The Role of Tissue Factor Pathway Inhibitor in Tumor Growth and Metastasis

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ABSTRACT

Clotting activation occurs frequently in cancer. Tissue factor (TF), the most potent initiator of coagulation, is expressed aberrantly in many types of malignancy and is involved not only in tumor-associated hypercoagulability but also in promoting tumor angiogenesis and metastasis via coagulation-dependent and coagulation-independent (signaling) mechanisms. Tissue factor pathway inhibitor (TFPI) is the natural inhibitor of TF coagulant and signaling activities. Studies have shown that TFPI exhibits antiangiogenic and antimetastatic effects in vitro and in vivo. In animal models of experimental metastasis, both circulating and tumor cell–associated TFPI are shown to significantly reduce tumor cell–induced coagulation activation and lung metastasis. Heparins and heparin derivatives, which induce the release of TFPI from the vascular endothelium, also exhibit antitumor effects, and TFPI may contribute significantly to those effects. Indeed, a non-anticoagulant low-molecular-weight heparin with intact TFPI-releasing capacity has been shown to have significant antimetastatic effect in a similar experimental mouse model. The evidence supporting the dual inhibitory functions on TF-driven coagulation and signaling strengthen the rationale for considering TFPI as a potential anticancer agent. This article primarily summarizes the evidence for antiangiogenic and antimetastatic effects of TFPI and describes its potential mechanisms of action. The possible application of TFPI and other inhibitors of TF as potential anticancer agents is described, and information regarding potential antitumor properties of TFPI-2 (which has structural similarities to TFPI) is also included.

KEYWORDS: Tissue factor pathway inhibitor, cancer, metastasis, angiogenesis, tissue factor

HYPERCOAGULABILITY AND CANCER

It is now well established that many cancer patients exhibit a hypercoagulable state typically manifested as low-grade disseminated intravascular coagulation. Thrombosis occurs frequently in cancer patients and represents the second most important cause of death from malignancy. Not all tumor types are equally associated with thromboembolic events; patients with tumors of the lung, pancreas, and gastrointestinal tract tend to be more hypercoagulable than those with breast

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or renal cancer. In addition, the prevalence of thromboembolic disease varies depending on the treatment protocol. Although the mechanism by which coagulation is activated in cancer is multifactorial, tissue factor (TF), the primary initiator of coagulation, has been traditionally recognized to play an important role in this process. Tumor cells of several types are known to constitutively express TF on their surfaces and possibly even trigger the production of TF by adjacent host cells (monocytes and endothelial cells). Recently, two forms of circulating TF have been described: an alternatively spliced soluble protein and a form associated with cell-derived microparticles (MPs). The latter has received special attention as it has been demonstrated in vitro that TF activity is secreted from cancer cells predominately in association with shed MPs, suggesting its potential contribution to the prothrombotic state observed in cancer. High levels of circulating TF have been found, in correlation with hemostatic markers, in the plasma of patients with different cancer types. A recent study showed remarkably high levels of microparticle-associated TF in a lung cancer patient with a severe form of Trousseau’s syndrome. However, in this case, TF activity in the patient’s plasma did not correlate with the high levels of circulating TF. In addition, the level of TF expression of certain tumors is not proportionately translated into a level of circulating TF. In addition, the level of TF expression is limited to extravascular sites (F) VII binds to exposed TF receptor and undergoes proteolytic activation to become FVIIa. TF can also bind to FVIIa (which comprises 1% of the total plasma FVII) directly. This TF/FVIIa complex acts in concert with anionic membrane phospholipid to convert circulating factors IX and X to IXa and Xa, respectively. FXa is the active catalytic component of the prothrombinase complex, which converts circulating prothrombin to thrombin. Thrombin in turn activates platelets and cleaves fibrinogen to produce an insoluble fibrin clot.

