Expression, Localization, and Activity of Tissue Factor Pathway Inhibitor in Normal and Atherosclerotic Human Vessels

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Abstract—Tissue factor (TF) pathway inhibitor (TFPI) is the major downregulator of the procoagulant activity of the TF-factor VIIa (FVIIa) complex (TF ∙ FVII). The active TF present in the atherosclerotic vessel wall is proposed to be responsible for the major complication of primary atherosclerosis, namely, acute thrombosis after plaque rupture, but our knowledge of the sites of TFPI expression in relation to TF remains fragmentary. The aim of this study was to investigate the expression, localization, and activity of TFPI and its relation to the activity and distribution of TF in the normal and atherosclerotic vessel wall. We applied a novel approach in which serial cross sections of human vascular segments were used to perform a complete set of assays: immunolabeling for TFPI and/or TF, in situ hybridization for the expression of TFPI mRNA, ELISA for the determination of TFPI antigen, and functional assay for the activity of TFPI and TF. In healthy vessels, TFPI protein and mRNA are present in luminal and microvascular endothelial cells (ECs) and in the medial smooth muscle cells (SMCs). In atherosclerotic vessels, TFPI protein and mRNA frequently colocalized with TF in ECs overlying the plaque and in microvessels, as well as in the medial and neointimal SMCs, and in macrophages and T cells in areas surrounding the necrotic core. At the ultrastructural level, immunogold electron microscopy confirmed the localization of TFPI in ECs, macrophages/foam cells, and SMCs. In ECs and SMCs, the gold particles decorated the plasmalemma proper and the caveolae. ELISA on cross sections revealed that atherosclerotic tissues contain more TFPI than do the healthy vessels. TFPI was functionally active against TF ∙ FVIIa-induced coagulation, and its activity was higher in those tissues that display less TF. The largest amount of TFPI and TF were detected in complicated arterial plaques. By immunofluorescence, TFPI colocalized with platelet- and fibrin-rich areas within the organized thrombi. Atherosclerotic vessel sections promote activation of factor X, which is dependent on the presence of TF and enhanced by preincubation of the sections with anti-TFPI IgG. Taken altogether, our results suggest that TFPI is largely expressed in the normal vessel wall and enhanced in the atherosclerotic vessel, in a manner suggesting a significant role of TFPI in the regulation of TF activity. (Arterioscler Thromb Vasc Biol. 2000;20:1362-1373.)

Key Words: atherosclerosis ■ tissue factor pathway inhibitor ■ anticoagulant activity ■ immunocytochemistry ■ thrombosis

Endothelial cells (ECs) play a central role in the regulation of hemostasis by ensuring the cellular control of procoagulant and anticoagulant mechanisms. Anticoagulant and profibrinolytic functions predominate in the quiescent state of the endothelium, thus maintaining blood fluidity (for a review, see References 1 and 2). Blood coagulation is initiated when factor VII/VIIa (FVII/VIIa) in plasma gains access to tissue factor (TF) at sites of blood vessel injury and the resulting TF ∙ FVIIa complex activates factor X (FX) to factor Xa (FXa) and factor IX (FIX) to factor IXa (FIXa), leading to thrombin generation and the formation of a fibrin clot.1 In the normal state, vascular TF is located on cells in the adventitia and variably on cells in the media.4,5 Healthy ECs do not show detectable TF,5 although expression of endothelial TF occurs in vitro after perturbation of ECs with different agonists6–9 and in vivo during sepsis,10 within the tumor vasculature,11 or in ECs overlying the atherosclerotic plaques.12 Significant TF has been reported to be deposited in the extracellular matrix surrounding mRNA-positive cells adjacent to cholesterol clefts and within the necrotic core of advanced atherosclerotic plaques,5 suggesting that TF contributes to the hyperthrombotic state of human atherosclerotic vessels.5 Fissuring and rupture of an atherosclerotic plaque with the subsequent formation of occlusive thrombi are thought to represent the central cause of acute ischemic syndromes.13-15 Increased levels of TF have been detected in
unstable angina, correlating with the presence of large areas rich in macrophages and smooth muscle cells (SMCs). Therefore, the macrophage-rich atherosclerotic plaques (soft plaques), which have a high risk for rupture, are at the same time prone to severe thrombosis because of the high expression of TF in macrophages/foam cells.

The most significant inhibitor of the TF·FVIIa complex is the Kunitz-type tissue factor pathway inhibitor (TFPI), whose effect becomes manifest after the generation of limited quantities of FXa. TFPI uses the tandem Kunitz-type domains in its structure to form a quaternary complex with FXa bound to TF·FVIIa and thus prevents further production of FXa and FIXa through the TF-dependent pathway. The major pool of TFPI resides in the endothelium, which constitutively expresses the protein in normal conditions. In addition to ECs, vascular SMCs, megakaryocytes, platelets, freshly isolated monocytes, and macrophages in certain tissues also express TFPI.

Little is known about the functional role of TFPI in vivo, but it has been proposed that TFPI is important for the early inhibition of TF-dependent procoagulant activity.

