Endogenous Tissue Factor Pathway Inhibitor Modulates Thrombus Formation in an In Vivo Model of Rabbit Carotid Artery Stenosis and Endothelial Injury

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Background—Tissue factor pathway inhibitor (TFPI) is the sole known inhibitor of the extrinsic coagulation pathway of physiological importance; however, its role in modulating thrombosis in vivo is still unclear. Methods and Results—Intravascular thrombosis was initiated by placing an external constrictor around endothelially injured rabbit carotid arteries (n = 10). Carotid blood flow velocity was measured by a Doppler flow probe. After placement of the constrictor, cyclic flow reductions (CFRs), due to recurrent thrombosis, developed at the site of stenosis. Transstenotic TFPI plasma activity was measured in blood samples before induction of CFRs and after 30, 60, and 180 minutes of CFRs. TFPI plasma activity distal to the site of thrombosis was significantly lower than the corresponding proximal values at 30, 60, and 180 minutes of CFRs. In addition, a progressive decrease in TFPI plasma activity was observed in both the proximal and the distal samples, indicating consumption of TFPI during thrombus formation. In 10 additional rabbits, CFRs were abolished by administration of aspirin (10 mg/kg). In the animals in which aspirin abolished CFRs, endogenous TFPI was depleted by a bolus of a polyclonal antibody against rabbit TFPI, and the effects on restoration of CFRs were monitored. In 5 of 6 animals in which aspirin abolished CFRs, depletion of endogenous TFPI activity caused full restoration of CFRs. Conclusions—The data of the present study support the involvement of endogenous TFPI in the process of thrombus formation in vivo and its active role in modulating arterial thrombosis.

Key Words: inhibitors • thromboplastin • coagulation • blood flow

A ccumulating evidence indicates that the complex tissue factor (TF)–activated factor VIIa (FVIIa) is a key initiator of arterial thrombosis in vivo. Coronary thrombosis generally occurs at sites of a preexisting atherosclerotic plaque, often precipitated by fissuration or ulceration of the plaque.1 Because atherosclerotic plaques are rich in TF-synthesizing cells, such as monocytes, foam cells, and mesenchymal-like cells, plaque rupture may lead to exposure of TF to flowing blood,2 binding of factor VII/VIIa to TF, and activation of TF-dependent coagulation. Several studies have identified both TF antigen and activity within human atherosclerotic plaques and have suggested that it might represent an important determinant of thrombogenicity after plaque rupture.3–5 Furthermore, a number of experimental studies have demonstrated that blocking the procoagulant activity of TF usually results in inhibition of intravascular thrombus formation in a variety of animal models.6–9

An important regulator of TF-mediated coagulation pathway is tissue factor pathway inhibitor (TFPI), a multivalent protease inhibitor with 3 Kunitz-type domains; TFPI inhibits TF-dependent coagulation cascade initially by binding factor Xa (FXa) via its Kunitz II domain and subsequently by binding to FVIIa catalytic complex via its Kunitz I domain.10 This prevents further production of factor IXa and FXa, as well as autoactivation of FVII in the FVII/TF complex.11 The formation of a quaternary complex, TF/FVIIa/TFPI/Xa, dampens ongoing coagulation and may allow modulation of thrombosis in vivo. However, the pathophysiological role of TFPI during thrombus formation in vivo is still unclear. Although recombinant TFPI administered in pharmacological concentrations has been clearly shown to inhibit in vivo thrombosis,12 to the best of our knowledge there is no evidence that TFPI at its physiological plasma concentrations might affect FVIIa-TF activity during arterial thrombosis in vivo.

Therefore, the aim of this study was to investigate the role of endogenous TFPI in modulating arterial thrombosis in an in vivo model of rabbit carotid artery thrombosis. To test this,
in stenotic and endothelially injured arterial vessels affected by recurrent cycles of thrombus formation, we measured transtenotic plasma TFPI activity. In addition, to demonstrate the role of endogenous TFPI in modulating arterial thrombosis, we verified whether the neutralization of TFPI anticoagulant activity would turn the hemostatic balance toward a prothrombotic state with the consequent restoration of recurrent arterial thrombosis previously inhibited by aspirin.

