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Review Article

Tissue factor pathway inhibitor in thrombosis and beyond: role of heparin

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Abstract

Tissue factor (TF) plays a crucial role in the pathogenesis of thrombosis, angiogenesis and inflammatory disorders, and the inhibition of this membrane protein thus provides a unique therapeutic approach for the prophylaxis and/or treatment of various diseases. In the past few years, TF pathway inhibitor (TFPI), the only endogenous inhibitor of the TF/FVIIa complex, has been characterized biochemically and pharmacologically. Studies in patients have demonstrated that both TF and TFPI may be indicators for the course and the outcome of cardiovascular and other diseases. Based on experimental and clinical data, TFPI may become an important drug or target for several clinical indications. Tissue factor pathway inhibitor is expected to inhibit the development of postinjury intimal hyperplasia and thrombotic occlusion in atherosclerotic vessels, as well as to be effective in acute coronary syndromes such as unstable angina and myocardial infarction. Of special interest is the inhibition of TF-mediated processes in sepsis and disseminated intravascular coagulation. At present, clinical studies with TFPI are rather limited, so the clinical potential of the drug cannot be properly assessed. However, TFPI and its variants are expected to undergo further development and to find indications in various clinical states.

Introduction

Tissue factor pathway inhibitor (TFPI) is a single-chain glycoprotein present in plasma in trace amounts. It was previously known as extrinsic pathway inhibitor or lipoprotein-associated coagulation inhibitor because of its binding to plasma lipoproteins. The translated amino acid sequence revealed that TFPI has a highly negatively charged *N*-terminus followed by three tandemly repeated Kunitz-type domains, as well as a highly positively charged *C*-terminus. The mature molecule consists of 276 amino acid residues, including 18 cysteines, all involved in disulfide bonds (Fig. 1).

Tissue factor pathway inhibitor recognizes factor Xa (FXa) most effectively when the enzyme is in the prothrombinase complex, which contains FXa, factor Va (FVa), calcium ions and phospholipids. This ability to inhibit prothrombinase is predominantly responsible for the prolongation of one-stage coagulation assays when exogenous TFPI is added to plasma *in vitro*. Heparin enhances, at least in part, the inhibition of FXa by TFPI through a template mechanism in which the simultaneous binding of FXa and TFPI to the same heparin molecule increases their interaction. Factor Xa-dependent inhibition of factor VIIa (FVIIa)/TF by TFPI involves the formation of a quaternary complex containing FXa/TFPI/FVIIa/TF, in which the second Kunitz domain of TFPI binds FXa and the first Kunitz domain binds FVIIa. This inhibitor complex could result from the initial binding of FXa to TFPI, with subsequent binding of the FXa/TFPI complex to FVIIa/TF, or alternatively, TFPI could bind to a preformed FXa/FVIIa/TF complex. Thus, at physiological concentrations, TFPI mediates feedback inhibition of the FVIIa/TF complex, but it does not inhibit the activity of FVIIa/TF until the complex has produced some FXa (Fig. 2). The requirement of FXa for the inhibition of FVIIa/TF by TFPI, however, is not absolute, and TFPI at higher concentrations inhibits the activation of factor IX (FIX) by FVIIa/TF in the absence of FXa.

Our understanding of the cascade, or waterfall, model of blood coagulation is undergoing certain changes with regard to the role of cellular components for the clotting process. In the so-called cell-based model, hemostasis

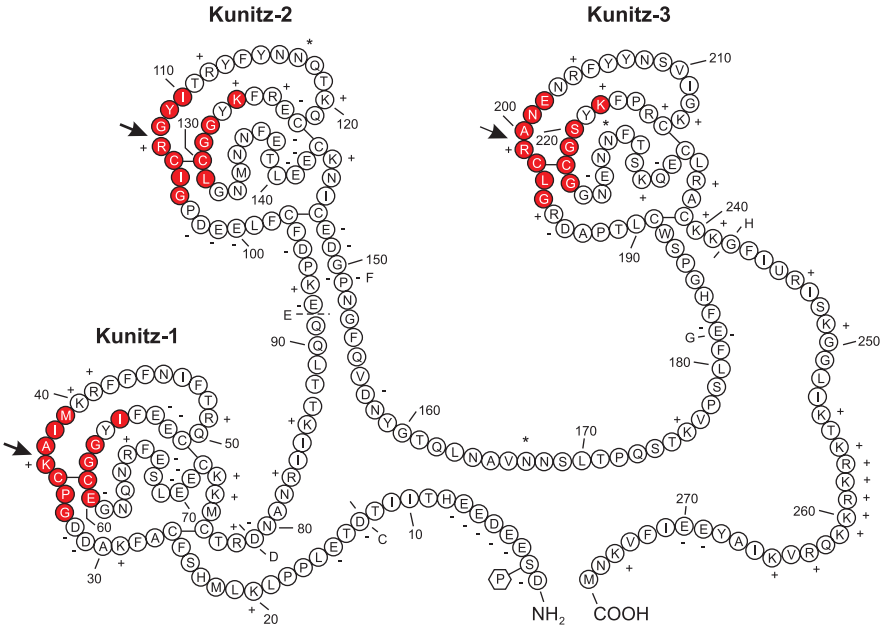

Fig. 1. Diagrammatic sketch of the different binding domains for tissue factor pathway inhibitor. Amino acid residues are numbered and the Kunitz-type domains are listed. The charges of the amino acid side chains are shown. Residues indicate the three inhibitory clefts of the Kunitz domain.

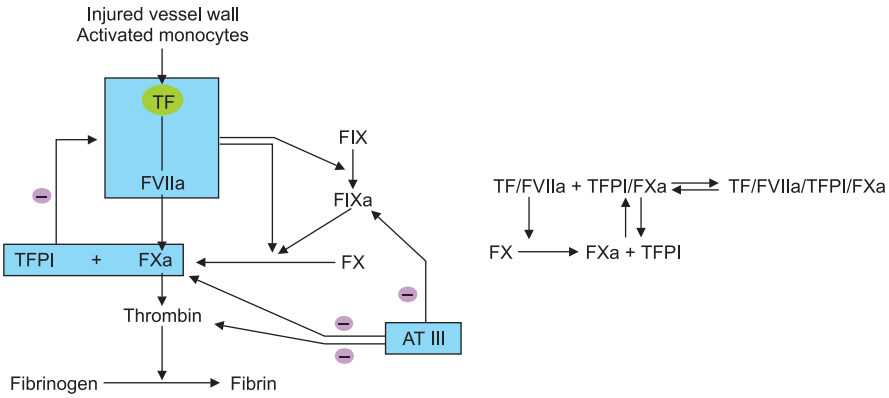

Fig. 2. Scheme of the initiation of the clotting process by the tissue factor (TF) pathway and mechanism of action of tissue factor pathway inhibitr (TFPI). AT = antithrombin.

is considered to occur in three overlapping phases: initiation, which occurs on TF-bearing cells or particles; amplification, in which platelets and cofactors are activated, leading to thrombin generation; and propagation, in which large amounts of thrombin are generated on the platelet surface (1). In both the cascade and the cell-based models of hemostasis, TF (which is a member of the cytokine receptor superfamily) is considered to be the primary physiological initiator of blood coagulation. The clotting process proceeds when TF is brought into close proximity to activated platelets and coagulation factors.

