Expression of Tissue Factor Pathway Inhibitor by Cultured Endothelial Cells in Response to Inflammatory Mediators

By Afshin Ameri, Mohan N. Kuppuswamy, Sudip Basu, and S. Paul Bajaj

We recently proposed that endothelium may represent the primary physiologic site of synthesis of the tissue factor pathway inhibitor (TFPI). In support of this conclusion, we have now found that the poly(A)⁺ RNAs obtained from rabbit and bovine lung tissues contain abundant amounts of TFPI messenger RNAs (mRNAs), whereas the poly(A)⁺ RNAs obtained from the liver of these animals contain less than 5% of that found in the lung tissues. Because inflammatory mediators are known to upregulate tissue factor (TF) expression by the endothelium, we have examined the effect of these agents on the TFPI expression by the cultured endothelial cells. When cultured human umbilical vein endothelial cells were stimulated (in 10% fetal bovine serum) with phorbol myristate acetate (PMA), endotoxin, interleukin-1, or tumor necrosis factor-α, the TF mRNA increased 7- to 10-fold within 2 to 4 hours. Unstimulated cells constitutively expressed TFPI mRNA and its levels either did not change or increased slightly (up to 1.5-fold) upon stimulation with these inflammatory agents. TF mRNA abruptly declined to a negligible level and the TFPI mRNA returned essentially to the basal level at ≈24 hours. The membrane-bound TF clotting activity of induced cells peaked between 4 and 8 hours, and finally declined. The cumulative TFPI activity secreted into the media was either unchanged or slightly higher in the induced cell cultures as compared with that present in the noninduced cultures. Endothelial cells were also cultured in 10% heat-inactivated human serum derived from plasma or whole blood. TFPI secreted into the media containing whole blood serum was consistently higher (≈1.5-fold at 8 hours) than that secreted into the media supplemented with serum obtained from plasma lacking the formed elements; these cells also expressed similarly increased levels of TFPI mRNA. Moreover, PMA-stimulated cells cultured in whole blood serum expressed modestly increased levels of TFPI mRNA (≈1.5-fold); supernatants from these cells also contained similarly increased TFPI activity. Cumulatively, our data indicate that, unlike thrombomodulin and fibrinolytic enzymes synthesized by the endothelial cells, TFPI synthesis is not downregulated and may be slightly upregulated during an inflammatory response. Inspection of the 5’ flanking region of the TFPI gene showed a conserved GATA-binding motif located ≈400 bp upstream of the proposed transcription initiation site(s). This motif by binding to the GATA-2 transcriptional factor may keep the endothelium in an ‘on’ state for constitutive expression of TFPI. A modest increase in TFPI expression by treatment of cells with whole blood serum or PMA may be due to the presence of two transforming growth factor-β–like responsive elements and of two phorbol ester-like responsive elements located in the 5’ end of the gene.

© 1992 by The American Society of Hematology.

IN THE LAST 2 DECADES, substantial evidence has been accumulated that indicates the tissue factor pathway (or the extrinsic pathway) plays a dominant role in initiating blood coagulation in vivo.¹⁻³ This pathway begins by exposure of blood to tissue factor (TF) at an injury site and formation of the complex between TF and plasma factor VII/VIIa. The TF/VIIa complex formed then activates both factors IX and X, leading to thrombin generation and fibrin formation.³ The primary physiologic inhibitor of TF/VIIa complex is a multivalent Kunitz-type inhibitor referred to as the lipoprotein-associated coagulation inhibitor or the extrinsic pathway inhibitor.¹⁻³ Recently, this inhibitor has been termed the tissue factor pathway inhibitor (TFPI) by the Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. As per recommendation of this Subcommittee, the inhibitor is referred to as TFPI in this communication. TFPI consists of a highly negative aminoterminal segment, three tandemly repeated Kunitz-type inhibitor domains, and a highly positive carboxyterminal segment. The inhibition of the catalytic activity of TF/VIIa complex occurs in two steps.¹⁻² In the first step, the middle Kunitz domain of TFPI binds to factor Xa. In the second step, the first Kunitz domain of TFPI binds to factor VIIa in the TF/VIIa complex.

