Regulation of Functions of Vascular Wall Cells by Tissue Factor Pathway Inhibitor
Basic and Clinical Aspects

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Abstract—Tissue factor pathway inhibitor (TFPI) is a Kunitz-type protease inhibitor that inhibits the initial reactions of blood coagulation. A major pool of TFPI is the form associated with the surface of endothelial cells, which is speculated to play an important role in regulating the functions of vascular wall cells. TFPI consists of 3 tandem Kunitz inhibitor domains, the first and second of which inhibit the tissue factor–factor VIIa complex and factor Xa, respectively. Recent findings indicate that TFPI has another function, ie, the modulation of cell proliferation. This function is based on the interaction of the C-terminal region of TFPI with these cells. In addition to endothelial cells, it has been shown that many other vascular wall cells can synthesize TFPI, eg, mesangial cells, smooth muscle cells, monocytes, fibroblasts, and cardiomyocytes. TFPI is associated with these cells mainly through heparan sulfate proteoglycans on their surface. However, recent findings suggest that there are several other candidates for TFPI-binding proteins on these cells. On the other hand, studies on plasma levels of TFPI in patients with various diseases suggest that TFPI may be a marker of endothelial cell dysfunction. An increasing number of reports suggest that recombinant TFPI may attenuate thrombosis and prevent restenosis. Clinical trials are needed to explore these possibilities. Recent reports also indicate that the application of recombinant TFPI or TFPI gene transfer prevents restenosis in addition to thrombosis after arterial injury in the animal model; corroboration of these reports awaits clinical investigation. (Arterioscler Thromb Vasc Biol. 2002; 22:539-548.)

Key Words: tissue factor pathway inhibitor ■ blood coagulation ■ thrombosis ■ restenosis ■ vascular wall cells

Blood coagulation is initiated by the interaction of factor VII in plasma with tissue factor (TF) at the site of blood vessel injury, and the TF–factor VIIa complex activates factor X and factor IX, leading to the generation of thrombin. Vascular wall cells play a central role in the regulation of hemostasis by the cellular control of procoagulant and anticoagulant mechanisms. Normal endothelial cells do not show detectable levels of TF, although expression of endothelial TF occurs in vitro after perturbation with different agonists. On the other hand, increased TF levels have been detected in atherosclerotic plaque, probably reflecting secretion by smooth muscle cells, monocytes/macrophages, and endothelial cells.

The TF pathway inhibitor (TFPI) is a Kunitz-type protease inhibitor that inhibits the initial reactions of blood coagulation. A major pool of TFPI is the form associated with the surface of endothelial cells, which is speculated to play an important role in the regulation of the functions of vascular wall cells. Recent findings indicate that TFPI has another function, ie, the modulation of cell proliferation. Many reviews on TFPI have been published.1–5 The structure and biology of TFPI have recently been reviewed.6 Recent findings related to regulation of the functions of vascular wall cells by TFPI in physiological and pathological conditions will be summarized in the present review.

Functional Domains of TFPI

Ever since the determination of the amino acid sequence of human TFPI 14 years ago,7 a number of studies have been published on the structure and functions of TFPI. The amino acid sequences of TFPI from monkeys, rabbits, dogs, rats, and mice have been reported. Many investigators have examined the properties of TFPI by using recombinant proteins. As shown in Figure 1, TFPI mRNA is expressed from the TFPI gene, which consists of 9 exons. Mature TFPI protein consists of 3 tandem Kunitz inhibitor domains, the first and second of which inhibit TF-VIIa and Xa, respectively. The third domain has no inhibitory activity toward proteases. Heparin-binding sites of TFPI are located in the C-terminal basic region of TFPI. The inhibitory effect of TFPI on the initiation of blood coagulation and the enhancement by heparin are caused by the interaction of these domains of TFPI. When TF is exposed on the surface of vascular wall cells under pathological condi-
Tissue factor pathway inhibitor (TFPI) is supposed to inhibit the initiation of the coagulation cascade by binding with the complex of TF, factor Xa, and factor VIIa and also with proteoglycans on the cells, as shown in Figure 2. In addition to a full-length TFPI, it has been shown that truncated forms lacking most of their C-terminal domains are present in plasma and that they exhibit reduced affinity for vascular wall proteoglycans. In vitro experiments indicate that TFPI can be cleaved into partially degraded forms by various proteases, such as thrombin, plasmin, factor Xa, and cell-derived matrix metalloproteinases, as summarized in Figure 3. It remains to be established how the truncated forms of TFPI are generated in vivo by these proteases and by other proteases under physiological and pathological conditions.