TF-dependent reactions are regulated by the natural plasma protein inhibitor TFPI, which suppresses coagulation by neutralizing the catalytic activity of FXa and/or forming a quaternary inhibitory complex with TF, FVIIa, and FXa on the cell membrane.

In vivo, TFPI is present in three pools: (1) the endothelium, (2) platelets (~8 ng/mL), and (3) associated with plasma lipoproteins (~100 ng/mL). Endothelium is the major source of TFPI, and heparin treatment of cultured endothelial cells induces the release of TFPI from intracellular stores without affecting its surface concentration. TFPI levels are elevated in advanced cancer, and cancer patients show an enhanced release of TFPI in response to heparin. In vitro, it has been shown that with increasing inflammatory stimuli (which enhance TF expression and activity), the expression of TFPI cannot keep pace. This may be the case in malignancy. In vivo, recombinant human TFPI is cleared from the circulation in a biphasic manner with a rapid α-phase half-life ($t_{1/2a}$) of 2.4 minutes and a terminal β-phase half-life ($t_{1/2b}$) of 19.5 minutes. From a therapeutic standpoint, this quick clearance is clinically inconvenient and potentially costly in conditions such as cancer for which continuous or regular administration would be required.

As a result of alternative mRNA splicing, two different forms of TFPI are produced: TFPI-α and TFPI-β. TFPI-α and -β migrate with the same apparent molecular weight (46 kDa) in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) but differ in the degree of carbohydrate sialylation. TFPI-α is a soluble glycoprotein that consists of an acidic N-terminal region followed by three tandem Kunitz-type protease inhibitor domains and a basic C-terminal region. The Kunitz-1 and -2 domains bind and inhibit TF-FVIIa complex and FXa, respectively. The Kunitz-3 domain (which lacks proteinase inhibitory activity) as well as the C-terminal domain of TFPI have been shown to be involved in TFPI cell-surface localization. In addition, a peptide within the C-terminal domain (residues 254 to 265) has been shown to exhibit anticoagulant activity by acting on TF and prothrombinase complex independently of the Kunitz domains. In TFPI-β, Kunitz-3 and the C-terminal domains (of TFPI-α) are replaced with an unrelated C-terminal region that directs membrane anchoring. Although TFPI-β comprises ~20% of

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the cell surface TFPI, it is responsible for most of the cellular FXa-dependent FVIIa/TF inhibitory activity of TFPI.25

**TF INVOLVEMENT IN TUMOR ANGIOGENESIS**

TF is aberrantly expressed in many tumor cell types including colon cancer,33 pancreatic cancer,34 glioma,35 breast cancer,36 and non–small cell lung cancer.37 Furthermore, increased TF expression in tumors is associated with increased angiogenesis and higher tumor grades.33,38 TF promotion of the angiogenic phenotype may be due to the upregulation of vascular endothelial growth factor (VEGF; a potent angiogenic factor) and the downregulation of thrombospondin, an antiangiogenic factor.39,40 In vitro studies have shown a direct correlation between TF and VEGF production by melanoma and breast cancer cells.41

TF influences tumor angiogenesis through both coagulation-dependent and coagulation-independent mechanisms. The coagulation-dependent pathway involves TF activation of FVIIa followed by thrombin generation, subsequent platelet activation, and fibrin clot formation. Coagulation-independent mechanisms involve (a) phosphorylation of the cytoplasmic domain of TF and downstream signaling events40 and (b) protease-activated receptor 2 (PAR-2)-dependent signaling. After ligand binding, the cytoplasmic domain of TF has been shown to bind actin-binding protein 280 (ABP-280), an interaction that appears to promote cell adhesion and migration by regulating the phosphorylation of focal adhesion kinases.42 The other coagulation-independent mechanism involves the activation of PAR-1 and PAR-2 by either the TF/VIIa complex or the TF/VIIa/Xa complex leading to an acceleration of angiogenesis.43 Recently, Ahamed and colleagues demonstrated that TF/VIIa-mediated coagulation and signaling involve distinct cellular pools of TF and that TF signaling can be inhibited without any significant effect on the coagulant function of TF.44

