Expression of Tissue Factor Pathway Inhibitor in Vascular Smooth Muscle Cells and Its Regulation by Growth Factors

Noel M. Caplice, Cheryl S. Mueske, Laurel S. Kleppe, Timothy E. Peterson, George J. Broze, Jr, Robert D. Simari

Abstract—Tissue factor pathway inhibitor (TFPI) in vivo is thought to be synthesized mainly by endothelial cells. To date, no significant regulator of TFPI synthesis has been described. Vascular smooth muscle cells (VSMC) express tissue factor in vitro and in vivo, which may contribute to vascular thrombosis. We hypothesized that VSMC might also express TFPI. To determine this, we examined growth-arrested coronary VSMC in culture and found that VSMC secreted an amount of TFPI similar to that seen in endothelial cells. Immunohistochemistry of normal human coronary arteries showed TFPI staining throughout the media and intima of the vessel with localization to VSMC and endothelial cells. To determine regulation of TFPI expression in VSMC, we examined the effects of serum stimulation on TFPI secretion and found that FBS induced a 5-fold increase in TFPI antigen and activity levels in conditioned medium at 48 hours (P<0.001) when compared with serum-free conditions. A similar stimulatory effect was seen with 10% pooled human serum. Moreover, epidermal growth factor and platelet-derived growth factor-B increased TFPI secretion by 4- to 5-fold and 2- to 3-fold, respectively (P<0.05), and these growth factors accounted for ~50% of the TFPI secretion effects of human serum. The serum effect was associated with a 3-fold increase in TFPI mRNA 24 hours after release from growth arrest and a 50% decrease in TFPI secretion after treatment with actinomycin D. Taken together, this study suggests that there is significant TFPI expression in VSMC in culture and in VSMC within the intima and media of the normal coronary artery wall. We present the first evidence for TFPI regulation by serum in VSMC and more specifically by its constituent growth factors, epidermal growth factor and platelet-derived growth factor-B. (Circ Res. 1998;83:1264-1270.)

Key Words: tissue factor inhibitor smooth muscle regulation

Arterial thrombosis is a significant complication of primary atherosclerosis. The complex of tissue factor (TF) and factor VIIa is one of the most potent initiators of in vivo arterial thrombosis, leading to activation of factors IX and X.1 Several studies have shown that TF is expressed in the adventitia of normal human arteries,2,3 intima of human atherosclerotic plaque, and neointima of the balloon-injured rat arteries.4 An important inhibitor of TF-mediated coagulation is tissue factor pathway inhibitor (TFPI), a multivalent protease inhibitor with 3 Kunitz-type domains that inactivates factor Xa generation by binding initially via its Kunitz II domain to factor Xa and subsequently by its Kunitz I domain to TF-VIIa catalytic complex.5 The formation of a quaternary TF-VIIa-TFPI-Xa complex dampens ongoing coagulation and may allow modulation of thrombosis in vivo. TFPI was purified initially from conditioned medium of a hepatoma cell line (HepG2)6 and since has been described in platelets, microvascular endothelium, and stimulated blood monocytes.7-10 The endothelium currently is thought to be the principle site of intravascular TFPI synthesis.7 Vascular smooth muscle cells (VSMC) are known to express TF in vivo, which may contribute to vascular thrombosis.4,11 Little is known about TFPI expression within VSMC and, in particular, within coronary VSMC. Bajaj and coworkers7 previously showed that human VSMC synthesized only small quantities of TFPI, but coronary VSMC were not examined. Furthermore, previous studies have failed to identify any factors that might significantly regulate TFPI expression in either monocyte/macrophages or endothelial cells.10,12 The purpose of the current study was to examine the expression and regulation of TFPI in human coronary VSMC under culture conditions and within the normal vessel wall. TFPI expression within coronary VSMC in culture was compared with that of endothelial and HepG2 cells, and in vivo expression of TFPI within normal human coronary arteries was studied. Finally, regulation of TFPI expression by serum and its constituent growth factors was examined.

