Murine Hematopoietic Cell Tissue Factor Pathway Inhibitor Limits Thrombus Growth

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Objective—Tissue factor (TF)–factor VIIa initiates blood coagulation and is found on microparticles that accumulate within intravascular thrombi. Tissue factor pathway inhibitor (TFPI), a factor Xa (fXa)–dependent inhibitor of TF–factor VIIa, is produced by megakaryocytes and is present in platelets. We sought to determine the role of platelet TFPI in regulation of thrombus growth.

Methods and Results—Western blot analyses demonstrated that murine platelets produce TFPIα, the most evolutionarily conserved alternatively spliced isoform of TFPI. A mouse model of hematopoietic cell TFPI deficiency was developed by transplanting irradiated TFPI+/− mice with TFPI−/− fetal liver cells. Platelets from transplanted mice totally lack TFPI inhibitory activity. An electrolytic vascular injury model was used to assess thrombus growth in the femoral vein and carotid artery. Mice lacking hematopoietic TFPI developed larger femoral vein and carotid artery thrombi than TFPI+/− mice transplanted with TFPI+/+ hematopoietic cells, as evidenced by increased platelet accumulation.

Conclusion—Hematopoietic TFPI limits thrombus growth following vascular injury. Because platelets are the primary hematopoietic cell accumulating within a growing thrombus, these findings suggest that TFPI present within platelets functions to limit intravascular thrombus growth, likely through inhibition of the procoagulant activity of blood borne TF. (Arterioscler Thromb Vasc Biol. 2011;31:821-826.)

Key Words: blood coagulation ■ platelets ■ thrombosis ■ tissue factor pathway inhibitor ■ tissue factor

Tissue factor (TF) is the primary protein that initiates blood coagulation in vivo. It binds to factor VIIa, forming a catalytic complex that activates factors IX and X (fIX and fX), which lead to thrombin generation and the formation of fibrin. Under physiological conditions, TF is localized to extravascular cells, and hemostasis occurs when it is exposed to flowing blood following traumatic vascular injury.1 Under pathophysiological conditions, such as inflammation, cells within the vasculature can express TF, resulting in thrombotic disease.2–4 For example, TF released on microparticles from activated leukocytes can integrate with activated platelets and contribute to development of intravascular thrombosis.5–7 The potential detrimental effects of intravascular TF are dampened by tissue factor pathway inhibitor (TFPI), an anticoagulant protein primarily produced by microvascular endothelial cells and megakaryocytes.8–11

Three alternatively spliced isoforms of TFPI—TFPIα, TFPIβ, and TFPIγ—have been identified that differ in their C-terminal domain structure and mechanism for association with cell surfaces.12 Alternatively spliced isoforms of TFPI are produced in a tissue-specific manner in mice. All mouse tissues produce mRNA for all 3 isoforms, but in the vascular beds of adult tissues, only the TFPIβ mRNA is found to be translated into protein, whereas placenta and embryonic tissues produce TFPIα protein.13 We demonstrate that adult mouse platelets produce exclusively TFPIα protein, as previously shown for human platelets.14 The conserved expression of TFPIα in adult mouse platelets that occurs when all other tissues switch to production of TFPIβ suggests that platelet TFPIα may have a physiologically relevant role in limiting intravascular TF activity that is not performed by endothelial TFPI.

Novotny et al demonstrated that blood samples obtained from the site of a skin wound have progressively increasing concentrations (2- to 3-fold) of TFPI.10 They hypothesized that this increase is due to TFPI released from platelets that acts to limit TF and factor Xa (fXa) activity at the site of a tissue wound. Additionally, because platelets accumulate at the site of vascular injury, platelet TFPI is optimally localized to inhibit blood-borne forms of TF that incorporate within a growing intravascular thrombus. A mouse model of hematopoietic cell TFPI deficiency was developed to investigate the ability of platelet TFPI to locally modulate thrombus formation. Data obtained from this model demonstrate that platelet TFPI significantly moderates late-stage thrombus growth following vascular injury.
Methods

Western Blot Analysis
Mouse heart tissue and platelets were examined for TFPI isoform expression by Western blot analysis following precipitation with fXa and deglycosylation as previously described.13

