Role for Tissue Factor Pathway in Murine Model of Vascular Remodeling

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Abstract—Tissue factor (TF) is a low-molecular-weight glycoprotein that initiates the extrinsic clotting cascade and is considered a major regulator of arterial thrombogenicity. TF pathway inhibitor (TFPI) is a major physiological inhibitor of TF-initiated coagulation. The aim of this study was to define the complex interplay between TF and TFPI and the regulation of vascular thrombogenicity in a model of vascular remodeling. To determine the levels and pattern of vascular expression of TF and TFPI associated with vascular remodeling, a murine model of flow cessation was studied. TF activity of the arteries increased after ligation (P<0.05). Quantitative analysis of homogenates of remodeled carotid arteries revealed increased TF expression but unchanged TFPI expression compared with normal carotid arteries, resulting in enhanced TF activity. To determine the potential therapeutic role of TFPI in this thrombogenic state, mice were treated with intravascular adenoviral delivery of either murine TFPI (Ad-mTFPImyc) or a control adenovirus (Ad-ΔE1). Overexpression of TFPI decreased vascular TF activity compared with viral control (P<0.01). Overexpression of TFPI inhibited neointimal formation (P=0.038), resulting in enhanced luminal area (P=0.001) 4 weeks after flow cessation. In this murine model of vascular remodeling, an imbalance between TF and TFPI expression is generated, resulting in increased TF activity. Overexpression of TFPI in this model inhibits vascular TF activity and results in attenuation of vascular remodeling associated with flow interruption. (Circ Res. 2001;89:71-76.)

Key Words: thrombosis ■ arteriosclerosis ■ gene therapy

Arterial thrombosis is the proximate cause of myocardial infarction and stroke. Biochemical and clinical evidence suggests the importance of the thrombogenic nature of atherosclerotic vessels in this process. Tissue factor (TF), a low-molecular-weight glycoprotein that initiates the extrinsic clotting cascade, is considered a major regulator of coagulation, hemostasis, and thrombogenicity of atherosclerotic arteries.1–9 TF pathway inhibitor (TFPI), which provides physiological inhibition of TF-initiated coagulation by binding to factor Xa and the TF–factor VIIa complex in a 2-step process, is found in vascular endothelium and smooth muscle cells as well as in platelets, blood monocytes, and macrophages.10–14 In atherosclerotic carotid arteries, TF expression is abundant, whereas TFPI expression is limited in up to 30% of plaques, resulting in predominant TF activity.15 In plaque, where TFPI expression is the greatest, TF activity is attenuated. Thus, this imbalance between TF and TFPI expression in plaque may result in the prothrombotic phenotype associated with atherosclerosis. To investigate the development and potential regulation of this imbalance, a well-defined murine model of vascular remodeling, including neointimal formation, was studied.16

Materials and Methods

Animal Model

The murine model of vascular remodeling associated with carotid flow cessation as described by Kumar and Lindner16 was used. All procedures complied with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, Md). Briefly, via a ventral longitudinal incision, the left common carotid of adult C57BL/6 mice was identified and ligated with 4-0 silk just proximal to the bifurcation. The midline incision was closed with 6-0 vicryl, and the skin was closed with a Nexaband topical skin closure kit supplied by Veterinary Products Laboratories. Warmed lactated Ringer’s solution (1 mL SC) and Enrofloxacin (Bayer) (0.01 mL IM) were injected, and the mice were allowed to recover on a warm hydrothermal pad. At 1, 2, 3, and 4 weeks, the ligated carotid arteries were harvested as fresh frozen specimens for protein analysis, or they were harvested after perfusion fixation at physiological pressure with 10% formalin. Animals were treated with intravascular adenoviral delivery of either murine TFPI (Ad-mTFPImyc) or a control adenovirus (Ad-ΔE1). Overexpression of TFPI decreased vascular TF activity compared with viral control (P<0.01). Overexpression of TFPI inhibited neointimal formation (P=0.038), resulting in enhanced luminal area (P=0.001) 4 weeks after flow cessation. In this murine model of vascular remodeling, an imbalance between TF and TFPI expression is generated, resulting in increased TF activity. Overexpression of TFPI in this model inhibits vascular TF activity and results in attenuation of vascular remodeling associated with flow interruption.