The availability of data concerning the in vivo distribution and function of TFPI in the normal and atherosclerotic vessel wall is still limited. Despite recent reports analyzing this issue, the picture remains incomplete, and the findings are sometimes inconsistent or contradictory, probably because of the limited number of specimens analyzed. We tried to overcome similar problems by using a broad range of vascular tissue samples. To make a proper correlation between TFPI and TF, we applied an original approach, in which we used serial sections of the same segment of vessel to carry out a complete set of assays: determination of TFPI and TF antigen and activity, immunostaining for TFPI and TF, and in situ hybridization for TFPI mRNA. In these conditions, we found TFPI protein and mRNA widely present and functionally active, although variable in amount and function of TFPI in the normal and atherosclerotic vessel wall. For further analysis, we used type III to VI atherosclerotic plaques: preatheroma (type I), with small pools of extracellular lipid accumulation and foam cells; atheroma (type IV), with confluent core of extracellular lipid; fibroatheroma (type Va), with prominent connective tissue cap covering the lipid core; and complicated lesions (type VI), displaying surface disruptions and thrombosis.

**Immunohistochemistry**

For bright-field immunohistochemical staining, the avidin-biotinylated peroxidase complex (Vectastain ABC kit, Vector Laboratories Inc.) technique was applied. Cryosections were mounted on gelatin-coated cover slips, fixed with 3% (wt/vol) PFA in PBS (1 hour at room temperature), quenched with 0.1 mol/L glycine in PBS (30 minutes), and incubated with the first antibody (1 hour at room temperature). After that, all steps were performed according to the manufacturer’s instructions. To correlate the localization of different antigens, each specific immunostaining was performed on consecutive sections.

**Immunofluorescence and Confocal Microscopy**

The topographical relation between TFPI and TF or cell-specific markers of the normal or atherosclerotic vessel wall was studied by double immunofluorescence labeling in conjunction with confocal microscopy. The tissue sections were fixed and quenched as described above, then treated with 0.1% (wt/vol) Triton X-100 in PBS for 10 minutes, blocked for nonspecific binding, and incubated overnight at 4°C with cocktails of the primary antibodies at the concentrations recommended by the manufacturers. TFPI was identified with the polyclonal anti-TFPI IgG, coupled to 10-nm gold was from the Department of Cell Biology, University of Utrecht. All the reagents used for electron microscopy were from TAAB Laboratory Equipment Ltd. Human coagulation FVIIa, FX, and FXa were purchased from Enzyme Research Laboratories Ltd. Chromogenic substrate S-2337 was from Quadratech. Secondary antibodies conjugated to horseradish peroxidase, BSA, ortho-phenylenediamine hydrochloride, HEPES, Tris, ovalbumin, paraformaldehyde (PFA), Triton X-100, rabbit brain thromboplastin with calcium (No. T7280), EDTA, and all other reagents were purchased from Sigma Chemical Co unless otherwise stated.

**Preparation of Normal and Pathological Vascular Tissues**

Human primary carotid endarterectomies were collected from bypass vascular surgery. Atherosclerotic coronary arteries were collected from hearts that were removed after heart transplantation. Heavily atherosclerotic popliteal arteries containing large areas of organizing thrombi, as well as occluded saphenous vein grafts, were retrieved from orthopedic surgery. Small specimens of apparently normal internal mammary artery and saphenous vein retrieved during surgery for aortocoronary bypass and healthy aortas from liver transplant surgery retrieved within 12 hours of removal from donors were used as healthy controls. All specimens were harvested after the informed consent of the donors, in accordance with protocols approved by the Institutional Ethics Committee. Specimens were embedded in OCT compound (Miles Scientific), snap-frozen in isopentane, cooled in liquid nitrogen, and stored at −70°C. Hema-toxylin-eosin–stained sections from each specimen were examined to establish the histological characteristics of the plaques, in accordance with the classification of Stary et al. For further analysis, we used type III to VI atherosclerotic plaques: preatheroma (type I), with small pools of extracellular lipid accumulation and foam cells; atheroma (type IV), with confluent core of extracellular lipid; fibroatheroma (type Va), with prominent connective tissue cap covering the lipid core; and complicated lesions (type VI), displaying surface disruptions and thrombosis.