**ANTIANGIOGENIC EFFECTS OF TFPI**

Using a B16 melanoma subcutaneous primary tumor model, Hembrough et al have shown that peritumoral injection of TFPI inhibited tumor growth.45 However, after 13 days, tumors exhibited similar growth kinetics to the controls possibly due to the secretion of a B16 melanoma protease that degrades TFPI. TFPI has also been reported to inhibit the growth of endothelial cells in vitro but not that of tumor cells.46 In the latter study, because TF is not expressed on quiescent endothelial cells, it was suggested that the antiproliferative effect of TFPI is not dependent on its anti-TF activity but mediated through interaction with the very-low-density lipoprotein (VLDL) receptor. Subsequently, a 23-amino-acid peptide corresponding with the basic carboxyl terminus of TFPI that binds to VLDL was shown to inhibit endothelial cell proliferation through an apoptotic mechanism.47 This peptide also exhibited a dose-dependent antiangiogenic effect. In our laboratory, the synthetic peptide KTSDKRKKQKVRK, corresponding with residues 254 to 265 within the TFPI C-terminus, exhibited anticoagulant activity as well as an antiproliferative effect against A375 human malignant melanoma and EAhy926 endothelial cells.48

The role of TFPI in regulating TF signaling through the PARs is complex. As mentioned above, TF-dependent signaling could take place via FXa in the ternary TF/VIIa/Xa complex that signals through PAR-2 or PAR-1 or via the TF/VIIa complex signaling through PAR-2 only.49–51 Recent evidence suggests that endothelial-expressed TFPI controls TF-mediated signaling through PARs and that proper localization of TFPI on the cell surface may be crucial for efficient inhibition of TF initiation phase signaling.52 Membrane TFPI has been shown to translocate the TF initiation complex into caveolae.53 Importantly, soluble TFPI concentrations required to block TF signaling in conjunction with PARs are higher than those required to inhibit TF/VIIa-dependent FXa generation (the step that triggers the coagulation cascade).52 In contrast, endogenous membrane TFPI of human umbilical vein endothelial cells (HUVECs) is equally efficient in inhibiting both PAR signaling and TF-dependent procoagulant activity. Therefore, the anticoagulant potency of TFPI may not correlate with its antisignaling and potential antiangiogenic potency. This may explain at least in part the failure of TFPI to reduce the overall 28-day mortality in a phase clinical trial in sepsis,54 a condition characterized by both TF-driven coagulation activation and inflammation.

**THE ROLE OF TF IN HEMATOGENOUS METASTASIS**

Studies in mice have shown that both the cytoplasmic tail of TF and the proteolytic activity of TF/VIIa are required for TF-dependent metastasis.55,56 Research in our laboratory using mouse models of experimental metastasis has focused on events that occur when metastatic tumor cells enter the circulating blood. In this model, TF-expressing tumor cells are injected into the tail vein and rapidly become trapped in the microvasculature of the lungs, the organ of first encounter. Within minutes, evidence of intravascular coagulation (e.g., falling platelet count and fibrinogen level) can be detected. In addition, the presence of increased plasma hemoglobin levels heralds the onset of microangiopathic hemolytic anemia, which is caused by the formation of fibrin strands in the microvasculature.57

Tumor cell–induced
(TF-mediated) thrombin generation and platelet activation can cause the release of inflammatory cytokines, such as CD40 ligand (CD40L). Platelet CD40L can activate the vascular endothelium and provoke an inflammatory response characterized by the upregulation of TF and adhesion molecules in endothelial cells. These processes can potentiate experimental metastasis in this murine model. Because we have previously investigated the effect of several anticoagulant and anti-platelet agents on experimental lung metastasis, the rationale for studying TFPI was particularly strong due to its anticoagulant and anti-inflammatory effects.