Generation of a Murine Model of Platelet TFPI Deficiency
All animal experiments were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee. TFPI-heterozygous C57Bl/6J knockout mice1 were a gift from Dr George Broze, Jr. Fetal livers from TFPI+/− and TFPI−/− embryos were harvested at embryonic day 14.5, processed into single-cell suspensions, and cryopreserved in 90% fetal bovine serum, 10% dimethyl sulfoxide containing 67 nmol/L trehalose. C57/BL6 TFPI−/− recipient male mice, 8 to 12 weeks old, were lethally irradiated with 1100 cGy using a Gamma Cell 40 Exactor (Best Theratronic Ltd., Ottawa, Ontario, Canada), anesthetized using 120 mg/kg ketamine and 15 mg/kg xylazine intraperitoneally, and transplanted with TFPI−/−/− fetal liver cells via retroorbital injection. Genomic DNA from peripheral blood was used to genotypes transplanted mice.

Platelet TFPI Activity Assays
Platelets were isolated from transplanted mice using blood collected from the inferior vena cava. Blood was diluted 2-fold with buffered saline glucose citrate (BSGC; 129 mmol/L NaCl, 13.6 mmol/L sodium citrate, 11.1 mmol/L glucose, 1.6 mmol/L KH2PO4, 8.6 mmol/L NaH2PO4, pH 6.5) and centrifuged at 130g for 10 minutes to obtain platelet-rich plasma. Platelets were pelleted at 700g for 10 minutes, resuspended in BSGC buffer, and counted using an Animal Blood Counter (SCIL Animal Care Co., Gurnee, IL). Following multiple freeze-thaw cycles, platelet lysates were prepared and used at a final concentration of 9×10^6 platelets per well to analyze them for TFPI activity using fXa generation assays.11 In some experiments, lysates were preincubated with polyclonal anti-mouse TFPI antibody (a gift from Dr. Robert Simari) for 1 hour at 23°C.

Mouse Analysis
Blood was collected from the inferior vena cava of mice 8 weeks posttransplant in 10% (v/v) 0.13 mol/L sodium citrate. Complete blood count analyses were performed on whole blood using the Animal Blood Counter.

Mouse TFPI ELISA
MaxiSorp CS80 96-well plates (Thermo Fisher Scientific Inc, Waltham, MA) were coated with bovine fXa (10 μg/mL) in PBS (150 mmol/L NaCl, 10 mmol/L NaH2PO4, pH 7.5) overnight at 4°C. After being washed 3 times with PBS containing 0.05% Tween-20 (also used in subsequent washes), plates were blocked with 0.05% casein at 37°C for 1 hour. Plasma samples were diluted 1:10 in PBS and incubated at 37°C for 1.5 hours. Plates were washed, and polyclonal rabbit anti-mouse TFPI was incubated at room temperature for 1.5 hours. The plates were again washed and then blocked with casein containing 0.025% normal goat serum at room temperature for 30 minutes. The blocking buffer was discarded, and goat anti-rabbit Alexa Fluor 488 (Invitrogen, Eugene, OR) was incubated at room temperature for 30 minutes. The plate was washed, and fluorescence was measured using a Victor2 V analyzer (PerkinElmer, Waltham, MA).

Thrombin-Antithrombin Complex Assay
Plasma thrombin-antithrombin complex was determined using an ELISA according to the manufacturer’s instructions (Siemens, Deerfield, IL).

Venous and Arterial Injury Models
In vivo platelet function was evaluated in fetal liver recipient mice at 8 to 12 weeks posttransplant. Anesthesia was induced using intraperitoneal sodium pentobarbital (50 mg/kg). The thrombus induction models and fluorescence imaging system are described elsewhere (Cooley BC, submitted for publication, 2010). Briefly, mice were preinjected with platelets obtained from donor mice of the same genotype/transplant group and prelabeled with Vybrant DiD (Invitrogen); monoclonal antifibrin antibody (does not bind fibrinogen; hybridoma cells were a gift from Dr Marshall Runge) labeled with Alexa Fluor-532 (Invitrogen) was coinjected. Electrolytic injuries were created in femoral veins and carotid arteries using a steel microsurgical needle applied to the outer surface of each vessel, with positive direct current (1.5 volts for veins, 3.0 volts for arteries) delivered for 30 seconds. Vessels were illuminated uniformly with beam-expanded green (532 nm) and red (650 nm) laser light. Fluorescent images were captured over a 60-minute interval with a low-light video camera attached to an operating microscope at ×100 magnification; video images were taken every 2 minutes for analysis of relative fluorophore intensity (ImageJ software) within the thrombus zone and normalized for interanimal comparisons (Cooley, submitted for publication).

