Tissue Factor Pathway Inhibitor Blocks Angiogenesis via Its Carboxyl Terminus

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Objective—Tissue factor pathway inhibitor (TFPI) is the primary regulator of the tissue factor (TF) coagulation pathway. As such, TFPI may regulate the proangiogenic effects of TF. TFPI may also affect angiogenesis independently of TF, through sequences within its polybasic carboxyl terminus (TFPI C terminus [TFPIct]). We aimed to determine the effects of TFPI on angiogenesis and the role of TFPIct.

Methods and Results—Transgenic overexpression of TFPI attenuated angiogenesis in the murine hindlimb ischemia model and an aortic sprout assay. In vitro, TFPI inhibited endothelial cell migration. Peptides within the human TFPIct inhibited endothelial cell cord formation and migration in response to vascular endothelial growth factor (VEGF) 165 but not VEGF121. Furthermore, exposure to human TFPIct inhibited the phosphorylation of VEGF receptor 2 at residue Lys951, a residue known to be critical for endothelial cell migration. Finally, systemic delivery of a murine TFPIct peptide inhibited angiogenesis in the hindlimb model.

Conclusion—These data demonstrate an inhibitory role for TFPI in angiogenesis that is, in part, mediated through peptides within its carboxyl terminus. In addition to its known role as a TF antagonist, TFPI, via its carboxyl terminus, may regulate angiogenesis by directly blocking VEGF receptor 2 activation and attenuating the migratory capacity of endothelial cells. (Arterioscler Thromb Vasc Biol. 2012;32:704-711.)

Key Words: angiogenesis ■ hemostasis ■ thrombosis ■ murine models ■ tissue factor

The biological systems of coagulation and angiogenesis show considerable interdependence. Proteases and inhibitors within the “extrinsic” or tissue factor (TF) pathway of coagulation have emerged as potential regulators of angiogenesis.1 TF, a transmembrane glycoprotein present on the surface of most extravascular cells, is the primary cellular initiator of coagulation. TF, and particularly the TF-factor VIIa (TF/VIIa) complex, can also promote angiogenesis, both directly1 and indirectly,2 through regulation of thrombin generation and activation of intracellular signaling mediated by protease-activated receptors.3-5 Tissue factor pathway inhibitor (TFPI) is expressed on the endothelial cell surface6 and is the major physiological inhibitor of TF/VIIa.7 As such, TFPI is ideally situated to modulate these proangiogenic biological actions of TF/VIIa.

TFPI is a multivalent serine protease inhibitor with 3 independently folded Kunitz-type proteinase inhibitor domains8 and a highly basic, positively charged carboxyl terminus known to bind heparin.9 The first Kunitz domain (K1) binds TF/VIIa, and the second Kunitz domain binds factor Xa.10 It is the formation of this quaternary TF-VIIa-TFPI-Xa complex that dampens ongoing coagulation. The physiological plasma concentration of TFPI is between 2.5 and 5 nmol/L11; however, most TFPI is bound to vascular surfaces,12 and local levels may be higher.

The TF/VIIa protease complex promotes angiogenesis directly through protease-activated receptor 2 (PAR2) signaling, and TF is expressed in response to angiogenic stimuli.3-5,13 Endothelial cells overexpressing PAR2 and TF demonstrated reduced PAR2 signaling in the presence of recombinant TFPI.14 The concentration of exogenous TFPI required to inhibit PAR2 signaling is higher than that required to inhibit TF-dependent initiation of the coagulation cascade,14 possibly suggesting a divergent mechanism. However, the significance of TFPI antagonism to angiogenic TF/VIIa signaling in vivo or in natively expressing cells remains unclear. Indeed, there is some evidence that human TFPI can inhibit endothelial cell proliferation, at possibly supraphysiological concentrations (0.5 μmol/L), perhaps through peptides within its carboxyl terminus (TF pathway inhibitor C terminus [TFPIct]), resulting in antitumor activity.14-16 However, its role in regulating angiogenesis in ischemic tissue in vivo and the mechanism of
action for these proposed antiangiogenic effects are not understood.

