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Tissue factor pathway inhibitor as a multifunctional mediator of vascular structure

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1. ABSTRACT

Tissue factor pathway inhibitor (TFPI) is a potent regulator of tissue factor – factor VII-dependent activation of the tissue factor pathway. TFPI is a serine protease inhibitor that contains three Kunitz domains and a basic carboxyl terminus. TFPI is primarily expressed on endothelial cells, and murine models have demonstrated that its expression regulates vascular thrombosis. The localization of TFPI expression and the requirement for TFPI in development suggest a potential role in regulating vascular structure. Data from animal studies suggest that vascular expression of TFPI inhibits pathologic vascular remodeling and inhibits angiogenesis. The mechanism for these effects is diverse and includes tissue factor and factor Xa-dependent and -independent mechanisms.

2. INTRODUCTION

The vascular endothelium is a multimodal organ system that provides an integrating focus for the regulation of vascular structure and function (1). Endothelial-derived nitric oxide would serve as a paradigm for this principle, functionally acting acutely to locally vasodilate vessels but also chronically on the underlying tunica media to affect both vascular structure and function. Other endothelial-derived molecules demonstrate similar multimodal properties. As the interface with blood, the endothelium is exposed to circulating factors and cells, as well as physical forces, which regulate thrombosis. The endothelium integrates pro- and anti-thrombotic signals to regulate local thrombogenicity. Endothelial cells express tissue factor pathway inhibitor (TFPI), a serine protease inhibitor which

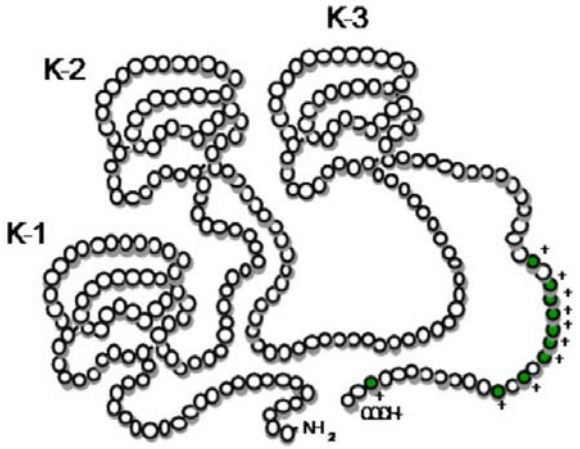

Figure 1. Schematic of TFPI demonstrating three Kunitz (K) domains and basic carboxyl terminus.

is the primary inhibitor of tissue factor (TF)-mediated coagulation. The functionality of TFPI, however, extends beyond this anticoagulant role to regulate different aspects of vascular biology. This review will highlight human and experimental data which suggest that TFPI regulates macro and microvessel structure in a diverse manner.

3. TFPI STRUCTURE AND FUNCTION

TFPI is a glycoprotein consisting of 276 amino acids. TFPI is a multivalent serine protease inhibitor with an acidic amino terminus, three independently-folded Kunitz-type proteinase inhibitor domains (2), and a highly basic, positively charged carboxyl terminus known to bind heparin (3) (Figure 1). The Kunitz 1 domain binds the TF/factor VIIa complex (4), the Kunitz 2 domain binds factor Xa. It is the formation of this quaternary TF-VIIa-TFPI-Xa complex which dampens ongoing coagulation. TFPI is also a direct inhibitor of factor Xa independent of the TF/factor VII complex (2). Recently, it has been identified that Protein S enhances this TF-independent effect through interaction with the third Kunitz domain (5, 6).

Multiple forms of TFPI are found in the circulation and in tissue, products of both proteolytic cleavage and alternate splicing (7). Full-length TFPI-alpha contains the three Kunitz domains and the carboxyl terminus as described above. Two alternatively spliced forms are present in mice and one in humans. TFPI-beta is an alternatively spliced form that does not contain the third Kunitz domain and has an alternative carboxyl terminus and has been identified in mice and humans. *In vitro*, endothelial cells express TFPI-beta at a ratio of 0.1 to 0.2 to that of TFPI (8). TFPI-beta contains a direct GPI anchor not present in TFPI-alpha. TFPI-alpha binds the cell surface through a yet to be identified indirect GPI anchor and binds to endothelial glycosaminoglycans via its carboxyl terminus. It is this latter interaction that is thought to result in heparin-induced increases in plasma TFPI found in humans.