Endothelial cells are involved in the regulation of both coagulation and fibrinolysis. Normal vascular endothelium produces components active in platelet aggregation, such as von Willebrand factor, and the components associated with the platelet antiaggregation properties, such as prostacyclin.⁴ Unperturbed endothelium also expresses components associated with anticoagulant properties such as heparan sulfate,⁴ thrombomodulin,² and TFPI.⁶ Moreover, the endothelium also secretes substances that promote fibrinolysis such as tissue plasminogen activator (t-PA) and the substances that inhibit fibrinolysis, such as plasminogen activator inhibitor-1 (PAI-1).⁵ These balanced properties of normal endothelium ensure natural anticoagulant checks against clotting.

Many cells that are normally not in contact with blood, such as fibroblasts and smooth muscle cells, constitutively express TF.⁷⁻⁹ On the contrary, endothelial cells that are in contact with blood either do not or express very little TF.⁷⁻⁹ However, upon stimulation with a variety of inflammatory agents such as endotoxin, phorbol 12-myristate acetate (PMA), interleukin-1 (IL-1), and tumor necrosis factor-α (TNF-α), endothelial cells can be induced to synthesize and

From the Departments of Medicine, Pathology and Biochemistry, St Louis University School of Medicine, St Louis, MO.

Submitted September 11, 1991; accepted February 25, 1992.

Supported by National Institutes of Health Grants No. HL36365 and 30572 (Project 5). S.P.B. is a recipient of an AHA Bristol-Myers Squibb Thrombosis Grant.

Address reprint requests to S.P. Bajaj, PhD, Division of Bone Marrow Transplantation, Oncology and Hematology, 3635 Vista Ave, PO Box 15250, St Louis, MO 63110-0250.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1992 by The American Society of Hematology.


3219
express TF on their surface.\textsuperscript{10-13} These inflammatory mediators also upregulate the expression of PAI-1 and downregulate the expression of TF-PA, prostacyclin, and thrombomodulin.\textsuperscript{1,12} Thus, during an inflammatory response, the ability of endothelium to provide proper anticoagulant checks against clotting is greatly impaired. In this context, it would be important to examine the influence of inflammatory mediators on the expression and secretion of TFPI by the endothelial cells and relate it to the changes in TF activity and messenger RNA (mRNA) expression. In the present study, we address this issue and report our findings. A preliminary account of this work has been presented.\textsuperscript{14}

\section*{MATERIALS AND METHODS}

\textbf{Materials.} Tissue culture media and supplements were obtained from Sigma Chemical Company (St Louis, MO). Collagenase (type 2) was obtained from Worthington Biochemical Corporation (Freehold, NJ) and fibronectin was purchased from Boehringer Mannheim Corporation (Indianapolis, IN). Endotoxin-lipopolysaccharide (LPS; \textit{Escherichia coli} 0.26.B6), PMA, bovine serum albumin (BSA), fetal bovine serum (FBS), and rabbit brain cephalin were obtained from Sigma. NaB\textsubscript{3}Ha was from Amersham.\textsuperscript{28} Nuclease (type 2) was obtained from Worthington Biochemical Corporation (Freehold, NJ) and fibronectin was purchased from Bio-Rad laboratories (Hercules, CA). A normal human fetal lung fibroblast cell culture (GM-1380) was obtained from the Coriell Institute for Medical Research (Camden, NJ).

\textbf{Proteins.} Human factor VII,\textsuperscript{16} factor IX,\textsuperscript{17} and factor X\textsuperscript{17} were purified as described. SI\textsubscript{3}H-factor IX (4 x 10\textsuperscript{8} cpn/mg) was prepared by the general technique of Van Lenten and Ashwell\textsuperscript{18} as described; the protein retained \textasciitilde{} 90\% of the clotting activity of the nonlabeled control.

\textbf{Cell culture.} Human umbilical vein endothelial (HUVE) cells were harvested essentially by the method of Jaffe et al.\textsuperscript{20} In brief, the umbilical cords were kept at 4\textdegree{}C in Eagles minimum essential medium containing 100 \textmu{}g/mL penicillin, and 100 \textmu{}g/mL streptomycin for up to 2 days before processing. HUVE cells obtained from five umbilical cords were routinely used for seeding 10-25 cm\textsuperscript{2} flasks coated with fibronectin (4 \textmu{}g/cm\textsuperscript{2}). The cells grew to confluence within 5 to 6 days in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15\% heat-inactivated FBS, 100 \textmu{}g/mL heparin, and 75 \textmu{}g/mL endothelial cell growth supplement. Our detailed culture conditions have been described.\textsuperscript{6,21} BPAE cells and GM-1380 fibroblast cells were cultured in DMEM containing 10\% FBS as described.\textsuperscript{6,21}

