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Expression of Tissue Factor Pathway Inhibitor by Endothelial Cells and Platelets

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Abstract

Tissue Factor Pathway Inhibitor (TFPI) is a potent anticoagulant protein that abrogates the activity of the tissue factor-factor VIIa catalytic complex that activates blood coagulation *in vivo*. The importance of TFPI in the regulation of blood coagulation is emphasized by its how its activity is modulated in human disease. Decreased TFPI activity contributes to the development of both arterial and venous thrombosis and has been implicated in the thrombotic events occurring in women using oral contraceptives and in patients with paroxysmal nocturnal hemoglobinuria. Both endothelial cells and platelets produce TFPI. Our laboratory is interested in the mechanisms for expression of TFPI on the surface of these cells to better understand how TFPI prevents intravascular thrombosis. Studies of cultured endothelial cells and human placenta have demonstrated that TFPI associates with the cell surface through a glycosyl phosphatidyinositol (GPI)-anchor in a manner that is not dependent on GAGs or altered by heparin. TFPI is not directly bound to the GPI-anchor; instead it appears to bind tightly to a GPI-anchored protein. This GPI-anchored protein appears to be necessary for proper trafficking of TFPI to the cell surface. An alternatively spliced form of TFPI, TFPIβ, is a truncated form of TFPI that is directly attached to a GPI-anchor. However, it is not clear that human endothelial cells produce TFPIβ. Platelets produce TFPI but not TFPIβ. TFPI is expressed on the platelet surface following dual activation with collagen plus thrombin, but not through a GPI-anchor. Studies using mouse models of TFPI deficiency are currently being conducted in our laboratory to determine if distinct physiological functions of endothelial and platelet TFPI exist *in vivo*.

Structure and Function of TFPI

Tissue factor pathway inhibitor (TFPI) is a 276 amino acid (~43 kDa) protein with an acidic N-terminal region followed by three tandem Kunitz-type serine protease inhibitory domains and a basic C-terminal region. As such, each individual Kunitz domain is homologous to aprotinin, the prototypical Kunitz-type serine protease inhibitor. TFPI abrogates blood coagulation by directly inhibiting factor Xa (fXa) with the second Kunitz domain and, in a fXa dependent manner, inhibiting TF-fVIIa with the first Kunitz domain.[1] The third Kunitz domain has not been shown to function as a protease inhibitor. Its unique mechanism for inhibition of both fXa and TF-fVIIa makes TFPI the only physiologically active inhibitor of the initiation of blood coagulation.[2]

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TFPI in the Pathophysiology of Disease

Treatment of Bleeding in Patients with Hemophilia

A classic question in the blood coagulation field has been: If TF-fVIIa is the primary initiator of blood clotting *in vivo* and also is a potent activator of fX, independent of fVIII or fIX, then why do hemophiliacs bleed? The discovery and characterization of TFPI activity has answered this question.[3] The rapid inhibition of fXa and TF-fVIIa and the extrinsic pathway by TFPI produces the requirement for propagation of coagulation through fVIIIa and fIXa of the intrinsic pathway. *In vitro* studies have demonstrated that reduction of the inhibitory effect of TFPI is a viable strategy for prevention of bleeding in patients with hemophilia.[4;5] *In vivo* animal studies have demonstrated that anti-TFPI antibody shortens the bleeding time in rabbits with antibody induced hemophilia A.[6] In the context of patients with hemophilia, the success of recombinant fVIIa therapy of patients with acquired inhibitors of fVIII or fIX has demonstrated the tissue factor pathway as an important target for treatment and suggests that therapeutic modulation of TFPI activity could be an attractive therapeutic target for the development of new therapies to prevent bleeding in patients with hemophilia.