Mice also express TFPI-gamma which also lacks the Kunitz 3 domain and has yet another distinct carboxy terminus from the other two forms. In mice, data suggests that TFPI-beta may be the dominant form in the adult while TFPI-alpha may be expressed through development (7). Consistent with these findings, mice have less heparin releasable TFPI than do humans.

Although alternatively spliced forms may account for some of the heterogeneity of TFPI forms *in vivo*, proteolysis may also be responsible for additional forms. Cleavage of human TFPI has been demonstrated in multiple settings. Recombinant TFPI is a substrate for plasmin cleavage, and this cleavage may predispose to rethrombosis in the setting of clot lysis (9). Belaaouaj and colleagues demonstrated that exposure of TFPI to matrix metalloproteinases including MMP-1, MMP-7, MMP-9, and MMP-12 results in decreased TFPI activity (10). They speculated that vascular inflammation with upregulation of MMPs may induce downregulation of TFPI activity through this mechanism. Of these, MMP-12 targets cleavage sites which would isolate the K3 and the carboxyl terminus of TFPI (TFPI-CT) and may continue to function independently of TF. Interestingly, Ohkura and colleagues identified that thrombin can cleave TFPI at multiple sites (11). *In vitro*, the initial product of thrombin-mediated cleavage is TFPI-CT. In concert with the prior studies, Yun and colleagues recently identified that TFPI is highly sensitive to bacterial omptins expressed by Gram negative bacteria (12). They suggest that this might be a mechanism by which pathogens induce a prothrombotic state.

TFPI activity is regulated in the vasculature and circulation by plasmin (13) and lipoprotein(a) [Lp(a)] (14) respectively. Our goal was to determine the effects of plasmin on TFPI on the cell surface and in extracellular matrix (ECM) of endothelial cells (ECs) and smooth muscle cells (SMC) *in vitro* and *in vivo*. Plasmin attenuated cell surface and matrix associated TFPI activity in ECs in culture. The proenzyme, plasminogen had no such effect on cell surface TFPI or matrix TFPI. TFPI antigen on the cell surface was also significantly reduced by plasmin. Plasmin also decreased TFPI activity of normal arteries in frozen sections of normal arteries while plasmin treatment of atherosclerotic plaques sections eliminated TFPI immunoreactivity of luminal EC and intimal SMC. Together, these studies demonstrated that plasmin cleaves the majority of surface and matrix EC-associated TFPI and may remove TFPI from vascular sources as well.

TFPI is known to bind lipoproteins in blood. We have studied the interaction TFPI with lipoprotein metabolism and the potential role in atherosclerotic disease prevention. Lp(a) has been demonstrated to have both antifibrinolytic and atherogenic effects, and it is thought that these effects are the mechanism by which Lp(a) is pro-atherosclerotic (15). To define the relevance of the interaction between TFPI and Lp(a), we studied the binding and functional effects of Lp(a), its constituents, apolipoprotein (a) [apo(a)] and low-density lipoprotein (LDL), and lysine-plasminogen (L-PLG) on TFPI. In these