\textbf{Cloning of human TF cDNA.} Total RNA from the GM-1380 fibroblast cells was isolated by a single step procedure of Chomczynski and Sacchi\textsuperscript{22} using the RNAzol B method as outlined in the Cinna/Biotec (Friendswood, TX) manual. A full-length TF cDNA was cloned using reverse transcription and the polymerase chain reaction (PCR). Two primers were used for this purpose: primer A, 5’-CGCTGCAATCAGGCTG-3’ and primer B, 5’-TATGGAAAGCTTTGAGTAGCTCGCCGCGAACTGCTGTA-3’. Primer A contains a Mbo I site and primer B contains a HindIII site. The restriction site sequences are italicized. The sequences in parentheses are the sequences complementary to TF cDNA regions.\textsuperscript{23-26}

Initially, 5 \textmu{}g of total RNA was used to reverse transcribe the TF mRNA using the primer B and the Moloney murine leukemia virus reverse transcriptase, as outlined.\textsuperscript{27} PCR amplification was performed using both primers essentially as described.\textsuperscript{28} Our procedure has been described in detail elsewhere.\textsuperscript{29} PCR amplified fragment was digested with Mbo I and HindIII restriction enzymes, cloned into BamHI and HindIII sites of pUC 18 vector and sequenced.\textsuperscript{27} The sequence of our TF clone differed from the published sequence\textsuperscript{23-26} at two places. These two nucleotide changes resulted in substitution of Glu-99 by Val (GAG \rightarrow GTG) and Asn-107 by Asp (AAC \rightarrow GAC). This may have resulted from reverse transcription and/or PCR errors. Sequence analysis also indicated the presence of triplet GCA coding for Ala-228\textsuperscript{26} instead of the triplet GTA coding for Val-228.\textsuperscript{23,24,26} This probably represents a sequence dimorphism. However, all three nucleotide changes in our TF cDNA clone are not expected to interfere with the Northern hybridization experiments described in this report.

\textbf{TF and TFPI functional assays.} TF activity was measured by a one-stage assay method in which 50 \textmu{}L of cell lysate was incubated with 50 \textmu{}L of normal citrated pooled plasma for 3 minutes at 37\degree{}C in a plastic tube. A 50-\textmu{}L aliquot of prewarmed 25 mmol/L CaCl\textsubscript{2} was then added and the clotting time recorded from the point of addition of CaCl\textsubscript{2}. Cell lysates were prepared by three freeze-thaw cycles (\textdegree{}7C/\textdegree{}37\degree{}C) of scrapes harvested cells (\textasciitilde{} 5 x 10\textsuperscript{7} cells/mL). TF activity of cells was expressed in units of human brain TF activity. A saline extract of human brain tissue,\textsuperscript{30} which clotted recalcified citrated normal plasma in 20 seconds, was arbitrarily assigned a value of 1 U of TF activity; a linear calibration curve was obtained when the log of clot times (25 to 140 seconds) was plotted against the log of human TF dilutions. In this assay, TF activity of unstimulated HUVE cells ranged from 15 to 20 mU/10\textsuperscript{6} cells and of stimulated cells ranged from 200 to 300 mU/10\textsuperscript{6} cells. Relative increases were calculated as the ratios of TF activities of stimulated versus unstimulated cells. TFPI assays were conducted as described.\textsuperscript{21} Cell culture supernatants were dialyzed against 0.05 mol/L Tris, 0.15 mol/L NaCl, pH 7.5, and centrifuged (12,000g for 10 minutes) before TFPI analysis. The results obtained on the same sample assayed on different days did not differ more than \pm{}10\%. The undiluted pooled human plasma was assigned a value of 1,000 mU/mL.

\textbf{Preparation of human whole blood serum (WBS) and cell-free plasma-derived serum (PDS).} WBS was obtained by allowing the blood to clot at 37\degree{}C for 2 hours. The serum was harvested and centrifuged at 10,000g for 10 minutes to remove insoluble components. The preparation was heat inactivated at 56\degree{}C for 30 minutes, centrifuged at 10,000g for 10 minutes, and filtered first through 0.45 \textmu{}m and then through 0.22 \textmu{}m low protein binding Millipore filters. The resulting WBS preparation (pool of five donors) was stored frozen in 10-mL aliquots at \textdegree{}20\degree{}C. PDS was prepared by initial centrifugation (6,000g for 15 minutes) of citrated blood to remove platelets, erythrocytes, and leukocytes. The plasma obtained was centrifuged at 10,000g for 30 minutes to remove any residual platelets. The platelet-depleted plasma was recalcified and allowed to clot at 37\degree{}C for 2 hours. The clot was removed by centrifugation and the serum was heat inactivated and filtered as outlined above for the WBS preparation. The fibroblast proliferation induced by the WBS preparation as determined by the \textsuperscript{3}H-thymidine incorporation assay was 15-fold greater than that of albumin or the PDS preparation.\textsuperscript{31}