**Materials**

Antibodies and suppliers were as follows: Rabbit anti-recombinant TFPI, IgG was developed in our laboratory, immunofluorescence-purified, and tested for specificity (50 μg IgG per mL, produced 95% inhibition of TFPI activity in normal human plasma, and the concentration of IgG that still produced 50% inhibition of TFPI activity was ~0.6 μg/mL; by immunofluorescence, the competition experiments, eg, staining in the presence of recombinant TFPI, were negative). Murine monoclonal antibody (mAb) against human TF (No. 4509), murine mAb against human FVII (No. 231, no recognition of FVIIa), rabbit IgG against human TF (No. 4502), and murine mAb against LDL receptor–related protein (LRP, No. 3402) were from American Diagnostica. Murine mAb against α-actin (No. 11488 818, marker for SMCs) and murine mAb against CD3 (marker for T cells) were from Boehringer-Mannheim. Murine mAb against von Willebrand factor (vWF, No. M616, marker for ECs), murine mAb against CD68 (marker for macrophages), and murine mAb against ααββ C (CD41, No. M 7057, marker for platelets) were from Dako Ltd. Murine mAb against fibrin II β-chain (TG, recognizes polymerized fibrin) was from Accurate Chemical and Scientific Corp. Secondary antibodies (horse anti-mouse IgG coupled to Texas red and goat anti-rabbit IgG conjugated with FITC) and Vectashield mounting medium were from Vector Laboratories Inc. Secondary antibodies conjugated to 5- or 10-nm colloidal gold and cold fish gelatin were from BioCell Research Laboratories, and protein A coupled to 10-nm gold was from the Department of Cell Biology, University of Utrecht. All the reagents used for electron microscopy were from TAAB Laboratory Equipment Ltd. Human coagulation FVIIa, FX, and FXa were purchased from Enzyme Research Laboratories Ltd. Chromogenic substrate S-2337 was from Quadratech. Secondary antibodies conjugated to horseradish peroxidase, BSA, ortho-phenylenediamine hydrochloride, HEPES, Tris, ovalbumin, paraformaldehyde (PFA), Triton X-100, rabbit brain thromboplastin with calcium (No. T7280), EDTA, and all other reagents were purchased from Sigma Chemical Co unless otherwise stated.
progressively lowered, and embedded in Lowicryl K4M (TAAB Laboratories Ltd). The immunogold labeling for TFPI was performed as described,25 with protein A labeled with 10-nm gold used for detection.

Double immunogold labeling of TFPI and TF was performed separately for each antigen on the 2 sides of each grid by using the polyclonal antibodies raised in rabbits for both proteins. In brief, sections laid on noncoated grids were first immunostained for TFPI,25 then dried, and covered with Formvar (TAAB). The immunostaining was repeated on the other side of the grid for TF; this time protein A conjugated with 15-nm gold was used for detection. In the end, the grids were fixed with glutaraldehyde, stained, and examined with a Philips 201 electron microscope.

Synthesis of TFPI Riboprobes

A 600-bp fragment of the 5' end of the TFPI coding sequence was cloned into pGEM3zf(+) vector containing T7 and SP RNA polymerase initiation sites. Sense and antisense TFPI and vWF antisense (positive control) riboprobes were produced by runoff transcription by using either T7 or SP RNA polymerases on linearized vector samples. The riboprobes were labeled by using 35S-UTP incorporation according to the Riboprobe system (Promega) protocol. Probes were purified by S-200 HR minicolumns (Pharmacia) and stored at −70°C until use.

In Situ Hybridization

The procedure was performed as described by Lupu and colleagues,34–36 with slight modifications. Tissue samples were fixed in 4% (wt/vol) PFA in PBS for 3 hours at room temperature, cryoprotected in 4% (wt/vol) sucrose in PBS overnight, and frozen in OCT compound. Serial sections (9-μm thickness) were taken onto Superfrost slides (BDH) and air-dried. Sections were fixed with 4% (wt/vol) PFA in PBS (3 minutes), delipidated with 0.1% (wt/vol) Triton X-100 in PBS (15 minutes), permeabilized with 0.5 mg/mL proteinase K in 0.1 mol/L triethanolamine buffer pH 8.0 (5 minutes), postfixed with 4% (wt/vol) PFA in PBS (3 minutes), and acetylated in 0.25% (vol/vol) acetic anhydride in 0.1 mol/L triethanolamine buffer pH 8.0 (10 minutes). Slides were rinsed in water and air-dried before to hybridization.

The riboprobes were denatured at 90°C for 2 minutes, diluted in hybridization buffer (1:1 mixture of hybridization buffer from Amersham and deionized formamide, supplemented with 0.2 mmol/L dithiothreitol), and added to the slides at 106 cpm per slide. The sections were covered with coverslips (Hybaid) and hybridized in a humid chamber at 54°C for 16 hours. The slides were washed in 2× SSC containing 0.1% (wt/vol) SDS and 80 μmol/L dithiothreitol (4 times for 10 minutes), then incubated with 10 mg/L RNase A in 2× SSC (20 minutes), and washed in SSC (diluted 1:10 [vol/vol]) supplemented with 0.1% SDS and 80 μmol/L dithioreitol, until the sense control slides showed no detectable radioactive activity and the antisense slides showed no further reduction of radioactivity between washes. Sections were dehydrated, then dipped in autodiographic emulsion (LM-1, Amersham), air-dried, and placed in dark boxes at 4°C. Slides were developed after 10 to 20 days, counterstained with hematoxylin, and mounted in DPX (BDH). The sections were analyzed with a Nikon Optiphot 2 microscope equipped with a mercury UV lamp and epipolarization filters.