To understand the mechanism of the specific interaction of these domains of TFPI with various proteases and proteoglycans, it is essential to determine the tertiary structure of TFPI. Although x-ray analysis of full-length TFPI has not yet been reported, we now know the tertiary structures of each of 3 Kunitz domains of TFPI. Tertiary structures of a complex of TF-VIIa with a basic pancreatic trypsin inhibitor (BPTI) mutant and a complex of K2 with Xa were studied by x-ray analysis. The structure of a complex of TF-VIIa-K1 was presented by computer modeling. We have determined the tertiary structure of K3 and the complex with heparin by a nuclear magnetic resonance technique, and we have identified the heparin-binding site of the K3 domain. We have also shown that the lack of the inhibitory activity of K3 is due to the absence of electrostatic interaction with factor Xa over a large surface area, whereas the K2 domain has the optimal distribution of the surface electrostatic potentials to make tight binding with factor Xa, as shown in Figure 4. A number of investigations have been made involving another heparin-binding site of TFPI, the C-terminal basic part. The cluster of
the positive charges in this domain is important for the interaction with heparin. We have also demonstrated that 12 amino acid residues in this domain are the minimal heparin-binding sequence.\textsuperscript{16}

Many other Kunitz-type inhibitors have been reported. Ikeo et al\textsuperscript{17} reported that the ancestral gene of the Kunitz-type inhibitor appeared \textasciitilde 500 million years ago. Then, \textasciitilde 450 million years later, the Kunitz domain was duplicated. One became the ancestral domain of group I, and the other became the domain of groups II and III. Approximately 400 million years ago, the ancestor of groups II and III was duplicated. The first and second domains of TFPI were in group II. On the other hand, the third domain of TFPI was in group III. The result supports the notion that the third domain of TFPI has a different function from the first and second domains.

\textbf{Synthesis of TFPI by Vascular Wall Cells}

As shown in Figure 1, the human TFPI gene consists of 9 exons with a promoter region. Although the nucleotide sequence of the promoter region containing several binding sites for transcription factors has been determined, it remains to be established how endothelial cells specifically synthesize TFPI and how the expression is regulated. It has been shown by several investigators that the level of TFPI mRNA was almost negligible in bovine and rabbit liver and in cultured hepatocytes from normal human liver. During studies on monkey TFPI, we found that TFPI mRNA was efficiently expressed in the hepatocytes of monkey liver as well as in the lung and kidney, in contrast to the expression in human, bovine, rabbit, and rat liver.\textsuperscript{18} We determined the nucleotide sequence of the 5'-flanking regions of the monkey and human TFPI genes. We found that the region up to \textasciitilde 138 in the monkey gene is important for the expression of monkey TFPI in hepatocytes.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Surface representation of the electrostatic potential of the protease domain of factor Xa, the TFPI K2 domain, and the K3 domain. (Reprinted with permission from Mine et al\textsuperscript{15}. Copyright [2002] American Chemical Society.) Red and blue represent negative and positive potentials, respectively. Residues involved in the binding interface between factor Xa and the TFPI K2 domain are labeled.}
\end{figure}
From the studies on hepatocytes from Macaque monkeys, crab-eating monkeys, rhesus monkeys, and cotton-top tamarins, we found that TFPI expression in hepatocytes is a specific event that developed during the evolution of nonhuman primates. In this sense, the nonexpression of TFPI in human hepatocytes is thought to be retrospective; ie, backward evolution occurred, which may have lowered the antithrombotic potential in humans.

