Presence of Tissue Factor Pathway Inhibitor in Human Atherosclerotic Plaques Is Associated With Reduced Tissue Factor Activity

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Background—Plaque disruption and exposure of subendothelial procoagulants such as tissue factor (TF) to circulating factor VII/VIIa (FVII/VIIa) lead to intravascular thrombosis. Tissue factor pathway inhibitor (TFPI) is an endogenous inhibitor of TF-induced coagulation that binds to factor Xa and the TF-FVIIa catalytic complex in a two-step process. The aim of this study was to determine the expression of TFPI within human atherosclerotic plaque and its role in modulation of TF activity.

Methods and Results—We measured the level of TFPI antigen in human carotid plaque and determined the relationship between TFPI and TF activity within plaque. Furthermore, we examined the biological activity and immunolocalization patterns of TFPI within carotid plaque. TFPI was detectable (TFPI+ group) in 22 of 34 specimens (mean±SEM, 404.4±91.8 pg/mg) and undetectable (TFPI− group) in 12 of 34 specimens. In the TFPI− group, normalized TF activity was significantly greater than that in the TFPI+ group (0.28±0.04 vs 0.14±0.02 U/pg, P=0.002). Furthermore, neutralization of TFPI activity using a polyclonal antibody resulted in an 8-fold increase in TF activity in the TFPI+ group (P=0.001) but had no effect in the TFPI− group. Immunostaining for TFPI showed localization to endothelial cells, vascular smooth muscle cells within the fibrous cap region of the plaque, and macrophages within the shoulder region of the plaque.

Conclusions—Taken together, these data suggest that biologically active TFPI is present within human atherosclerotic plaque and is associated with attenuated TF activity. (Circulation. 1998;98:1051-1057.)

Key Words: atherosclerosis ■ tissue factor ■ inhibitor ■ plaque

Acutethrombosis after atherosclerotic plaque disruption is a major complication of primary atherosclerosis, leading to acute ischemic syndromes and atherosclerosis progression.1 Endothelial disruption and damage to the normal vessel may expose subendothelial procoagulant molecules, including tissue factor (TF), which complexes with factor (F) VII/VIIa in flowing blood, cleaving FIX and FX with subsequent fibrin deposition.2–4 Several studies have identified TF in the intima of human atherosclerotic plaque and have suggested that it is an important determinant of thrombogenicity after plaque rupture.5–7 Marmur and colleagues5 recently demonstrated great variability among plaque TF activity, although a cause for this variability was not determined. Tissue factor pathway inhibitor (TFPI), which provides physiological inhibition of TF-initiated coagulation by binding to FXa and TF-VIIa complex in a two-step process, has been described in platelets, blood monocytes, macrophages, and vascular endothelium.8–11 We recently identified vascular smooth muscle cells as another significant source of TFPI synthesis in culture and in normal coronary arteries.12 Little is known about the functional role of TFPI in atherosclerosis. We hypothesized that TFPI present within atherosclerotic lesions might attenuate plaque TF activity. To test this, we measured TFPI antigen within the plaque and determined the relationship between plaque TFPI and TF activity levels. To confirm that the TFPI detected in plaque was biologically active, we determined the effect of a neutralizing antibody to TFPI on plaque TF activity. Finally, we examined the immunolocalization of TFPI within the plaque through the use of immunocytochemistry.

Methods

Patient Population
Thirty-four carotid plaque samples were obtained from patients at the time of surgical endarterectomy. Human tissue was obtained according to a protocol approved by our institutional review board. All specimens were identified as atherosclerotic plaque by the responsible clinical pathologist. Each plaque specimen was randomly divided into two separate portions at the time of collection; one portion was used for immunocytochemical localization of TFPI, and the other portion was assayed for TFPI antigen, TF antigen, and TF activity.