In addition to its essential role in physiological hemostasis, TF (which is exposed to the blood following vascular injury) also triggers a wide variety of thrombotic diseases and other hypercoagulable states. These include disseminated intravascular coagulation (DIC) induced by sepsis, as well as arterial thrombosis overlying an atherosclerotic plaque, a process that is known to be the final event in acute myocardial infarction (AMI) and unstable angina. New studies indicate that TF can function as a signaling receptor and, upon binding of FVIIa to TF, a signaling cascade can be initiated. Furthermore, it is suggested that this integral membrane protein exerts important nonhemostatic functions. Assembly of the TF/FVIIa complex on cellular surfaces may be important in mediating intimal hyperplasia, as well as for tumor metastasis and angiogenesis. Tissue factor has also been shown to play an important role in early embryonic development

(2-5). Because of the pathophysiological role of TF in various disease states, the inhibition of the TF pathway represents an attractive therapeutic target for the development of new pharmacological or biological agents (5).

Regulatory mechanisms of blood coagulation include antithrombin III (AT III), a plasma protease inhibitor that serves as a protease scavenger; any of the blood coagulation enzymes that move away from the growing clot rapidly form a complex with this protein, and their activities are neutralized. Protein C inactivates the active cofactor forms of factor VIII (FVIII) and factor V (FV), thus rapidly slowing blood coagulation.

Tissue factor pathway inhibitor represents another important regulatory mechanism of blood coagulation. Tissue factor-mediated plasmatic and cellular reactions can be influenced by TFPI, the only known physiologically significant inhibitor of the TF-initiated coagulation pathway. As described earlier, TFPI consists of 276 amino acid residues with 18 cysteine residues and 3 potential *N*-linked glycosylation sites, after removal of a 28-residue signal peptide. It contains an acidic *N*-terminal region followed by three tandemly repeated Kunitz-type serine protease-inhibitory domains and a basic *C*-terminal region (Fig. 1). Posttranslational modifications in the TFPI molecule include partial phosphorylation of serine-2, as well as *N*-linked glycosylation (6-8). These modifications can influence the pharmacokinetic/pharmacodynamic properties of TFPI. Recombinant TFPI (rTFPI) lacking posttranslational modifications may show functional differences compared to TFPI that is endogenously synthesized or released from endothelium (9).

Tissue factor pathway inhibitor produces an FXa-dependent feedback inhibition of the TF/FVIIa catalytic complex through the formation of a final, quarternary inhibitory complex consisting of TFPI/FXa/TF/FVIIa. In the first step, TFPI binds and directly inhibits activated FX via the second Kunitz domain. In the second step, the TFPI/FXa complex binds and inhibits FVIIa in the TF/FVIIa complex via the first Kunitz domain (Figs. 2 and 3) (10-15). A kinetic analysis of the regulation of the extrinsic pathway by TFPI suggested that FXa is initially inhibited when it is bound to or near TF/FVIIa on the membrane surface. This leads to an unexpectedly rapid inactivation of TF/FVIIa and the resulting inhibition of FX activation by the TF/FVIIa complex (16).

Extensive studies have been conducted to characterize TFPI regarding its biochemical actions and pharmacological properties (17, 18). During the last few years, the work on TFPI has been concentrated on the direct or indirect use of the inhibitor for various clinical indications, especially in the cardiovascular field. The aim of this paper is to review newer experimental and clinical results obtained with TFPI and further aspects of its use in clinical states. Data on human TFPI-2, a matrix-associated Kunitz inhibitor that inhibits the plasmin- and trypsin-mediated activation of zymogen matrix metalloproteinases (MMPs) involved in tumor progression, invasion and metastasis (19), are also presented.

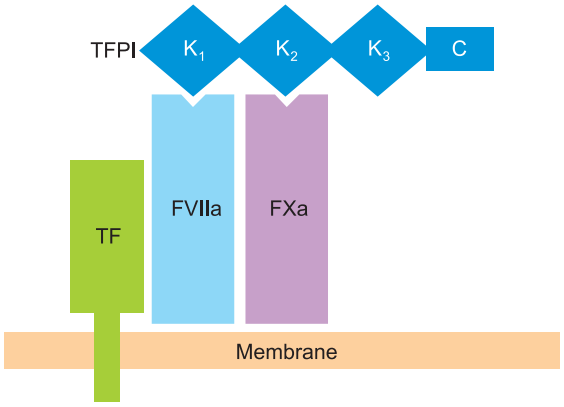

Fig. 3. Schematic illustration of the assembly of tissue factor pathway inhibitor (TFPI) binding to factor Xa (FXa) and tissue factor (TF)/factor VIIa (FVIIa).

TFPI structure and function

The plasma concentration of TFPI is low (≈ 2 nM), and much of the circulating TFPI is bound to lipoproteins, including low-density lipoprotein (LDL), high-density lipoprotein (HDL) and lipoprotein (a) (16, 17). The predominant forms of TFPI in plasma have molecular weights of 34 and 41 kDa, but less abundant forms of higher molecular mass are also present. This size heterogeneity of plasma TFPI reflects apparent proteolytic carboxy-terminal truncation of the molecule and the formation of mixed disulfide complexes with apolipoproteins (18). The major form of TFPI bound to LDL has a molecular weight of 34 kDa and lacks the distal portion of full-length TFPI, including at least part of the third Kunitz domain. The 41-kDa form of TFPI that circulates with HDL is apparently a similarly truncated form of TFPI linked to apolipoprotein AII (apo AII). Additional forms that have undergone less carboxy-terminal truncation and some full-length TFPI (43 kDa) also circulate in plasma.

Platelets carry approximately 10% of the total TFPI in blood and release their TFPI following stimulation by thrombin and other agonists (20). The concentration of TFPI in blood escaping from a superficial injury (template bleeding time) increases progressively and reaches levels 3-fold those of venous plasma obtained simultaneously. This additional TFPI is likely derived from platelets that aggregate at the site of the wound, although the potential contribution of other cells to the local increase in TFPI concentration cannot be excluded.

In vivo infusion of heparin increases the circulating levels of TFPI in plasma 2- to 4-fold (21, 22). Since the *ex vivo* addition of heparin to blood or plasma does not change the TFPI concentration, the *in vivo* effect of heparin appears to be mediated by the release of TFPI from intra- or extracellular stores. The source of this additional TFPI is thought to be the endothelium, where TFPI may be bound to heparan sulfate or other glycosaminoglycans at the endothelial surface. The TFPI released by heparin *in vivo* represents the full-length molecule. This

form possesses greater FXa- and prothrombinase-inhibitory activity and is enhanced to a greater extent by heparin than are the carboxy-terminal-truncated forms of TFPI that circulate in plasma. Thus, some of the anticoagulant and the antithrombotic effects of heparin therapy may be mediated by TFPI.