To define the role of TFPI in regulating angiogenesis, a murine transgenic model of vascular TFPI overexpression (TFPI\textsuperscript{TG}) was tested in relevant models of angiogenesis.\textsuperscript{17} We then performed further ex vivo studies on harvested aortic rings to verify the attenuated angiogenic capacity of this murine strain and to remove the effects of circulating factors focusing on the intrinsic properties of the harvested vessel. Complementary in vitro studies of endothelial cell function and vascular endothelial growth factor receptor 2 (VEGFR2) phosphorylation were then used. Our studies ultimately focused on residues within the polybasic TFPIct and its role in regulating angiogenesis and endothelial cell migration.

**Methods**

All animal experimental procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committees and conformed to the National Institutes of Health and Mayo Clinic institutional guidelines.

**Genetically Modified Mice**

We used a transgenic murine model of TFPI overexpression via a SM22\textalpha{} promoter (TFPI\textsuperscript{TG}).\textsuperscript{17} These mice (in C57BL/6 background) have increased vascular expression of full-length murine TFPI.\textsuperscript{18} Male, age-matched C57BL/6 mice were used as controls in all experiments.

**Aortic Ring Outgrowth Model**

Angiogenesis was measured using aortic ring studies as described previously.\textsuperscript{19} Briefly, thoracic aortas were harvested from 11- to 12-week-old TFPI\textsuperscript{TG} and age-matched male C57BL/6 control mice (n=4) and finely dissected to remove adventitia. The aorta was flushed thoroughly to eliminate any contamination with blood, and subsequently, the aorta was dissected into 1-mm-thick rings and flushed thoroughly to eliminate any contamination with blood. Aortic segments were then overlaid with endothelial cell medium containing 5% fetal bovine serum and 20 ng/mL of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDG), and fibroblast growth factor (FGF). The medium was changed on a daily basis, and aortic sprouts were scored on day 4 in a blinded manner.

**Hindlimb Ischemia Model**

Mouse hindlimb ischemia model was performed as described previously.\textsuperscript{20} Mice were assessed before and during experiment using a clinical score (0=normal, 1=pale foot/gait, 2=gangrenous tissue <half foot, 3=two lower limb necrosis, 4=gangrenous tissue >half foot, 5=loss of half lower limb) and a foot movement score (0=normal [plantar/toe flexion on tail traction], 1=no toe flexion, 2=no plantar flexion, 3=dragging of foot), as well as laser Doppler perfusion imaging. In subsequent studies, C57BL/6 mice received intraperitoneal injections of either murine TFPIct (mTFPIct) (200 \mu g/day) or saline control. Histological analysis was performed using immunohistochemistry to von Willebrand factor (Dako, Carpinteria, CA) or control.

**TFPI C-Terminal Peptide Sequences**

Human TFPIct (consisting of residues 254–265 of human TFPI) was KTRKRKQRKQRVK; scrambled human TFPIct, KVKKRTRKQKK; alanine-substituted human TFPIct, ATAAAKAAAAAVQA; mTFPIct (consisting of residues 253–266 of murine TFPI), KNKSKGVVKIQQRK.

**Tube/Cord Formation**

Serum-starved human umbilical vein endothelial cells (HUVECs) (25,000 cells) were grown in endothelial growth medium (EGM)-2 medium and seeded onto 250 \mu L of Matrigel (BD Biosciences) in 24-well plates. Dishes were photographed (5/well) at 6 and 24 hours, and calculation of percentage area of Matrigel-covered area by complete endothelial “tubes” (ie, a complete circle of endothelial cords) was performed using the ImageProPlus program. Experiments were performed in triplicate. The same protocol was followed for HUVECs receiving prior TFPI C-terminal peptides.