Thrombosis Associated With Use of Oral Contraceptives

The plasma TFPI concentration decreases about 25% in women using oral contraceptives. The decrease in TFPI mediated anticoagulant activity in these women may to the increased risk (2- to 6-fold) of thrombosis associated with the use of oral contraceptives.[7–11] The physiological basis for the decrease in plasma TFPI is not known. Heritable thrombophilias are known to increase a woman's risk for thrombosis when using estrogen therapies. Of these, the factor V Leiden (FVL) mutation, an altered form of FV resistant to degradation by activated protein C, produces the most significant risk. In oral contraceptive users with FVL the risk of thrombosis is about 5 times that of either risk factor in isolation.[12;13] Animal models have demonstrated that decreased TFPI in the presence of FVL provides a key "second hit" that produces a severe thrombotic state. Mice with heterozygous deficiency of TFPI ($TFPI^{+/-}$) develop normally and do not suffer from spontaneous thrombosis.[14] Mice genetically altered to produce the FVL mutation have a mild prothrombotic phenotype, exhibiting occasional spontaneous thrombosis.[15] However, when the FVL mutation is bred into $TFPI^{+/-}$ mice, the mice suffer from severe disseminated thrombosis and nearly complete perinatal mortality.[16] These studies in genetically altered mice support the notion that decreased TFPI contributes to increased risk for thrombosis associated with oral contraceptive use.

Thrombosis Associated with Paroxysmal Nocturnal Hemoglobinuria (PNH)

Patients with PNH have a pronounced predisposition to intravascular thrombosis. PNH is an acquired clonal disease characterized by lack of glycosyl phosphatidylinositol (GPI)-anchored protein expression. The thromboses occur in an organ specific pattern. Most occur in the portal circulation (hepatic vein occlusion, also called the Budd-Chaiari Syndrome, occurs in 30% of patients) or in venous circulation of the brain.[17] As described below, TFPI is a GPI-anchored protein. Defective expression of TFPI in patients with PNH and may contribute to the organ specific thrombosis observed in this disease.[18]

Association of TFPI Deficiency with Arterial and Venous Thrombosis

TFPI null humans have not been identified suggesting that TFPI is required for human birth. However, low plasma levels of TFPI are weakly linked to disease in humans. Several studies have suggested that plasma TFPI levels may demonstrate a "threshold effect" where patients with free (non-lipoprotein bound) plasma TFPI concentration less than 10% of the normal mean value are at increased risk (~2-fold) for both deep venous thrombosis and myocardial infarction.[19–22] In these studies there is no difference in the mean free plasma TFPI level between the

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disease and control groups. In other published studies there is a considerable amount of conflicting data about the contribution of plasma TFPI levels and polymorphisms to the development of thrombosis.[23–26] This is likely a result of the wide normal range for plasma TFPI,[27] the various methods for measurement of plasma TFPI[28] and the poor correlation between the soluble plasma TFPI concentration and the amount of cell surface associated endothelial and/or platelet TFPI.[27]

TFPI is a GPI-anchored Protein on Endothelium

TFPI is present primarily on the endothelial surface[29;30] but is also present on the surface of monocytes,[31] within platelets[32] and circulating in plasma.[33;34] Studies of cultured endothelial cells have demonstrated that TFPI associates with the cell surface through a GPI-anchor in a manner that is not dependent on GAGs or altered by heparin.[35–37] However, heparin infusion results in a prompt 2- to 4-fold increase in circulating TFPI,[38] therefore, another mode for TFPI cell surface association is non-specific interactions with glycoaminoglycans (GAGs). Since primary endothelial cells can rapidly change phenotype in tissue culture, we measured heparin-releasable and GPI-anchored TFPI in fresh human placenta. We found 1) that GPI-anchored TFPI is present at 10 to 100 times the level of heparin-releasable TFPI; 2) that pretreatment of placenta with phosphatidylinositol specific phosopholipase C (PIPLC) increases the amount of heparin releasable TFPI by ~3-fold; and 3) that only small amounts of TFPI are released by heparin after PIPLC treatment.[39] These results indicate that TFPI is a GPI-anchored protein. In addition, heparin releasable TFPI likely represents only a small portion of the total TFPI on endothelium that remains attached to cell surface glycosaminoglycans after cleavage of the GPI-anchor by endogenous enzymes.