Materials and Methods

Cell Culture

Human coronary VSMC (Clonetics Inc) were cultured initially on 90-mm dishes in the presence of basal media (SmBm, Clonetics Inc)
and supplemented with 10% FBS (Gibco BRL), 100 μM penicillin, 100 mg/ml streptomycin, and glutamine (Sigma Chemical Co). HepG2 cells (American Type Culture Collection) were grown in MEM with 10% FBS, antibiotic supplements, and 1 mmol/L sodium pyruvate. Human coronary artery endothelial cells (Clonetics Inc) were grown in basal media (EcBm, Clonetics Inc) supplemented with 5% FBS, bFGF, vascular endothelial growth factor, and antibiotics. For all experiments, coronary VSMC were used between passages 4 and 8, HepG2 cells between passages 4 and 10, and coronary artery endothelial cells between passages 2 and 4. Immuno- staining of these coronary VSMC for smooth muscle α-actin was similar to that of primary cell cultures, suggesting that their phenotype was similar to early passage primary cultured VSMC. Human embryonic kidney cells (293, American Type Culture Collection) were grown in DMEM (Gibco BRL) supplemented with 10% FBS and antibiotics.

TFPI Antigen Measurement
A sandwich enzyme immunoassay was carried out using a commercial ELISA kit (American Diagnostica), which identifies the Kunitz I domain of the human TFPI antigen. Duplicate samples were examined in 3 separate experiments.

Tissue Processing
Three human coronary arteries obtained at the time of cardiac transplantation or at the time of autopsy (within 24 hours) were either freshly frozen in liquid nitrogen or snap frozen in Cryoform (Intermediate Equipment Co) in liquid nitrogen–cooled isopentane. Human tissue was obtained according to a protocol approved by the Institutional Review Board. Thawed tissue was homogenized subsequently 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% NP-40, 0.5% sodium deoxycholate) using a tissue homogenizer. A portion of each tissue sample was deproteinized and adventitial removal before analysis. The supernatant then was analyzed for protein content using a Bradford assay (BioRad).

Immunohistochemistry
Freshly frozen sections (5 μm) from normal human coronary arteries were fixed in acetone at −20°C for 10 minutes. Sections were washed in TBS, blocked with 10% normal donkey serum, and incubated for 1 hour at room temperature with a polyclonal rabbit anti-human TFPI antibody (gift from Dr L.V.M. Rao, University of Texas, San Antonio, Tex)14 at 1:100 dilution. Control sections were incubated with rabbit IgG (Sigma Chemical Co) at a similar dilution. Sections were washed and incubated with an anti-rabbit biotinylated antibody (Amersham Life Sciences) at 1:200 dilution for 30 minutes and then with a streptavidin alkaline phosphatase antibody (Vector Laboratories) at 1:300 dilution for an additional 45 minutes. Visualization of reaction product was carried out using Fast Red (Sigma Chemical Co) and counterstained with hematoxylin. Further serial sections were immunolabeled with α-actin (HHF 35, Dako Co) and anti-VWF (Dako Co) primary antibodies. These antibodies were labeled with secondary anti-mouse biotinylated antibodies at a dilution similar to that described previously. Anti-VWF was visualized with an alkaline phosphatase system using Vector Blue substrate (Vector Laboratories). Anti-α-actin was visualized with a horseradish peroxidase system using diaminobenzidine substrate.

Serum and Growth Factor Stimulation of Cultured VSMC
Human coronary VSMC were seeded at a density of 1 × 10^5 cells per well on a 6-well plate. After overnight attachment, cells were growth-arrested for 24 hours in serum-free medium. BrdU incorporation experiments confirmed negligible DNA synthesis consistent with growth arrest after 24 hours in serum-free conditions. Cells then were stimulated with medium containing 15% FBS, and conditioned media and cell lysates were processed in duplicate at 12, 24, and 48 hours after release from growth arrest. Control cells at similar density, treated with serum-free medium, were processed at similar time points. At each time point, cells were counted using a hemocytometer before lysis. In other experiments, cells were stimulated with 10% pooled human (AB) serum (Sigma Chemical Co), and conditioned media were collected at 24 hours after release from growth arrest.