**Migration Assay**

Serum-starved HUVECs were preincubated with TFPI peptides or control. Injury migration assay was performed as previously described;\textsuperscript{21} migration was assessed after 24 hours of stimulation with either VEGF165 or VEGF121 (10 ng/mL).

**VEGFR2 Receptor Tyrosine Residue 951 Phosphorylation**

HUVECs were serum-starved and then pretreated with (if receiving peptide) TFPI peptides before VEGF165 stimulation (10 ng/mL) for 5 minutes (37°C). Western blots were performed to quantitate VEGFR2 Tyr\textsuperscript{951} phosphorylation relative to total VEGFR2 expression.

**Statistics**

Results are expressed as means±SEM. Differences between groups were analyzed for statistical significance using an unpaired Student t test (2-tailed), and survival differences using log rank test and group differences in Western blot densitometric analysis were analyzed using 1-way ANOVA.

**Results**

**Vascular Overexpression of TFPI Inhibits Angiogenesis In Vivo**

To determine effects of vascular TFPI overexpression on angiogenesis, a murine model was used. Transgenic mice overexpressing full-length mTFPI (TFPI\textsuperscript{TG}) showed impaired recovery from hindlimb ischemia compared with wild-type mice as demonstrated by laser Doppler imaging and both clinical and foot movement scores (Figure 1). The limb perfusion ratio (perfusion in ischemic limb divided by perfusion in normal limb) was significantly reduced in the TFPI\textsuperscript{TG} mice as compared with controls after only 3 days (0.22±0.01 versus 0.35±0.03, P<0.01) (Figure 1c). Similar differences were found on day 7 (0.36±0.02 versus 0.56±0.04, P<0.01) and day 28 (0.54±0.09 versus 0.80±0.03, P<0.05). Functional recovery was also impaired in the TFPI\textsuperscript{TG} group. Histology revealed lower postischemic gastrocnemius capillary density (TFPI\textsuperscript{TG} 23.4±2.5 versus 64.9±6.0 capillaries/mm in controls) and lower capillary:muscle fiber ratio (0.67±0.05 in TFPI\textsuperscript{TG} versus 1.0±0.03 in controls) at 28 days in the TFPI\textsuperscript{TG} compared with the wild-type mice. Taken together, these results suggest that vascular overexpression of TFPI limits angiogenesis.

**Vascular Overexpression of TFPI Inhibits Aortic Sprouting**

To determine whether these effects were locally determined, a standard in vitro aortic ring assay was used.\textsuperscript{5,19} Aortic segments from TFPI\textsuperscript{TG} mice demonstrated less sprouting angiogenesis than did aortas from C57BL/6 mice in this model (Figure 2a and 2b). Additionally, recombinant full-length human TFPI (gift of Dr Alan Mast) inhibited sprouting in aortas from C57BL/6 mice (Figure 2c and 2d). These data suggest that the in vivo effects were locally determined and...
an effect of TFPI. As sprouting in this model has previously been shown not to be dependent on proteases within the clotting cascade; these results may suggest a TFPI-dependent mechanism independent of its known inhibition of factor X/Xa. As TF expression is upregulated in ischemic models and mediates sprouting in this model, these studies do not delineate between a TF-mediated effect of TFPI and a TF-independent effect. To do so, further studies were done in vitro.