Tissue factor pathway inhibitor is rapidly cleared from the plasma with an initial half-life of ~2 min and a terminal half life of ~70 min. Tissue distribution studies showed that the liver is the predominant site of TFPI clearance, as early as 3 min. The clearance of TFPI produced in eukaryotic cells (glycosylated), as well as TFPI produced in *Escherichia coli* (nonglycosylated), is identical in rabbits. The liver, via receptor-mediated endocytosis, serves as the clearance organ for many circulating blood proteins and particles, including TFPI (23). Receptor-mediated endocytosis provides the major pathway for the trafficking of extracellular molecules into the cell. This involves the binding of a ligand to a specific cell-surface receptor, clustering of the ligand-receptor complexes in coated pits, invagination and pinching off of the coated pits to form coated vesicles, and delivery of coated vesicles to discrete membrane-limited cytoplasmic sorting organelles, the endosomes. Within these endosomes, ligands and receptors are each targeted to their appropriate cellular destination (*e.g.*, lysosome, cytoplasm, opposite cell surface). Two dominant receptor clearance systems are active in the liver: the asialoglycoprotein receptor and the LDL receptor-related protein (LRP). The asialoglycoprotein receptor governs the clearance of plasma glycoproteins whose carbohydrate groups terminate in galactose. Upon ligand binding, ligand and receptor are rapidly internalized and delivered to endosomes. The LRP governs the clearance of a wide variety of plasma proteins and particles, including plasma TFPI. Like the asialoglycoprotein receptor, LRP is internalized together with its ligands. Because the clearance *in vivo* of TFPI is independent of carbohydrate, it is unlikely that the asialoglycoprotein receptor is important in TFPI clearance.

A third pathway for TFPI uptake has also been suggested by Sevinsky *et al*. (24), who examined the fate of TF upon quaternary complex formation with FVIIa, FXa and TFPI on umbilical endothelial cells. Their cell fractionation studies suggest that the complex may transit the noncoated plasmalemmal vesicles, which are called caveolae. This pathway may serve to regulate cell-surface TF/FVIIa procoagulant activity. The relative contributions of these last two pathways (TFPI/FXa, TF/FVIIa/FXa/TFPI) to the overall cellular uptake of TFPI remain to be precisely determined.

Role of TFPI in disease

Low levels of TFPI are occasionally seen in septicemia and DIC, but more often TFPI concentrations are normal (25). The progression of DIC in the presence of a normal plasma level of TFPI is consistent with the fact that TFPI, at physiological concentrations, inhibits FVIIa/TF effectively only after FXa has been generated. Thus, TFPI dampens, but does not prevent, the coagulation process when continuing generation of TF occurs. Animal studies have shown that the depletion of endogenous TFPI sensitizes rabbits to DIC induced by TF or endotoxin infusion (26, 27). Conversely, the infusion of high therapeutic concentrations of TFPI, predicted to directly inhibit the FVIIa/TF complex in the absence of FXa, ameliorates the intravascular coagulation induced by TF in rabbits and prevents mortality in a baboon model of *E. coli* sepsis (28, 29).

Although TFPI deficiency might be expected to cause a prothrombotic phenotype, no individual with TFPI deficiency has yet been identified. The low plasma levels of TFPI found in abetalipoproteinemic patients (< 20% of normal individuals) appear to simply reflect the absence of LDL, a carrier of TFPI in plasma. The total TFPI in these patients, as estimated by plasma TFPI levels following heparin infusion, is similar to that of normal individuals (25). However, recent studies with knockout mice have shown that mice with a modified TFPI first Kunitz domain, leading to the expression of inactive protein, do not survive the neonatal stage, probably owing to unregulated TF/FVIIa hyperactivity, with the consequent consumptive coagulopathy and bleeding; this suggests that human TFPI-deficient embryos may suffer a similar fate (30).

Role of TFPI in vascular and cardiovascular disorders

The role of TFPI in modulating arterial thrombosis cannot be discussed without underlining the importance of the TF pathway in triggering intravascular thrombosis. The importance of TF-dependent activation of the coagulation cascade is suggested by several clinical and experimental studies. Results showed that the selective inhibition of TF/FVII complex formation by a monoclonal antibody and recombinant active site-blocked activated FVII results in marked antithrombotic effects in different *in vivo* models of arterial thrombosis (31, 32). Tissue factor has been identified in human atherosclerotic coronary and carotid plaques by immunohistochemistry in several cell types, such as monocytes, foam cells and fibroblasts (33, 34), and it has been shown that TF present in human plaques retains its procoagulant properties (35). More importantly, TF antigen and procoagulant activity have been shown in human atherectomy specimens obtained from patients with clinical evidence of acute coronary syndromes in significantly higher concentrations compared to patients with stable angina (36, 37). In addition, a recent study reached the same conclusions in carotid endoarterectomy specimens from patients with and without clinical evidence of cerebral ischemia (38). In that study, TF antigen and activity in specimens from patients with stroke or transient ischemic attacks were far higher than those obtained from patients with significant carotid stenosis due to an atherosclerotic plaque but without clinical evidence of cerebral ischemia. Interestingly, plaques

with detectable TFPI antigen by enzyme-linked immunosorbent assay (ELISA) or immunohistochemistry showed significantly reduced TF activity, suggesting that, within atherosclerotic plaques, TFPI is capable of modulating TF activity and plaque thrombogenicity (38, 39).

Role of TFPI in atherosclerosis/restenosis

The most important underlying mechanism for the development of acute coronary syndromes are atherosclerotic plaques. Spontaneous plaque rupture or acute interventions –such as balloon angioplasty, coronary atherectomy or stent placement– may increase the procoagulant activity of the vessel wall and especially expose TF to circulating blood, resulting in initiation of the clotting process with the formation of intravascular thrombi. As already mentioned, the effects of TF *in vivo* are very complex, and this membrane protein may play an important role in inflammatory processes associated with atherosclerosis and restenosis, among others. Because TF is upregulated after vascular injury and in atherosclerotic plaques, TFPI is expected to inhibit the development of postinjury intimal hyperplasia and thrombotic occlusion in atherosclerotic vessels.

Activation of inflammatory and procoagulant mechanisms is thought to contribute significantly to the initiation of restenosis, a common complication after balloon angioplasty of obstructed arteries. During this process, expression of TF represents one of the major physiological triggers of coagulation that results in thrombus formation and the generation of additional signals, leading to vascular smooth muscle cell (VSMC) proliferation and migration. Overexpression of TFPI suppressed the autocrine release of platelet-derived growth factor BB (PDGF-BB), monocyte chemoattractant protein-1 (MCP-1) and MMP-2 in response to FVIIa and FXa from VSMCs *in vitro*, and it inhibited monocyte TF activity. These results suggest that TFPI exerts its action through hemostatic and non-hemostatic mechanisms (40, 41).

Various studies on the role of TFPI in hyperplasia of VSMCs have been done using cell culture systems. In human pulmonary arteries, the expression of TFPI in SMCs is upregulated by treatment with serum or basic fibroblast growth factor (bFGF)/heparin, indicating that growth factors that can stimulate the vessel wall *in vivo* might locally regulate TFPI expression. The upregulation of TFPI could be pathophysiologically important for the regulation of TF-mediated coagulation within the vessel wall, and thus, for the course of hyperplasia associated with pulmonary hypertension and atherosclerosis (20). Increased expression of TFPI after initial expression of TF was also found in serum-stimulated cultured fibroblasts, VSMCs and cardiac myocytes (21).

The *in vitro* findings suggest that TFPI not only regulates TF-initiated clotting, but is also involved in inhibiting VSMC proliferation. Various studies have demonstrated the antiproliferative effects of TFPI both *in vitro* and *in vivo* (Table I). Tissue factor pathway inhibitor was found to inhibit the FVIIa/TF-induced migration of cultured VSMCs (22), the proliferation of cultured human neonatal aortic SMCs (23) and the growth of human umbilical vein endothelial cells (HUVEC) (42). More recent studies have demonstrated that the antiproliferative activity of TFPI is mediated by the very-low-density lipoprotein (VLDL) receptor. In addition, the *C*-terminal region of TFPI seems to be responsible for inhibition of the proliferation of bFGF-stimulated endothelial cells because a truncated form of TFPI that contains only the first two Kunitz-type inhibitor domains is not nearly as effective (43).