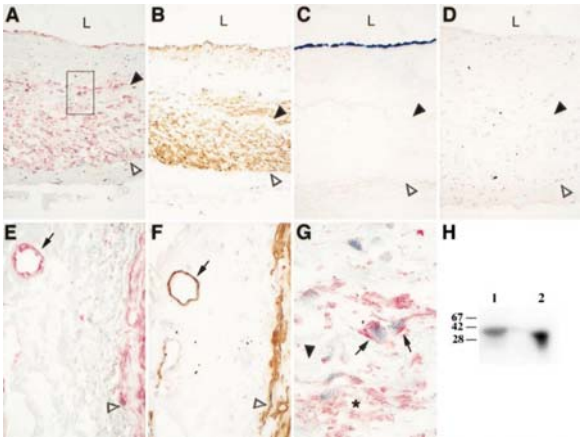

Figure 2. Photomicrographs of fibrocellular atherosclerotic plaque from human carotid endarterectomy specimens. A, Hematoxylin and eosin staining of the intima of the plaque (original magnification x20). Arrow denotes internal elastic laminae in A-D. B, Adjacent section showi α-smooth muscle actin staining in the intima (immunoperoxidase with DAB substrate; original magnification x20). C, Adjacent section showing TFPI staining throughout the intima (alkaline phosphatase with Vector Blue substrate; original magnification x20). D, Adjacent section using an isotype-matched IgG control antibody (original magnification x20). E, TFPI staining along the endothelium and throughout the intima of a fibrocellular plaque (alkaline phosphatase with Vector Blue substrate; original magnification x20). Arrow denotes adventitial vessel in E and F. F, vWF staining of adjacent section showing intact endothelium (alkaline phosphatase with fast red substrate; original magnification x20). G, Double immunostaining f α-smooth muscle actin (immunoperoxidase with DAB substrate) and TFPI protein (alkaline phosphatase with Vector Blue substrate) in the intima of a fibrocellular plaque (original magnification x100). Arrow indicates cell-associated staining; closed arrowhead, external elastic lamina. *Extracellular staining for TFPI... H, Western blot of human coronary artery homogenate (lane 1) and conditioned media from 293 cells transfected with pCMV-TFPI plasmid (lane 2). Reproduced with permission from (42).

studies, we showed that Lp(a), apo(a), and PLG but not recombinant TFPI (rTFPI) which was bound to LDL *in vitro*. We also demonstrated that apo(a) bound to a region within the C-terminus of TFPI (the last 37 amino acid residues). The binding affinity for TFPI was higher for Lp(a) (K_D ~150 nM) compared to PLG (K_D ~800 nM), and nanomolar concentrations of apo(a) (500 nM) inhibited PLG binding to TFPI. Furthermore, we showed that Lp(a) inhibited rTFPI activity and endothelial cell surface TFPI activity *in vitro*. In human atherosclerotic plaque, apo(a) and TFPI coexist in SMC-rich areas of the intima.

Taken together, these studies demonstrate that multiple forms of TFPI exist as a result of alternative splicing and proteolysis. This diversity allows for regulation of the antithrombotic actions of TFPI but may also impact on the non-antithrombotic activities of TFPI as well. This may include generation of truncated forms which may have unique functions.

4. EXPRESSION OF TFPI

Expression of TFPI is essential for murine development (16). Genetic deletion of the Kunitz 1 domain results in intrauterine lethality (E9.5- E11.5) associated with yolk sac and placental abnormalities. Evidence of intravascular thrombosis and hemorrhage suggest a consumptive coagulopathy. These abnormalities result from an imbalance between pro- and anti-coagulant proteins as rescue of these mice can be achieved through deletion of factor VII (17) or reductions in murine TF expression (18). Furthermore, these TFPI-$K1^{+/-}$ mice have been bred into a strain expressing factor V Leiden resulting in lethality due to perinatal thrombosis (19).

No human deficiencies of TFPI have been described. In human plasma, TFPI exists in small quantities (<5%) as a free full-length protein but predominantly in association with lipoproteins (20-23). The normal physiological concentration of TFPI in plasma is between 2.5 and 5 nM (24) with a half life of between 1 to 2 hours (25). The lipoprotein associated TFPI is a truncated form which has been shown to be less active *in vivo* than full-length TFPI.(26) Although originally identified in a hepatoma cell line (27), the primary source of TFPI expression *in vivo* is the endothelium. Our laboratory utilized a targeted Cre-Lox strategy to delete the Kunitz 1 domain in a tissue specific manner (28). In these studies, endothelial cells are responsible for approximately 50% of circulating forms of TFPI while myelomonocytic cells account for 20%. Although the endothelium is the primary cellular source of TFPI protein expression in the adult, TFPI mRNA are found in abundance in many tissues including the placenta, the heart, platelets, and the vasculature (18, 29).

Although no deficiencies have been identified, TFPI levels may be altered in disease. In a retrospective study, elevated levels of free circulating TFPI have been associated with unfavorable outcomes in unstable angina (30). However, two prospective clinical studies indicate that low circulating levels of TFPI are associated with increased risk of vascular disease and thrombosis. In the PRIME study (31), low circulating levels of TFPI were associated with a two-fold increased risk of hard coronary heart disease events (fatal and nonfatal myocardial infarction). Furthermore, low levels of TFPI were associated with risk of deep venous thrombosis in the Leiden Thrombophilia Study (32).