ANTIMETASTATIC EFFECTS OF TFPI

The potential therapeutic value of TFPI has been suggested by its ability to suppress coagulation activation in a variety of clinical scenarios including arterial balloon injury and sepsis. Although most experiments have used intravenous administration of purified TFPI, gene transfer of a TFPI expression vector into rabbits prevented arterial thrombosis due to shear stress or balloon injury.

In our laboratory, using three experimental approaches, we investigated whether TFPI could inhibit experimental metastasis as well as tumor cell–induced coagulation activation. First, murine recombinant TFPI (rTFPI; 0.7 μg/mouse) was injected intravenously in C57/BL6 mice shortly before the injection of B16 metastatic mouse melanoma cells. This almost completely abolished tumor cell–induced coagulation activation (Fig. 1A) and significantly reduced experimental lung metastasis (Fig. 1B). Second, B16 cells, which do not express TFPI, were transfected with a murine sense plasmid for TFPI. This significantly reduced the procoagulant activity of the tumor cells (Fig. 2) without

![Figure 1](image1.png)

**Figure 1** The effect of recombinant TFPI (rTFPI) injection on (A) tumor cell–induced thrombocytopenia and (B) experimental lung metastasis. Results suggest that rTFPI protected animals against tumor cell–induced coagulation activation and reduced tumor nodule formation in the lungs.

![Figure 2](image2.png)

**Figure 2** The effect of TFPI (sense, antisense, vector control) transfection of B16 melanoma cells on cellular procoagulant activity as measured by a one-stage clotting assay. Fourteen sense (S), two antisense (AS), and one vector-only cell lines were prepared and compared with untransfected wild-type B16 cells. S10 and AS1 were used for the experimental metastasis studies. Lung seeding 14 days after intravenous injection of \(2.5 \times 10^5\) B16 cells was assessed (inset). As shown, TFPI-expressing B16 cells produced significantly fewer pulmonary tumor nodules than TFPI-negative cells.
altering TF antigen expression on the surfaces of these cells. Furthermore, compared with nontransfected or antisense-transfected B16 cells, these cells failed to produce a significant fall in platelet count after their injection and formed significantly fewer lung tumors (Fig. 2, inset). Interestingly, although subcutaneous injection of TFPI (+) or TFPI (−) tumor cells produced similar-size primary tumors, two of five animals with TFPI (−) tumors developed spontaneous lung metastases compared with zero of five animals bearing TFPI (+) tumors. These results suggest that both circulating and tumor cell–associated TFPI appear to play a role in hematogenous metastasis. In our third approach, we performed transient intravenous gene transfer in C57/BL6 mice. In these experiments, TFPI sense, antisense, or vector plasmids were combined with a transfection agent and rapidly injected into mouse tail veins. Twenty-four hours later, the mice were injected intravenously with nontransfected B16 cells and subsequent lung metastasis measured. This approach resulted in a threefold increase in plasma TFPI levels 24 hours after transfection in animals injected with TFPI sense plasmid. In addition, mice transfected with sense plasmid exhibited a greater TFPI response to an unfractionated heparin injection. Sense plasmid–transfected mice developed significantly fewer lung tumor nodules (experimental metastases) than those receiving control plasmids.

The strong antimetastatic effect of a relatively small amount of rTFPI in our mouse model, though perhaps surprising, is comparable with the effects of small increases in TFPI resulting from transfection, either of cells or animals, and also with the effects of modest increases in circulating TFPI after heparin administration. In a later study, using a similar mouse model, Hembrough et al reported a significant reduction in B16 experimental lung metastasis by intraperitoneal injection of human TFPI (1 mg/kg). However, in this study, TFPI was injected daily after tumor cell injection. In addition, the latter study showed that another inhibitor of TF/FVIIa, the nematode anticoagulant protein rNAPc2, exhibited similar antimetastatic effect compared with TFPI, whereas rNAP5, a second nematode anticoagulant protein that specifically inhibits FXa, did not have significant antimetastatic activity. These results suggest that the proteolytic activity of TF/FVIIa may promote experimental metastasis by mechanisms independent of FXa activation. However, Donnelly et al have shown that subcutaneous injection of SCID mice with a specific active-site inhibitor of human FXa, rAcAP, prior to tail vein injection of human melanoma cells results in a dose-dependent reduction of experimental lung metastasis. Thus, the precise role of FXa in the antimetastatic effect of TFPI requires further study.