**TFPI Can Inhibit Angiogenesis In Vitro via the Heparin Binding, Highly Charged Region of its C Terminus**

To determine the mechanism by which TFPI might inhibit angiogenesis, in vitro studies were performed with human endothelial cells. Migration of endothelial cells was significantly inhibited by exposure of cells to exogenous recombinant full-length human TFPI (Figure 3a–3c). Furthermore, the TFPIct peptide inhibited endothelial cell migration and cord formation at a dose of 10 nmol/L (Figure 3d–3f). Thus, TFPIct peptides inhibit VEGF165-induced migration and cord formation at nanomolar concentrations mimicking the effect of exogenous recombinant full-length human TFPI application. To test the hypothesis that TFPIct interferes with cell surface heparan sulfate proteoglycan augmentation of VEGF165 signaling through its heparin-binding domain, migration studies were performed with HUVECs stimulated with VEGF121, which lacks this heparin-binding domain. Thus, TFPIct inhibition of VEGF165-induced migration is dependent on the 44 amino acids, containing the heparin-binding domain, that are not present in VEGF121.
TFPI Inhibits Phosphorylation of VEGFR2 at Tyrosine 951 (Tyr951) via Its C Terminus

VEGFR2 Tyr951 phosphorylation has been shown to be the critical event in VEGF-induced endothelial cell migration.24 Following VEGF165 stimulation, Western blot analysis demonstrated that exogenous addition of TFPIct 20 minutes before VEGF165 stimulation dose-dependently inhibited phosphorylation of the VEGFR2 Tyr951 down to a nanomolar concentration (Figure 4a and 4b). Scrambled and alanine substituted TFPIct peptides did not demonstrate this effect (Figure 4c–4f). Therefore, inhibition of the VEGFR2 receptor phosphorylation by TFPIct is evident at nanomolar concentration and appears to be sequence specific.

Murine TFPIct Inhibits Angiogenesis In Vivo

To determine whether the TFPIct might have independent effects on angiogenesis in vivo, mice received intraperitoneal injections daily with mTFPIct peptide or saline. Mice receiving mTFPIct failed to demonstrate untoward effects and demonstrated significantly delayed angiogenesis in the hindlimb ischemia model compared with control mice (Figure 5). This difference was significant at 7 and 14 days (perfusion ratio in mice receiving mTFPIct daily at 7 days was 0.30±0.04 versus 0.56±0.04 in controls and after 14 days 0.6±0.03 versus 0.79±0.03, n=6, P<0.01) and confirmed by histology (Figure 5c–5e) following limb harvest after 2 weeks (capillary density mTFPIct left gastrocnemius muscles was 23.4±2.5/mm³ versus 64.9±6.0/mm³ in controls, P<0.05) and clinical functional scoring (Figure 5f and 5g).

Discussion

The biological systems of hemostasis and angiogenesis are fundamental to all living, multicellular organisms. Defining the coordinate regulation of these pathways may provide unique pathophysiological insight and offer novel pharmacological opportunities. This is the first study to examine the angiogenic effects of TFPI overexpression in vivo. The TFPI TG mouse overexpresses TFPI in vascular smooth muscle cells. TFPI expression is increased locally in vascular smooth muscle cells, but also at the endothelial surface, as evidenced by increased heparin-releasable TFPI and systemically, with raised plasma TFPI antigen levels. Heparin-releasable TFPI must come from the intravascular, endothelial surface. We demonstrated a clear inhibition of angiogenesis in recovery from hindlimb ischemia in these mice. Our in vitro studies support an antiangiogenic effect independent of the potential effect of TFPI on TF through its polybasic carboxyl terminus. Furthermore, we were then able to replicate the antiangiogenic effect seen in the TFPI TG mice simply with administration of a peptide from the murine TFPI C terminus.

TF in complex with factor VIIa is a well-established proangiogenic complex. Previous in vitro data suggest that TFPI can inhibit downstream proangiogenic signaling via the PAR2 receptor. TFPI binds TF/VIIa via its K1 domain. The C terminus of TFPI is thought to bind cell surface heparans and enable TFPI binding to cell surface membranes and circulating lipoproteins. It should be noted that this TFPIct is distinct from that of TFPIB; a splice variant of the TFPI gene that lacks the Kunitz 3 domain of TFPI and has a unique carboxyl terminus that unlike TFPI contains a direct
glycosylphosphatidylinositol (GPI) anchor. The second major finding of this study is that peptide derivatives of the C terminus of TFPI can account for a significant portion of the antiangiogenic effect of TFPI previously reported in vitro. These TFPIct studies definitively exclude a TF-dependent effect. We have shown inhibition of endothelial cell migration and cord formation at concentrations as low as 10 nmol/L. This replicates the in vitro action of the full-length molecule precisely and reinforces our findings from the in vivo studies that TFPI can act independently of TF to inhibit angiogenesis.