Tissue Processing and Analysis

The carotid arteries were obtained from mice, thoroughly rinsed in saline, and freshly frozen in liquid nitrogen. thawed tissue was subsequently homogenized in 50 mmol/L Tris HCl, pH 8.0, by using a tissue homogenizer. The homogenate then was analyzed for protein content by using a Bradford assay (Bio-Rad).
Computer-aided morphometric analysis (Image Pro Plus) was performed on hematoxylin and eosin-stained sections by two investigators blinded to the treatment. Briefly, after perfusion fixation and rinsing, the entire carotid artery was embedded and cut at 250-μm intervals from the ligature. Because of the nature of the remodeling process, contiguous sections starting at a fixed distance from the ligature after fixation and embedding (500 μm) were analyzed morphometrically. These sections were uniformly free of thrombus. The areas subtended by the external and internal elastic laminae and lumen were measured, and means were compared between the treatment groups.

Adenovirus Generation

A first-generation recombinant adenoviral vector encoding for murine TFPI (Ad-mTFPImyc) was generated by using standard techniques. Briefly, the cDNA for murine TFPI (mTFPI) was kindly obtained from Dr George Broze at Washington University, St. Louis, Mo. A myc tag was placed on the 3′ end of the coding region to provide a unique epitope for detecting transgene expression. The mTFPImyc cassette containing a cytomegalovirus promoter/enhancer was cloned into a shuttle plasmid containing LoxP sites and recombined with a cosmid containing the Ad5 genome in the presence of Cre recombinase. This solution was then transfected into 293 cells, resulting in recombinant adenoviral generation. Final preparations were purified by using standard double–cesium chloride techniques. A control adenoviral vector without cDNA insert (Ad-ΔE1) was used as a control for adenoviral infection, and an adenovirus expressing Escherichia coli β-galactosidase from the cytomegalovirus promoter/enhancer (Ad-LacZ) was used as a reporter construct.

Immunohistochemical Staining for β-Galactosidase

Five-micron-thick transverse frozen sections of mouse carotid arteries harvested 2 days after delivery of Ad-LacZ were fixed with acetone for 10 minutes at −20°C, washed with PBS, and blocked for 30 minutes with 10% normal donkey serum. The sections were then incubated 1 hour with rabbit anti-β-galactosidase antibody (Molecular Probes) at a concentration of 10 μg/mL, washed with PBS containing Triton and normal donkey serum, and incubated with a biotinylated donkey anti-rabbit antibody (Amersham Pharmacia Biotech) for 30 minutes. After a PBS wash containing Triton and normal donkey serum, the sections were incubated for 45 minutes with streptavidin–alkaline phosphatase (Vector Laboratories). The reaction product was visualized by using Vector Blue with levamisole (Vector Laboratories). Nontransduced arteries failed to show immunoreactivity when studies were performed in parallel. The rabbit anti-mTFPI antiserum was prepared commercially (Cocalico Biologicals) after inoculation of rabbits with 1 mg recombinant mTFPI made in eukaryotic cells. Preimmunization serum was used as a control. A rooster anti-serum to murine TF was used (gift from the first. Ten microliters of the viral solution containing either Ad-LacZ, Ad-mTFPImyc, or Ad-ΔE1 (without cDNA insert) was also used as a control for adenoviral infection, and an adenovirus expressing Escherichia coli β-galactosidase from the cytomegalovirus promoter/enhancer (Ad-LacZ) was used as a reporter construct.

Immunoblot Analysis

TFPI protein was analyzed in ligated carotid arteries obtained at 1, 2, and 4 weeks. Equal amounts of protein were denatured by boiling for 5 minutes and resolved by electrophoresis on a 9% SDS-polyacrylamide gel. Transfer of protein to a nitrocellulose membrane was carried out for 3 hours at 4°C. Immunoblotting was performed by using the above-described rabbit anti-mouse TFPI antiserum at 1:500 dilution in nonfat milk/Tris-buffered saline. After washes, the membrane was probed subsequently with a rabbit secondary antibody conjugated to horseradish peroxidase (Amersham Life Sciences) at 1:500 dilution and developed with chemiluminescence (Supersignal, Pierce). The membrane then was exposed to x-ray film (Kodak) and subsequently developed. Immunoblotting for TF expression was performed by using rooster anti-mouse TF antiserum at 1:500 dilution in nonfat milk/Tris-buffered saline and by subsequently probing with a rabbit secondary antibody conjugated to horseradish peroxidase. The tissue homogenates from the mice treated with Ad-mTFPImyc or Ad-ΔE1 (without cDNA insert) were also immunoblotted with monoclonal anti-myc antibodies to differentiate between native and adenovirus-induced TFPI.