TFPI is expressed in the vasculature, in luminal endothelial cells, endothelial cells in the adventitia, and in vascular smooth muscle cells (Figure 2). In disease, advanced atherosclerotic plaques contain macrophages expressing TFPI including those in the “shoulder regions” of plaque associated with the sites of plaque rupture and thrombosis (Figure 3). Several studies have shown that TF is expressed in the adventitia of normal human arteries (33-

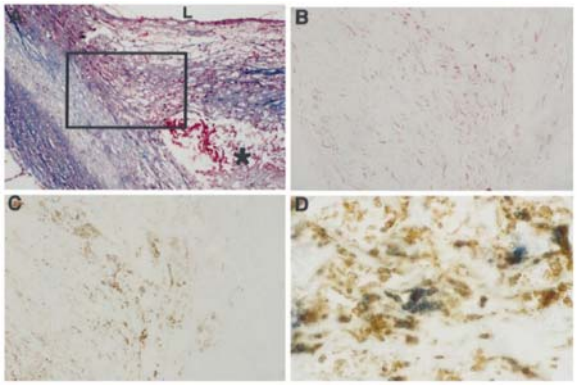

Figure 3. Photomicrographs of advanced atherosclerotic plaque from human carotid endarterectomy specimens. A, Masson trichrome staining of an advanced atherosclerotic plaque with a typical lipid-rich necrotic core (*) (original magnification x20). The inset outlines an area in the shoulder of the plaque shown enlarged in B and C. B, Adjacent section showing TFPI staining in the shoulder region of the plaque (alkaline phosphatase with fast red substrate; original magnification x40). C, Adjacent section showing CD68 staining for macrophages in the shoulder region of the plaque (immunoperoxidase with DAB substrate; original magnification x40). D, Double immunostaining for CD68 (immunoperoxidase with DAB substrate) and TFPI protein (alkaline phosphatase with Vector Blue substrate) in the shoulder region of the plaque (original magnification x100). Reproduced with permission from (29).

35), also in the intima of human atherosclerotic plaques (36), and the neointima of balloon injured arteries (37). It has been suggested that intraplaque TF is an important determinant of thrombogenicity following plaque rupture (38-40). TF and TFPI expression is linked to sites of fibrin deposition in advanced plaques (41). We have demonstrated that TFPI expression in plaque regulates the thrombotic potential of TF-mediated plaque TF thrombogenicity (29).

TFPI expression is regulated by growth factors found within atherosclerotic plaques. *In vitro*, TFPI expression in VSMC is stimulated by fetal bovine and human serum induced (5-fold increase in TFPI antigen and activity) (42). Additionally, PDGF B and EGF increased TFPI secretion similarly and account for approximately 40% of the TFPI secretion effects of human serum. This effect is due in part to modest transcriptional effects of these growth factors. The serum effect was associated with a 3-fold increase in TFPI mRNA 8 hours following release from growth arrest and a 70% decrease in TFPI secretion following treatment with actinomycin D. These studies suggest that there is significant TFPI expression in VSMC *in vitro* and *in vivo* which may account for the remainder of TFPI expression. Furthermore, these studies demonstrate that TFPI is expressed at sites of active vascular remodeling (i.e. atherosclerotic plaques).