In separate experiments, cells were growth-arrested for 24 hours and then stimulated with platelet-derived growth factor (PDGF)-A, PDGF-B, αFGF, bFGF, epidermal growth factor (EGF), and transforming growth factor (TGF)-β1 (all from R&D Systems) over a concentration range of 2 log-folds on either side of the mitogenic ED₅₀ as determined by the manufacturer for that particular growth factor. Conditioned medium was processed and cell counts determined as previously described at 48 hours after release from growth arrest. Duplicate wells were measured from at least 3 separate experiments.

Measurement of TFPI Activity
Functional TFPI activity in conditioned medium from serum-free and serum-treated VSMC was measured using a commercial Achromo TFPI activity assay (American Diagnostica). This assay measures the inhibition by TFPI of factor Xa generation after activation by TF/VIIa catalytic complex and was carried out at 37°C. Duplicate samples were measured from 3 separate experiments.

Western Blot Analysis
TFPI protein in conditioned media and tissue homogenates was analyzed using Western blot analysis. After protein determination, 1 mmol/L DTT (Sigma Chemical Co) and lysis buffer were added to equal amounts of protein, and samples were denatured by boiling for 5 minutes and resolved by electrophoresis on a 12% SDS-polyacrylamide gel. Transfer of protein to a nitrocellulose membrane was carried out over 3 hours at 4°C. Immunoblotting was performed using the previously described polyclonal rabbit anti-human TFPI antibody14 at 1:200 dilution in nonfat milk/TBS buffer. After washes, the membrane was probed subsequently with a rabbit secondary antibody conjugated to horseradish peroxidase (Amersham Life Sciences) at 1:5000 dilution and developed with chemiluminescence (Supersignal, Pierce). The membrane then was exposed to x-ray film (Kodak) and subsequently developed.

Growth Factor Neutralization
Human VSMC were growth-arrested for 24 hours on 6-well plates as previously described. Cells then were stimulated with medium enriched with 10% pooled human serum in the presence or absence of neutralizing antibodies to EGF and PDGF-B (R&D Systems). Each neutralizing antibody was used at a ~100-fold excess (20 μg/ml) compared with each growth factor concentration in 10% human serum. The antibodies also were used in combination. An isotype-matched IgG at the same concentration was used as a control. The 10% human serum-enriched medium was preincubated with the neutralizing antibody for 2 hours at 37°C before incubation with the cells in culture. Conditioned medium then was collected at 48 hours after release from growth arrest. In each case, TFPI activity of the conditioned medium was measured in duplicate in at least 3 separate experiments.

Northern Blot Analysis
Human coronary VSMC were grown in 150-mm dishes and growth-arrested for 48 hours in serum-free medium. Cells then were stimulated with medium containing 15% FBS and harvested at 0, 4, 8, 12, and 24 hours after release from growth arrest. Total RNA was isolated using RNeasy Kit (Tel-Test Inc). Northern blot analysis was performed using standard techniques (Northern Max, Ambion Inc). Briefly, 20 μg of total RNA was electrophoresed on a 1% denaturing formaldehyde gel and transferred to a nitrocellulose membrane using the Turbo Blot (Schleicher and Schuell) method of capillary transfer. Membranes then were crosslinked and prehybridized for 2 hours at 42°C followed by hybridization overnight at 42°C with a random prime–labeled (α-32P dCTP) cDNA probe (Prime It II Random Prime Caplice et al December 14/28, 1998
standard methods. The conditioned medium from these cells was used as a positive control obtained for an additional 24 hours in this medium. Conditioned medium and cell lysate from these cells were processed as described in Methods. TFPI antigen levels are shown for (A) conditioned medium and (B) cell lysates from HepG2, VSMC, and endothelial cells, 24 hours after serum starvation. Data are the mean of 2 experiments performed in duplicate.

Labeling Kit, Stratagene). TFPI mRNA bands subsequently were visualized on x-ray film. To control for variations in RNA loading, membranes were stripped of previously labeled TFPI probe by boiling in 1% SDS solution and rehybridized with a cDNA probe to mouse GAPDH (Ambion Inc).

**Inhibition of Transcription With Actinomycin D**

Human coronary VSMC, after 24 hours in serum-free medium, were released from growth arrest as previously described with addition of 15% FBS in the presence or absence of 2 μg/mL actinomycin D (Boehringer Mannheim). Conditioned medium then was processed at 12, 24, and 48 hours after serum stimulation, and cell counts were obtained from each well as previously described. Wells were measured in duplicate in 3 separate experiments.