Determination of TF Activity

TF activity was determined as described previously. Diluting the samples with Tris HCl equalized the protein concentration in the samples. Thirty microliters of sample was added to a fibrin cup in a BBL Fibrosystem Fibrometer (Becton Dickson). This was followed by the addition of 30 μL of rabbit brain cephalin diluted 1:10 in 0.85% NaCl and 30 μL of 0.02 mol/L CaCl2. Thirty microliters of reconstituted mouse plasma was added immediately thereafter. The fibrometer was started, and the clotting time was recorded.

Statistical Analysis

Groups were compared by using unpaired Student t tests. A value of P<0.05 was considered significant.

Results

Vascular Remodeling Results in Enhanced Vascular Thrombogenicity

As previously described by Kumar and Lindner, ligation of the left carotid artery in C57BL/6 mice resulted in vascular remodeling associated with neointimal formation. To determine whether vascular remodeling results in increased vascular thrombogenicity, vascular TF activity was determined by measuring murine plasma clotting times in the presence of tissue homogenates from ligated arteries at baseline and at 1, 2, and 4 weeks after surgery. There was a significant decrease in the clotting time in the

![Figure 1. TF activity after arterial injury. Plasma clotting time of homogenates from arteries at baseline and 1, 2, and 4 weeks after ligation. *P<0.05 vs baseline.](Image 307x594 to 538x718)
ligated arteries at each time point after ligation \( (P < 0.05) \) compared with baseline (Figure 1). These results suggest that vascular remodeling in this murine model is associated with enhanced vascular thrombogenicity.

**Vascular Remodeling Leads to Imbalance Between TF and TFPI**

To define the mechanism of altered thrombogenicity of vascular homogenates, the levels and pattern of vascular expression of TF and TFPI associated with vascular remodeling were studied. Immunoblotting for TF and TFPI expression was performed in arterial homogenates from ligated arteries at 1, 2, and 4 weeks after surgery. Quantitative analysis of the immunoblots revealed that TF expression was increased in the ligated carotid arteries of every animal \( (n = 3) \) at each time point compared with normal carotid arteries (Figure 2). Expression of TF was maximal at 2 weeks after flow cessation. In contrast, TFPI expression was unchanged in ligated arteries compared with normal arteries. Thus, a pattern of gene expression favoring a thrombogenic phenotype associated with vascular remodeling was seen in this model as in previous studies of human tissue.\(^{15}\)

**Overexpression of Murine TFPI**

To determine whether the enhanced TF activity can be attenuated by heterologous vascular expression of TFPI, a replication-deficient adenoviral vector expressing mTFPI was generated (Ad-mTFPImyc). To overexpress mTFPI in ligated arteries, a direct in vivo method of gene transfer was established. To confirm gene transfer, immunostaining of arteries 2 days (at peak expression) after delivery of Ad-LacZ demonstrated transgene expression in the transduced arteries (Figures 3A and 3B). With this technique of distending luminal delivery, transgene expression was detected in cells within the adventitial portions of the arteries.

To further define the ability to overexpress mTFPI in ligated arteries, either Ad-mTFPImyc or Ad-ΔE1 was delivered at the time of ligation, and the animals were euthanized at 1 week. Immunoblotting revealed specific overexpression of the mTFPImyc construct by enhanced immunoreactivity to anti-mTFPI antiserum (Figure 3C). To determine whether overexpression of mTFPI would attenuate the enhanced TF activity associated with vascular remodeling, TF activity was measured in the ligated arteries \( (n = 5 \text{ per group}) \) treated with
Ad-mTFPImyc or Ad-ΔE1 at 1 week. There was a 26% increase (P<0.001) in the homogenous plasma clotting time in the Ad-mTFPImyc group compared with the viral control group. These data suggest that the imbalance between TF and TFPI results in increased TF activity with vascular remodeling and that this increase can be modulated by enhancing vascular TFPI expression.

**TFPI Overexpression Attenuates Vascular Remodeling**

To determine whether overexpression of TFPI resulting in inhibition of vascular TF activity might affect vascular remodeling in this murine model, the effects of Ad-mTFPImyc infection on vascular remodeling were studied and compared with control adenoviral infection (Ad-ΔE1). Four weeks after ligature, the Ad-mTFPImyc group demonstrated less neointimal formation, as measured by the neointimal/medial ratio (0.35±0.18 versus 1.7±0.50 in Ad-ΔE1, P=0.038) (Figure 4). There was no difference between the groups in external elastic laminae or medial areas. However, the luminal area was greater in the Ad-mTFPImyc group compared with the viral control group (2101±1254 μm² for Ad-ΔE1 versus 15482±2049 μm² for Ad-mTFPImyc, P=0.0014). Taken together, these data suggest that the TF pathway plays a role in the development of vascular remodeling in this murine model.

