Expression and Localization of Tissue Factor Pathway Inhibitor-2 in Normal and Atherosclerotic Human Vessels

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Abstract—Tissue factor pathway inhibitor-2 (TFPI-2) is a Kunitz-type, serine protease inhibitor with inhibitory activity toward activated factor XI, plasma kallikrein, plasmin, certain matrix metalloproteinases, and the tissue factor:activated factor VII complex. In this study, we investigated TFPI-2 expression and localization in normal and atherosclerotic human arteries by using in situ hybridization and immunohistochemical techniques. In healthy human blood vessels, TFPI-2 was detected in the vascular endothelium alone. In human atherosclerotic tissues, TFPI-2 expression was assigned to macrophages, T cells, endothelial cells, and smooth muscle cells. Western blot analysis for TFPI-2 confirmed its production by cultured human aortic smooth muscle cells, U937 cells (monocytes), and Jurkat (T cell) cell lines. Reverse transcription–polymerase chain reaction revealed similar TFPI-2 expression levels in both monocytes and macrophages in culture. Electron microscopic study with immunogold labeling revealed the association of TFPI-2 antigen with both the extracellular matrix and plasma membranes. TFPI-2 antigen was detected in some areas of atheroma that also stained positively for both tissue factor and factor VII. Moreover, detection of TFPI-2 in close spatial proximity to plasmin/plasminogen on macrophages, on endothelial cells, and in matrix-rich areas highlighted its possible functional significance in the regulation of plasmin activity and downstream proteolytic mechanisms that occur in the atherosclerotic lesion. (Arterioscler Thromb Vasc Biol. 2002;22:218-224.)

Key Words: atherosclerosis • matrix metalloproteinases • tissue factor pathway inhibitor-2 • plasmin • tissue factor

Originally identified as placental protein 5, TFPI-2 was renamed owing to its homology with tissue factor pathway inhibitor (TFPI). As a member of the same Kunitz-type, serine protease inhibitor family as TFPI, TFPI-2 contains 3 tandemly arranged Kunitz domains with a high degree of conservation. The highly basic carboxy terminus of TFPI-2 mediates ionic interactions that associate this protein with glycosaminoglycans in plasma membranes and the extracellular matrix (ECM).

See cover

A variety of human tissues, including the placenta, liver, skeletal muscle, heart, kidney, and pancreas, express TFPI-2. In culture, it is constitutively secreted by several endothelial cell (EC) types in 3 alternatively glycosylated forms of varying molecular weight (33, 31, and 27 kDa). TFPI-2 gene activation has been reported in these cells in response to various inflammatory mediators, including tumor necrosis factor and phorbol ester (PMA).

With respect to the vascular system, the in vitro inhibitory potential of TFPI-2 toward plasmin, matrix metalloproteinases (MMPs), and the tissue factor (TF):activated factor VII (FVIIa) complex activity are of primary interest. In 1 or more of these roles, TFPI-2 might have important regulatory function in healthy and/or atherosclerotic vessels. In vitro, the inhibition of plasmin activity by TFPI-2 can retard cell migration and favor matrix accumulation as a consequence of reduced downstream MMP activation. Interestingly, recent data have highlighted the ability of TFPI-2 to inhibit selected MMPs directly.

Although the importance of TFPI-2 as an inhibitor of thrombin generation remains unclear, a recent report has implicated the interaction of TFPI-2 with TF:FVIIa in promoting cell migration, although the physiological relevance of this process is yet to be elucidated. A further action of TFPI-2 as a vascular smooth muscle cell (VSMC) mitogen has also been suggested.

Taken together, these data indicate that TFPI-2 could play roles in the regulation of ECM turnover, cell migration/proliferation, and thrombin generation within the vessel wall. Because atherosclerosis is a chronic inflammatory condition intimately linked to these processes, we performed a detailed study of the expression of TFPI-2 in normal and atherosclerotic vessels to examine the possible significance of its in vitro functions.
Methods
Methods can be found online at http://atvb.ahajournals.org.