Methods

Rabbit Model of Thrombosis

The rabbit model of recurrent intravascular thrombus formation has been described in detail elsewhere and represents a modification of the canine model originally described by Folts et al. Briefly, New Zealand White rabbits of either sex were anesthetized with a mixture of ketamine (35 mg/kg IM) and xylazine (5 mg/kg IM). Anesthesia was maintained during the course of the experiment by an intravenous infusion of ketamine sufficient to abolish the corneal reflex. Through a median incision of the neck, either the left or the right carotid artery was exposed and carefully isolated from the surrounding tissue. Polyethylene catheters were inserted into a jugular vein for drug administration and also into the abdominal aorta via a femoral artery for continuous blood pressure monitoring. A segment of the exposed carotid artery was injured by gentle squeezing of the artery between a pair of rubber-covered forceps. An external plastic constrictor was positioned proximal to the damaged site. Carotid blood flow velocity was measured continuously by a Doppler flow probe positioned proximal to the constrictor. Induction of arterial damage and stenosis produced cyclic fluctuations of arterial blood flow (cyclic flow reductions [CFRs]).

CFRs were monitored in all animals for 30 minutes. CFR frequency (cycles per hour) and severity (carotid blood flow at its nadir, expressed as a percent of baseline values), heart rate, and arterial blood pressure were monitored continuously throughout the experiment. In all animals, heparin was not used so as to avoid heparin-induced release of TFPI from the endothelium.

Experimental Protocol: Group 1A

In this group of rabbits (n = 10), CFRs were monitored for 180 minutes. Serial blood samples were taken from the abdominal aorta (proximal site) and from the stenosed and endothelially injured carotid artery distal to the site of thrombus formation to measure transtenotic TFPI plasma activity at the following time points: baseline (ie, before CFRs were induced) and 30, 60, and 180 minutes after initiation of CFRs. Blood samples (1 mL) were drawn simultaneously in a plastic syringe containing 100 μL of 3.8% sodium citrate with a 24-gauge needle inserted distal to the site of stenosis and endothelial damage and from the polyethylene catheter placed in the aorta. The sites of blood sampling were then sutured with a 7-0 silk. Blood samples were immediately placed on ice, centrifuged at 2000g for 10 minutes at 4°C to separate the plasma, and stored at −80°C. TFPI plasma activity was measured as described below.

Experimental Protocol: Group 1B

This group of rabbits (n = 6) was included to rule out the possibility that surgery and/or suture sites could have caused multiple thrombosis and affected TFPI activities per se. Rabbits were subjected to the surgical procedure described above, but the carotid artery was not damaged, and the constrictor was not positioned around it. Blood samples were obtained at baseline and after 30, 60, and 180 minutes simultaneously from the catheter placed in the abdominal aorta and from the carotid artery. The sites of sampling were sutured with a 7-0 silk.

Experimental Protocol: Group 2

Ten rabbits were included in this arm of the study. After 30 minutes of CFRs, aspirin was administered as an intravenous bolus of 10 mg/kg. Animals in which aspirin was not effective in abolishing CFRs were excluded from the study. To test the hypothesis that endogenous TFPI activity is important in modulating thrombus formation, 30 minutes after CFRs were completely abolished by aspirin, the endogenous activity of TFPI was neutralized by administration of a bolus of a polyclonal antibody against rabbit TFPI (see below) at a dose of 1 mg/kg IV. This dose was calculated on the basis of in vitro experiments (see below). After administration of anti-TFP antibody, animals were followed up for 1 hour. To determine whether the antibody was indeed effective in neutralizing endogenous TFPI activity, venous blood samples were obtained before and after antibody administration to measure total plasma TFPI activity.

Goat Anti-Rabbit TFPI Polyclonal Antibody

Anti-rabbit TFPI polyclonal antibody was raised in goats. The antigen used for immunization (purchased from PRIMM SpA) was a 10-amino-acid polypeptide conjugated with ovalbumin, the sequence of which corresponds to amino acids 106 to 116 of rabbit TFPI; this sequence represents the binding site of TFPI to FXa on the second Kunitz domain. The immunization protocol has been described elsewhere. Goat anti-rabbit TFPI IgGs were purified by immunoaffinity chromatography with a commercial kit according to the manufacturer’s instructions (QuickPure affinity column, Sterogene Bioseparations, Inc). Purified antibody was dialyzed against PBS, filter-sterilized, and stored in aliquots at −80°C. By the TFPI plasma activity assay described below, 15 μg of goat anti-rabbit TFPI polyclonal antibody inhibited the TFPI activity contained in 1 mL of rabbit plasma by >95%. The antibody recognized rabbit TFPI in Western blot experiments as a major band of ~34 kDa and minor bands of higher molecular weight, as previously described (data not shown).