The effect of TFPI on the proliferation of VSMCs, as well as on atherosclerotic processes, was investigated in experimental animal models (Table I). Tissue factor pathway inhibitor was shown to reduce angiographic restenosis and intimal hyperplasia in a rabbit atherosclerotic femoral artery injury model (44), to inhibit neointima formation and stenosis in pigs after deep arterial injury of the carotid artery (45), as well as to reduce the procoagulant activity and the upregulation of TF at injured sites (46) and to inhibit mural thrombus formation, neointima formation, and growth after repeated balloon angioplasty of the rabbit thoracic aorta (47). In a model of small autografts in rabbit femoral artery, topically applied TFPI significantly increased the patency rates of the grafts, reduced the intimal area and reduced the percentage of stenosis (48).

In the last few years, experimental studies have demonstrated the antithrombotic and antiproliferative effectiveness of overexpression of TFPI achieved by local gene transfer (49-54). Retrovirus-mediated *TFPI* gene transfer to the arterial wall efficiently inhibited intravascular thrombus formation in stenotic and injured rabbit carotid arteries (49). Adenovirus-mediated local expression of TFPI inhibited shear stress-induced recurrent thrombosis in balloon-injured carotid arteries of rabbits (50) and pigs (51). The combination of brief irrigation with rTFPI and adenovirus-mediated *TFPI* gene transfer fully suppressed TF activity and reduced fibroproliferative changes in rabbit carotid arteries, as shown by the reduction of intima/media area ratios (52). Recently published results obtained in a model of balloon-injured atherosclerotic arteries in rabbits demonstrated that overexpression of TFPI markedly inhibited intimal hyperplasia (53). In a murine model of flow cessation, adenoviral delivery of *TFPI* decreased vascular TF activity and inhibited neointima formation (54). Tissue factor pathway inhibitor overexpression by gene transfer and the resulting effects on thrombosis and restenosis were achieved without impairment of systemic hemostasis or excess bleeding. The local regulation of TF activity might be considered a target for gene transfer-based antirestenosis therapy. The development of atherosclerosis in mice with homozygous apolipoprotein E (apo E) deficiency and additional heterozygous TFPI deficiency was significantly greater than that seen in apo E knockout mice with a normal TFPI genotype, indicating that TFPI protects from atherosclerosis and is an important regulator of thrombotic events in atherosclerosis (55).

Table I: In vitro and in vivo studies on the effect of tissue factor pathway inhibitor on vascular smooth muscle cells.

Effect
In vitro
• Synthesis and secretion of TFPI by human pulmonary SMCs and upregulation of TFPI expression by serum or bFGF (20)
• Increased expression of TF and TFPI by serum-stimulated fibroblasts, VSMCs and cardiac myocytes (21)
• Inhibition of TF/FVIIa-induced migration of cultured VSMCs (22)
• Inhibition of the proliferation of cultured human neonatal aortic SMCs (mediated by the *C*-terminal region of TFPI) (23)
• Inhibition of the growth of cultured HUVECs by inducing apoptosis (no inhibition of DNA synthesis) (42)
• Expression of TFPI protein and mRNA in atherosclerotic arteries, in internal carotid plaques from patients undergoing endarterectomy and in fatty-streak lesions in rabbits fed a high-cholesterol diet (56)
• Presence of biologically active TFPI and co-localization with TF in human atherosclerotic plaques of carotid and coronary arteries (38, 57, 58)
In vivo
• Reduction of angiographic restenosis and decrease of neointimal hyperplasia in a rabbit atherosclerotic femoral artery injury model (44)
• Inhibition of neointimal formation and stenosis in minipigs after deep arterial injury of the carotid artery (45)
• Inhibition of the procoagulant activity and the upregulation of TF at balloon-induced arterial injury in pigs (46)
• Inhibition of mural thrombus formation and neointimal growth after repeated balloon angioplasty of the rabbit thoracic aorta (47)
• Reduction of intimal thickness and increase of long-term patency of small arterial autografts in rabbits (48)
• Inhibition of intravascular thrombus formation and recurrent thrombosis in carotid arteries of rabbits and pigs by gene transfer-mediated overexpression of TFPI (49-51)
• Inhibition of intimal hyperplasia in balloon-injured atherosclerotic arteries in rabbits by gene transfer of TFPI (52, 53)
• Inhibition of neointima formation by adenovirus-mediated overexpression of TFPI in a murine model of vascular remodeling (54)
• Increase of atherosclerotic changes in arteries of apo E knockout mice with a heterozygous TFPI deficiency (55)

bFGF = basic fibroblast growth factor; HUVECs = human umbilical vein endothelial cells; SMCs = smooth muscle cells; TF = tissue factor; TFPI = tissue factor pathway inhibitor; VSMCs = vascular smooth muscle cells.

Studies in human vessels

Active TF is present in the atherosclerotic vessel wall, where it is thought to be especially responsible for acute thrombosis after plaque rupture, the major complication of primary atherosclerosis. Besides TF, TFPI is also expressed in atherosclerotic plaques (38, 56-58). In atherosclerotic lesions of human coronary arteries, a co-localization of TF and TFPI was found in endothelial cells, macrophages, macrophage-derived foam cells and SMCs in intimal lesions. In type III and IV atherosclerotic lesions, the number of TF- and TFPI-positive cells is increased, accompanied by extracellular localization of TF and TFPI in the lipid core of atherosclerotic plaques (38). In human carotid atherosclerotic plaques, biologically active TFPI was found in endothelial cells, VSMCs and macrophages (57). Similar results were seen in other studies using human coronary arteries, popliteal arteries and saphenous vein grafts. In the atherosclerotic vessels, TFPI frequently co-localized with TF in endothelial cells overlying the plaque and in microvessels; this also occurred in the medial and neointimal SMCs, as well as in macrophages and T-cells in areas surrounding the necrotic core (58). Tissue factor pathway inhibitor was found to be largely expressed in the normal vessel wall and enhanced in atherosclerotic vessels. The inhibitor was active against TF-dependent procoagulant activity, suggesting a significant role in the regulation of TF activity. It is assumed that upregulation of TFPI in atherosclerotic plaques may control their thrombogenicity, or even prevent the complications associated with plaque rupture (58). Figure 4 illustrates the release of TFPI from endothelium by heparin and its circulation in free and bound forms.

In the *TFPI* gene, three polymorphisms that are associated with a significant variation in plasma TFPI levels have been identified recently (59, 60). However, studies in patients who underwent angioplasty, with or without stent implantation, revealed that, despite significant variations in TFPI levels, there was no evidence that the polymorphism of the *TFPI* gene influences the risk of angiographic restenosis after angioplasty (61).