Results
TFPI-2 in Healthy Human Vessels
TFPI-2 was detected in a variety of apparently healthy vascular tissues (n=9, see Methods) by in situ hybridization (Figure 1a) and immunoperoxidase staining (reddish brown; Figures 1b through 1f). In situ hybridization revealed TFPI-2 mRNA in the ECs of healthy human arteries (Figure 1a), which were identified on serial section by using anti–von Willebrand factor (vWF) antibodies (not shown). Markedly lower signal was detected in the VSMCs of these tissues in comparison with ECs. Low yet detectable amounts of TFPI-2 antigen were detected in the endothelium of all normal mammary (n=3) (Figure 1b), coronary (n=1) (Figure 1c), aortic (n=2) (Figure 1d), and splenic (n=1) (Figure 1e) artery specimens examined. In healthy saphenous veins (n=2) (Figure 1f) and microvascular ECs (not shown) processed on the same slides as arterial specimens, stronger staining for TFPI-2 was observed. In some instances, low-level staining of the acellular areas immediately underlying the EC layer was seen. In none of the healthy arterial or venous specimens analyzed was TFPI-2 antigen detected by immunoperoxidase staining in VSMCs.

TFPI-2 Antigen in Atherosclerotic Coronary Arteries
Double immunofluorescence was used to establish the distribution of TFPI-2 in relation to certain cell type–specific markers in each of the atherosclerotic artery specimens (n=14) (Figures 1g through 1k; coronary artery). TFPI-2 staining is shown in green, cell markers in red, and areas of colocalization in yellow. TFPI-2 was detected in CD31-positive arterial and microvessel ECs (g and h). Macrophages (i, arrows) and T cells (k, arrows), identified by CD68 and CD3 immunoreactivity, respectively, also produced TFPI-2. TFPI-2 was detected in medial VSMCs (j, asterisk) and pericytes surrounding microvessels (j, arrow) that stained positively for α-actin. Double-immunofluorescence results were confirmed by the low-level staining observed in the no-primary-antibodies control (l). L indicates lumen; EEL, external elastic lamina; Adv, adventitia; and Med, media. Bars=50 μm.

TFPI-2 Expression in Atherosclerotic Coronary Arteries
TFPI-2 expression was detected in the majority of ECs in all atherosclerotic coronary (n=5), aortic (n=3), and carotid (n=3) arteries examined. Both TFPI-2 antigen (Figures 2a, 2h, and 2k) and mRNA (Figures 2c and 2i) were detected on serial sections in cells showing immunoreactivity with anti-
vWF antibodies (not shown). Cell typing was further confirmed by the specific binding of a vWF probe to ECs (Figure 2b), which also served as a positive control for the in situ hybridization.

CD68-positive macrophages (Figure 2e; blue asterisk) and CD3-positive T cells (Figure 2f; black asterisk) showed, on serial sections, comparatively high levels of both TFPI-2 mRNA and antigen (Figures 2d and 2g, respectively). Macrophages in the adventitia (Figures 2j and 2k) were indistinguishable from those around the necrotic core with respect to their levels of TFPI-2 expression. TFPI-2 antigen was detected not only within cells but also in areas rich in ECM. Cells showing immunoreactivity to anti-α-actin antibodies (not shown) were identified as VSMCs. The staining for TFPI-2 in these cells was heterogeneous between different atherosclerotic specimens. Most of the VSMCs identified exhibited variable low-level staining for TFPI-2 antigen, whereas the majority exhibited detectable levels of TFPI-2 mRNA. Medial VSMCs between the necrotic core and adventitia produced the highest amounts of TFPI-2 antigen and mRNA (Figures 2d and 2g; green asterisk) in this cell type, though still in lower amount by comparison with the macrophages and T cells in these tissues. As a general observation from all atheromas studied (types IV through VI17), those VSMCs closest to the atherosclerotic lesions expressed higher amounts of TFPI-2. Conversely, staining for TFPI-2 in VSMCs distant from lesions was often low or absent. The probe used for the in situ hybridization was tested by Northern blot analysis of placental RNA. These data (not shown) revealed 2 distinct transcript sizes of 1.2 and 1.8 kb, in accordance with previous reports.5 Specimens hybridized with a sense probe exhibited very low-level probe binding (Figure 3g), indicating that the signal observed on the antisense slides was specific and represented sites of potential TFPI-2 synthesis.