**Transfection of 293 Cells With pCMV-TFPI**

Transfection of 293 cells, grown to 90% confluence on 90-mm dishes, was carried out with 10 μg of pCMV-TFPI plasmid (a eukaryotic expression plasmid that expresses full-length human TFPI from the cytomegalovirus immediate-early promoter/enhancer) in the presence of 10 μg of liposome (GAP DLRIE-DOPE, kindly provided by Gibco BRL) in OPTI-MEM (Gibco BRL) according to standard methods. The conditioned medium from these cells was collected at 48 hours after transfection and used as a positive control for TFPI in human artery homogenate Western blot experiments.

**Statistical Analysis**

Data are presented as mean±SEM. Comparisons were made using unpaired Student t test (2-tailed) or ANOVA with Fisher post hoc test. P<0.05 was considered statistically significant.

**Results**

**TFPI Antigen Levels in VSMC, Endothelial Cells, and HepG2 Cells**

To quantify TFPI expression in human coronary VSMC, the levels of TFPI antigen in VSMC, endothelial cell, and HepG2 cell lysates and conditioned media assessed by ELISA were compared (Figure 1). HepG2 cells synthesized and secreted 3-fold greater amounts of TFPI per cell than either endothelial or VSMC. Similar amounts of TFPI were secreted from coronary VSMC compared with coronary endothelial cells. Approximately 10% of TFPI was cell-associated in endothelial cells and VSMC. A nonvascular cell line (293 cells) had undetectable TFPI in cell lysates or conditioned medium under basal cell culture conditions.

**Immunolocalization of TFPI in Human Coronary Arteries**

To determine whether TFPI is expressed by human coronary VSMC in vivo, immunostaining of TFPI was performed on normal coronary arteries (n=3) with diffuse intimal thickening. TFPI staining was found within cells in the subendothelial intima and throughout the media of the coronary artery (Figure 2A) and in association with adventitial microvessels (Figure 2E). To examine whether VSMC were the cells associated with TFPI staining, serial sections were stained with α-smooth muscle actin antibody (Figure 2B and 2F) and VWF antibody (Figure 2C). This showed that the predominant cells within the coronary artery staining positive for TFPI were smooth muscle cells and luminal and microvessel endothelial cells. No staining was seen using a rabbit IgG control antibody at a concentration similar to that of the TFPI antibody (Figure 2D). Staining appeared to be both cell-associated and extracellular (Figure 2G).

Western blot analysis of homogenized coronary artery with the same polyclonal TFPI antibody revealed a protein of similar size to that expressed in conditioned medium of cultured coronary VSMC, suggesting that the TFPI seen within the coronary vessel was the full-length form (Figure 2H). In addition, nanogram quantities of TFPI were found in all coronary artery homogenates (denuded and nondenuded) by ELISA (data not shown).

**Regulation of TFPI Synthesis and Activity**

**Serum-Induced TFPI Secretion**

The effects of serum stimulation on growth-arrested VSMC were examined at 12, 24, and 48 hours after stimulation. Conditioned medium was analyzed for TFPI antigen, activity, and protein size using an ELISA, an activity assay, and Western blot analysis, respectively. TFPI antigen secretion from human VSMC in culture over 12, 24, and 48 hours in the presence of serum measured by ELISA increased 5-fold when compared with serum-free treatment (Figure 3A). TFPI activity as measured by inhibition of factor X activation by TF-VIIa catalytic complex was increased similarly at 48 hours after serum treatment (Figure 3B). On Western blot analysis, a protein consistent with full-length human TFPI was identified in serum-treated conditioned medium over the 48-hour time period (Figure 3C). This protein was not detected in serum-free conditioned medium.

TFPI antigen also was measured in conditioned medium of cells after 24 hours of stimulation with 10% pooled human serum. Pooled human serum–treated cells showed an 8-fold increase in TFPI antigen at 24 hours after serum stimulation (Figure 3D).