Role of TFPI in unstable angina and myocardial infarction

Several clinical studies have been performed to evaluate the significance of measuring various parameters with regard to their diagnostic and prognostic value for cardiovascular disorders, especially for ischemic heart diseases such as unstable angina and AMI. In patients with ischemic heart diseases, excess thrombin formation was demonstrated by increased plasma concentrations of prothrombin fragment F1.2 and thrombin/AT III complexes. This could be related to the elevated levels of circulating TF also found. In these patients, plasma levels of TFPI were also increased but were not sufficient to interrupt the TF-induced coagulation activation (62, 63). Increased plasma concentrations of both total and free TFPI measured in AMI patients may result from their release by ischemic tissues (64). Patients with AMI show

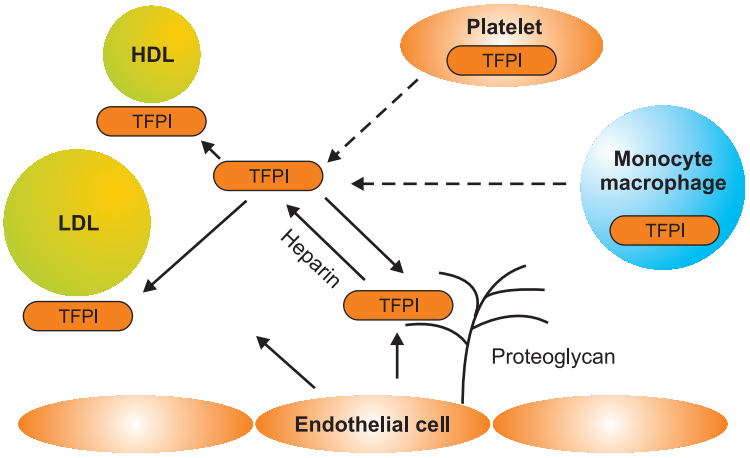

Fig. 4. Endothelial release of tissue factor pathway inhibitor (TFPI) by heparin and its distribution in free and bound forms. HDL = high-density lipoprotein; LDL = low-density lipoprotein.

increased procoagulant activity of monocytes, which is thought to be caused by upregulation of TF and can be partially inhibited by surface-bound TFPI; this suggests that direct inhibition of TF activity by a specific therapy might be particularly effective for the treatment of AMI (65).

The prognostic importance of TF and TFPI for recurrent coronary events was investigated in a long-term follow-up study over 4 years in patients after AMI (66). There were no statistical differences in TF or in total and free TFPI levels between patients and controls, demonstrating that the TF/TFPI system is not a useful prognostic marker. On the other hand, in middle-aged men without a history of coronary heart disease, a significant positive correlation was found between free TFPI plasma levels and atherogenic lipids, as well as FVII and fibrinogen; this can be considered as a compensatory increase in plasma free TFPI to the occurrence of risk factors for atherothrombotic diseases in apparently healthy men (67). A large population study on the association between plasma levels of free and total TFPI, conventional cardiovascular risk factors and endothelial cell markers showed that plasma levels of free TFPI correlated poorly with those of total TFPI, indicating that free and lipid-bound TFPI are regulated differently. Free TFPI strongly correlates with endothelium-derived molecules, such as thrombomodulin, von Willebrand factor and tissue-type plasminogen activator (tPA), whereas total TFPI is related more to conventional risk factors such as LDL cholesterol (68).

Role of TFPI in sepsis and DIC

Severe sepsis is associated with the activation of various inflammatory pathways and especially with the activation of the coagulation system. Due to endotoxin and the production of proinflammatory cytokines, TF is expressed on activated monocytes and vascular endothelial cells. Tissue factor-mediated coagulation activation can lead to microvascular thrombosis and further endothelial activation, which seems to play an important role in the development of the multiple organ failure associated with severe sepsis. Based on the role of TF in sepsis and DIC, it is expected that TFPI can provide a new therapeutic rationale for the treatment of sepsis.

Experimental findings suggested a significant role for TFPI in DIC and severe sepsis. Rabbits immunodepleted of plasma TFPI activity are sensitized to substantial intravascular coagulation induced by an infusion of TF at doses that were essentially ineffective in control animals (26). Similar results were found after an injection of endotoxin that induced extensive DIC in immunodepleted rabbits but only minimal or moderate intravascular clotting in animals with normal TFPI levels (27). The effectiveness of TFPI in DIC and septic shock has been investigated in various animal models (17, 18, 69). The results suggest that TFPI may offer benefits when used to treat severe sepsis.

In studies in humans, plasma concentrations of TF and TFPI have been determined to define the pathophysiological role of these molecules in DIC and septic shock. In patients with DIC, plasma concentrations of both TF and TFPI were significantly higher than in patients without DIC (70, 71). The increase in plasma TF in DIC patients is followed by an increase in TFPI, which is most likely based on its release from vascular endothelium due to endothelial cell injury (70). In patients with DIC, plasma concentrations of truncated TFPI were significantly elevated as compared to pre- and non-DIC patients. Reduced levels of the intact form of TFPI found in pre-DIC patients may suggest a hypercoagulable state in those patients due to consumption of TFPI (72, 73).

In animal models of sepsis, TFPI was able to completely block the coagulant response and to prevent death, as well as to reduce the cytokine response. In a human model of endotoxemia, human rTFPI effectively and dose-dependently attenuated the endotoxin-induced

coagulation activation. However, it did not influence leukocyte activation, chemokine release, endothelial cell activation or the acute-phase responses. Thus, the complete prevention of coagulation activation by TFPI does not inhibit activation of inflammatory pathways during human endotoxemia (74, 75). In posttrauma patients with DIC, the TF-dependent coagulation activation could not be sufficiently prevented by TFPI, which remained at normal levels. Disseminated intravascular coagulation was associated with thrombotic and inflammatory responses, leading to multiple organ dysfunction and a poor outcome in these patients (76).

In normal volunteers, TFPI was well tolerated without clinically significant bleeding (69). Recently, rTFPI (Chiron) was examined in small phase II clinical studies in patients with severe sepsis (69, 77). In the first trial, some of the patients showed an increase in the incidence of serious adverse events involving bleeding that might be caused by the relatively high doses of TFPI (0.33 and 0.66 mg/kg/h) administered. In the following studies, lower doses of TFPI (0.025-0.1 mg/kg/h) were used; adverse events did not differ between placebo and TFPI groups (69). A phase II study in 210 patients with sepsis showed a trend toward a relative reduction in day-28 all-cause mortality in TFPI-treated patients as compared with placebo. There was also an improvement in selected organ dysfunction scores and biochemical evidence of TFPI activity in these patients (69, 77). However, in a large, international phase III study in severe sepsis, treatment with rTFPI had no effect on all-cause mortality (78).

Role of TFPI in other disorders

The role of TFPI as a possible diagnostic or prognostic marker or as a pathogenetic factor was also investigated in other diseases (18). In patients with ischemic stroke, TFPI activity was significantly lower in atherothrombotic and lacunar infarction than in control subjects, which might be attributed to atherosclerotic changes in endothelial cells. In patients with venous thrombosis, an association between TFPI deficiency and thrombosis has not been clearly demonstrated. Increased TFPI levels in patients with end-stage chronic renal failure are considered to be a compensatory mechanism for the activation of the clotting process. Increased levels of TFPI in patients with non-insulin- and insulin-dependent diabetes mellitus could indicate an imbalance between procoagulant and anticoagulant mechanisms in diabetic patients, or reflect vascular damage with endothelial dysfunction. The increased TFPI levels seen in patients with malignancies cannot yet be explained.