In addition to endothelial cells, many other vascular wall cells can synthesize TFPI, eg, mesangial cells, smooth muscle cells, monocytes, fibroblasts, and cardiomycocytes. Studies on the expression of TFPI by these cells indicate that TFPI is constitutively synthesized. However, several reports have indicated a marked upregulation of TFPI by serum and specific growth factors or downregulation by lysophosphatidylcholine. The role of the GATA motif in TFPI gene expression in malignant cells has been reported. We examined how vascular wall cells enhance the synthesis of TFPI in inflammatory conditions and found that rat cardiomycocytes significantly enhanced the expression of TFPI mRNA in the presence of interleukin-1β and endotoxin. We also demonstrated that cardiomycocytes from human heart specimens from patients with sarcoidosis, myocarditis, and myocardial infarction were stained with anti-TFPI antibody. These results indicate a role of TFPI in the protection of the heart in inflammatory situations.

Distribution of TFPI in Blood Stream in Physiological and Pathological Conditions

The distribution of TFPI in vivo is complicated, as shown in Figure 5. In the blood stream, TFPI exists in a free form and also in lipoprotein-associated forms. Endothelial cells, platelets, and monocytes secrete TFPI into the blood stream. A complex of TFPI with factor Xa and/or factor VIIa is also present in pathological conditions. These forms of TFPI are mainly metabolized by hepatocytes in the liver. However, other vascular wall cells can also incorporate TFPI. TFPI mediates the permanent downregulation of cell-surface TF in monocytic cells and fibroblasts via internalization and degradation by LDL receptor–related protein. On the other hand, the major pool of TFPI is the endothelial cell–associated form. On the infusion of heparin, most of the associated form is released into the blood stream. Therefore, it is believed that TFPI binds to heparan sulfate proteoglycans on the endothelial cells, such as syndecans and glypicans. In fact, these proteoglycans can bind to TFPI in vitro. Glypican-3, a glycosyl phosphatidylinositol–anchored proteoglycan on the HepG2 cell surface, binds specifically to TFPI. Studies involving the subcellular localization and metabolism of TFPI in human umbilical vein endothelial cells (HUVECs) indicate that these TFPI-binding proteoglycans are in caveolae. Endothelium-associated TFPI binds to glycosyl phosphatidylinositol–anchored proteins and can be released by phospholipase treatment. However, it is possible that other proteins on the cells can bind to TFPI. Treatment of the cells with heparinase III and chondroitinase ABC lyase did not influence the binding of TFPI with the cells. Many other reports suggest that TFPI binds with cells in heparin-releasable and unreleasable forms, indicating that many other proteins on the cell surface participate in the binding of TFPI with cells. Recently, 1 of the TFPI-binding proteins was identified to be platelet thrombospondin-1. TFPI binds specifically and saturably to thrombospondin-1 purified from platelet α granules. The data reveal a mechanism for the recruitment and localization of TFPI to extravascular surfaces within a bleeding wound, where it efficiently downregulates the procoagulant activity of TF and allows subsequent aspects of platelet-mediated healing to proceed.

On the other hand, a marked redistribution of TFPI occurred in parallel with an acute release of TFPI in the cell medium on treatment of cultured HUVECs with thrombin. The result (ie, experimentally induced generation of thrombin in baboons caused the acute release of TFPI) indicates a possible novel function for thrombin in the downregulation of the coagulation process, which is potentially relevant to the outcome of disseminated intravascular coagulation. Furthermore, the synthesis and secretion of TFPI in endothelial cells were enhanced by heparin. Unfractionated heparin (UFH)
increased TFPI mRNA expression in endothelial cells and released TFPI into the medium. Increased secretion and redistribution of cellular TFPI were also induced by UFH and low molecular weight heparin in endothelial cells in culture. TFPI plays an important role in the antithrombotic effect of UFH and low molecular weight heparin, which are clinically used for the prevention of thrombosis. Infusion of heparin into the bloodstream raises the TFPI level in plasma and inhibits the blood coagulation pathway. The binding of heparins with antithrombin and TFPI causes the major antithrombotic effect of heparin. On the other hand, it has been reported that TFPI can inhibit activated protein C in the presence of heparin. The significance of the contradictory effect is not known.