5. TFPI REGULATION OF MACROVASCULAR REMODELING

Attempts to define TFPI function have included depletion and deletion studies. Early studies using a neutralizing antibody in rabbits showed that a reduction in TFPI sensitized rabbits to DIC (43). We and others have used murine models to address the role of TFPI in macrovascular remodeling. These studies have tested varied levels of TFPI expression in models of acute and chronic vascular injury and remodeling. Initially, we used an arterial flow cessation model to determine the effects of overexpression and heterozygotic deletion of TFPI on vascular remodeling. We demonstrated that in this model, ligation of the carotid artery results in enhanced TF expression (44). To study overexpression of TFPI, we generated an adenoviral vector to overexpress murine TFPI. Mice were treated with intravascular adenoviral delivery of the virus expressing murine TFPI or a control adenovirus. Overexpression decreased vascular TF activity compared to viral control and inhibited neointimal formation and resulted in enhanced luminal area. We also used this model to study mice which are heterozygous for the genetic deletion of the first Kunitz domain of TFPI (TFPI-$K1^{+/-}$) or wildtype (WT) littermates. There was greater neointimal formation and smaller luminal areas in the TFPI-$K1^{+/-}$ mice compared to TFPI-$K1^{+/+}$ littermates. This increased neointimal formation was associated with increased cellular proliferation in TFPI-$K1^{+/-}$ mice compared to TFPI-$K1^{+/+}$ mice. Similar beneficial effects were seen with TFPI protein delivery in a rabbit model of arterial restenosis (45). Adenoviral overexpression of TFPI inhibited a rabbit model of restenosis (46). This effect was thought mediated through the thrombotic effects of TFPI as well as through inhibition of monocyte activity. Taken together, these studies suggest that TFPI expression is an important mediator of the vascular remodeling response through thrombotic and nonthrombotic mechanisms.

To establish an experimental animal model of modulated vascular smooth muscle cell-derived TFPI and examine its effects on chronic vascular remodeling, transgenic mice in which a cDNA-encoding murine TFPI is expressed from the murine SM22-alpha promoter were generated (47). Arterial expression of transgenic mRNA was 4-fold higher than the level of endogenous TFPI mRNA. *In situ* hybridization confirmed that expression of the transgene was limited to medial vascular smooth muscle cells. In a ferric chloride-induced model of carotid thrombosis, homozygotic transgenic mice demonstrated resistance to thrombotic occlusion compared to wildtype littermates.

To determine the role of TFPI in the development of atherosclerosis, we bred SM22-alpha TFPI into the $ApoE^{-/-}$ background (48). On a high fat diet, SM22-alpha TFPI/$ApoE^{-/-}$ mice were shown to have less aortic plaque burden compared to $ApoE^{-/-}$ mice (Figure 4). Similarly, TFPI-$K1^{+/-}$ mice had more atherosclerotic plaque when bred into the $ApoE^{-/-}$ background (49). However, quite unexpectedly, the SM22-alpha TFPI/$ApoE^{-/-}$ mice had lower plasma cholesterol levels compared to $ApoE^{-/-}$ mice.