In our experience, in studies in which anticoagulant agents are introduced to mice before the intravenous injection of tumor cells, experimental metastasis is reduced significantly. These agents include Coumadin (warfarin), heparins, and hirudin. In addition, fate studies in our laboratory and that of others have demonstrated that after injection, tumor cells persist in the lung vasculature for a shorter period of time in anticoagulated or fibrinogen-deficient animals. Therefore, in this type of model, it is reasonable to suggest that any anticoagulant strategy that diminishes thrombin production or activity will have a similar effect, as the early events of the experimental metastatic process are coagulation– and platelet–dependent. However, if anticoagulant treatment (e.g., TFPI) were to be initiated after tumor cell inoculation, when tumor cell lodgment is complete, it may be possible to determine potential coagulation-independent antitumor effects. Understanding the role of TFPI in the metastatic process becomes more challenging when we consider that TFPI bound to extracellular matrix has been shown to act as a support for the TF/VIIa complex in promoting tumor cell adhesion and migration. Thus, it is possible to suggest that, within the circulation, therapeutic TFPI would exhibit both anticoagulant and antimetastatic effects, whereas in scenarios in which the extracellular matrix is exposed, TFPI can act as a prometastatic protein.

**POTENTIAL CONTRIBUTION OF TFPI TO THE ANTIMETASTATIC EFFECTS OF HEPARINS**

Heparins have proved to be effective in preventing thromboembolic complications in cancer patients, but more interestingly, their use may possibly be associated with beneficial effects in delaying cancer progression and prolonging survival. A recent prospective study designed to determine the potential value of long-term low-molecular-weight heparin (LMWH) therapy to improve survival in cancer patients suggested a striking survival advantage for heparin treatment in a subgroup of cancer patients with good prognosis.

Potential mechanisms of antitumor effects of heparins include (a) anti-FXa and antithrombin functions, (b) the release of TFPI from the endothelial cells, and (c) inhibition of matrix-degrading enzymes. In a recent study, we sought to assess the potential antimetastatic effect of a novel non-anticoagulant low-molecular-weight heparin (NA–LMWH) in a mouse model of experimental metastasis. NA–LMWH had no anti-Xa or anti-IIa activity compared with enoxaparin but had similar capacity to induce TFPI release from human endothelial cells in vitro (Fig. 3). Intravenous injection of B16 melanoma cells resulted in a significant and rapid fall (>50%) in the platelet counts of control mice previously injected with phosphate buffered saline (PBS). This reduction in platelet count was completely abolished.
in mice treated with enoxaparin. In contrast, the NA-LMWH had no effect on tumor cell–induced thrombocytopenia (Fig. 4A). However, a pretumor cell injection dose of NA-LMWH or enoxaparin followed by daily doses (for 14 days) reduced experimental lung metastasis by 70% (Fig. 4B, C). Because enoxaparin and NA-LMWH were equally effective at inducing the release of TFPI from vascular endothelial cells, this could represent a key mechanism by which LMWH or NA-LMWH exert their antimetastatic effect.