**Growth Factor–Induced TFPI Secretion**

To evaluate whether growth factors within human or FBS might be contributing to TFPI secretion, concentration response experiments were performed using several serum–derived growth factors. EGF and PDGF-B significantly increased TFPI secretion by VSMC 24 hours after release.
from growth arrest (Figure 4A). Furthermore, ∼30% and 40% of the human serum’s stimulatory effect on TFPI secretion could be abolished by using neutralizing antibodies to PDGF-B and EGF, respectively (Figure 4B). An irrelevant isotype-matched control antibody at a similar concentration had no effect on TFPI activity. Combining EGF– and PDGF-B–neutralizing antibodies caused a 50% reduction in the serum effect, suggesting a nonadditive antibody effect perhaps because of convergent intracellular signaling from these growth factors.16 None of the other growth factors studied including PDGF-A, aFGF, bFGF, or TGF-β, had any significant effect on TFPI secretion (data not shown).

**Quantitation of Serum-Induced mRNA for TFPI**
To determine whether serum stimulation of VSMC is associated with an increase in steady-state levels of TFPI mRNA, quantitative assessment of Northern blots (n = 4) was performed (Figure 5). Analysis of RNA at baseline and 4, 8, 12, and 24 hours after serum stimulation with 15% FBS demonstrated a 3-fold increase in TFPI mRNA (normalized to GAPDH) 24 hours after stimulation (P = 0.0019).

**Inhibition of Transcription With Actinomycin D**
To determine the contributions of transcription to serum-induced increases in TFPI antigen, cells were stimulated by serum in the absence and presence of actinomycin D, and the secreted antigen was measured in the conditioned medium by ELISA. Exposure to actinomycin D resulted in a ∼50% reduction (P < 0.01) in TFPI antigen secretion within the conditioned medium over the 24-hour time period.

**Discussion**
In the present study, we show that human coronary VSMC in culture synthesize and secrete an amount of TFPI similar to that by coronary endothelial cells. We have shown for the first time TFPI immunostaining in vivo within VSMC throughout the intima and media of the coronary artery wall. We present experimental evidence for significant serum-derived regulation of TFPI synthesis in VSMC in the form of EGF and PDGF-B. In addition, serum-induced stimulation of TFPI synthesis is associated with an increase in steady-state
levels of mRNA and is inhibited partially by actinomycin D, suggesting that TFPI is regulated, in part, at the mRNA level.

Endothelial cells previously have been thought to be the major source of TFPI within the vasculature. We show that coronary VSMC in culture synthesize similar amounts of TFPI when compared with coronary endothelial cells. In this study, TFPI predominantly was secreted with only 10% being cell-associated in culture. The relative levels of TFPI secretion by endothelial and HepG2 cells in the current study are similar to those previously reported for these cell types. However, the levels of TFPI antigen in VSMC are much greater in the current study than previously reported by Bajaj and colleagues. This difference might be explained by different sources of VSMC or different methods used to measure TFPI antigen levels.

Few data currently exist on the distribution of TFPI within large- to medium-sized vessels. Drew et al recently showed TFPI immunostaining within the adventitia of normal human aorta and renal artery but not within the medial layer or luminal endothelium. However, Werling and colleagues found no evidence of TFPI within larger vessels, although they found TFPI in the endothelium throughout the microvasculature. In our study, TFPI staining was found throughout the media of normal coronary arteries and in the subendothelial VSMC within diffuse intimal thickenings of these arteries. TFPI staining also was seen along the endothelium of these vessels. The current study differs from the other studies in that we examined TFPI staining patterns in isolated coronary arteries, whereas Werling and coworkers looked at smaller vessels within several organs, and Drew and coworkers did not specifically examine the coronary circulation. In the current study, the presence of TFPI protein in coronary artery homogenates on Western blot analysis and ELISA using both polyclonal and monoclonal antibodies to TFPI is consistent with the presence of significant amounts and a wider intracellular and extracellular distribution of TFPI within the coronary vasculature.