Statistics
For plasma assays, the Student t test and ANOVA were performed using GraphPad Prism software (San Diego, CA). For the vascular injury model, 10, 20, 30, and 60 minutes were selected as representative of the growth and stabilization of the clots for use in statistical analysis. Quantitative data were analyzed with ANOVA, using the post hoc Student least significant differences test for comparisons between platelet genotypes at each time point. A probability value of <0.05 was used to assign statistical significance.

Results

Murine Platelets Make TFPIα
Platelet TFPI isoform production was examined using Western blot analysis following protein deglycosylation because glycosylated TFPIα and TFPIβ migrate at the same molecular weight.13,14 Deglycosylated mouse platelet TFPI migrates as a single band at the molecular weight of TFPIα (Figure 1). In a simultaneously run control reaction to assess the activity of the deglycosylation enzymes, mouse heart TFPI migrated at the molecular weight of TFPIβ, demonstrating that the deglycosylation reactions were effective. Thus, the slower migration of deglycosylated platelet TFPI is due to its larger size (that of TFPIα) and not to incomplete deglycosylation of another TFPI isoform.

Generation of a Mouse Model of Hematopoietic TFPI Deficiency
A model of hematopoietic TFPI deficiency was generated to investigate the contribution of circulating blood cell TFPI in the regulation of intravascular TF activity and thrombus formation. Because TFPI-null mice die during embryonic development,15 embryonic day 14.5 fetal livers were used to produce mice lacking functional hematopoietic TFPI. Genotyping of whole blood from the TFPI−/− recipient mice confirmed the presence of either TFPI+/− or TFPI−/− blood cells and lack of detectable TFPI−/− blood cells (Figure 2A). There were no significant differences in hematocrit, white blood cell count, or platelet count between mice transplanted with TFPI+/− or TFPI−/− fetal liver cells (Figure 2B). Platelet TFPI activity assays confirmed that the platelets from mice transplanted with TFPI−/− fetal liver cells lacked...
Loss of hematopoietic TFPI does not significantly alter the plasma TFPI concentration

An ELISA that pulls down TFPI with fXa was used to detect functionally active plasma TFPI. We have previously demonstrated that fXa-precipitated plasma from TFPI<sup>+/−</sup> mice contains only full-length TFPI<sup>13</sup> and not both the full-length and the K1 deleted forms that are detected when pulling down with anti-TFPI antibody.<sup>15</sup> No significant differences in the plasma TFPI concentration were found between nontransplanted TFPI<sup>+/+</sup> mice and TFPI<sup>+/−</sup> mice transplanted with either TFPI<sup>+/+</sup> or TFPI<sup>+/−</sup> fetal liver cells (Figure 3A). Although there is a trend for lower plasma TFPI in mice with TFPI<sup>−/−</sup> cells, there is not a trend for higher plasma TFPI in mice with TFPI<sup>+/+</sup> cells, which is consistent with the hematopoietic cells not contributing to active plasma TFPI.

Hematoipoietic TFPI limits thrombus size following vascular injury by reducing platelet accumulation within the growing thrombus

In the venous electrolytic injury model, mice with TFPI<sup>+/+</sup> or TFPI<sup>+/−</sup> platelets had essentially identical rates of platelet accumulation during the first 12 minutes following injury (Figure 4A and 4B). Thereafter, platelet accumulation continued in the mice with TFPI<sup>+/−</sup> platelets, whereas it slowed, peaked, and began to decrease at 14 minutes in the mice with TFPI<sup>+/+</sup> platelets. At 30 minutes, the mice transplanted with TFPI<sup>−/−</sup> platelets had significantly more platelets in the thrombus than the mice with TFPI<sup>+/+</sup> platelets (<i>P</i>=0.039). An associated difference in the amount of fibrin was not observed.