**Discussion**

Human studies suggest that vascular thrombogenicity is regulated by vascular expression of prothrombotic and anti-thrombotic factors. TF plays a central role in this process in spite of vascular expression of its inhibitor, TFPI. There are three lines of evidence that support the potential for TFPI to modulate vascular TF activity. First, studies in explanted human tissue suggest that expression of TFPI is related to attenuated TF activity. Second, studies have shown that inherent or recombinant TFPI alters local vascular TF activity and neointimal formation. Finally, gene transfer studies of TFPI have resulted in local antithrombotic effects. However, insights into the establishment and local regulation of this imbalance between TF and TFPI have not been defined. To do so, an animal model that mimics important aspects of human vascular disease without acute endothelial denudation was used. Unlike endothelial injury models of remodeling, this model results in the development of neointima in the absence of direct endothelial injury as a result of changes in shear stress and stasis. Thus, this model attempts to model the pathophysiological situation of lesion development in the absence of noticeable endothelial denudation.

A key finding of the present study is that the arterial wall developed a prothrombotic phenotype in this murine model concurrent with neointimal formation and vascular remodeling. This thrombogenic phenotype was due to increased vascular TF expression without a concomitant increase in TFPI expression. This imbalance resulted in increased vascular TF activity, which mirrors the imbalance demonstrated in human atherosclerotic plaque. Furthermore, vascular overexpression of mTFPI was performed by using a novel direct-delivery system. Overexpression of mTFPI attenuated the enhanced TF activity and attenuated vascular remodeling. Taken together, these data support the concept that this model of vascular remodeling is associated with a thrombogenic phenotype and that inhibition of TF activity by TFPI may inhibit vascular remodeling.

These data clearly identify a relative vascular deficiency of TFPI and an increase in TF in this model. The regulation of TF expression is well defined, and a number of stimuli for TF production in vascular cells have been identified, including thrombin, growth factors, and cytokines. In this model, TF is expressed within the media and the growing neointima from vascular and blood-borne sources. The lack of parallel increases in TFPI may be due to differences between the vascular regulation of TF and TFPI and the recruitment of nonvascular sources of TF. These differences favored robust TF expression in the setting of uninducible TFPI expression. Thus, the imbalance that was identified in the present study may be an ultimate result of these regulatory differences. Attenuation of TF activity by a TFPI transgene driven by viral regulatory sequences supports this contention.

The present study provides a mechanism to explain the results of two prior studies of TFPI gene transfer that demonstrated inhibition of vascular thrombosis in animal models. Distinct from these studies, the present study determines that TFPI overexpression is capable of modulating vascular TF activity and uses a species-matched form of the protein. The delivery technique used in the present study resulted in adventitial transgene expression.
and the intimal effects seen likely represent the potent secreted nature of the TFPI transgene affecting intravascular and/or intraluminal TF activity. Thus, the ability of TFPI expressed in the vasculature to inhibit TF activity likely explains the previously defined effects of TFPI overexpression in distinct animal models of intravascular thrombosis. To further delineate whether the effects seen in the present study are due to intravascular or endoluminal expression, studies with genetically determined alterations in vascular, systemic, or blood-derived TFPI expression will be performed.

Perhaps the most interesting finding of the present study is the effect of TF inhibition on the structural response to flow cessation without endothelial denudation. Previous studies have identified the importance of growth factors and P-selectin in the development of remodeling in this model. There are several potential mechanisms for TF and TFPI as regulators of vascular structure. Because intravascular or intramural fibrin deposition may act as a scaffold for plaque growth, the TF pathway may play an indirect role. In addition, TF may act via thrombin and factor Xa as a mitogen for vascular smooth muscle cells. TFPI has been shown to inhibit vascular smooth muscle cells in vitro. A role for TF in cellular migration has been proposed. Thus, inhibition of TF may have multifactorial effects on vascular remodeling.

Taken together, the findings of the present study clearly establish that vascular remodeling results in a prothrombotic vascular phenotype. This prothrombotic phenotype is, in part, due to an imbalance between TF and TFPI, which results in increased vascular TF activity. Attenuation of this increased thrombogenicity and neointimal formation by overexpression of TFPI provides evidence for therapeutic possibilities of mitigating both the enhanced thrombogenicity and vascular remodeling associated with vascular disease.

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