Immunolocalization of TFPI-2 antigen in atheromas derived from different locations in the vasculature (including aortic, carotid, and splenic arteries and a failed saphenous vein graft; data not shown) highlighted staining comparable with that in the atherosclerotic coronary arteries depicted.

**TFPI-2, TF, and FVII Antigens in Atherosclerotic Coronary Atheromas**

TFPI-2, TF, and FVII were detected by immunoperoxidase staining on serially cut sections of human coronary atheromas (n=5). Relatively strong staining for each of these antigens was observed on and around macrophages in the shoulders of the atherosclerotic plaques (Figures 3a through 3c), identified by immunoperoxidase staining for CD68 on serially cut sections (not shown). Here, TFPI-2, TF, and FVII were detected in areas similar to each other. Whereas TF was predominantly cell associated on macrophages, some ECs, and variably on VSMCs (Figure 3b), FVII was more diffusely distributed through the vessel wall (Figure 3c). TFPI-2, as previously stated, was most strongly detected in both cell- and matrix-rich locations around macrophages (Figure 3a). TFPI-2 was also detected in lower quantities in medial VSMCs in regions of some atherosclerotic specimens also staining positively for TF and FVII.

**TFPI-2 and Plasminogen Antigens in Atherosclerotic Coronary Arteries**

Immunoperoxidase staining on serially cut sections of human atherosclerotic coronary arteries showed that TFPI-2 (Figure 3d) was present in areas that also stained positively for plasminogen (Figure 3e). Cell-specific immunostaining on serial sections identified these cells as macrophages and VSMCs adjacent to the necrotic core. As before, TFPI-2 was detected in both cellular and acellular areas, whereas plas-
minogen staining was largely, but not exclusively, associated with areas around cells.

Double immunofluorescence for TFPI-2 and plasminogen revealed that both proteins were also localized in association with macrophages in the atherosclerotic intima (Figure 3f). In this view, staining appeared strongly cell associated. Arterial ECs and some adventitial microvessel ECs also showed staining for both of these antigens (data not shown). The reliability of the observations from all immunostaining was consistently confirmed by the lack of staining seen in the control slides without primary antibody (Figure 1l) and the sections incubated with nonimmune mouse or rabbit serum (Figure 3h).

Electron Microscopic Localization of TFPI-2 and Plasminogen in Atherosclerotic Coronary Arteries

Immunogold electron microscopy revealed the presence of TFPI-2 antigen within and around arterial ECs (Figure 4a). TFPI-2 detected within ECs did not appear in any defined storage granules and was located mainly toward the abluminal side of the cell, where cell-surface TFPI-2 also predominated. TFPI-2 was detected in relatively high amounts in association with the ECM, identified by the characteristic fibrils (Figures 4a and 4c) throughout the human atherosclerotic coronary arteries studied. VSMCs of coronary atheromas showed immunoreactivity to TFPI-2 antibodies, which to a large extent appeared on or close to the extracellular surface of plasma membranes (Figure 4b). However, owing to the abundance of actin fibers in VSMCs, definition of the plasma membranes was markedly reduced in these cells. Double labeling of TFPI-2 and plasmin/plasminogen with 2 sizes of colloidal gold (TFPI-2, 15 nm; plasminogen, 10 nm) localized the 2 proteins in close proximity, mainly in association with the ECM (Figure 4d).

Western Blotting

We examined the production of TFPI-2 in cultured cell-type analogues by Western blotting (Figure 5a). Total cell lysates of unstimulated and PMA (100 ng/mL)-stimulated human aortic VSMCs, Jurkat cells, and U937 cells were analyzed along with samples normalized with respect to total protein (10 μg protein per lane). Western blot analysis revealed that TFPI-2 was detected in 3 forms, ranging from ≈33 to 27 kDa, as reported previously.7 Whereas TFPI-2 was detected in unstimulated human aortic VSMCs, U937 cells, and Jurkat cells, these levels were increased after stimulation with PMA (100 ng/mL) for 8 hours.