The arterial injury model produced the same general pattern of thrombus growth observed in the venous model (Figure 4C and 4D). The rate of platelet accumulation was approximately the same for the first 6 minutes following injury before they began to diverge. Total platelet accumulation at 30 minutes was significantly higher in the mice with TFPI<sup>−/−</sup> platelets (<i>P</i>=0.024). Again, an associated difference in the amount of fibrin was not observed.
Discussion

Endothelial cells are thought to be the major source of intravascular TFPI. However, other cells within the vasculature make TFPI, including platelets and monocytes. We have demonstrated that mouse platelets produce TFPIα. Thus, in regards to the production of alternatively spliced isoforms of TFPI, platelets are similar to placenta and embryonic tissues that also produce TFPIα, rather than adult tissue vascular beds that produce TFPIβ. The third Kunitz domain and basic C-terminal region present in TFPIα, which are lacking in TFPIβ, have been evolutionarily conserved from fish to primates, over 430 million years, suggesting that platelet TFPI may have a physiological role in limiting thrombus formation following vascular injury.

A mouse model of TFPI deficiency was developed by transplanting fetal liver cells from TFPI+/− or TFPI−/− embryos into lethally irradiated adult TFPI+/− recipients. The TFPI+/− and TFPI−/− recipient mice had comparable hematocrit, white blood cell count, and platelet count. TFPI activity assays performed using washed platelet lysates demonstrated the absence of functional TFPI in the mice transplanted with TFPI−/− fetal liver cells, definitively confirming successful generation of mice lacking platelet TFPI.

TFPI−/− mice were chosen as transplant recipients because they have one-half the plasma TFPI concentration of wild-type mice, and their use limits confounding effects of plasma TFPI on thrombus growth in the vascular injury models while maximizing the effects of platelet TFPI. There was no difference in the active plasma TFPI concentration in the mice transplanted with TFPI+/− or TFPI−/− fetal liver cells, demonstrating that hematopoietic cells do not significantly contribute to the active TFPI in mouse plasma. This finding is consistent with mouse plasma containing predominantly TFPIβ with little to no TFPIα.

Plasma TAT levels were not elevated in mice transplanted with TFPI−/− fetal liver cells, demonstrating that lack of hematopoietic TFPI does not produce a procoagulant state. This finding is consistent with the cell surface expression and release of TFPI from only highly activated platelets and suggests that hematopoietic TFPI acts locally at the site of vascular injury rather than as a systemic anticoagulant.

Figure 3. A, Platelet TFPI-null mice have normal plasma TFPI concentration. Plasma TFPI concentration was measured using a mouse TFPI ELISA. There were no significant differences between nontransplanted TFPI+/− mice (TFPI-HET; ▲) and TFPI−/− mice transplanted with TFPI+/− (▼) or TFPI−/− (●) fetal liver cells. The plasma TFPI concentration of wild-type mice is shown for comparison (■). B, Absence of platelet TFPI does not promote a generalized prothrombotic state. Plasma TAT concentration was measured using a commercially available ELISA. There were no significant differences in plasma TAT levels of TFPI+/− mice transplanted with TFPI+/− (TFPI-HET; ■) or TFPI−/− (▲) fetal liver cells. WT indicates wild-type.

Figure 4. Mice with TFPI−/− platelets form enlarged thrombi following venous or arterial electrolytic injury. The femoral vein (A and B) and carotid artery (C and D) were studied in an electrolytic vascular injury model. The relative normalized fluorescence intensity of platelets (A and C) and fibrin (B and D) within the thrombi were measured over time. TFPI+/− mice transplanted with TFPI−/− fetal liver cells (▼) produced larger thrombi than those transplanted with TFPI+/− fetal liver cells (○) in both the venous (n = 7 for mice with TFPI+/− platelets; n = 5 for mice with TFPI−/− platelets) and arterial (n = 5 for mice with TFPI+/− platelets; n = 7 for mice with TFPI−/− platelets) model systems, primarily because of increased numbers of platelets accumulating within the growing blood clot (P = 0.039 for the venous model, P = 0.024 for the arterial model). There were no significant differences in the amount of fibrin present within the thrombi of the different groups of mice in either the venous or the arterial model system.
Platelets accumulate at the site of microvascular injury in 3 distinct phases. In the first phase, there is a net accumulation of platelets within the thrombus. During the second phase, there is a net loss of platelets. During the third phase, the platelets stabilize at a plateau. Formation of a fibrin mesh network is necessary for stabilization of the platelet thrombus. TF present on microparticles released from activated leukocytes incorporates into the growing blood clot via interactions between P-selectin expressed on the surface of activated platelets and P-selectin glycoprotein ligand-1 present on the microparticles. The importance of blood-borne TF in thrombus development has been demonstrated in a ferric chloride vascular injury model performed in mice with hemophilia A, where microparticle-associated TF significantly improved the kinetics of clot formation.