Immunohistochemical Quantification of TFPI

TFPI antigen present in the tissue sections was measured by the direct ELISA that we have established and described for ECs in culture.23 The vascular tissue cryosections (9-μm thickness) were laid on the bottom of a 24-well culture plate, fixed with 4% (wt/vol) PFA in PBS (1 hour), quenched with 0.1 mol/L glycine in PBS containing 1% (wt/vol) H2O2 (15 minutes), permeabilized with 0.5% Triton X-100 in PBS (10 minutes), and blocked in a mixture of 50 g/L nonfat dry milk, 10 g/L cold fish gelatin, and 1:100 diluted normal goat serum (1 hour). After this, the sections were processed as described for ECs in culture.23

Activity Assays

The quantification of the inhibitory activity of TFPI against TF · FVIIa was performed as described for ECs in culture23 by using the 2-stage amidolytic chromogenic assay. In brief, nonfixed cryosections were adhered on coverslips and laid in 24-well plates as described above and incubated for 30 minutes at 37°C with 300 μL of combined reagent containing (all final concentrations) 2.5 mg/L FVIIa, 5 U/L FXa, 1:80 diluted rabbit brain thromboplastin (from 1 vial reconstituted with 2 mL distilled water), and 15 mMol/L CaCl2. The supernatants overlaying each tissue section were divided into 2 portions (duplicates) and transferred into the wells of a 96-well microtiter plate, to which a mixture of 0.4 U/mL FX and 0.3 mMol/L chromogenic substrate S-2337 was added. The rate of substrate cleavage was monitored over 25 minutes at 37°C in a microplate reader (Molecular Devices THERMOMax, Alpha Laboratories Ltd) by using the dual kinetic mode (A405 nm = A650 nm + A500 nm, A, absorbance).

The TFPI activity in the sections was extrapolated from a standard curve constructed with serial dilutions of normal human plasma, which was assigned a TFPI functional potency of 1 U/mL.

To confirm the specificity of the assay, control experiments were performed by incubating adjacent tissue sections with anti-TFPI IgG (60 μg/mL) for 1 hour at 4°C before the assay.

For TF-dependent activation of FX, we modified an assay that measures the proteolytic activity of TF · FVIIa toward FX by a 2-stage chromogenic assay,37 as described in detail for ECs in culture.

In antibody-blocking experiments, a preincubation step with anti-TFPI IgG was performed for 1 hour at room temperature before the assay. For all these assays, the values obtained were normalized to the surface area (in square centimeters) of consecutive sections stained with hematoxylin-eosin and measured under the microscope.

Statistical Analysis

Determination of TFPI antigen by ELISA and of TFPI activity by functional assay was performed individually on 4 specimens of carotid endarterectomy, 3 of saphenous vein failed graft, and 2 specimens each of popliteal artery, mammary artery, and healthy saphenous vein. TF-dependent activation of FX was carried out on 2 specimens each of carotid endarterectomy, saphenous vein failed graft, mammary artery, and healthy saphenous vein.

All the assays were repeated 3 times; 6 serial section replicates from individual tissue samples were used every time for each particular determination. The assays were carried out on the same tissue blocks on which the immunolabeling was performed to ensure a good correlation between the different parameters examined. The optical readings made in duplicate were averaged separately for each section. Results from replicate experiments were grouped for each tissue specimen separately and compared by a nonparametric Mann-Whitney U test. We did not observe significant differences between equivalent vessel specimens originating from different donors (P>0.5 in all the cases); therefore, the values for individual specimens were grouped under the generic vessel category. Descriptive statistics include mean±SD or median value and range.

The differences between types of vessels or time points were considered significant at a value of P<0.05.

Results

Localization of TFPI Antigen and mRNA in Normal Human Vessels

Results of the immunostaining for TFPI in healthy vessels are illustrated for the mammary artery (Figure 1a), abdominal aorta (Figure 1b and 1c), coronary artery (Figure 1c), and the saphenous vein (Figure 1d). TFPI protein was found in cells located in the tunica intima, throughout the tunica media, and in adventitial microvessels in arteries (Figure 1a through 1d) and veins (Figure 1e and 1i).

The cell types associated with TFPI staining were confirmed either by immunoperoxidase staining on adjacent sections (Figure 1e, α-actin) or by double immunofluores-
cence labeling with anti-TFPI IgG and either anti–α-actin IgG (Figure 1g) or anti-vWf IgG (Figure 1i). Luminal and microvascular ECs stained positively for TFPI within all the specimens of arteries and veins examined. SMCs also showed strong labeling, but the distribution of TFPI was heterogeneous, with medial SMCs in the mammary and coronary arteries (Figure 1a and 1d) apparently exhibiting stronger staining than SMCs in the aorta or the saphenous vein (Figure 1b and 1e). The pericytes surrounding the adventitial microvessels also stained positively for TFPI (Figure 1i).

Analysis of serial sections of mammary arteries by in situ hybridization and immunocytochemistry for cell-specific markers revealed high levels of expression of TFPI mRNA by medial SMCs (Figure 1g and 1h) and luminal and adventitial ECs (Figure 1j through 1m).