TFPIβ is an alternatively spliced form of TFPI

Endothelial cells also appear to make an alternatively spliced, truncated form of TFPI that is directly attached to a GPI-anchor.[40;41] This form of TFPI, called TFPIβ, contains the first two Kunitz domains present in full-length TFPI and, therefore, is theoretically able to inhibit both factor Xa and factor VIIa/TF. The alternative splice occurs following exon 7 that encodes the connecting region between the second and third Kunitz domains of full-length TFPI. In human TFPIβ exon 8 encodes a stretch of 12 amino acids followed by a GPI anchor attachment sequence that is removed in the endoplasmic reticulum. Thus, TFPIβ has only 12 unique amino acids that are not present in TFPI. Real time PCR measurements of cultured endothelial cells indicate that TFPIβ mRNA is present at about one-tenth that of TFPI mRNA.[41]

TFPI Is Indirectly GPI-anchored; TFPIβ Is Directly GPI-anchored

GPI-anchored proteins have N-terminal leader sequences that direct them to the endoplasmic reticulum. Within the ER, addition of the GPI-anchor to the C-terminal end of the protein is associated with removal of the C-terminal 17 to 30 amino acids of the protein. There is not a specific signal sequence among the C-terminal peptides cleaved from proteins during GPI-anchor attachment. However, a region of 15 to 20 hydrophobic residues at the C-terminus is a feature common to GPI-anchored proteins.[42] TFPIβ contains an appropriate signal for direct attachment of a GPI-anchor within its alternatively spliced C-terminal region (Figure 2B). In contrast, TFPI has a very basic C-terminal region without long stretches of hydrophobic amino acids. Importantly, we demonstrated by western blot analysis with an antibody directed against the final 12 amino acids of TFPI that the C-terminal region is intact in GPI-anchored TFPI.[39] It has been hypothesized by both us[39;43] and others[35;37;41] that TFPI is attached to the GPI-anchor indirectly by binding to an, as yet, unidentified GPI-anchored co-receptor. The GPI-anchor attachment of TFPI and TFPIβ localizes them to lipid raft microdomains on the cell surface.[41] Lipid rafts are areas of the plasma membrane rich in glycosphingolipids and

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cholesterol that have been implicated in several cellular functions including signal transduction.[44;45]

Most recently, our laboratory has focused on the expression of TFPI in endothelial cells and platelets to further our understanding of how TFPI present in these two cells regulates the development of intravascular thrombosis.

Expression of TFPI on Endothelial Cells

In order to further understanding how the GPI-anchored co-receptor and TFPI interact to produce GPI-anchored TFPI on the endothelial surface, we used aerolysin, a bacterial pore forming toxin that attacks eukaryotic cells by binding to the glycan core of the GPI-anchor to produce aerolysin resistant EaHy926 and ECV304 cell lines. These cell lines have served as a valuable tool for study of the cellular trafficking of TFPI; providing additional new evidence that TFPI associates with the endothelium indirectly by binding a GPI-anchored co-receptor.[18] Unlike wild type endothelial cells, aerolysin resistant cells do not express TFPI on their surface and have greatly decreased amounts of total cellular TFPI. Aerolysin resistant and wild type cells secrete equal amounts of TFPI into the conditioned media. Taken together, these data suggest that TFPI is degraded within the aerolysin resistant cells. Data obtained from confocal microscopy of permeabilized cells and experiments with different metabolic inhibitors suggests that TFPI is produced by aerolysin resistant cells, translocated into the ER, transported to the Golgi and then degraded within lysosomes along with directly GPI-anchored proteins.[18] Based on the data obtained with the aerolysin resistant cells, we propose that TFPI cellular trafficking and surface expression is controlled by its GPI-anchored co-receptor. The wild type and aerolysin resistant cells initially produce normal amounts of structurally sound TFPI and co-receptor that bind reversibly to each other in the ER/Golgi. In wild type cells the TFPI/co-receptor complex is expressed on the endothelial surface. In aerolysin resistant cells this complex is degraded in lysosomes. Lysosomal degradation occurs because of the inability to produce the GPI-anchor due to a defect in the PIG-A gene,[18] a key component of GPI-anchor biosynthesis. Thus, the aerolysin resistant cells are similar to those present in patients with PNH that also are not able to produce the GPI-anchor due to PIG-A defects. As described above, it is possible that TFPI also is degraded within cellular clones of these patients providing a potential explanation for the thrombotic complications associated with this disease. When TFPI is transfected into CHO or HEK293 cells it is secreted into the conditioned media instead of expressed on the cell surface, presumably because these cells do not make the GPI-anchored co-receptor.[41] This suggests that secretion into the conditioned media is the default cellular processing pathway for TFPI and is consistent with the presence of an N-terminal leader sequence and the absence of a C-terminal transmembrane domain or GPI-anchor attachment signal in TFPI. It is important to note that not all of the TFPI is bound to the co-receptor at any one time due to the reversible nature of the binding interaction. The unbound pool of TFPI is secreted in a manner similar to that of recombinant TFPI expressed within CHO or HEK293 cells thereby explaining the equal amounts of TFPI secreted by the wild type and aerolysin resistant cells.