**TFPI-2 AND CANCER**

TFPI-2 is a serine protease inhibitor with structural similarities to TFPI-1. Originally isolated from the human placenta as placental protein 5, it contains three tandem Kunitz-type domains with a short acidic amino terminal region and a highly basic C-terminal tail. TFPI-2 is synthesized by all vascular cells (endothelial cells, smooth muscle cells, and fibroblasts). It appears to function as a plasmin inhibitor, thus playing a major role in endothelial cell matrix (ECM) digestion and remodeling by preventing the release of growth factors and matrix metalloproteinases (MMPs), which leads to ECM degradation that facilitates tumor invasion and metastasis. Although TFPI-2 has homology to TFPI-1, it is known to only weakly inhibit coagulation via FVIIa/TF complex. Nonetheless, it is interesting to note that several studies have demonstrated that TFPI-2 behaves
as an antimetastatic, antiangiogenic, and proapoptotic agent thus making its function an area of intense study for cancer.79–81 In a study by Chand et al, TFPI-2–overexpressing fibrosarcoma cells when injected into athymic nude mice grew at a lower rate producing smaller tumors and generating less metastatic lung lesions in comparison with the mock-transfected cells. Also noteworthy is the finding that VEGF gene expression was downregulated in these cells indicating the possibility that TFPI-2 may be a key player in suppressing VEGF-mediated angiogenesis.79 In addition, other studies have shown high expression of TFPI-2 in benign tumors whereas lacking in malignant tumors.82 During tumor development, TFPI-2 is often downregulated in some aggressive cancer cells such as lung cancer, prostate cancer, and glioma.83–85 Kondraganti et al, using a malignant meningioma cell line, demonstrated that restoring TFPI-2 in these aggressive cells led to decreased invasiveness and a weak angiogenic potential both in vitro and in vivo.86 The role that TFPI-2 may play in poor tumor progression along with its antiangiogenic and antimetastatic properties, like TFPI-1, may potentially make it a promising protein for further studies in cancer biology.

CONCLUSION
Tissue factor is now recognized as a likely trigger for cancer-associated clotting activation, and its potential for promoting tumor angiogenesis and blood-borne metastasis is increasingly appreciated in the literature. Therefore, specific inhibition of TF activity in cancer may be beneficial not only by preventing tumor-associated thromboembolic complications but also by suppressing tumor growth and dissemination. Potentially, three types of therapeutics may be employed to specifically block TF coagulant and/or signaling functions. These are TFPI, anti-TF antibodies, and synthetic small-molecule TF inhibitors. Recent studies have identified TF-mediated coagulation and signaling pathways. TFPI possesses both anticoagulant and anti-TF signaling activities although higher concentrations may be needed in vivo for effective blockade of TF-initiated signaling. This dual inhibitory action of TFPI presents a clinical advantage in addressing the cancer patient’s hypercoagulable state as well as disease progression. However, TFPI’s short half-life presents a major obstacle (both in terms of in vivo concentrations and cost) for it to be administered directly and regularly in cancer patients. TFPI is released from the vascular endothelium in response to heparin and heparin derivatives; However, it is not clear to what extent this released TFPI can inhibit tumor progression in vivo. In animal studies, anti-TF monoclonal antibodies have been shown to inhibit experimental metastasis.57 Interestingly, an antibody developed by Ahamed and colleagues inhibits TF signaling but not the coagulant function.44 Such agents may be useful in adjunct treatment of human malignancies. Overall, targeting TF by antibodies or TFPI may be beneficial to cancer patients; however, preliminary studies are required to obtain bioavailability and safety profiles for such agents for their potential long-term use in human cancer therapy.

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ABBREVIATIONS
ABP 280 actin-binding protein 280
CD40L CD40 ligand
ECM endothelial cell matrix
F factor
HUVECs human umbilical vein endothelial cells
LMWH low-molecular-weight heparin
MMPs matrix metalloproteinases
MPs microparticles
NA-LMWH non-anticoagulant low-molecular-weight heparin
PAR protease-activated receptor
PBS phosphate buffered saline
rTFPI recombinant TFPI
SDS-PAGE sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TF tissue factor
TFPI tissue factor pathway inhibitor
VEGF vascular endothelial growth factor
VLDL very-low-density lipoprotein

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