\textbf{Treatment of HUVE cells with PMA, endotoxin, IL-1, or TNF-\alpha.} Primary cultures of HUVE cells were used for each experiment. Confuent monolayers in T-25 cm\textsuperscript{2} flasks were kept in DMEM supplemented with 10\% FBS (pretreatment media) for 24 hours, at which time control flasks received fresh pretreatment media, whereas the experimental flasks received pretreatment media containing the inflammatory agent. At selected time intervals, the supernatants were processed for TFPI activity and the cells were
processed either for TF activity or for RNA isolation. The
pretreatment media did not possess TFPI activity in our assay
system. For experiments in which human serum was used, the
pretreatment media at 24 hours was changed to DMEM containing
10% FBS or WBS with or without PMA.

**Northern blotting.** Total RNA from the cultured HUVE cells
(\( \approx 3 \times 10^6 \) cells/T-25 cm² flask) was extracted by the method of
Chomczynski and Sacchi. Total RNA (20 to 25 \( \mu \)g) samples were electrophoresed on 1.2% agarose
formaldehyde gels as outlined. Prehybridization and hybridiza-
tion was performed in 50% formamide at 42°C and the washing
temperature was 60°C (stringent conditions). The filters were
exposed to Kodak X-AR5 film (Eastman Kodak, Rochester, NY)
at \( \approx 70°C \) with one intensifying screen. The same filters were used
for hybridization with TFPI, TF, and \( \beta \)-actin cDNA probes. Before
rehybridization, the removal of the previous probe was confirmed
by autoradiography. Full-length TFPI cDNA probe was as de-
scribed earlier for the human Our data
was cloned in EcoRI/HindIII sites of plasmid
pGEM. Each cDNA probe was radiolabeled with \( ^32P \) using the
random hexamer primer method as outlined in the Dupont-New
England Nuclear Products Bulletin. The specific activity of each
probe was 4 to 6 \( \times 10^6 \) cpm/\( \mu \)g. Northern blotting of RNA
from BPAE cells, and from bovine and rabbit lung and liver tissues,
was performed under nonstringent conditions; prehybridization, hybrid-
ization, and washing were all performed at 37°C. Levels of mRNAs
were quantitated using a Pharmacia LKB (Piscataway, NJ) Ultro-
scan XL laser densitometer.

**RESULTS**

**Northern blotting of RNA from BPAE cells, and from rabbit
and bovine lung and liver tissues.** Our previous data using
human cells and tissues indicated that liver parenchymal
cells may not be the site of TFPI synthesis. In this report,
we have extended this observation to other species. BPAE
cells contained two TFPI mRNAs of \( \approx 4.0 \) and 1.3 kb sizes
(Fig 1, lane 2). Rabbit lung tissue rich in endothelium also
contained two TFPI mRNAs of \( \approx 4.0 \) and 1.4 kb sizes (Fig
1, lane 3). Rabbit liver, however, contained only about 3% of
TFPI mRNAs of that found in the lung tissue (Fig 1, lane
4). Similarly, bovine liver contained less than 5% of the
TFPI mRNAs as compared with that found in the lung (gels
not shown). The differences in the two mRNAs in the
rabbit and bovine species sizes probably reflect the pres-
ence of additional 3' untranslated regions in the \( \approx 4 \)-kb
bands as reported earlier for the human system. Our data
obtained in the rabbit and the bovine system are in
agreement with our earlier findings in the human system
that endothelium may indeed represent the primary site of TFPI
synthesis.