Expression of TFPI in Platelets

We extended our studies of TFPI on the endothelial surface by investigating how TFPI is expressed on the platelet surface. TFPI is present within platelets accounting for about 10% of the TFPI present in whole blood.[32] Platelet expression of TFPI may be physiologically important for regulation of circulating TF activity, such as that released in microvesicles from activated leukocytes or endothelial cells.[46] Incorporation of TF bearing microvesicles into the blood clot is thought to be necessary for effective stabilization of the clot within the vasculature.[47;48] However, inadequate regulation of the procoagulant activity of these microvesicles by

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TFPI may lead to propagation of a hemostatic plug into an occlusive thrombus. We found that TFPI is expressed on the surface of coated-platelets produced by dual agonist activation with convulxin and thrombin but not following activation with single agonists. These are the first data describing TFPI expression on the platelet surface. A brief summary of the results is as follows. Western blot analysis of gel filtered platelet lysates demonstrates the presence of full-length, 44 kDa TFPI but not TFPIβ indicating that full-length TFPI is either produced by megakaryocytes or selectively adsorbed from plasma by platelets. Data demonstrating TFPI production by megakaryocytes includes real time PCR analysis of cDNA produced from highly purified human platelets that identified transcripts for TFPI at 63% the level of RPL-19 and immunofluorescent staining of permeabilized cultured mouse megakaryocytes. Consistent with the western blot data, message for TFPIβ was not detected by real time PCR. TFPI is not present on the surface of quiescent platelets as assessed by flow cytometry. TFPI is not released from platelets or expressed on their surface following stimulation with the thrombin receptor activating peptide SFLLRN (TRAP) indicating that it is not stored as a soluble α-granule protein. This was confirmed by confocal microscopy experiments demonstrating that TFPI does not co-localize with VWF or fibrinogen within platelets. In experiments performed in collaboration with Dr. George Dale at the University of Oklahoma, we found that TFPI is consistently present on the surface of coated-platelets, a sub-population of platelets observed following dual stimulation with thrombin and collagen.[49;50] Differential centrifugation experiments demonstrated that TFPI is released from coated-platelets both in microvesicles and as a soluble protein. Platelet TFPI activity was measured in multiple individuals by determining the rate of fX activation by fVIIa-TF. Comparison of quiescent and coated-platelets demonstrates a significant increase in TFPI activity on the surface the coated-platelets (11.0+/−0.3 vs. 38.7+/−17.2 pmole/10 million platelets; $p<0.005$), but no significant increase in platelets activated with 20 or 200 μM TRAP. Thus, platelets differ from endothelial cells in regards to TFPI expression in the following ways: 1) platelets contain only TFPI while endothelial cells have both TFPI and TFPIβ; and 2) platelets express TFPI on their surface only following dual activation with collagen and thrombin while endothelial cells constitutively express surface TFPI. Studies using mouse models of TFPI deficiency are currently being conducted in our laboratory to determine if distinct physiological functions of endothelial and platelet TFPI exist *in vivo*.

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