Localization of TFPI Antigen and mRNA in Atherosclerotic Human Vessels

In most of the atherosclerotic arteries studied, TFPI staining was seen alongside the luminal endothelium overlying the plaques (Figure 2a, carotid with type IV lesions) and in the ECs of the neointimal microvessels, which sometimes displayed a characteristic multilayered “onionskin” appearance\(^3^8\) (Figure 2d, popliteal artery containing type VI lesions; double immunolabeling for TFPI and vWF). The topographical relation between TFPI and TF was investigated by double immunolabeling. In atherosclerotic carotids displaying type Va lesions (Figure 2b), TFPI colocalized with TF in ECs covering the plaque (white arrow), in some elongated cells with the morphological characteristics of SMCs throughout the cap of the plaque (fc in panel b), and consistently in macrophage-rich areas located in the shoulder of the atheroma (asterisk in panel b). The necrotic core itself was positively stained only for TF (nc in panel 2b). Immunostaining of serial sections of atherosclerotic carotid with anti-FVII and anti-TFPI IgGs (not shown) indicated colocalization in SMC-rich areas but not in the luminal endothelium, in which only TFPI was observed, or in the necrotic core itself, in which only FVII was present (not shown).

Within the fibrous cap of complicated lesions, TFPI immunoreactivity colocalized with the intimal SMCs (Figure 2e, double staining for TFPI and α-actin). Positive staining for TFPI was observed in the cells surrounding the necrotic core, colocalizing with LRP and the macrophage marker CD68 (Figure 2f and 2g).

Within type VI plaques (popliteal artery with intramural thrombus), large areas of organizing thrombus were observed, as demonstrated by the dense staining for the fibrin monomer II (Figure 2h, red). The same panel indicates a partial association between TFPI (green) and the fibrin strands in a pattern that resembles that observed for platelet-rich areas of the thrombus when double immunolabeling for TFPI and the platelet marker α\(_\text{IIb}β\_3\) was performed (Figure 2i).
In the occluded saphenous vein graft (Figure 2c), the anti-TFPI IgG labeled both the luminal EC (white arrow) and the microvessels (white arrowheads), as well as the SMCs within the thickened intima and the media. The TF immunostaining was mainly confined to the adventitia, with some dispersed labeling in the medial SMCs. Almost no colocalization between TFPI and TF was evident.

By in situ hybridization, the signal for TFPI mRNA was detected in the ECs bordering the lumen (Figure 3a through 3c, coronary artery with type III to IV lesions) and the adventitial microvessels (Figure 3a through 3c). Similar to normal arteries, SMCs located in the tunica media of the atherosclerotic coronary arteries expressed high levels of TFPI mRNA (Figure 3a and 3b). The neointima-located SMCs were also positive for TFPI mRNA (Figure 3a [arrowheads], 3b [arrowheads], 3d, and 3e), although the signal seemed to be weaker than for medial SMCs and more heterogeneous, because not all of the α-actin–stained cells expressed TFPI mRNA (Figure 3d [arrow] and 3e).

Macrophages in the thickened intima of coronary arteries (Figure 3a, black asterisk), identified by positive staining for CD68 on consecutive sections (not shown), also expressed TFPI mRNA (Figure 3b, black asterisk). Macrophages/foam cells within the shoulder and rim of the necrotic core of advanced atherosclerotic plaques also expressed TFPI mRNA, as revealed by the large amount of silver grains overlying cells stained in consecutive sections by the macrophage marker CD68 (Figure 3f and 3g, type Va lesion in the carotid).

Interestingly, we also observed that cells positive for CD3, a T-lymphocyte–specific antigen, displayed specific staining for TFPI antigen and expressed TFPI mRNA as well (Figure 3h to 3j).

Immunogold Electron Microscopy
The distribution of TFPI at the subcellular level was examined by electron microscopy after labeling with specific antibodies and colloidal gold probes.

In the healthy mammary arteries, TFPI-immunogold labeling was observed mainly in ECs and to a smaller extent in SMCs, consistently associated with caveolae and subapical vesicles (Figure 4a and 4b, open arrowheads; mammary artery).

In atherosclerotic coronary arteries, the ECs that border the lumen displayed strong immunostaining for TFPI (Figure 4c). The gold particles were seen to be mostly associated with the cell surface or within intracellular structures with apical polarization, including the Golgi complex and vesicles (Figure 4c and 4e, arrowheads). The cell surface label was irregular, with noticeable variations between cells and different parts of the same cell. In this respect, the luminal surface of ECs consistently displayed more gold labeling than did the...
abluminal front (Figure 4f, small size gold labeling). Some ECs that also showed signs of activation (eg, cell contraction and plasmalemmal projections) displayed staining for TFPI all over the cell body without any specific polarization (Figure 4d, type Va lesion). Although the abluminal staining was less prominent, a fair amount of gold labeling was observed in the junction areas (Figure 4f, gold labeling at J), as well as in the extracellular matrix, especially in the subendothelial space (Figure 4c inset and 4f). Where present, caveolae were often immunolabeled for TFPI, mainly on the apical surface of ECs (Figure 4c and 4e, gold labeling at open arrowheads).

Compared with ECs, SMCs exhibited less immunostaining, but the labeling was also heterogeneous. The myofilament-rich (contractile) SMCs in the tunica media of the coronary arteries showed staining mainly associated with the cell surface on the plasmalemma proper and in caveolae (Figure 5a). The synthetic type of SMCs present in the fibrous cap of type Va lesions displayed staining over the rough endoplasmic reticulum areas, confirming the production of TFPI by these cells (Figure 5b). The SMC-derived foam cells exhibited intense gold labeling for TFPI with less polarization over the cell surface and intracellularly (Figure 5c).