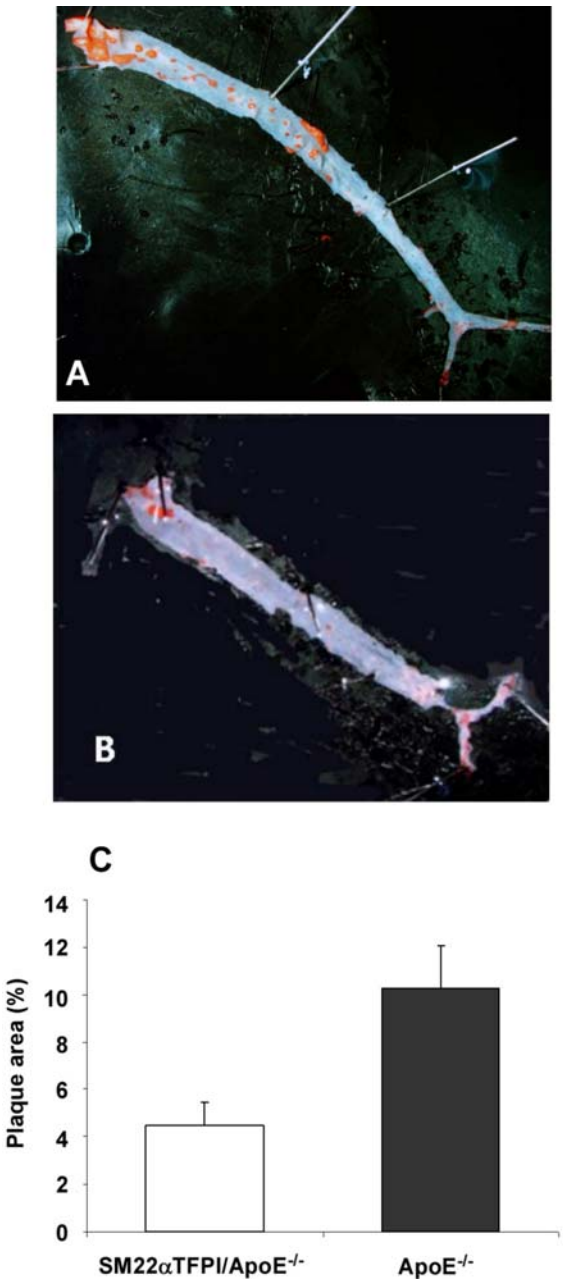

Figure 4. Vascular directed overexpression of TFPI reduces aortic plaque burden in ApoE-deficient mice. Atherosclerotic plaques in $ApoE^{-/-}$ (A) and $SM22\alpha$-$TFPI/ApoE^{-/-}$ (B) mice stained with Sudan IV for en face analysis. C, Comparison of plaque areas in aorta from $ApoE^{-/-}$ (n=13) and $SM22\alpha$-$TFPI/ApoE^{-/-}$ mice (n=12). *P<0.02. Reproduced with permission from (48)

Furthermore, SM22-alpha TFPI mice fed a high fat diet had lower cholesterol levels than did wildtype mice.

Given that TFPI associates with lipoproteins and its carboxy terminus (TFPI-CT) has been shown to be a ligand for the VLDL receptor, we hypothesized that TFPI overexpression may alter lipoprotein distribution. We quantified VLDL binding and uptake *in vitro* in mouse aortic smooth muscle cells (mASMCs) from SM22-alpha TFPI and wildtype mice. mASMCs from SM22-alpha TFPI mice demonstrated higher VLDL binding and internalization compared to those from wildtype mice. Since SM22-alpha TFPI mice have increased circulating levels of TFPI antigen, we examined whether TFPI-CT may act to alter lipoprotein distribution. *In vitro*, TFPI-CT increased VLDL binding, uptake, and degradation in murine embryonic fibroblasts. *In vivo*, administration of TFPI-CT lowered cholesterol levels in $ApoE^{-/-}$ mice. These studies suggest that TFPI overexpression lowers plasma cholesterol through the interaction of its carboxy terminus with lipoproteins.

Another pathologic state in which vascular remodeling is a key feature is pulmonary hypertension (PH). We hypothesized that inhibition of the tissue factor pathway would result in attenuation of pathophysiologic parameters typically associated with hypoxia-induced PH. We tested this hypothesis using a chronic hypoxia-induced murine model of PH utilizing SM22-alpha TFPI mice that have increased pulmonary TFPI expression compared to wildtype (WT) mice (50). In WT mice, hypoxia (28 days at 10% O2) resulted in increased systolic right ventricular and mean pulmonary arterial pressures. These pressures were significantly reduced in SM22-alpha TFPI mice. These physiologic changes were associated with structural changes as well. These included pulmonary vascular muscularization in WT mice which was significantly reduced in SM22-alpha TFPI mice. SM22-alpha TFPI mice had less pulmonary fibrin deposition following exposure to hypoxia consistent with antithrombotic effects of TFPI. Finally, SM22-alpha TFPI mice had a reduced number of proliferating pulmonary VSMCs consistent with *in vitro* findings. However, in mice in which the K1 domain is deleted from endothelial cells, there was no worsening of pulmonary pressures compared to control mice perhaps suggesting a noncoagulant effect of overexpression.

Taken together, these studies suggest that local expression of TFPI regulates macrovascular remodeling (Figure 5). Of course, as local fibrin generation is an important feature of vascular remodeling, we cannot exclude the contribution of a TF-dependent mechanism. However, data *in vivo* and *in vitro* suggest an independent role for the unique basic carboxyl terminus.