Tissue Processing
Carotid endarterectomy specimens were either freshly frozen in liquid nitrogen or embedded in OCT compound in liquid nitrogen--
chilled 2-methylbutane. Tissue segments for TF and TFPI assays was subsequently homogenized in lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.02% sodium azide, 0.1% SDS, 100 µg/mL PMSF, 1 µg/mL aprotinin, 1% Nonidet P-40, and 0.5% sodium deoxycholate) using a tissue homogenizer (Omni International). Protein concentrations of the plaque homogenates were determined using a Bradford assay (BioRad) with BSA as a standard.

**TFPI Antigen ELISA**

To detect TFPI antigen in plaque homogenates, a sandwich ELISA was carried out using a commercial ELISA kit (American Diagnostica) that identifies the Kunitz 1 domain of human TFPI.

**TF Antigen ELISA**

A sandwich ELISA (TF Imubind; American Diagnostica) was used to detect TF antigen in plaque homogenates. The ELISA kit used a mouse monoclonal anti-human TF antibody for antigen capture. The captured TF then was detected using a biotinylated antibody that specifically recognized bound TF with subsequent detection using a streptavidin– horseradish peroxidase and TMB substrate system. Absorbance was measured at 405 nm with the generation of a standard curve from known dilutions of human TF (American Diagnostica).

**TF Activity Assay**

Homogenized samples were assayed for TF activity with the use of a chromogenic assay. Twenty microliters of sample was incubated with 20 µL FVIIa (50 ng/mL; American Diagnostica) in the presence of 20 µL FX (1.5 U/mL; American Diagnostica) for 30 minutes at 37°C. The reaction was stopped by the addition of 20 µL of 0.2 mol/L EDTA. Then, 20 µL of spectrozyme FXa chromogenic substrate (American Diagnostica) was added. This substrate releases a p-nitroaniline chromophore on cleavage of FX. Absorbance at 405 nm was measured, and TF activity was determined from a standard curve established by plotting the V_{max} values of known dilutions of human TF (American Diagnostica). Twenty microliters of TF solution at 6.25 ng/mL were defined to have 1000 arbitrary activity units. TF activity for each sample was expressed in activity units per milligram of protein.

**TFPI Neutralization**

The presence of biologically active TFPI was determined by neutralization of TFPI antigen within the plaque homogenates. All plaque homogenates were incubated for 1 hour at 37°C with a neutralizing anti-human TFPI antibody at a concentration of 25 µg/mL (10 000-fold in excess of the highest level of TFPI antigen measured in any homogenate sample). This neutralizing polyclonal antibody was raised in rabbits was purified according to published methods and was specific for human TFPI, capable of completely inhibiting plasma TFPI. Control homogenate samples were similarly incubated for 1 hour at 37°C in the absence of TFPI antibody. TF activity then was assayed in all samples in the presence or absence of TFPI antibody neutralization.

**Histological and Immunocytochemical Analyses**

Specimens were freshly frozen in OCT compound in liquid nitrogen and subsequently sectioned to 5-µm thickness and fixed in cold acetone (-20°C) before immunolabeling. All sections were routinely stained with hematoxylin and eosin and with a combined Masson trichrome stain. Immunocytochemical staining was performed on sections to localize TFPI antigen and to identify smooth muscle cells, macrophages, and endothelial cells using polyclonal anti-TFPI (a gift from Dr L.V.M. Rao, University of Texas, Tyler), monoclonal anti– smooth muscle cell actin (DAKO Corporation), monoclonal anti–CD68 (DAKO Corporation), and monoclonal anti– von Willebrand factor (vWF; DAKO Corporation) antibodies, respectively, each at 1:100 dilution. Sections were initially blocked with 10% normal goat or donkey serum, depending on whether a mouse or rabbit primary antibody was used. After TBS/Trition X-100 washes, sections were incubated with the appropriate dilutions of primary antibody for 1 hour at room temperature. The sections then were incubated with an anti-mouse or anti-rabbit biotinylated antibody (1:200 dilution) for 30 minutes and subsequently a streptavidin– alkaline phosphatase enzyme conjugate (1:300 dilution) for 45 minutes at room temperature. The reaction product was visualized using a fast red (Sigma Chemical) or Vector Blue with levamisole (Vector Laboratories) substrate. The sensitivity and specificity of the TFPI antibody were determined with a dot blot analysis using recombinant TFPI probed with the previously mentioned rabbit polyclonal antibody. Isotype-matched IgG served as a negative control for each antibody used.