Measurements of Plasma TFPI Activity

TFPI activity in rabbit plasma was determined by a 2-step colorimetric assay, as previously described, based on the ability of the antibody to inhibit FXa activity. Briefly, in the first step, a dilution of the test sample is incubated with a saturating concentration of FVIIa, a limiting concentration of TF, a low concentration of FXa, and calcium ions. In the second step, a high concentration of FX is added to the reaction mixture as a substrate for residual FVIIa-TF catalytic activity; the FXa generated is measured with a specific chromogenic substrate (Chromozym X, Boehringer Mannheim). The principles of the assay are summarized in Figure 1. Absorbance was read at 405 nm. Linear standard curves were obtained with different dilutions of reference plasma (pooled normal rabbit plasma). All test samples were assayed at a 1% dilution. This assay is able to detect TFPI activity present in 0.8 μL of control rabbit plasma, which corresponds to ~80 pg of TFPI, assuming a TFPI concentration in normal plasma of ~100 ng/mL. Results are expressed as percent of TFPI activity in pooled rabbit plasma.

Statistical Analysis

All values are expressed as mean ± SEM. The rate of CFR restoration by anti-TFPI polyclonal antibody was evaluated by Fisher’s exact test. A 1-way ANOVA with a design for repeated measurements was used to compare hemodynamic variables and plasma TFPI activity, followed, when appropriate, by a Student’s t test with Bonferroni’s correction. A value of P < 0.05 defined significant differences between populations.

Results

Group 1

CFR Induction and Hemodynamic Parameters

After arterial injury and placement of the constrictor, CFRs developed in all group 1 rabbits (n = 10), with a mean...
frequency of 19.2±2.5 cycles per hour. The severity of CFRs, expressed as the nadir of carotid blood flow velocity (the lowest blood flow velocity recorded before flow restoration), averaged 5±1% of baseline. No significant differences in averaged 5 lowest blood flow velocity recorded before flow restoration), expressed as the nadir of carotid blood flow velocity (the frequency of 19.2

2.5 cycles per hour. The severity of CFRs, expressed inversely related to amount of TFPI present in test sample, is amount of FXa generated, which is inversely related to amount of TFPI present in test sample, is measured with a specific chromogenic substrate (Chromozym X). Absorbance was read at 405 nm. Linear standard curves were obtained with different dilutions of reference plasma (pooled normal rabbit plasma).

Transstenotic TFPI Plasma Activity

In group 1A animals, under baseline conditions, ie, before the artery was damaged, no significant differences were seen in TFPI plasma activity in blood samples obtained proximal and distal to the site of subsequent thrombus formation, indicating absence of nonspecific changes in plasma TFPI activity at different sites of blood sampling. After 30 minutes of continuous CFRs, plasma TFPI activity was significantly lower in samples obtained distal to the site of thrombus formation than in the samples obtained from the proximal site, 91.1±3.2% versus 103±3.7% of reference pooled plasma activity, respectively, P<0.05. This difference persisted and actually increased at 60 and 180 minutes, 83.9±6.0% versus 90.3±3.9% and 77.3±4.6% versus 83.5±3.8%, respectively (Figure 2). In addition, a progressive reduction in TFPI activity was observed in both the proximal and the distal samples, demonstrating a progressive consumption of TFPI at the site of recurrent thrombus formation (Figure 2).

In group 1B animals, TFPI plasma activities in blood samples obtained from the carotid artery did not differ significantly from those measured in blood samples taken from the abdominal aorta at each time point, indicating that the surgical procedure had no effect on TFPI activity per se (Table).

Discussion

The major findings of the present study are that during intravascular thrombus formation, TFPI activity was significantly lower in samples obtained from carotid artery distal to site of thrombus formation with respect to corresponding proximal samples at 30, 60, and 180 minutes of cyclic flow variations. Furthermore, TFPI activity progressively decreased over time in both proximal and distal samples, indicating consumption of TFPI at site of thrombus formation. *P<0.05 vs corresponding proximal value; #P<0.05 vs corresponding baseline value.