The effect of hematopoietic TFPI on thrombus growth was assessed in models of large vessel arterial and venous injury in the platelet TFPI-null mice, using models similar to previously published models. Both vessel types demonstrated similar patterns of thrombus growth. The initial phase of platelet accumulation within the thrombus was not affected by the presence of TFPI or TFPI-/- platelets. However, in mice with TFPI-/- platelets the platelet accumulation phase lasted longer, generating a larger thrombus when compared with mice with TFPI-/+ platelets. This pattern of thrombus growth is consistent with platelet TFPI altering the kinetics of clot formation by limiting platelet accumulation and the thrombus stabilization that occurs as a result of blood-borne TF accumulating within the developing thrombus.

Inhibition of the later stages of thrombus formation by platelet TFPI suggests that it may act to limit thrombus propagation and prevent clinical diseases, such as myocardial infarction and deep venous thrombosis, which are a result of total vessel occlusion.

Fibrin deposition within thrombi produced in both the venous and arterial injury models was essentially identical in both groups of mice. This is somewhat unexpected result because a rational hypothesis would be that reduced inhibition of the blood-borne TF as it incorporates into a growing clot would result in a measurable increase in the amount of fibrin within the clot. However, studies performed using a laser-induced arteriole injury model have demonstrated that in wild-type mice, most of the fibrin deposition in the thrombi occurs near the vessel wall/platelet thrombus interface, with lesser amounts farther from the vessel wall. Furthermore, it has been demonstrated that thrombin cleavage of platelet PAR4 is necessary for propagation of the platelet thrombus at a distance from the vessel wall. Interpretation of the data presented here in the context of these previously published results suggests that blood-borne TF that incorporates within a growing thrombus at a distance from the vessel wall may function primarily to generate thrombin that promotes platelet activation through PAR4 cleavage rather than generating additional fibrin within the clot.

Because there are no differences in the active TFPI concentration in the 2 groups of mice, plasma TFPI is not responsible for the differences in thrombus size observed. TFPI has been identified on the surface of human monocytes, a leukocyte that would be expected to be altered by fetal liver transplantation. Monocytes lacking TFPI are unlikely to contribute to the procoagulant phenotype observed because they account for only 1.4% of the white blood cells in C57Bl/6J male mice (Mouse Phenome Database, http://Jax.org) and do not initially accumulate at sites of vascular injury. It is possible that increased procoagulant activity on microparticles derived from monocytes lacking TFPI may contribute to the increased thrombus size observed in the mice transplanted with TFPI-null fetal liver cells. However, mice lacking monocyte TFPI do not produce increased clot volume following arterial ferric chloride injury. Thus, platelets that are directly accumulating at the site of vascular injury are likely the predominant source of TFPI contributing to the difference in thrombus growth observed in the vascular injury models.

Mouse and human platelets both produce TFPIα, and similar to human platelets, mouse platelets express 10- to 20-fold more TFPI on their surface following dual activation with thrombin and calcium ionophore than with either agonist alone (S.A. Maroney and A.E. Mast, unpublished data). Nevertheless, interpretation of the data presented here in the context of human disease is somewhat limited because the predominant plasma isoforms of TFPI are different between mice (TFPIβ) and humans (TFPIα). In addition, humans have a large pool of heparin-releasable TFPI that is not present in mice. Because TFPIα and TFPIβ may have different anticoagulant potencies when circulating in plasma, it is not clear whether or not the human plasma pool of TFPI would compensate for a lack of platelet TFPI to a greater degree than is observed in the mouse model presented here. However, the localization of platelets at the site of vascular injury suggests that platelet TFPI is an important anticoagulant protein that acts to regulate thrombus growth independently of the presence of plasma or endothelial pools of TFPI.

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