Quantification of TFPI Antigen and Activity

In direct correlation with the immunostaining, we determined the amount of TFPI antigen and the functional activity of the inhibitor on adjacent cross sections of healthy and atherosclerotic vessel segments.

The amount and the activity of TFPI were normalized to the surface area of the sections and represented as scattergrams containing ranges and median values (Figure 6a and 6b) under 2 main categories: healthy tissues (mammary artery and saphenous vein) and atherosclerotic samples (carotid endarterectomy, popliteal artery, popliteal artery with thrombus, and saphenous vein).

The atherosclerotic tissues always displayed more TFPI than did their healthy counterparts, as follows: 2.2-fold for carotid endarterectomy ($P=0.0007$), 1.8-fold for popliteal artery ($P=0.008$), and 2.3-fold for atherosclerotic saphenous vein ($P=0.0002$). The TFPI antigen determined in the saphenous vein was lower than for any of the arterial tissues analyzed, including healthy and atherosclerotic specimens ($P<0.008$ in all cases). The amount of TFPI antigen measured on sections of popliteal arteries containing complicated plaques with intramural and surface thrombi (type VI lesions) was in distinct areas: anti-TFPI IgG stained mainly the luminal cell surface, whereas the TF-specific labeling was confined to the abluminal front of the cells and the subendothelial extracellular matrix, with hardly any TF at all on the apical plasmalemma (Figure 4e and 4f). As with TFPI, TF appeared located in vesicles/caveolae (Figure 4f, open arrowhead), sometimes colocalized with TFPI (Figure 4f, arrows). SMCs also displayed positive staining for TF, which appeared over the apical surface and colocalized with TFPI in caveolae (Figure 5d and 5d inset).

Double labeling for TFPI (10-nm gold particles) and TF (15-nm gold particles) in coronary arteries indicated the simultaneous presence of the 2 proteins in ECs, either luminal or microvascular (Figure 4e and 4f). On the EC surface, the gold was seen in distinct areas: anti-TFPI IgG stained mainly the luminal cell surface, whereas the TF-specific labeling was confined to the abluminal front of the cells and the subendothelial extracellular matrix, with hardly any TF at all on the apical plasmalemma (Figure 4e and 4f). As with TFPI, TF appeared located in vesicles/caveolae (Figure 4f, open arrowhead), sometimes colocalized with TFPI (Figure 4f, arrows). SMCs also displayed positive staining for TF, which appeared over the apical surface and colocalized with TFPI in caveolae (Figure 5d and 5d inset).

**Figure 3.** In situ hybridization and immunolocalization of TFPI in atherosclerotic human vessels. Consecutive cross sections of atherosclerotic arteries were immunostained for TFPI (a and i), hybridized with the antisense TFPI probe (b, e, g, and j), or immunostained for cell-specific markers: vWF (c), α-actin (d), CD68 (f), and CD3 (i). Panels a through e show coronary artery containing type III to IV lesions (blue asterisks show extracellular lipid accumulation in the intima). Panels f through j show carotid artery with type Va lesions. In panels a and b, arrowheads indicate neointima-located SMCs; black asterisks show macrophages in the subendothelium expressing TFPI mRNA. In panel h, double immunofluorescence for TFPI (green, as for Figure 1) and CD3 (red, as for Figure 1) shows colocalization (yellow) in T cells within the fibrous cap of carotid complicated plaques. Bars=100 μm.
Figure 4. Postembedding immunogold staining for TFPI and TF in healthy and atherosclerotic human arteries. Immunogold labeling for TFPI (rabbit anti-TFPI IgG followed by protein A/10-nm gold) in healthy mammary artery reveals gold labeling on the plasmalemmal surface, in open caveolae (open arrowheads), and in cytoplasmic vesicles with apical polarization (arrowheads) in ECs (a, ec) and in SMCs (b, smc). Panels c through f show atherosclerotic coronary artery. In panel c, ECs overlying type III to IV lesions exhibit TFPI labeling similar to normal ECs. In the panel c inset, TFPI in the extracellular matrix (ecm) is shown. In panel d, ECs that show morphological signs of activation (type Va lesions) display large amounts of gold particles over the cell body without any polarization. Panels e and f show double immunogold labeling for TFPI (as above) and TF (rabbit anti-TF IgG followed by protein A/15-nm gold) performed sequentially on the separate sides of the same section. Panel e shows microvascular ECs (mvec). Panel f shows luminal ECs. Open arrowheads indicate caveolae. Arrows show colocalization between TFPI and TF. J indicates junction areas; and bl, basal lamina. Bars=100 nm.
Figure 5. Postembedding immunogold staining for TFPI and TF in atherosclerotic human vessels. Immunogold labeling for TFPI (as for Figure 4) on sections of coronary artery reveals gold particles in SMCs (smc), macrophages, and foam cells (fc). Panel a shows medial SMCs. TFPI in caveolae is indicated by open arrowhead. Panel b shows neointimal SMCs in fibroatheroma. RER indicates rough endoplasmic reticulum. Panel c shows spindle-shaped cells with characteristics of lipid-laden SMCs in the intima of type III lesions. ld indicates lipid droplets. Panel d shows double immunolabeling for TFPI (10-nm gold) and TF (15-nm gold), as for Figure 4. Panel d inset shows colocalization of TFPI and TF in caveolae (open arrowhead). Macrophages (e) and foam cells (f) in the intima of type III to IV plaques display TFPI immunostaining over the cell surface and in the cytoplasm. N indicates nucleus. Bars = 100 nm.
Figure 6. Quantification of TFPI antigen and activity on sections of healthy and atherosclerotic vessel segments. Panel a shows the amount of TFPI antigen determined by ELISA. Panel b shows the activity of TFPI measured in the chromogenic assay. For both panels, healthy tissues are mammary artery (MA) and saphenous vein (SV); atherosclerotic vessels are carotid endarterectomy (CE), saphenous vein (ASV), popliteal artery (PA), and popliteal artery with thrombus (PAT). Antigen and activity values are normalized to the surface area of each section. Scattergrams illustrate the distribution of individual values and the median for each group of data. Panels c and d show TF-FVIIa-dependent activation of FX on vessel cross sections. Normal and atherosclerotic vascular segments promote TF-dependent generation of FXa in the presence of FVIIa in a time-dependent manner. The activation of FX is shown in parallel for controls (solid symbols) and for sections preincubated with anti-TFPI IgG (open symbols). For both panels, healthy tissues (MA and SV) are represented by squares, and atherosclerotic vessels (CE, and ASV failed graft) are represented by circles. Values are expressed as mean±SD.