Although recombinant protein has been used in these studies, it has been demonstrated that endogenous TFPI and recombinant TFPI differ in their distribution on the cell surface. A full-length TFPI, expressed in mouse C127 cells, was neither internalized nor degraded via the TFPI endocytic receptor, LDL receptor–related protein. Therefore, care should be taken for the interaction of recombinant TFPI with cells in experiments in vitro.

**Plasma TFPI Levels in Various Diseases**

To evaluate the plasma level of TFPI in various diseases, a functional assay of TFPI was established by the measurement of factor Xa activity generated after the incubation of the mixture of TF-VIIa and factor X with diluted plasma. However, this method is controversial because of the heterogeneity of TFPI in plasma and is still to be improved. On the other hand, enzyme immunoassays for the various forms of TFPI are now available; these immunoassays can distinguish between the free forms (full length and truncated), lipoprotein-associated forms, and endothelial cell–associated forms. Methods for measuring the TFPI-Xa complex have recently been reported. With the use of these methods, TFPI levels in various diseases have been reported. It is clear that the TFPI concentration in plasma is increased in patients with acute myocardial infarction. There are also reports on the plasma levels of TFPI in relation to disseminated intravascular coagulation and to other diseases, such as diabetes mellitus, renal diseases, and cancer. The relation of TFPI synthesis to lipid metabolism has also been suggested.

We examined lipid profiles and TFPI levels before and after heparin infusion and lipoprotein lipase (LPL) after heparin infusion in 156 patients with coronary arterial disease. Preheparin and postheparin free TFPI levels were inversely correlated with HDL cholesterol levels and postheparin LPL levels. It has been speculated that HDL improves endothelial function by opposing the oxidation and expression of adhesion molecules. We then postulated that the inverse relationship between HDL cholesterol and preheparin and postheparin free TFPI in the patients also reflects compensatory augmentation of antithrombotic properties of the vascular wall to maintain normal endothelial function. The inverse relationship between postheparin LPL and free TFPI suggests competitive sharing of the binding sites of hepatic sulfate proteoglycans on the endothelial surface. The significance of TFPI as a cardiovascular risk factor and endothelial cell marker has been further supported by recent reports that examined the plasma levels of TFPI in patients with cardiovascular risks. These results indicate that the free form of TFPI in plasma may be a marker of endothelial cell dysfunction.

Reports of mutation and polymorphism of TFPI are increasing. One important factor in the change in the plasma level of TFPI is the genetic background of the patients. Mutation of P151L in TFPI has been reported by a German group; 342 patients with venous thrombosis were screened, and 4 individuals were identified as being heterozygous for the TFPI mutation. These patients had no other genetic defects of clotting proteins. Among 5120 randomly chosen unrelated blood donors, 10 were found to be heterozygous for the mutation. From a calculation of potential risk for venous thrombosis in carriers of the mutation, the authors concluded that this mutation is a significant risk factor for venous thrombosis. However, a Spanish group has recently reported that this mutation is not associated with an increased risk for venous and arterial thrombosis.

Another mutation of TFPI, V264M, has been reported by a French group. To assess the effect of V264M on plasma TFPI levels of patients with acute coronary syndrome, 5 V/M and 13 V/V patients were examined. Total and free TFPI antigen levels were lower in the patients heterozygous for the V264M mutation than in patients with the wild-type variant. TFPI activity levels were also lower, but the difference was not significant. Their study did not support an association between TFPI polymorphism and acute coronary syndrome. The same authors reported no link between this mutation and venous thromboembolic disease or restenosis after coronary angioplasty. Polymorphism in the promoter region of TFPI was not associated with the plasma level of TFPI or with venous thrombosis.

More investigation into the relationship between polymorphism of the TFPI gene and various diseases is required to clarify the significance of the polymorphism.