Interactions between TFPI and unfractionated heparin and LMWH

Tissue factor pathway inhibitor is synthesized in vascular endothelium and is released from there after injection of either unfractionated heparin (UFH) or low-molecular-weight heparins (LMWHs), thus providing high concentrations of TFPI at sites of tissue damage and ongoing thrombosis (Fig. 4). Endogenous, cell-associated TFPI is possibly more important for maintaining the anticoagulant properties of the endothelium than is circulating TFPI (79). Endothelial cell-released TFPI, which, in contrast to plasma TFPI, still contains the basic *C*-terminal tail, is considered to contribute significantly to the anticoagulant effect of heparin.

Unfractionated heparin and LMWHs, which were originally developed for the prophylaxis of venous thromboembolic diseases, are now also used for the treatment of thrombotic disorders of both the venous and especially the arterial type (80, 81). Intravenous or subcutaneous administration of heparin reduced the elevated levels of TF observed in patients with unstable angina and AMI, and increased the concentrations of free TFPI (82-84). The antithrombotic effectiveness of different LMWHs has been demonstrated in large, randomized clinical trials (85-91). Low-molecular-weight heparins were observed to be superior to UFH in the treatment of both arterial and venous thrombosis, an effect that may particularly be based on the differential action of UFH and LMWHs on intravascular pools of TFPI (92, 93). At therapeutic doses, UFH, but not LMWHs, is associated with progressive depletion of both circulating and endothelial-bound TFPI, which may lead to a strong rebound activation of coagulation after cessation of treatment (94-97). Depletion of intracellular TFPI was also observed in endothelial cell culture systems, where the depletion was also significantly stronger with UFH than with LMWHs (79). This difference might be responsible for the higher antithrombotic efficacy of LMWHs as compared to UFH.

The cellular mechanisms of the effect of heparin on endothelium-associated TFPI seem to involve not only a simple displacement of TFPI from the cell surface, but also a more specific mechanism that includes increased secretion as well as redistribution of cellular TFPI induced by heparin (79). *In vitro* studies on the effect of UFH on the synthesis and secretion of TFPI in endothelial cells demonstrated that heparin concentration-dependently increased the expression of TFPI mRNA in endothelial cells, followed by a synthesis-dependent increase in TFPI release (98). After heparin stimulation, the major portion of TFPI seems to be secreted from intracellular stores and not displaced from the membrane surface. An alternative explanation would be that TFPI that was displaced from the membrane surface is rapidly replaced from intracellular stores (98).

In contrast to the differences between UFH or LMWH described above, a recently published study comparing the TFPI-releasing effect of therapeutic doses of UFH and the LMWH dalteparin in hospitalized patients demonstrated that dalteparin caused significantly less TFPI induction; thus, it is suggested that TFPI may not be a major contributor to the antithrombotic effect of heparin (99). In patients with acute coronary syndromes such as unstable angina and AMI, the measurements of markers

of thrombin generation, endothelial function and acute-phase reactions revealed similar responses to UFH and the LMWH enoxaparin. The increase in plasma levels of total TFPI also did not show significant differences between UFH and the LMWH. In particular, after both heparins, a depletion of TFPI was observed, which may indicate that the constitutive synthesis of TFPI is surpassed by its elimination in such patients (100). The pharmacodynamic effects of the LMWH tinzaparin on plasma TFPI in healthy volunteers demonstrated a rapid onset and sustained elevation in plasma TFPI (101).

Role of TFPI and other TF/VIIa inhibitors in cancer

The importance of TF in tumor biology has been highlighted by studies suggesting its involvement in cell signaling, metastasis and angiogenesis. An association between cancer and thrombosis has been recognized for more than a century. However, the manner by which tumor growth is regulated by coagulation *in vivo* remains unclear. To assess the role of coagulation on tumor growth *in vivo*, coagulation inhibitors specific for either TF/FVIIa complexes or FXa were tested for antitumor activity. Inhibitors of TF/FVIIa, TFPI and the nematode anticoagulant protein rNAPc2 inhibited both primary and metastatic tumor growth in mice. In addition, rNAPc2 was shown to be a potent inhibitor of angiogenesis. In contrast, rNAP5, a second nematode anticoagulant protein that specifically inhibits FXa, did not exhibit antitumor activity. Because the hemostatic activity of TF/FVIIa is mediated through activation of FXa, these data suggest that the proteolytic activity of TF/FVIIa promotes tumor growth and angiogenesis through a novel proangiogenic mechanism that is independent of hemostasis (102).

TFPI and TFPI-modulating agents in comparison with other anticoagulants/antithrombotics

Antithrombotic agents such as heparin and aspirin have been used for a long time for the prophylaxis and therapy of thromboembolic disorders in both the venous and arterial systems. New drugs developed in recent years represent a wide spectrum of natural, synthetic, semisynthetic and biotechnologically produced agents, and they include, in particular, LMWHs, serine proteinase inhibitors (such as direct and indirect thrombin and FXa inhibitors), endogenous anticoagulants (such as AT III) and platelet function inhibitors (such as glycoprotein IIb/IIIa receptor antagonists or adenosine diphosphate receptor blockers).

With special emphasis on TFPI, it must be stated that, based on the central role of the TF/FVIIa complex in the initiation of blood coagulation, other therapeutic strategies besides TFPI for regulating the TF pathway are currently being evaluated. Recombinant inactivated FVIIa (FVIIai), which has similar or even higher binding capacity for TF than FVIIa but blocks the catalytic activity of the TF/FVIIa complex, was investigated in various animal models (103, 104). Given intravenously or topically, FVIIai prevented or diminished immediate thrombus formation after vessel wall injury both in the venous and arterial systems; it also reduced the long-term intima thickening and narrowing of the vessel lumen. Furthermore, it attenuated coagulant and inflammatory responses in septic shock and reduced experimental ischemia/reperfusion injury.

In humans, FVIIai was studied in dose-escalating trials in healthy volunteers and as an adjunct to heparin in patients undergoing percutaneous transluminal coronary angioplasty. The combination with FVIIai revealed a trend toward a reduction in the number of ischemia events, as well as a lowering of heparin doses required to reach the same effect (104).

Recombinant NAPc2 (rNAPc2), a potent inhibitor of the TF/FVIIa complex (105, 106), demonstrated antithrombotic efficacy after subcutaneous administration in a multicenter dose-response study for the prevention of venous thromboembolism in patients undergoing knee replacement (107). In healthy human volunteers, the anticoagulant action of rNAPc2 could be reversed by infusion of recombinant FVIIa, resulting in increased generation of thrombin. It is assumed that FVIIa might be used as an antidote for inhibitors of the TF/FVIIa complex in case of adverse effects, such as bleeding events (108).

In addition to the TF pathway, other anticoagulant pathways (*i.e.*, the AT pathway and the protein C system) are also defective in sepsis and DIC (109). Because the natural anticoagulants exhibit specific cellular interactions that appear to provide antiinflammatory as well as anticoagulant activities, it is expected that restoration of the anticoagulant pathways might be associated with an improved clinical outcome in patients with sepsis and DIC (110). The protein C system seems to play a central role in the pathogenesis of severe sepsis due to its antithrombotic/profibrinolytic actions, the modulation of vascular functions and antiinflammatory properties (111, 112). In various animal models of sepsis and DIC, activated protein C (APC) has been shown to be an effective therapeutic agent (113). In humans, the efficacy and safety of recombinant human APC for severe sepsis was studied in a multicenter phase III trial involving patients with systemic inflammation and organ failure due to acute infection. Administration of APC significantly reduced the rate of death from all causes at 28 days in patients with a clinical diagnosis of severe sepsis, but this may be associated with an increased risk of bleeding (114).

