with (A) glypican-1 (green), (B) syndecan-4 (green) (C) thrombospondin (THBS green) (D) laminin-5 (green). TFPIα did not colocalize with (E) vWF (green), (F) perlecan (green), (G) agrin (green) and (H) collagen IV (green).

Table 1.

TFPIα C terminal peptide binding proteins identified in Ea.hy926 conditioned medium.

Gene	Description	Band	%Coverage	# Unique peptides	Score Sequest HT
AGR	Agrin	1	54	117	2616.1
HSPG2	Perlecan	1	39	115	1498.5
COL18A	Collagen XVIII	2	31	117	3242.2
LAMA5	Laminin subunit a5	2	31	76	993.38
SDC4	Syndecan-4	2	25	5	42.35
GPC1	Glypican 1	3	29	6	23.1
SEMA3C	Semaphorin-3C	3	34	23	285.17
GPC1	Glypican 1	3	29	10	101.5
THBS1	Thrombospondin-1	3	14	2	69.58
LAMC1	Laminin subunit gamma-1	3	11	13	64.02
LAMB2	Laminin subunit beta-2	3	15	20	93.7
TINAGL1	Tubulointerstitial nephritis antigen	4	36	15	174.06
COL4A1	Collagen alpha-1(IV)	4	2	5	33.02
VWA1	von Willebrand factor A domain	4	22	5	26.82
DDOST	Dolichyl glycosyltransferase	4	13	5	22.76
NID1	Nidogen-1	4	5	6	22.3
F10	Coagulation factor X	4	4	2	7.73
ANGPTL4	Angiopoietin- related protein	4	8	3	7.03