**Potential Roles of TFPI in Atherogenesis**

In addition to having anticoagulant activity, TFPI was recently found to inhibit the proliferation of vascular wall cells by inducing apoptosis. The new function is based on the interaction of the C-terminal region of TFPI with these cells. A synthetic peptide with C-terminal Lys254-Met276 inhibited the binding of TFPI with human smooth muscle cells. The recent finding indicates that the synthetic peptide prevents the proliferation of the cells, probably by inhibiting mitogen-activated protein kinase kinase in the signal transduction pathways in the cells. Although the inhibitory mechanism of TFPI on the proliferation of vascular wall cells remains to be established, it is quite reasonable that the binding of TFPI with these cells triggers the signal transduction cascade and induces the expression of several genes related to the regulation of cell growth. The new function of TFPI should be underlined in connection with many reports on the regulation of atherogenesis by TFPI.
Atherosclerosis is a process of chronic inflammation initiated and sustained in response to injury of the vascular wall. Several reports have demonstrated the colocalization of TFPI and TF in atherosclerotic plaques, suggesting a significant role for TFPI in the regulation of TF activity. Thrombosis after plaque disruption is the immediate cause of most acute myocardial infarcts and may contribute to the progression of atherosclerosis. Therefore, one of the roles of TFPI is the attenuation of the thrombogenicity of the atherosclerotic plaque. Endothelial cells, monocytes, T lymphocytes, smooth muscle cells, and platelets are the cells responsible for these processes. Key molecules in the processes are oxidized lipoproteins, Lp(a), cell adhesion molecules, cytokines, chemokines, growth factors, extracellular matrix proteins, and matrix metalloproteases. As described below, TF and TFPI have been demonstrated to be involved in the interaction of most of these cells and key molecules.

Monocytes and most vascular wall cells, such as endothelial cells, smooth muscle cells, and cardiomyocytes, can synthesize TF and TFPI by various stimulations. Regulation of monocyte procoagulant activity by TFPI was found to be important in acute myocardial infarction. The secreted TFPI from these cells may be associated within plaque through the binding with proteoglycans and other TFPI-binding proteins on the cells. Overexpression of TFPI was shown to reduce thrombus formation after vascular injury in animal models. Reduced endogenous TFPI activity may enhance thrombus formation. The administration of recombinant TFPI or of the TFPI gene into animal models has demonstrated that TFPI is a useful anticoagulant for preventing the thrombogenicity of vascular wall cells. The relationship of TFPI with oxidized LDL and with Lp(a) has been demonstrated.

On the other hand, it is well known that thrombosis is closely associated with the proliferation of vascular wall cells, vascular remodeling, angiogenesis, and restenosis. The proteases generated in the blood coagulation cascade, such as the TF–factor VIIa complex, factor Xa, and thrombin, stimulate the inflammatory response of vascular wall cells, leading to the expression of adhesion molecules, leukocyte activation, and the migration of smooth muscle cells. Therefore, it is possible that the prevention of thrombosis can also prevent the proliferation of neointima, ie, restenosis. In fact, many reports have demonstrated that the administration of recombinant TFPI or of the TFPI gene is capable of reducing the restenosis after balloon injury of the vascular wall, in addition to preventing the immediate thrombosis. Recombinant TFPI can prevent thrombosis and restenosis after arterial injury in cell culture systems in vitro and in various animal models. There are also reports that endogenous TFPI is involved in the process of thrombus formation in vivo and that it plays an active role in modulating arterial stenosis. These results indicate that the intravenous administration of recombinant TFPI protein prevents thrombosis at an early stage and restenosis at a later stage. Local gene transfer of an adenovirus encoding human TFPI into rabbits and pigs has been reported to prevent platelet-dependent thrombosis and restenosis. Because all the results on the inhibition of restenosis by TFPI have been obtained from animal experiments, a pioneering work on atherosclerotic patients is required to prove the clinical effect of TFPI.

The mechanism of the novel function of TFPI is still obscure. As described above, one can conclude that the inhibition of the initial reactions of blood coagulation and the subsequent thrombin generation by TFPI prevented the proliferation of vascular wall cells. However, it is also possible that TFPI directly inhibited the proliferation of vascular wall cells, inasmuch as several reports have demonstrated that TFPI binds to endothelial cells and smooth muscle cells and regulates the function of these cells. As recently reported, the VLDL receptor is a candidate for the receptor of TFPI.