Reverse Transcription–Polymerase Chain Reaction

To establish the relative expression level of TFPI-2 in macrophages by comparison with their precursors (monocytes), we performed reverse transcription–polymerase chain
reaction (RT-PCR) for TFPI-2 on poly-A mRNA isolated from either freshly isolated monocytes or macrophages (Figure 5b). Monocyte-derived macrophages were obtained by culturing monocytes for 21 days. In these semiquantitative experiments, we found no significant difference in the amount of TFPI-2 mRNA detected by this method, as determined by the intensity of the 440-bp band (Figure 5b, lanes 1 and 2). The similarity of the band intensities from RT-PCR performed by using control glyceraldehyde-3-phosphate dehydrogenase (GAPDH)–specific housekeeping gene primers. No apparent change in TFPI-2 expression was detected between monocytes and macrophages.

**Discussion**

TFPI-2 is a protease inhibitor that could play a regulatory role in several processes that are relevant to the physiology of healthy vessels and to the pathogenesis of atherosclerosis. In vitro, TFPI-2 can inhibit extracellular proteolytic activity and TF-mediated coagulation and may also influence cell migration and proliferation. Dysfunction of these processes has been reported to contribute to atherogenesis. Thus, to elucidate the potential role of TFPI-2 in vascular disease, we investigated the expression of TFPI-2 in healthy and atherosclerotic vessels.

In this study, we have demonstrated that under normal conditions, TFPI-2 antigen is located in the vascular endothelium. The relative degree of staining between different vascular tissues suggests that arterial ECs express lower quantities of TFPI-2 when compared with ECs of veins and microvessels, consistent with in vitro data that demonstrate that cultured ECs of the microvasculature express nearly 3-fold more TFPI-2 antigen than do cultured, arterial ECs.6 The expression of TFPI-2 in normal vascular tissues suggests that this protease inhibitor participates in the physiological functions of the healthy arterial wall.

In atherosclerotic tissues, the expression pattern of TFPI-2 is altered. Besides the arterial and microvascular ECs, TFPI-2 was expressed also by macrophages, T cells, and VSMCs of atherosclerotic tissues. Western blot analysis confirmed the production of TFPI-2 by cultured human aortic VSMCs, monocyte-like U937 cells, and T-cell–like Jurkat cells. Furthermore, RT-PCR suggested that monocytes and macrophages express TFPI-2 at similar levels.

VSMCs, which we found not to express TFPI-2 normally, produced both TFPI-2 mRNA and antigen in some medial areas of atherosclerotic tissues, albeit at lower levels than did...
other cell types in these tissues. TFPI-2 expression in VSMCs was most noticeable in the vicinity of the atheroma, where greatest VSMC activation occurs. The presence of TFPI-2 in this cell type is interesting in light of the data demonstrating a mitogenic response in cultured human aortic VSMCs after stimulation with recombinant TFPI-2. It was suggested that this response was probably transduced by a specific receptor. However, it remains to be established whether TFPI-2 indeed plays a role as a VSMC mitogen in vivo, and if so, what its contribution might be relative to other VSMC mitogens present in the atherosclerotic vessel wall.19

The strongest expression of TFPI-2 was found in macrophages and T cells throughout the human atheromas studied. However, a recent report, based on immunostaining of vascular specimens and Western blot analysis of isolated monocytes, described the apparent downregulation of TFPI-2 in macrophages of atherosclerotic tissues.16 Our observations, based on immunostaining, in situ hybridization, Western blot, and RT-PCR analyses, would indicate that TFPI-2 is produced by both monocytes and macrophages in comparable amounts. We therefore believe that macrophages and also T cells, which were not identified in the aforementioned report,10 most likely represent a significant source of this inhibitor during atherosclerosis.