**Effect of PMA, endotoxin, IL-1, or TNF-\( \alpha \) on the TFPI
expression by cultured HUVE cells in 10% FBS.** These data
are presented in Fig 2. When PMA (100 ng/mL) was used as
the inflammatory agent, the cumulative TFPI activity
secreted by the stimulated cells was modestly increased as
compared with that secreted by the nonstimulated cells.
The difference became apparent after about 8 hours and
was \( \approx 1.5 \)-fold at 16 hours and \( \approx 1.3 \)-fold at 24 hours (Fig
2A). There was also a modest increase in the TFPI mRNA
levels of stimulated cells at 4 hours but not at 8 or 24 hours
(data not shown). The TF activity of stimulated cells was
highest (\( \approx 15 \)-fold) at 4 hours and then sharply declined
over time (Fig 2A, top). The TF mRNA levels peaked
between 2 and 4 hours and also declined over time. Similar
results were obtained when experiments were repeated
using 50 ng or 200 ng/mL PMA. Thus, when there was a
sharp increase in TF synthesis by the PMA stimulated cells,
only a slight increase in TFPI synthesis was evident.

When those inflammatory mediators that may be active
during sepsis, such as endotoxin, IL-1, and TNF-\( \alpha \), were
used to stimulate endothelial cells, no significant increase
or decrease in the cumulative TFPI activity secreted into
the media was observed. There was also no significant
difference between the TFPI mRNA levels of stimulated
cells versus nonstimulated cells. The data obtained when
endotoxin was used as the stimulus are presented in Fig 2B.
In the bottom panel of Fig 2B, the cumulative TFPI activity
secreted into the media in the presence or absence of 5
\( \mu \)g/mL of endotoxin is presented as a function of time. The
analysis of these data show no observable differences in the
secreted TFPI activity of control versus stimulated cultures.
Northern blot analysis of RNA isolated at 2, 4, and 8 hours
also showed no significant differences in the TFPI mRNA
levels of nontreated versus treated cultures. As reported
earlier, the TF activity of stimulated cells peaked (\( \approx 15 \)-
fold increase) between 4 and 8 hours and the TF mRNA
levels peaked between 2 and 4 hours. Similar results (both
for TFPI and TF) were obtained when 1, 3, or 10 \( \mu \)g/mL of
endotoxin was used. Thus, when a dramatic increase in TF
expression was noted, a corresponding increase in TFPI
expression was not evident.

The effect of two cytokines, namely, IL-1 and TNF-\( \alpha \), on the
TFPI expression is shown in Fig 2C and D. IL-1 at 10
Fig 2. Effect of PMA, endotoxin, IL-1, or TNF-α on the expression of TF and TFPI by cultured HUVE cells. In all experiments confluent primary cultures in 10% FBS were used. At indicated intervals, TF activity associated with the cells and TFPI activity secreted into the media was determined in the control and stimulated cultures as described in Materials and Methods. The cells were stimulated with 100 ng/mL PMA (A), 5 μg/mL endotoxin (B), 10 U/mL IL-1 (C), or 0.5 nmol/L TNF-α (D). The activity represented is the mean of three experiments performed in duplicate. The top panel for each stimulus (A through D) shows the relative change in TF activity over time and the inset in this panel shows the Northern blot of TF mRNA (2.2 kb) at indicated times for stimulated cells. As reported earlier by several groups, the TF activity in cells was minimal and remained essentially constant within the experimental error; thus, a straight line was drawn to illustrate this low level of TF activity in each control experiment. As observed earlier, the TF activity of stimulated cells increased and then declined; a line connecting the data points was drawn to illustrate this point in each experiment. The bottom panel for each stimulus (A through D) shows the cumulative TFPI activity secreted into media over time and the inset in this panel shows the Northern blot of TFPI mRNAs (4 kb and 1.4 kb) at indicated times for control as well as stimulated cells. A random scatter was noted in the cumulative TFPI activity for both the control and the stimulated cells and connecting the data points yielded cumbersome plots. A second order regression analysis statistically appropriate for such curvilinear plots was performed and a computer fitted line was drawn for each TFPI activity graph. (○) Unstimulated control (CNTL) cultures; (●) cultures stimulated with the indicated inflammatory agent. LPS (lipopolysaccharide) represents endotoxin. Twenty micrograms of total RNA was used for each lane in Northern blotting experiments. The exposure time varied from 2 to 4 days depending on the specific activity of TF or TFPI cDNA probe.
TFPI expression by endothelial cells

TFPI expression by endothelial cells was stimulated with a combined dose of 0.5 nmol/L TNF-α at 0.5 nmol/L (Fig 2D) or at 0.3, 1, and 5 nmol/L did not result in a decrease or increase in the TFPI expression. In both IL-1- and TNF-α-treated cells, the TF activity peaked (≈ 15-fold increase) at 8 hours and the mRNA levels peaked between 2 to 4 hours. Moreover, when cells were stimulated with a combined dose of 0.5 nmol/L TNF-α and 10 U/mL IL-1, again no significant increase or decrease in the cumulative TFPI activity or the TFPI mRNAs levels was observed. Thus, the cytokines, which have been known to upregulate TF expression and which have been identified to play a pathologic role in the inflammatory/septic process, did not appear to influence TFPI expression by cultured endothelial cells.