The activity of TFPI within the cross sections, as determined by the chromogenic assay, was also increased for atherosclerotic tissues compared with healthy tissues (Figure 6b): 2.7-fold for carotid endarterectomy (P<0.0001), 2.4-fold for popliteal artery (P<0.001), 3.5-fold for popliteal artery with thrombus (P<0.0001), and 2.1-fold for atherosclerotic saphenous vein (P=0.0002). In contrast with the antigen values, the activity of TFPI against TF·FVIIa was significantly higher on the saphenous vein segments, either healthy or atherosclerotic, than on the arterial samples (P<0.003), except for the popliteal artery with thrombus (P=0.9).

The novelty of our approach relates to the use of serial sections through the same portion of the vessel segment to perform all the different analyses described: immunocytochemistry, in situ hybridization, quantification by ELISA or functional assay, and TF-dependent activation of FX. In this way, we overcame previous limitations, such as those arising from the use of plaque homogenates or different portions of atherosclerotic plaques for different types of assays. Besides, we were able to correlate the amount, distribution, and activity of TFPI with the type and nature of the vessels (arteries/veins, healthy/atherosclerotic), with the type of lesion, and with the distribution and activity of TF.

Discussion
We have presented a comprehensive study that integrates for the first time the topographical localization of TFPI and TF within the vessel wall with the amount of TFPI antigen, the inhibitory activity of TFPI against TF·FVIIa, and the expression of TFPI mRNA in different human vascular tissues. At the ultrastructural level, we investigated the subcellular distribution of TFPI within the vessel wall layers.

Overall, the cells located in the thickened intima of diseased coronary arteries and saphenous veins seemed to display less TFPI than did the medial SMCs, but local
variations were always visible within the samples. As observed at the ultrastructural level, the luminal ECs with morphological signs of activation were also heavily immunogold-stained for TFPI. This raises the interesting possibility that TFPI may become overexpressed in vivo in pathological states in which the endothelium is activated (eg, inflammation and tumor vasculature).

The macrophages/foam cells, present in large numbers in coronary arteries and in severely stenosed carotid and popliteal arteries, also express TFPI antigen and mRNA. TFPI was colocalized with the macrophage-specific marker CD68 and with LRP, the clearance receptor that was previously detected by us on macrophages and SMCs and that is also involved in the clearance of TFPI.

Quantitatively, cross sections of atherosclerotic arteries and veins displayed significantly more TFPI antigen (2- to 3-fold increase) than did the healthy controls. However, the failed saphenous vein grafts consistently showed 20% to 40% less TFPI antigen than did the carotid endarterectomies or the popliteal arteries, a difference that can be probably attributed to the high proportion of TFPI-expressing macrophages present in the arterial lesions.

The role of macrophages in atherosclerosis is not restricted to lipid accumulation, but macrophages are also associated with coagulant activities and intravascular thrombotic complications and with local immune responses in cooperation with the T cells. The latter, as we show in the present study for the first time, also express TFPI mRNA and antigen.

In view of the role played by TFPI as the main physiological inhibitor of the TF-initiated pathway of coagulation, we considered it essential to establish the topographical relation between TFPI and the other components of the complex, ie, TF and FVIIa/FVa. It was even more important to assess whether TFPI in the vessel wall was active against the TF·FVIIa complex. Previous staining for active TF with digoxigenin-labeled FVIIa or FVIIa-FITC (F.L., unpublished data, 2000) revealed a positive signal in macrophages, SMCs in the fibrous cap, endothelium overlying the lesion, and the lipid-rich core of advanced arterial plaques. Coronary plaques with a high content of macrophages may have a high risk of rupture and a propensity for thrombosis that are related to the expression of TF in macrophages/foam cells.