Double immunolabeling was carried out through modification of a previously described technique. After visualization of the first antibody (α-actin or CD68) using a biotin streptavidin-peroxidase system with 3,3’-diaminobenzidine (DAB), yielding a brown reaction product, sections were washed several times in TBS and blocked with streptavidin at a 1:200 dilution (Amersham) to bind unoccupied biotin sites from the first antibody run. Sections then were blocked with 10% donkey serum for 30 minutes, followed by incubation of the previously mentioned polyclonal TFPI antibody at a 1:100 dilution for 1 hour at room temperature. Biotinylated anti-rabbit antibody and streptavidin– alkaline phosphatase enzyme conjugate were used as described previously with subsequent visualization of the second primary antibody with Vector Blue substrate and levamisole (Vector Laboratories).

**Statistical Analysis**

Data are presented as mean±SEM. Comparisons between TFPI antigen subgroups were made with an unpaired Student’s t test. Differences in patient characteristics between groups were made with a χ2 test. A value of P<0.05 was considered statistically significant.

**Results**

**TFPI Antigen Levels in Atherosclerotic Plaque**

To quantify TFPI levels in human carotid plaque, TFPI antigen was measured in plaque homogenates using an ELISA. The variation among TFPI levels is shown in Figure 1A. Twelve samples had no detectable TFPI antigen on ELISA. In the remaining 22 samples, TFPI antigen levels ranged from 90 pg/mg to 2.4 ng/mg of protein (mean±SEM, 404.4±91.8 pg/mg). In subsequent analyses, the plaque homogenate samples were separated into two subgroups based on whether TFPI antigen was detectable within the sample.

**TF Antigen and Activity Levels in Atherosclerotic Plaque**

TF antigen in each plaque homogenate was measured using an ELISA. Unlike TFPI, TF antigen was detectable in all samples, with levels ranging from 245 pg/mg to 2.1 ng/mg of protein (mean±SEM, 682.8±74.5 pg/mg) (Figure 1B). TF activity in each plaque homogenate was measured with an amidolytic assay. TF activity was similarly detected in all samples with a similar degree of variation in TF activity levels, ranging from 12.6 to 454 U/mg of protein (mean±SEM, 130.7±21.5 U/mg of protein) (Figure 1C).

**Relationship Between Plaque TFPI Antigen and TF Activity**

TF activity in plaque homogenates measured by an ex vivo amidolytic assay is influenced by the amount of TF present in the homogenate but also may be influenced by the amount of biologically active TFPI present in the same sample. To
clarify the contribution of TFPI to the variation in plaque TF activity, TF activity was corrected for the amount of TF antigen. Normalized TF activity was significantly greater in samples with undetectable TFPI antigen (n=12) than in samples with detectable TFPI antigen (n=22) (0.28±0.04 vs 0.14±0.02 U/pg, P=0.002) (Figure 2). Analysis of TF activity independent of TF antigen demonstrated a trend (P=0.08) toward significantly higher activity in samples with undetectable compared with detectable TFPI antigen. To confirm the relationship between TFPI antigen and normalized TF activity and to confirm that TFPI antigen detectable within the plaque was biologically active, a polyclonal anti-TFPI antibody was used to completely neutralize TFPI activity in plaque homogenates. TF activity again was measured in each homogenate after neutralization with TFPI antibody. Normalized TF activity in plaque homogenates with previously undetectable TFPI antigen levels did not change significantly after TFPI neutralization (0.28±0.04 vs 0.33±0.06 U/pg) (Figure 3A). However, in homogenates with detectable TFPI levels, normalized TF activity increased 8-fold after TFPI neutralization (0.14±0.02 vs 1.12±0.3 U/pg, P=0.001) (Figure 3B). These same relationships with regard to antibody neutralization were seen with TF activity independent of TF antigen (data not shown).