Effect of human serum on the TFPI expression by cultured HUVE cells. To examine the effect of a moiety or moieties present in human WBS, confluent cells were incubated with either 10% WBS or 10% PDS and the TFPI activity secreted into the media was determined (see Materials and Methods). The dialyzed samples of 10% WBS or 10% PDS/DMEM contained 85 to 90 mU/mL and 10% PDS/DMEM contained 70 to 75 mU/mL of TFPI activity. The reason for the decrease in the cumulative TFPI activity or the TFPI mRNAs levels was observed. Thus, the cytokines, which have been known to upregulate TF expression and which have been identified to play a pathologic role in the inflammatory/septic process, did not appear to influence TFPI expression by cultured endothelial cells.

Effect of human serum on the TFPI expression by cultured HUVE cells. To examine the effect of a moiety or moieties present in human WBS, confluent cells were incubated with either 10% WBS or 10% PDS and the TFPI activity secreted into the media was determined (see Materials and Methods). The dialyzed samples of 10% WBS or 10% PDS/DMEM contained 85 to 90 mU/mL and 10% PDS/DMEM contained 70 to 75 mU/mL of TFPI activity. The reason for the decrease in the cumulative TFPI activity or the TFPI mRNAs levels was observed. Thus, the cytokines, which have been known to upregulate TF expression and which have been identified to play a pathologic role in the inflammatory/septic process, did not appear to influence TFPI expression by cultured endothelial cells.

Effect of human serum on the TFPI expression by cultured HUVE cells. To examine the effect of a moiety or moieties present in human WBS, confluent cells were incubated with either 10% WBS or 10% PDS and the TFPI activity secreted into the media was determined (see Materials and Methods). The dialyzed samples of 10% WBS or 10% PDS/DMEM contained 85 to 90 mU/mL and 10% PDS/DMEM contained 70 to 75 mU/mL of TFPI activity. The reason for the decrease in the cumulative TFPI activity or the TFPI mRNAs levels was observed. Thus, the cytokines, which have been known to upregulate TF expression and which have been identified to play a pathologic role in the inflammatory/septic process, did not appear to influence TFPI expression by cultured endothelial cells.

Effect of human serum on the TFPI expression by cultured HUVE cells. To examine the effect of a moiety or moieties present in human WBS, confluent cells were incubated with either 10% WBS or 10% PDS and the TFPI activity secreted into the media was determined (see Materials and Methods). The dialyzed samples of 10% WBS or 10% PDS/DMEM contained 85 to 90 mU/mL and 10% PDS/DMEM contained 70 to 75 mU/mL of TFPI activity. The reason for the decrease in the cumulative TFPI activity or the TFPI mRNAs levels was observed. Thus, the cytokines, which have been known to upregulate TF expression and which have been identified to play a pathologic role in the inflammatory/septic process, did not appear to influence TFPI expression by cultured endothelial cells.

Effect of human serum on the TFPI expression by cultured HUVE cells. To examine the effect of a moiety or moieties present in human WBS, confluent cells were incubated with either 10% WBS or 10% PDS and the TFPI activity secreted into the media was determined (see Materials and Methods). The dialyzed samples of 10% WBS or 10% PDS/DMEM contained 85 to 90 mU/mL and 10% PDS/DMEM contained 70 to 75 mU/mL of TFPI activity. The reason for the decrease in the cumulative TFPI activity or the TFPI mRNAs levels was observed. Thus, the cytokines, which have been known to upregulate TF expression and which have been identified to play a pathologic role in the inflammatory/septic process, did not appear to influence TFPI expression by cultured endothelial cells.

Effect of human serum on the TFPI expression by cultured HUVE cells. To examine the effect of a moiety or moieties present in human WBS, confluent cells were incubated with either 10% WBS or 10% PDS and the TFPI activity secreted into the media was determined (see Materials and Methods). The dialyzed samples of 10% WBS or 10% PDS/DMEM contained 85 to 90 mU/mL and 10% PDS/DMEM contained 70 to 75 mU/mL of TFPI activity. The reason for the decrease in the cumulative TFPI activity or the TFPI mRNAs levels was observed. Thus, the cytokines, which have been known to upregulate TF expression and which have been identified to play a pathologic role in the inflammatory/septic process, did not appear to influence TFPI expression by cultured endothelial cells.