6. TFPI REGULATION OF ANGIOGENESIS

Evidence has accumulated that TF and the TF-VIIa complex can promote tumor growth, tumor metastasis, and angiogenesis (51-53). Although TF is not normally expressed on endothelial cells, its expression is upregulated on endothelial cells within breast cancer and elevated levels of TF correlate with an invasive carcinoma phenotype (54). TF expression in tumor cells correlates with the ability of tumors to secrete vascular endothelial growth factor (VEGF) and consequently induce an angiogenic response when implanted in immunodeficient mice (55). The TF/VIIa protease complex may also promote angiogenesis

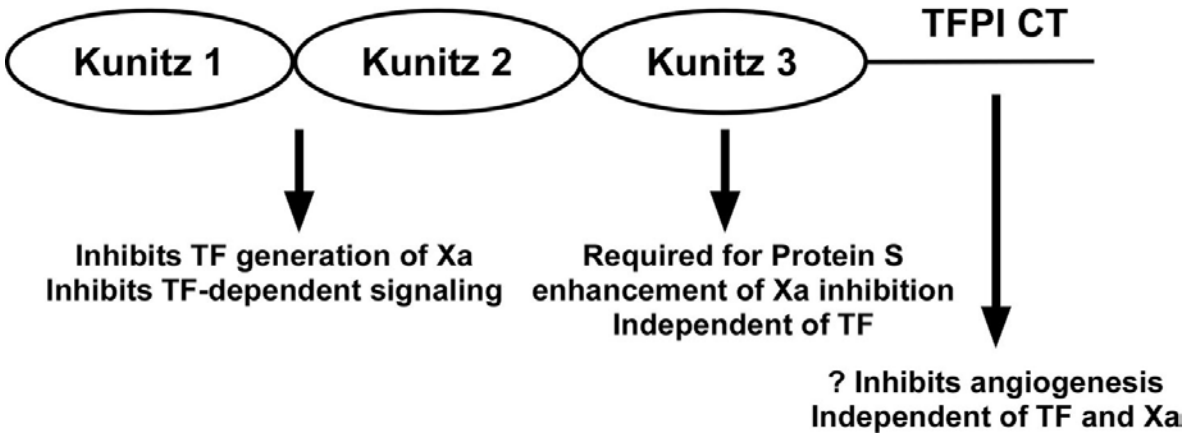

Figure 5. Schematic demonstrating the multiple mechanisms by which TFPI may affect vascular structure.

through protease-activated receptor-2 (PAR-2) signaling (56-58). Tissue factor pathway inhibitor (TFPI) is the physiological inhibitor of TF-mediated coagulation, its role in inhibiting TF signaling has been studied.

The broad functionality of TFPI includes TF-dependent and TF-independent inhibition of factor Xa, inhibition of TF-FVIIa dependent activation of PAR-2 signaling, and includes emerging evidence of independent effects of TFPI-CT. As TFPI is expressed on endothelial cells, its potential to affect angiogenesis has also been studied. Endothelial cells overexpressing PAR2 and TF demonstrated reduced PAR2 signaling in the presence of recombinant TFPI (59).

TFPI has known to exert anti-tumor effects. Hembrough demonstrated that direct injection of TFPI surrounding B16 melanoma tumors inhibited growth (60). However, while TFPI did not affect proliferation of B16 cells cultured *in vitro* (61), it did inhibit proliferation of endothelial cells, albeit at supraphysiologic concentrations (0.5 μM), indicating that TFPI may act indirectly on tumor growth by inhibiting angiogenesis. A truncated form of TFPI, containing only the first two Kunitz domains, had no anti-proliferative activity. Hembrough (62) in later studies demonstrated inhibition of proliferation using TFPI-CT at supraphysiological (40 uM) concentrations via an apoptotic mechanism. The mechanism by which TFPI may inhibit angiogenesis at nanomolar concentrations (5-20 nM) and *in vivo* has not yet been established. The importance of concentration was highlighted by Provencal who demonstrated inhibition of *in vitro* endothelial cell migration induced by S1P by full-length TFPI at 20 nM but no effect on proliferation (63). Taken together, these studies support a role for the TFPI-CT in the regulation of endothelial function and angiogenesis.

7. SUMMARY

TFPI is a remarkable molecule of endothelial quiescence; ensuring not only an anti-coagulant internal vascular surface but also an anti-angiogenic and anti-atherogenic vasculature. These diverse properties are important to our understanding of basic animal physiology as well as the pathophysiology of the major causes of death and disease worldwide: atherosclerosis and cancer. Further work is necessary, and on going, to delineate the molecular mechanisms and pathways behind the effects described above and discover further aspects to the biology of this unique molecule.

8. ACKNOWLEDGEMENTS

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