Theoretically, replacement therapy with AT III, which is an important inhibitor of thrombin and other proteases of the coagulation system, could be useful for the treatment of DIC and severe sepsis (115, 116). However, there are no convincing clinical trial results demonstrating the efficacy of AT III in these diseases. As shown in a multicenter, randomized, placebo-controlled, double-blind phase II trial (as well as in a meta-analysis of all trials with AT III in patients with severe sepsis), there was a positive trend with AT III for reduction of mortality and organ failure, but no statistically significant difference between

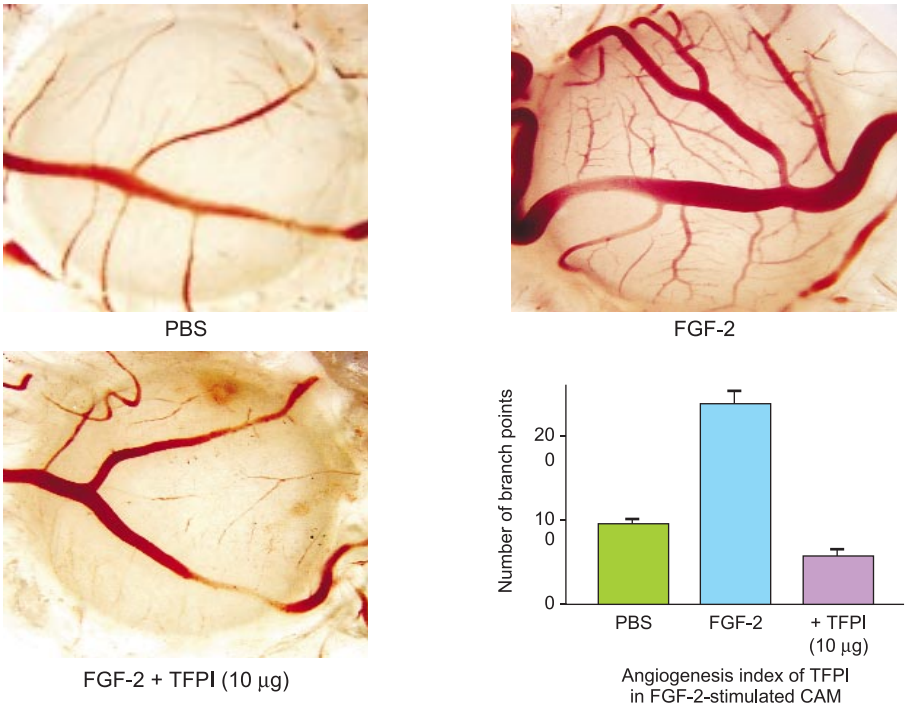

Fig. 5. Potent inhibition of fibroblast growth factor-2 (FGF-2)-induced angiogenesis by recombinant tissue factor pathway inhibitor (TFPI) in the chick chorioallantoic membrane (CAM) model. PBS = phosphate-buffered saline.

AT III- and placebo-treated groups (117). In another study, AT III replacement therapy significantly reduced mortality only in the subgroup of septic shock patients (118). In the clinical trials, a relatively low number of patients were included. Therefore, final assessment of the therapeutic value of AT III for DIC and severe sepsis requires additional and larger clinical trials (119).

Tissue factor pathway inhibitor demonstrated potent antiangiogenic efficacy against many growth factors that might mediate heparin angiogenic effects (Fig. 5).

TFPI-2

Tissue factor pathway inhibitor-2 (TFPI-2) is a recently described serine proteinase inhibitor. Human and murine TFPI-2 share about 50% homology. The cellular localization of human and murine TFPI-2 in the liver and the regulation of their expression during acute inflammation were investigated. Northern blot, *in situ* hybridization and studies on isolated hepatocytes demonstrated high-level expression of TFPI-2 in murine hepatocytes. On the other hand, very little TFPI-2 mRNA expression could be detected in human liver. Studies with isolated human liver cells suggested that TFPI-2 expression in human liver was mainly observed in liver sinusoidal endothelial cells rather than in hepatocytes. Murine liver TFPI-2 expression was greatly increased after lipopolysaccharide (LPS) administration, with delayed kinetics as compared to α_1-acid glycoprotein, a classical acute-phase reactant. Accordingly, studies with isolated cells showed that the increase in TFPI-2 transcripts occurred in nonhepatocytic cells. Moreover, the LPS response was abolished in mice with a hepatocyte-specific knockout for the gp130 receptor, thus indicating that a mediator from hepatocytes is involved in the upregulation of TFPI-2 in nonparenchymal cells. In conclusion, murine TFPI-2 is highly expressed in hepatocytes in the normal liver and is upregulated in nonparenchymal cells in the context of inflammation. The large difference in the level of liver expression of human and murine TFPI-2 suggests that, despite significant sequence similarities, these proteins presumably have different functions in the two species (120).

Human TFPI-2 is a matrix-associated Kunitz inhibitor that inhibits the plasmin- and trypsin-mediated activation of zymogen MMPs involved in tumor progression, invasion and metastasis. To directly assess its role in tumor growth and metastasis *in vivo*, we used stably transfected HT-1080 fibrosarcoma cells expressing either fully active wild-type human TFPI-2 (WT) or inactive R24Q TFPI-2 (QT), and examined their ability to form tumors and metastasize in athymic mice in comparison to mock-transfected cells (MT). Mock-transfected and QT fibrosarcoma tumors grew two to three times larger than WT tumors. Tumor metastasis was confined to the lung and was observed in 75% of mice treated with either MT or QT cells, whereas only 42% of mice treated with WT cells developed lung metastases. Real-time quantitative reverse transcriptase-polymerase chain reaction

Table II: Clinical relevance of tissue factor pathway inhibitor and potential therapeutic applications (17, 18).

Acute coronary syndromes (unstable angina, acute myocardial infarction)
• Prevention of reocclusion and restenosis after successful percutaneous transluminal coronary angioplasty or lysis
• Disseminated intravascular coagulation and sepsis
• Prophylaxis and therapy of deep vein thrombosis
• Microvascular anastomosis
• Ischemic stroke (especially atherothrombotic and lacunar infarction) and ischemia-reperfusion injury

analyses of each tumor group revealed three- to six fold lower levels of murine vascular endothelial growth factor gene expression in WT tumors in relation to either MT or QT tumors. Comparative tumor gene expression analysis revealed that several human genes implicated in oncogenesis, invasion, metastasis, apoptosis and angiogenesis had significantly altered levels of expression in WT tumors. Ruf *et al*. demonstrated that secretion of inhibitory TFPI-2 by a highly metastatic tumor cell markedly inhibits its growth and metastasis *in vivo* by regulating pericellular extracellular matrix remodeling and angiogenesis (121).

Vasculogenic mimicry (VM), which is the formation of matrix-rich vascular-like networks in three-dimensional culture corresponding with the expression of vascular cell-associated genes, as well as the lining of matrix-rich networks *in situ*, has been observed in highly aggressive and malignant melanoma. However, little is known about the molecular underpinnings of this phenomenon. On the basis of gene profiling, protein detection and immunohistochemistry, aggressive relative to poorly aggressive melanoma showed upregulation of *TF*, *TFPI-1* and *TFPI-2*, critical genes that initiate and regulate the coagulation pathways. The procoagulant function of TF in highly aggressive melanoma is shown to be regulated by TFPI-1 but not by TFPI-2. Thus, aggressive melanoma exhibits endothelial cell-like anticoagulant mechanisms that may contribute to the fluid-conducting potential of melanoma cell-lined networks, as studied by correlative *in vivo* Doppler flow measurements.