Effect of human serum on the TFPI expression by cultured HUVE cells. To examine the effect of a moiety or moieties present in human WBS, confluent cells were incubated with either 10% WBS or 10% PDS and the TFPI activity secreted into the media was determined (see Materials and Methods). The dialyzed samples of 10% WBS or 10% PDS/DMEM contained 85 to 90 mU/mL and 10% PDS/DMEM contained 70 to 75 mU/mL of TFPI activity. The reason for the decrease in the cumulative TFPI activity or the TFPI mRNAs levels was observed. Thus, the cytokines, which have been known to upregulate TF expression and which have been identified to play a pathologic role in the inflammatory/septic process, did not appear to influence TFPI expression by cultured endothelial cells.

Effect of human serum on the TFPI expression by cultured HUVE cells. To examine the effect of a moiety or moieties present in human WBS, confluent cells were incubated with either 10% WBS or 10% PDS and the TFPI activity secreted into the media was determined (see Materials and Methods). The dialyzed samples of 10% WBS or 10% PDS/DMEM contained 85 to 90 mU/mL and 10% PDS/DMEM contained 70 to 75 mU/mL of TFPI activity. The reason for the decrease in the cumulative TFPI activity or the TFPI mRNAs levels was observed. Thus, the cytokines, which have been known to upregulate TF expression and which have been identified to play a pathologic role in the inflammatory/septic process, did not appear to influence TFPI expression by cultured endothelial cells.

Effect of human serum on the TFPI expression by cultured HUVE cells. To examine the effect of a moiety or moieties present in human WBS, confluent cells were incubated with either 10% WBS or 10% PDS and the TFPI activity secreted into the media was determined (see Materials and Methods). The dialyzed samples of 10% WBS or 10% PDS/DMEM contained 85 to 90 mU/mL and 10% PDS/DMEM contained 70 to 75 mU/mL of TFPI activity. The reason for the decrease in the cumulative TFPI activity or the TFPI mRNAs levels was observed. Thus, the cytokines, which have been known to upregulate TF expression and which have been identified to play a pathologic role in the inflammatory/septic process, did not appear to influence TFPI expression by cultured endothelial cells.
abundant amounts of TFPI mRNAs. Because other cells of the vasculature synthesize only small amounts of TFPI and because endothelium of capillaries, venules, and lymphatic channels stain positive in immunohistochemical studies, we believe that a majority of the TFPI mRNA, obtained form the lung tissue is derived from the endothelium and that it represents the primary source of plasma TFPI.

Endotoxin and the two prominent mediators (TNF-α and IL-1) of septic shock and inflammation did not significantly decrease or increase the TFPI mRNA expression and protein secretion by endothelial cells in culture. The concentrations of cytokines used in our experiments were within the limits found in human endotoxemia and primate bacteremia. The concentrations used are also within the range known to induce TF in endothelial cells. As reported earlier, in all of our experiments, the TF mRNA expression preceded the manifestation of TF activity, which peaked between 4 and 8 hours and then declined. These inflammatory mediators, in addition to upregulating endothelial TF and fibrinolytic inhibitors, also downregulate thrombomodulin and fibrinolytic enzymes (see above). Thus, it would appear that TFPI is the only known endothelial anticoagulant protein the expression of which may not be downregulated during an inflammatory response.

The endothelial cells cultured in PDS constitutively synthesized and secreted TFPI. The synthesis of TFPI under these conditions reflects the ability of these cells to synthesize TFPI in a plasma milieu devoid of releasates from formed elements of blood. The cells cultured in WBS invariably expressed and secreted higher levels (≈ 1.5-fold) of TFPI than the cells cultured in PDS. This observation suggests that there are additional serum factors released from the formed elements such as platelets, which modestly upregulate synthesis and secretion of TFPI from endothelial cells. The WBS elements have been previously noticed to induce synthesis of TF in murine BALB/c 3T3 and AKR-2B fibroblasts and in human fetal lung fibroblasts. However, the significance of slight upregulation of TFPI by WBS in endothelial cells and induction of TF in fibroblasts by FBS remains to be established. Moreover, we also found that endothelial cells cultured in WBS expressed twofold increased levels of TFPI than the cells cultured in FBS. The reason(s) for this is not readily evident but may be related to species differences.