Given the critical role of growth factor-mediated signaling in angiogenesis, we investigated this as a mechanism of action by which TFPI could directly inhibit angiogenesis. There is a striking similarity between the TFPIct, which contains numerous positively charged lysine and arginine residues and other peptides known to interact with the VEGF receptor and inhibit angiogenesis. Transactiva-

![Figure 3](image-url)

Figure 3. Tissue factor pathway inhibitor (TFPI) and TFPI C terminus (TFPIct) inhibit vascular endothelial growth factor (VEGF) 165-mediated endothelial cell migration and tube/cord formation. a and b, Photographs: baseline (top row) and 24 hours after VEGF165 stimulation (bottom row); Cells moving into the wounded area were counted/×10 field. c, Quantification of assay at 24 hours (*P<0.001). d, Human TFPIct (hTFPIct) inhibited endothelial cell migration compared with control peptides. e, HUVECs on Matrigel with hTFPIct. f, Quantification reveals dose-dependent inhibition of tube formation. *P<0.01. g, rTFPIct did not inhibit VEGF121-induced endothelial cell migration. aa indicates amino acids; FBS, fetal bovine serum; rTFPIct, recombinant full-length human TFPI.
tor of transcription (TAT) polypeptide is encoded by the HIV-1 virus. TAT causes transactivation of the HIV-1 long terminal repeat and is essential in HIV viral replication and gene expression. TAT has been shown to be involved in the growth of the highly vascular lesions seen in Kaposi sarcoma, as well as the general microangiopathy associated with AIDS. Peptides derived from TAT corresponding to the most basic, cysteine-rich domains have been shown to directly interact with the VEGF receptor. HIV TAT peptides inhibit VEGF binding to VEGFR2, VEGF-dependent extracellular signal regulated kinase activation, and in vitro assays of angiogenesis. The TFPIct also resembles the heparin-binding domains of several growth factors, including VEGF. Peptides derived from the heparin-binding region of snake venom VEGF-F have been shown to directly inhibit binding of VEGF to its receptor and inhibit endothelial cell proliferation.

We demonstrated that TFPIct impairs the phosphorylation of the VEGFR2 receptor at tyrosine-951 by endothelial cells in response to VEGF165 stimulation, a residue known to be critically important to endothelial cell migration. Heparan sulfate proteoglycans augment binding and activation of many proangiogenic growth factors to their receptors, including those of VEGF165 to VEGFR2. We hypothesize that TFPI acts via its C terminus to interfere with this heparin-dependent augmentation. To further support this hypothesis, we demonstrated that TFPIct has no effect on VEGF121-induced migration. VEGF121 does not contain a heparin-binding domain.

In conclusion, we have demonstrated an inhibitory role for TFPI in angiogenesis, independent of TF, in complementary

Figure 4. Effect of tissue factor pathway inhibitor (TFPI) on VEGF165-induced VEGFR2-Tyr951 phosphorylation. a. Western blots: β-actin (top), total VEGFR2 (middle), and VEGFR2-Tyr951 phosphorylation (bottom) after 5 minutes of VEGF165 stimulation. b. Density quantification of Tyr951-phosphorylation bands normalized to total VEGFR2 relative to controls. TFPI C terminus (TFPIct) dose-dependently inhibited response (*P<0.05, n=4). c to f, Effect of scrambled and substituted peptides.
This antiangiogenic effect has for the first time been further localized to the heparin-binding carboxyl terminus of TFPI, which acts to inhibit VEGF signaling.

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Disclosures
None.

References


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