Furthermore, the role of TFPI in atherogenesis has been straightforwardly indicated by the studies on TFPI knockout mice. Most of the knockout mice without a K1 domain of TFPI, ie, TFPIK1−/− mice, died during embryonic days 9.5 and 11.5, with signs of yolk sac hemorrhage. None of the mice survived to the neonatal period. Factor VII deficiency rescued the intrauterine lethality in the TFPI knockout mice. A recent study on the double knockout mice, ie, TFPI+/−ApoE−/− mice, indicates that TFPI protects from atherosclerosis and is an important regulator of the thrombosis that occurs in the setting of atherosclerosis.

Future Prospects for TFPI

One of the goals from the intensive studies on TFPI is a clinical application of TFPI for the prevention of thrombosis and other cardiovascular diseases. The use of TFPI for the prevention of thrombosis in sepsis is now the most promising clinical application, because patients with sepsis frequently have reduced TFPI levels and because leukocyte elastase can cleave TFPI, thereby reducing its activity. A recent phase II clinical trial by the Chiron Corp has shown a trend toward a reduction in 28-day all-cause mortality in TFPI-treated patients, in addition to the significant reduction in thrombin generation. A phase III clinical trial in patients with severe sepsis is ongoing by the company. The administration of the recombinant protein to humans with endotoxemia has been reported. After an intravenous administration of endotoxin, TFPI dose-dependently inhibited coagulation activation without influencing the fibrinolytic and cytokine responses during human endotoxemia. The administration of endotoxin resulted in the generation of thrombin, as reflected by increases in the plasma levels of the prothrombin fragment F1 +2 and TATc (the thrombin-antithrombin complex). High-dose TFPI almost completely prevented the increase in prothrombin fragment F1 +2 and TATc. The endotoxin-induced changes in the fibrinolytic system and cytokine levels were not affected by either low-dose or high-dose TFPI. The authors concluded that TFPI effectively and dose-dependently attenuates the endotoxin-induced coagulation in humans without influencing the fibrinolytic and cytokine response. In addition to the clinical trials, it has recently been demonstrated that extremely low doses of TFPI decrease mortality in a rabbit model of septic shock. These studies will also stimulate the clinical application of TFPI in severe sepsis.

In spite of the recent findings, there is still much to learn about TFPI. New findings have added to our knowledge of...
the anticoagulant properties of TFPI. For example, antibodies to β2 glycoprotein I associated with antiphospholipid syndrome suppressed the inhibitory activity of TFPI. This result indicates that the C-terminal region of TFPI and the phospholipid binding domain of β2 glycoprotein I compete to bind phospholipid on the surface of the cells. We have still only a poor understanding of the mechanism by which TFPI inhibits the proliferation of vascular wall cells. A breakthrough involving the identification of the receptors for TFPI on the vascular wall cells and of the genes expressed in the cells may occur. As described in the present review, TFPI binds to cells in heparin-dependent and -independent manners. Heparin-dependent binding of TFPI is evidently mediated by heparan sulfate proteoglycan. Its role has been clarified by studies in syndecan knockout mice. In addition, the discovery of other receptors for TFPI may reveal a connection between the receptors and gene expression and the activation of signal transduction pathways in the cells and may also reveal the mechanism of the effect of TFPI on the function of vascular wall cells. The future prospects for the clinical application of TFPI as a new marker of endothelial cell dysfunction and as a new drug for thrombosis and restenosis are more promising. On the other hand, an increasing number of findings have been reported on another type of TFPI, TFPI-2. Although the roles of TFPI-2 seem to be quite different from those of TFPI, these studies encourage us to clarify the in vivo significance of TFPI.

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Arterioscler Thromb Vasc Biol. April 2002


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Arterioscler Thromb Vasc Biol. 2002;22:539-548; originally published online February 28, 2002;
doi: 10.1161/01.ATV.000013904.40673.CC
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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