By immunostaining, we observed large amounts of TF and FVII in the lipid-rich core of the arterial plaques, but TFPI was absent in the same areas. This finding suggests that unimpeded TF activity may account for the thrombotic complications after plaque rupture. Within fibroatheromas (carotid with type Va lesions), the presence of TFPI in the luminal ECs, SMCs, and T cells in the fibrous cap and macrophages around the necrotic core in the same locations as TF suggests that TFPI may regulate the TF-dependent procoagulant activity. This is further confirmed with the use of neutralizing anti-TFPI IgG, which causes inhibition of TFPI activity against TF·FVIIa and an increase of TF-dependent FXa generation on the vessel sections. The subcellular colocalization of TF and TFPI in caveolae in ECs or SMCs also indicates an active role for TFPI, in view of the fact that the formation and translocation of the inhibitory complex in caveolae downregulate the TF-procoagulant activity. Our findings reinforce previous reports by Kaikita et al suggesting that the presence of TFPI within the luminal surface of atherosclerotic lesions without disruption may play an active role in the prevention of thrombotic complications.

Working on carotid plaque homogenates, Caplice et al have also suggested that TFPI could modulate the activity of TF. Our approach of using consecutive sections of fresh-frozen vessel segments for different assays has several advantages over the use of homogenates. One of the most important is that the activity of TFPI and TF can be correlated with the distribution of the antigens detected by immunohistochemistry in the same vessel segments. This becomes particularly important when discrepancies between values for TFPI antigen and activity are found, such as the ones observed for the saphenous vein failed graft. In this case, the difference is probably due to the low amount of TF present in the samples (F.L., unpublished data, 2000). This was confirmed by the immunohistochemistry, which showed weak staining for TF topographically separated from TFPI, and by the assay of TF activity, which indicated a low capacity of the sections to generate FXa. Nevertheless, the TF-dependent activation of FX on the diseased saphenous veins proves that TF is active in this location. The lack of colocalization between TFPI and TF suggests again that unimpeded TF activity might have borne the responsibility for the local formation of fibrin deposits within the grafts, which could have led to the graft failure.

The presence of fibrin deposits and of fibrin degradation products within the atherosclerotic vessel wall is well documented, even though it is not known whether fibrin deposits are locally generated by the clotting of fibrinogen within the intima or whether they originate from the incorporation of mural clots.

There are few reports pointing to a possible interaction between TFPI and fibrin. Accordingly, reconstituted TFPI associates with fibrin when topically applied in a model of balloon angioplasty, reducing the neoendothelium formation and thrombosis. Blocking TFPI in a rabbit model of fibrin-dependent glomerulonephritis augments the deposition of glomerular fibrin and renal injury. Whether native TFPI also associates with fibrin in vivo and, if it does, whether it preserves the functional activity after fibrinolysis are issues that require elucidation. In advanced complicated plaques (popliteal arteries with thrombus, type VI lesions), we observed strong colocalization between TFPI and the platelet marker GPIIb, or the fibrin monomer II, which prevails over fibrin monomer I in the thrombotic regions of the plaques. The amount of TFPI antigen determined by ELISA in complicated plaques is significantly larger than that found on segments of similar arteries without thrombus. The activity of TFPI is also enhanced, but not to an equal extent, because of the simultaneous presence of high amounts of TF (F.L., unpublished data, 2000). We have previously observed that in vitro stimulation of platelets with thrombin leads to the release of TFPI from granules and accumulation of the inhibitor on the periphery of platelet aggregates (F.L., unpublished data, 2000). We suggest that early inhibition of active TF by platelet TFPI at sites of vessel injury may repress the evolution of thrombosis. The procoagulant activity of injured arteries containing actively evolving thrombi that are subjected to thrombolytic therapy has been attributed to fibrin-bound TF, FXa, or both, leading to persistent local generation of thrombin. In this context, a potential associ-
ation between TFPI and fibrin within intimal fibrin deposits or occlusive thrombi in stenosed arteries would provide a natural reservoir of anticoagulant molecules readily made available when either endogenous fibrinolysis or thrombolysis/recanalization of the thrombus occurs.

Our results confirm that human atherothrombotic vessels contain the factors responsible for the activation of the coagulation cascade that may lead to the generation of thrombin and fibrin deposition within the narrowed vessel wall. Likewise, the presence of biologically active TFPI within arterial plaques in locations physically close to TF and FVII suggests that TFPI plays an active role against the TF-driven pathway of coagulation during lesion development and after plaque rupture.

In conclusion, our findings are consistent with the presence of significant amounts of TFPI in a wide intracellular and extracellular distribution across the human vascular tissues examined. There may be local variations as well as differences among arteries and veins, but TFPI is ubiquitously present and, more important, is active against the TF-dependent procoagulant activity. This reinforces the idea that upregulation of TFPI may control thrombogenicity or even prevent the complications associated with atherothrombotic plaque rupture.

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