Antibody inhibition experiments reveal that TFPI-2 is required for VM *in vitro*, but plasmin is an unlikely target protease of TFPI-2. Blockade of TFPI-2 suppressed MMP-2 activation; therefore, TFPI-2 appears to regulate an essential pathway of VM. The TFPI-2 is synthesized by endothelial and tumor cells, which deposit TFPI-2 into extracellular matrices. Culturing poorly aggressive melanoma cells on a three-dimensional matrix containing recombinant TFPI-2 produces some of the phenotypic changes associated with aggressive, vasculogenic melanoma cells. Thus, TFPI-2 contributes to VM plasticity, whereas TFPI-1 has anticoagulant functions of relevance for perfusion of VM channels formed by TF-expressing melanoma cells.

Clinical perspectives

It can be expected that, due to the various biological functions of TF and the role of this protein in the pathogenesis of many diseases, the inhibitor TFPI may have a broad spectrum of therapeutic use. However, until now, TFPI has only found indications in severe sepsis, where defined trials have been carried out demonstrating the effectiveness of the drug by improved outcome in terms of surrogate markers and mortality reduction in the patients.

Numerous studies in patients suffering from cardiovascular and other disorders showed that both TF and TFPI may be indicators for the course and the outcome of certain diseases. Based on experimental and clinical findings, it may be assumed that TFPI could become an important drug for several clinical indications (Table II). The prevention of thrombotic complications of atherosclerosis after plaque rupture, and thus the development of acute coronary syndromes, might represent a very promising indication for TFPI. In addition, in animal studies, TFPI was found to protect vascular sites from atherosclerosis. Topical application of TFPI has been shown to improve capillary blood flow, which could be of benefit for patients with microangiopathic disorders. This is consistent with the observation that defibrotide enhances the release of TFPI. Tissue factor pathway inhibitor affects the inflammation cycle that contributes to microvascular thrombosis, and may therefore be of value in acute respiratory distress syndrome. Tissue factor pathway inhibitor could also be useful for the prevention of thrombosis associated with stroke or transient ischemic attacks, as an antithrombotic drug in microvascular surgery and for the reduction of ischemia/reperfusion injury (18). Although TFPI may have important therapeutic value for various clinical indications, it is important to recognize that the preclinical and especially the clinical investigations of TFPI are rather limited, so that the clinical potential of the drug can not yet be properly assessed and requires further studies.

Conclusions and future directions

Tissue factor plays a crucial role in the pathogenesis of thrombotic, vascular and inflammatory disorders (Fig. 6). Targeting of this mediator provides a unique therapeutic approach to influence the underlying pathophysiological processes and to prevent or treat the respective diseases (Fig. 7). The development of rTFPI and its molecular variants has provided a new dimension in the management of various disorders, such as atherosclerosis, acute coronary syndromes, thrombotic stroke and microangiopathic disorders, as well as for

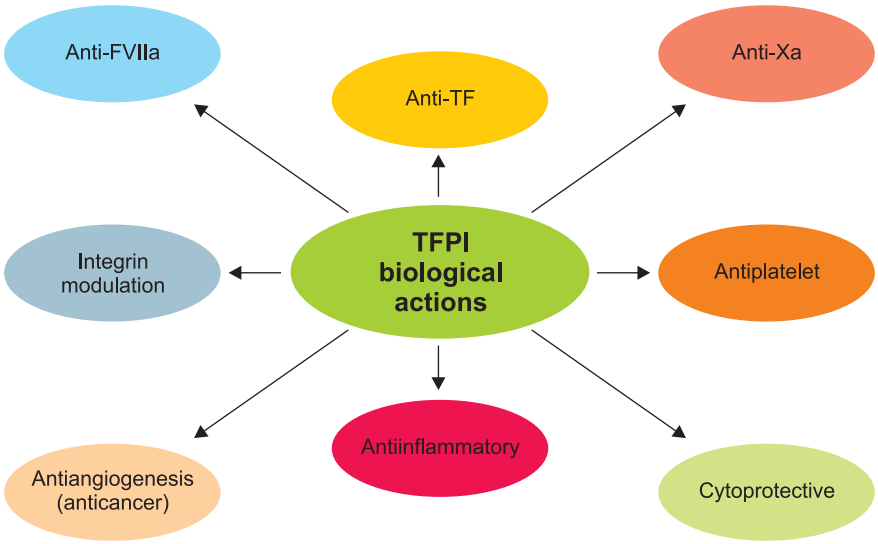

Fig. 6. Biological actions of tissue factor pathway inhibitor (TFPI) based on the inhibition of FXa and the TF/FVIIa complex.

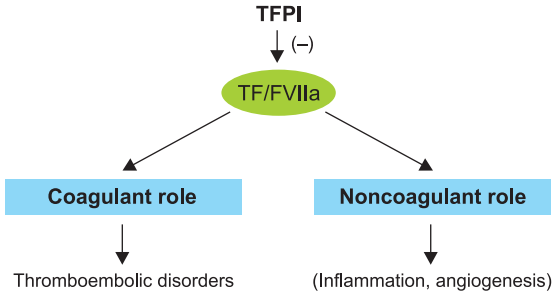

Fig. 7. Inhibition of TF/FVIIa-mediated coagulant and noncoagulant effects by tissue factor pathway inhibitor (TFPI).

inflammation-associated pathogenetic processes. Molecular manipulation of the currently available TFPI forms will provide drugs with varying pharmacokinetic and pharmacodynamic properties, as well as with relatively stronger affinity for endothelial or subendothelial target sites. As TFPI is present in several forms, the identification of these forms and their differential function may lead to the development of additional TFPI variants for specific indications. In addition, TFPI conjugates with drugs and chemicals may provide agents with a broader spectrum for the management of various diseases and a desirable duration of action. Because of the unique nature of TFPI genomic manipulation of the vascular system to enhance the endogenous synthesis and release of TFPI, this approach might be of future application in introducing the *TFPI* gene at various sites. Additionally, heparin or heparin derivatives that are capable of releasing vascular TFPI could represent another strategy.

The inhibition of the TF pathway seems to be one of the most promising pharmaceutical interventions to stop the thrombotic process. A number of experimental studies have demonstrated a potent antithrombotic effect against TF/FVIIa for TF-inhibiting compounds, such as rTFPI and active site-blocked activated FVII (30, 43-47). Preclinical and clinical studies in humans are warranted to elucidate the potential antithrombotic effects of these substances. Several demonstrations that inhibition of the TF pathway at an early step is also capable of inhibiting neointimal hyperplasia in an experimental model (54) shed light on additional potential applications of TFPI as a pharmacological agent in coronary interventional procedures. However, the marked prolongation of bleeding times seen in experimental studies underline that safety, effective dose and route of administration are the most important issues to resolve; the possibility of local delivery of the gene or drug during interventional procedures seems intriguing, but the feasibility of this approach has yet to be demonstrated. More interestingly, novel agents such as heparin or heparin derivatives that modulate vascular TFPI synthesis and release might be other alternative pharmacological tools.

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