Group 2

Effects of Anti-TFPI Antibody Administration on CFR Inhibition by Aspirin

In this group (n=10), the administration of aspirin 30 minutes after CFRs were induced caused complete inhibition of recurrent thrombosis in 6 of 10 rabbits. The remaining 4 rabbits, in which aspirin failed to inhibit CFRs, were excluded from the study. After CFRs had been abolished for 30 minutes, a polyclonal antibody against rabbit TFPI was administered to inhibit endogenous TFPI activity. Administration of this antibody markedly decreased TFPI plasma activity, from 106.0±13.9% (% of reference plasma activity) to 1.4±1.8% (P<0.01, Figure 3). This inhibition of endogenous TFPI activity resulted in a spontaneous restoration of CFRs in 5 of 6 animals, indicating that the inhibition of the anticoagulant activity of endogenous TFPI turned the hemostatic balance toward a prothrombotic state with a consequent restoration of recurrent thrombosis (Figure 4). These data further underline the importance of endogenous TFPI in modulating in vivo arterial thrombosis.

Figure 1. Schematic of 2-step colorimetric assay used to measure TFPI plasma activity. In step 1, a dilution of test sample is incubated with saturating concentration of factor VII (FVII), limiting concentration of tissue factor (TF), low concentration of factor Xa (FXa), and calcium ions. After formation of TF/FVII complex, amount of TFPI present in test sample can bind to FXa present in reaction mixture and thereafter to TF/FVII complex, forming quaternary complex TF/FVII/TFPI/FXa devoid of enzymatic activity. In step 2, a high concentration of FX is added to reaction mixture as substrate for residual TF/FVII catalytic activity not inhibited by TFPI; amount of FXa generated, which is inversely related to amount of TFPI present in test sample, is measured with a specific chromogenic substrate (Chromozym X). Absorbance was read at 405 nm. Linear standard curves were obtained with different dilutions of reference plasma (pooled normal rabbit plasma).

Figure 2. Transstenotic TFPI plasma activity during intracarotid thrombus formation. TFPI activity was significantly lower in samples obtained from carotid artery distal to site of thrombus formation with respect to corresponding proximal samples at 30, 60, and 180 minutes of cyclic flow variations. Furthermore, TFPI activity progressively decreased over time in both proximal and distal samples, indicating consumption of TFPI at site of thrombus formation. *P<0.05 vs corresponding proximal value; #P<0.05 vs corresponding baseline value.

The importance of TF-dependent activation of the coagulation cascade is suggested by several clinical and experimental studies. It has been demonstrated that atherosclerotic plaques are rich in TF-synthesizing cells, such as monocytes, foam cells, and fibroblasts. Therefore, it is possible to speculate that plaque fissuration may lead to TF exposure to flowing blood, with the consequent activation of the extrinsic

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pathway and intravascular thrombosis. Indeed, TF antigen has recently been demonstrated in human atherectomy specimens obtained from patients with clinical evidence of acute coronary syndromes in significantly higher concentrations with respect to patients with stable angina. In addition, other studies have demonstrated significant TF-dependent procoagulant activity localized in human atherosclerotic plaques. Finally, previous studies from our own group have demonstrated that inhibition of formation of TF/FVII complex results in marked antithrombotic effects.

TFPI is a Kunitz-type serine protease inhibitor of \( \approx 34 \) kDa. TFPI is a potent inhibitor of the factor VIIa/TF complex in the presence of factor Xa and is also a direct inhibitor of factor Xa. The inhibitory mechanism is currently thought to involve, in a first step, the formation of a TFPI-FXa complex, and in a second step, the formation of a quaternary TFPI-FXa/FVIIa/TF complex. In vivo, TFPI is confined to 3 different pools. A major pool of TFPI is bound to the endothelial surface, and this fraction may be released by heparin. Plasma contains a second smaller pool of TFPI (10% to 50% of the endothelial pool), mostly complexed with lipoproteins, whereas only <10% is carrier-free. A third pool of TFPI is confined to platelets (<10% of the plasma pool). The biological roles of these pools are still uncertain, but some evidence suggests that carrier-free TFPI is biologically most active.