**Patient Characteristics**

Patient characteristics are summarized in Table 1. There was no significant difference in clinical or laboratory values between patients with undetectable plaque TFPI and those with detectable plaque TFPI levels.

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**Figure 1.** A, Frequency distribution of TFPI antigen (Ag) in homogenized human carotid plaque specimens with an interval width of 50 pg/mg of protein. The solid bar indicates samples with undetectable TFPI Ag (n=12); stippled bars, samples with detectable TFPI Ag (n=22). B, Frequency distribution of TF Ag in homogenized carotid plaque specimens with an interval width of 100 pg/mg of protein. C, Frequency distribution of TF activity in homogenized carotid plaque specimens with an interval width of 50 U/mg of protein.

**Figure 2.** Scatterplot of normalized TF activity in homogenized carotid plaque specimens with undetectable TFPI antigen (□, TFPI Ag –) and detectable TFPI antigen (○, TFPI Ag +). Mean±SEM values are presented adjacent to the individual data points.
Immunolocalization of TFPI Within Atherosclerotic Plaque

All endarterectomy specimens consisted of intact intima and a small portion of media. All specimens (n = 22) that had TFPI antigen detectable on ELISA also had immunostaining performed for TFPI. TFPI staining was seen along the endothelial lining of the plaque sample and throughout the intima and remaining medial portion of each specimen studied. There was consistent cellular TFPI staining along the fibrous cap of advanced atherosclerotic plaque. The features of TFPI staining in the plaque intima are shown in Figure 4. Staining of serial sections with anti-TFPI, anti-actin, and anti-vWF antibodies showed the distribution of TFPI staining to be predominantly in smooth muscle cell–rich areas of the intima and along the endothelium of plaque. No staining was seen using isotype-matched IgG control antibodies at a similar concentration. Double immunolabeling showed that TFPI colocalized to α-actin, staining smooth muscle cells within the fibrous cap of the plaque. TFPI staining also was seen in the shoulder regions of the plaque in macrophage-rich areas (Figure 5). In these regions, double immunolabeling confirmed TFPI colocalization to macrophages. Double immunolabeling patterns in all cases were consistent with their single labeling counterparts for each antibody studied. In four specimens containing a discrete necrotic core, no TFPI staining was seen in the area of the necrotic core. TFPI staining was consistently seen in the medial portion of each specimen.

Discussion

This study is the first to our knowledge to quantify TFPI antigen within human atherosclerotic plaque. We demonstrate that TFPI within human carotid plaque is biologically active and modulates plaque TF activity. TFPI was immunolocalized to endothelial cells, vascular smooth muscle cells within the fibrous cap, and macrophages within the shoulder region of the plaque.

TFPI is a potent inhibitor of FVIIa-TF catalytic complexes formed in suspension, on the surface membrane of cultured cells, and in the subendothelial matrix. The administration of recombinant TFPI in vivo has been shown to prevent venous thrombosis and repeat thrombosis after successful arterial thrombolysis. In these clinical states, the common event is exposure of TF to circulating blood. Rupture and fissuring of unstable atherosclerotic lesions are thought to similarly expose TF and perhaps other procoagulant plaque proteins to circulating blood. TFPI present within these plaque samples at the time of TF exposure may play a key role in the regulation of TF-induced thrombosis.

In this study, we demonstrate that TFPI is present within human atherosclerotic plaque and may modulate plaque thrombogenicity by attenuating TF activity. We found significant variations among the plaque samples in the levels of TFPI antigen, TF antigen, and TF activity. The variations in TF antigen and TF activity seen in the present study are consistent with those seen in other studies, although the levels of plaque TF activity in the present study are significantly higher, which might reflect differences among these studies in the source of plaque and the assays that were used. To evaluate the effect of TFPI on plaque TF activity, we corrected for variations in the level of TF antigen in the samples by expressing TF activity per picogram of TF antigen. Interestingly, normalized TF activity was signifi-
significantly lower in plaque homogenates with detectable TFPI antigen than in those with undetectable TFPI antigen, suggesting that TFPI present in these plaque samples may reduce the level of TF activity for any given amount of TF antigen. This was confirmed with the use of neutralizing TFPI antibodies, which caused a significant increase in normalized TF activity in plaque homogenates in which TFPI antigen was detectable. Taken together, these data suggest for the first time that active TFPI within the plaque may be another important determinant of TF activity. This may have significant in vivo implications because variations in the level of TF activity are thought to account in part for the varying incidence of thrombosis after balloon injury and spontaneous plaque rupture.5