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**Figure 3.** Secretion of TFPI by human VSMC in culture. Cells were growth-arrested for 24 hours. Cells then were either maintained in serum-free medium or stimulated with 15% FBS over 48 hours. Conditioned medium was analyzed for TFPI antigen, TFPI activity, and TFPI protein size. A, Time course of secretion of TFPI antigen by human VSMC in culture in the presence (•) or absence (○) of 15% FBS. Data represent the mean of 3 experiments performed in duplicate. *P<0.05 for serum-treated vs serum-free cells. B, Time course of TFPI activity in human VSMC–conditioned media in the presence (□) or absence (○) of 15% FBS. Data represent the mean of 3 experiments performed in duplicate. *P<0.05 for serum-treated vs serum-free cells. C, Western blot of conditioned medium from serum-free treated cells at 12, 24, and 48 hours after growth arrest (lanes 1 to 3) and conditioned medium from 15% FBS-treated cells at 12 (lane 5), 24 (lane 6), and 48 (lane 7) hours after release from growth arrest. Lane 4 is a molecular weight marker (kDa), and lane 8 is nonconditioned medium with 15% FBS added. D, TFPI antigen in conditioned medium of 10% pooled human serum–treated cells 24 hours after release from growth arrest. *P<0.01 compared with control serum-free cells.
To date, no significant regulator of TFPI expression has been identified in either endothelial cells\textsuperscript{12} or monocyte/macrophages\textsuperscript{10} despite the use of a range of inflammatory cytokines, PMA, LPS, and human serum. In the current study, we have shown that FBS and human serum caused a significant increase in TFPI protein secretion from smooth muscle cells after their release from growth arrest. This increase in TFPI antigen within the culture medium was accompanied by a similar increase in TFPI anticoagulant activity. Furthermore, Western blot analysis showed that full-length TFPI was secreted after serum stimulation. The association of full-length TFPI with high levels of anticoagulant activity is consistent with previous studies.\textsuperscript{18}

By Northern analysis and transcription-inhibition experiments, we also showed that the serum-induced increase in TFPI protein secretion at least partly was regulated at the RNA level with peaking of steady-state TFPI mRNA at 24 hours after release from growth arrest. Serum is also known to rapidly induce transcriptional activation of TF in VSMC within 1 hour of release from growth arrest, with mRNA returning to baseline at 8 hours.\textsuperscript{5} It is interesting to speculate whether an “immediate early” effect of serum on TF mRNA induction within VSMC is related to the later induction of TFPI mRNA seen in the present study.

Ameri and colleagues\textsuperscript{12} previously showed a modest (1.5-fold) upregulation of TFPI in endothelial cells by whole blood serum over that of plasma-derived serum and suggested that the increase might be due to TGF-\( \beta \) from platelets. Two TGF-\( \beta \)-like response elements are present in the 5' end of the TFPI gene.\textsuperscript{19–21} In the current study, we found that TGF-\( \beta \) had no effect on TFPI secretion by VSMC, whereas other serum-derived growth factors, PDGF-B and EGF, had significant effects on TFPI secretion. These data suggest there are differences in regulatory mechanisms for TFPI secretion between VSMC and other cells within the vessel wall. However, consistent with other studies on endothelial cells,\textsuperscript{12} we found that tumor necrosis factor-\( \alpha \) did not appear to significantly affect TFPI secretion by VSMC (data not shown).

Taken together, our data suggest that VSMC are a significant in vitro and in vivo source of TFPI. The levels of TFPI synthesized by VSMC under culture conditions in our study are equivalent to those of endothelial cells previously thought to be the major cellular pool of vascular TFPI. The evidence from our study of regulation of TFPI by serum and its constituents PDGF-B and EGF and the presence of TFPI staining within the media and intima of coronary arteries may have significant and related in vivo implications. Several serum-derived growth factors are known to be upregulated locally and released in atherosclerosis\textsuperscript{22–24} and after coronary angioplasty in human subjects.\textsuperscript{25} It is possible that growth factors within the vessel wall might locally regulate TFPI synthesis and secretion. Future studies will need to assess regulation of TFPI in vascular disease states where TF/factor VII activation plays a major role in thrombosis formation.

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Figure 5. Northern analysis of TFPI mRNA after serum stimulation. A, Representative Northern blot of mRNA from VSMC at 0, 4, 8, 12, and 24 hours after serum stimulation with 15% FBS. The 2 bands (4.0 and 1.4 kb) represent TFPI with and without 2.6-kb untranslated sequence.7 B, Densitometric analysis of Northern blots (n=4) for TFPI (1.4 kb) normalized to GAPDH. *P=0.0019 vs 0 hours.

References
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