Despite several indications of the role of the extrinsic coagulation pathway in arterial thrombosis, little is known about the in vivo contribution of TFPI to the regulation of coagulation during intravascular thrombus formation. For example, important evidence for TFPI as a natural anticoagulant in vivo is offered by the finding that plasma depletion of TFPI by polyclonal antibody sensitizes rabbits to TF- or endotoxin-induced disseminated intravascular coagulation. In other studies, exogenous recombinant TFPI was effective in inhibiting intravascular thrombosis, but this effect was achieved at doses of exogenous TFPI far higher than those present physiologically in plasma. Thus, to date there is no evidence that endogenous TFPI may be involved in the process of intravascular thrombus formation.

The results of the present study demonstrate that TFPI is involved in the regulation of arterial thrombosis in vivo. We have demonstrated that during thrombus formation, plasma TFPI activity measured distal to the site of thrombus formation is significantly lower with respect to plasma obtained at a proximal site, suggesting a direct involvement of TFPI at the site of thrombosis. An additional finding of the present study is that systemic plasma TFPI activity decreases during recurrent thrombosis, indicating a progressive consumption of this protein during the process of thrombus formation. A possible explanation for this finding may be related to the mechanisms regulating the cell surface TF/FVIIa proteolytic activity. A recent work by Sevinsky et al in fact did show that TF procoagulant activity is downregulated in the cells expressing TF by a translocation of the complex TF/FVIIa into noncoated plasmalemma vesicles. Interestingly, this translocation of TF is mediated by cell-associated TFPI, indicating that formation of the quaternary complex TF/FVIIa/Xa/TFPI is necessary for the transport of TF in the cytoplasm. Therefore, it can be speculated that the progressive consumption of TFPI at the site of thrombosis observed in the present study might be explained by an increased translocation of the quaternary complex TF/FVIIa/Xa/TFPI from the cell surface to the cytoplasm.

Another important finding of this study is that the endogenous activity of circulating TFPI plays an important role in modulating activation of the extrinsic pathway. This conclusion arises from the observation that depletion of circulating TFPI activities measured in control, sham-operated rabbits with no CFRs at different time points.

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<th>TFPI, % pooled plasma</th>
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<td>Proximal</td>
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<td>94.7±6.3</td>
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Proximal indicates samples obtained from the catheter placed in the abdominal aorta; distal, samples obtained from the carotid artery.

Figure 3. Effects of anti-rabbit TFPI polyclonal antibody (Ab) on plasma TFPI activity. After administration of antibody, a marked decrease in plasma TFPI was observed. *P<0.01.

Figure 4. Effects of depleting endogenous TFPI activity on spontaneous restoration of CFRs. In 6 rabbits, CFRs were inhibited by administration of aspirin (10 mg/kg). Thirty minutes after CFRs were abolished, a goat polyclonal anti-rabbit TFPI antibody (Ab) was injected (1 mg/kg IV). Within 30 minutes, CFRs spontaneously restored in 5 of 6 animals. CFV indicates cyclic flow variation.
TFPI by a polyclonal antibody determined a spontaneous restoration of CFRs previously abolished by aspirin. This finding may be explained by viewing hemostatic function in vivo as a balance of prothrombotic and antithrombotic stimuli. After endothelial damage, this balance is altered, as procoagulant stimuli, including TF exposure, prevail, leading to the activation of the extrinsic coagulation pathway and the activation of circulating platelets, with consequent release of mediators such as thromboxane A₂, serotonin, platelet-activating factor, and ADP. These platelet-derived mediators, recruiting other platelets, further promote platelet aggregation; thus, platelets and coagulation cascade interact strictly in the process of thrombus formation. Inhibiting ≥1 procoagulating or proaggregating factors, as we did in the animals treated with aspirin, creates a new equilibrium between activating and inhibiting stimuli, with consequent inhibition of thrombosis. However, if this equilibrium is altered again (for instance, eliminating ≥1 antithrombotic factors, as we did by blocking basal TFPI activity), thrombus formation at the site of arterial stenosis and endothelial damage may start again, because of a relative predominance of prothrombotic factors.

In conclusion, our study demonstrates the involvement of endogenous TFPI in the process of thrombus formation and its active role in modulating arterial thrombosis in vivo. Additional studies aimed at demonstrating this phenomenon in patients with acute coronary syndromes are warranted.

References

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