TFPI-2 may be involved in the pathogenesis of atherosclerosis in several ways. In vitro, TFPI-2 can inhibit TF:FVIIa, plasmin, and certain MMP activity.5,10 The inhibition of thrombin generation by TFPI-2 occurs via the binding and inactivation of the TF:FVIIa complex. Importantly though, this inhibition, unlike that of TFPI, is not augmented by the presence of FXa, which results in reduced inhibitory potency in comparison with TFPI.3 During atherosclerosis, an upregulation of TF expression occurs in ECs, VSMCs, and macrophages,20 and permeability of the vessel wall to plasma-borne clotting factors also increases.21 Therefore, through its interaction with the TF:FVIIa complex, TFPI-2 might assist in modulating thrombin generation. Immunoperoxidase staining for TFPI-2, TF, and FVII on serially cut sections served to establish the relative distribution of TFPI-2 with respect to potential sites for the TF:FVIIa complex. Our results revealed that in atherosclerotic tissues, macrophages and, to a lesser extent, medial VSMCs stained positively for TFPI-2, TF, and FVII, highlighting the potential availability of the TF:FVIIa complex to bind TFPI-2 in vivo. We have previously documented the expression of TFPI in ECs, VSMCs, macrophages, and T cells in atherosclerotic tissues and its activity therein.16 Given the reduced anticoagulant potency of TFPI-2 in comparison with that of TFPI, it might seem unlikely that TFPI-2 modulates thrombin generation significantly during atherosclerosis. However, the interaction of TFPI-2 with TF:FVIIa may, as recently suggested, have possible relevance to MMP activation,11 in which plasmin plays a pivotal role.12 Plasminogen permeates the vasculature more readily at atheromatous locations. Here, it can be activated to plasmin by plasminogen activators (for a review, see Collen23). In the vessel wall, plasminogen binds to the ECM and cell membranes with low affinity but high capacity by way of lysine residues in its kringle domains.24 In these locations, plasmin can degrade certain matrix components as well as potentiate MMP activation. Plasminogen activators, plasmin, and MMPs have all been detected in atherosclerotic lesions, where they are believed to play important roles in the control of cell migration and matrix turnover.25–27 Therefore, as an inhibitor of plasmin, MMP activation, and also, as recently reported, a direct inhibitor of MMP-1 and MMP-13 activity,10 TFPI-2 could have important implications in plaque development and stability by its modulation of extracellular proteolytic mechanisms. Our immunoperoxidase staining demonstrated that both TFPI-2 and plasminogen antigens were present in cell- and matrix-rich areas. Immunogold labeling showed TFPI-2 and plasminogen to be present in association with matrix fibers and cell membranes in atherosclerotic coronary arteries. Double-immunofluorescence staining for TFPI-2 and plasminogen also showed that these proteins colocalized during atherosclerosis on macrophages. These cells can upregulate the number of plasminogen-binding sites and thus, potentially concentrate plasmin on their surfaces.28 Macrophages represent the primary source of many of the factors associated with tissue remodeling29; consequently, our observations support the hypothesis that TFPI-2 acts on plasmin in vivo and could modulate ECM turnover in the atherosclerotic vessel wall.

For TFPI-2 to interact with either plasmin or TF:FVIIa, it requires an extracellular location. TFPI-2 is secreted by cultured ECs and binds, by ionic interactions, to glycosaminoglycans.3 Our electron microscopic results confirmed that in vivo, more TFPI-2 is bound to the ECM and lesser amounts to plasma membranes. Furthermore, we also observed that TFPI-2 predominates on the abluminal surface of arterial ECs of atherosclerotic tissues. The lack of TFPI-2 in plasma30 and its association with the ECM and plasma membranes suggest that these structures probably represent the primary site of TFPI-2 activity.

In this article, we have documented the upregulation of TFPI-2 expression in human atherosclerotic coronary arteries in comparison with the healthy state. This may provide new insights into the understanding of the control of matrix turnover and TF-dependent functions, 2 important processes involved in the evolution of the atherosclerotic plaque.

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