In our studies, PMA treatment of cells cultured in FBS or WBS resulted in a slight upregulation of TFPI expression. Although a small increase in TFPI synthesis in PMA-treated cells may not be significant, we did not observe a decrease in TFPI secretion, as reported earlier; however, the reason(s) for this is not readily apparent at this time. Moreover, the increase in TFPI mRNAs induced by WBS or by PMA was transient. This may be related to the presence of AUUU-rich sequences in the 3' end of the TFPI mRNAs that may, in part, contribute to its instability. Such A and U-rich sequences present in other genes, such as TF42 and inflammatory mediators, have been reported to render the mRNA susceptible to degradation.

TFPI synthesis by endothelial cells and its slight upregulation by PMA and WBS is consistent with the transcriptional enhancer elements present in the 5' flanking region of the gene. Two groups have reported the structural organization of the human TFPI gene. Our analysis of the 5' end of the gene showed a perfect GATA consensus element (AGATAA, bp number 75-80 in Girard et al) located approximately 400 bp upstream of the transcription initiation site(s). This core DNA motif is thought to be essential for expression of certain genes by erythroid cells, megakaryocytes, and endothelial cells. As is the case with the endothelin-1 gene expression, binding of the TFPI gene GATA motif to the GATA-2 transcriptional factor (which is also synthesized by the endothelium) may be necessary for constitutive expression of TFPI by the endothelium. A slight upregulation observed by treatment of cells with PMA may be attributed to the presence of two AP-1-like motifs located in the 5'-end of the TFPI gene. The first motif (AGAGTCA, bp numbers 426-432 in Girard et al) has A instead of T at position 1 of the consensus AP-1 sequence and probably is nonfunctional. The second motif (TGCTCTCA, bp numbers 91-97 in Girard et al) has T instead of A at position 1 of the consensus AP-1 sequence. Because replacement of A by T at position 3 in

Fig 4. Effect of PMA on the TFPI expression by cultured HUVE cells in 10% WBS. The concentration of PMA used was 100 ng/mL. The cumulative TFPI activity secreted into the media is plotted against time of treatment. The data represent the mean ± SD of two experiments performed in duplicate using separate cell isolates. The inset shows the Northern blot analysis of RNA isolated from control cells and the PMA stimulated cells at 4 and 8 hours. The probe used was TFPI cDNA and the exposure time was 2 days.
this motif has been shown to be functional.\textsuperscript{50} we suspect that this second AP-1-like motif in the TFPI gene may be responsible for the PMA response observed in our study. This second AP-1-like motif may also be responsible for the PMA induction of TFPI synthesis in a monocytoid cell line.\textsuperscript{51}

The modest upregulation of TFPI expression observed for endothelial cells cultured in WBS in part, be due to the presence of transforming growth factor-\(\beta\) (TGF-\(\beta\)) released from platelets. Two TGF-\(\beta\)-like responsive elements, termed NF-1,\textsuperscript{52} are present in the 5' end of the TFPI gene.\textsuperscript{35,46} One element (TGGGCN\(\_\)GCCAA, bp number 14-29) has a six-nucleotide spacer instead of the normal five and the other element (TGGGCN\(\_\)GCCAA, bp numbers 459-472) has a four-nucleotide spacer and CCCAA sequence instead of the consensus GCCA sequence. Although the second NF-1–like motif is thought to be active,\textsuperscript{45} whether one or both of these motifs are functional remains to be experimentally tested. Similarly, whether the GATA consensus sequence and the AP-1-like consensus sequences are actually involved in the regulation of TFPI gene expression is an interesting subject for further study. One should also note that there are two additional GATA consensus motifs that occur downstream of the transcription initiation site(s) (bp numbers 534-540 and 652-657 in Van der Logt et al\textsuperscript{45}). Their function in TFPI gene regulation also remains to be elucidated.

\textbf{ACKNOWLEDGMENT}

The authors thank Mark Hoyer for technical assistance and Beth Haase for preparation of the manuscript.

\textbf{REFERENCES}

2. Bauer KA, Kas BL, ten Cate H, Hawiger JJ, Rosenberg RD: Factor IX is activated in vivo by the tissue factor mechanism. Blood 76:731, 1990
Expression of tissue factor pathway inhibitor by cultured endothelial cells in response to inflammatory mediators

A Ameri, MN Kuppuswamy, S Basu and SP Bajaj