TFPI was undetectable in ~30% of the plaque samples, suggesting that there may be plaque that is relatively deficient in TFPI. Furthermore, these samples had significantly higher normalized TF activity levels, suggesting that deficiency of TFPI relative to TF may cause the plaque to be more thrombogenic. The failure to detect TFPI antigen in these samples was unlikely to be due to sampling bias because the morphology of these plaque samples was similar in all respects to that of the plaque samples with detectable TFPI antigen. Interestingly, no significant differences in clinical symptoms were seen between patients with undetectable TFPI levels and those with detectable plaque TFPI levels, although this may be due in part to the pathophysiology of symptoms in carotid artery disease, in which thromboembolic events often occur distant from the atherosclerotic lesion rather than locally at the site of plaque rupture. In carotid artery disease, local plaque conditions (plaque TF and TFPI levels) therefore may have less effect on thrombotic outcomes than in the case of occlusive coronary artery disease.

Little data exist on the distribution of TFPI within human atherosclerotic lesions. Drew and coworkers29 recently demonstrated TFPI protein and mRNA in three of six human carotid endarterectomy specimens and TFPI immunostaining within the cap region of the plaque and in foci of macrophage accumulation within the body of the plaque consistent with the TFPI antigen and immunostaining patterns seen in the present study. In contrast, Werling and colleagues10 found TFPI only within the microvascular endothelium, with no evidence of staining within medium-sized vessels. In the present study, we found cellular TFPI staining throughout the intima and media of all endarterectomy specimens with detectable TFPI antigen by ELISA. TFPI staining was seen particularly in the cap region of the plaque, with colocalization to smooth muscle cells on double immunolabeling. We have previously shown that smooth muscle cells are a significant vascular source of TFPI,12 and the staining patterns for TFPI within plaque seen in this study are consistent with that observation. The present study differs from that of Drew and colleagues29 in that cellular colocalization of TFPI was carried out using double immunolabeling rather than single labeling of serial sections. Furthermore, Drew and colleagues29 did not stain for smooth muscle cell markers, so it is difficult to exclude smooth muscle cells as a source of TFPI in their study. In the present study, TFPI also colocal-
ized to CD68+ macrophages in the shoulder regions of the plaque, which is consistent with other studies.10,29

Study Limitations
A comparison of TFPI antigen levels between different plaque segments collected during surgical endarterectomy is limited by the potential for sampling bias. In this study, there was no difference in histological features between those specimens with detectable and those with undetectable TFPI antigen levels. In particular, there were similar intima and media area proportions within specimens in each subgroup, suggesting that specimen sampling was uniform across both groups. Furthermore, correlation of TFPI antigen and TF activity data with TFPI immunostaining patterns was limited in this study by use of separate portions of the plaque for quantitative TFPI and TF assays and immunocytochemistry. Finally, the use of total TF activity in homogenized samples to assay plaque thrombogenicity in this study is at best an in vitro model of TF bioavailability and thrombogenicity in vivo, and we do not know what portion of total plaque TF is exposed to flowing blood after plaque rupture in humans.

In summary, TFPI is differentially expressed in human atherosclerotic plaque. This differential TFPI expression is associated with attenuation of plaque TF activity in plaque with TFPI. Neutralization experiments confirmed TFPI to be biologically active, and immunocytochemistry experiments demonstrated that TFPI colocalized with endothelial cells, smooth muscle cells, and macrophages within the plaque. Taken together, these data suggest that TFPI is associated with attenuated TF activity and may modulate thrombogenicity in human atherosclerotic plaque. Additional studies are needed to determine whether overexpression of TFPI by techniques such as gene transfer might further inhibit TF activity and its sequelae.30

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