Quantification and Characterization of Human Endothelial Cell–Derived Tissue Factor Pathway Inhibitor-2

Masaki Iino, Donald C. Foster, Walter Kisiel

Abstract—Tissue factor pathway inhibitor-2 (TFPI-2), also known as placental protein 5, is a serine protease inhibitor consisting of three tandemly-arranged Kunitz-type protease inhibitor domains. While TFPI-2 is a potent inhibitor of trypsin, plasmin, kallikrein, and factor Xa in the test tube, the function of this inhibitor in vivo remains unclear. In the present study, we investigated the synthesis and secretion of TFPI-2 by cultured endothelial cells derived from human umbilical vein, aorta, saphenous vein, and dermal microvessels to gain insight into its biological function. While all endothelial cells examined synthesized and secreted TFPI-2, dermal microvascular endothelial cells synthesized threefold to sevenfold higher levels of TFPI-2. Approximately 60% to 90% of the TFPI-2 secreted by endothelial cells was directed to the subendothelial extracellular matrix (ECM). When cultured human umbilical vein endothelial cells were stimulated with inflammatory mediators such as phorbol 12-myristate,13-acetate; endotoxin; and tumor necrosis factor-α, TFPI-2 synthesis by these cells increased twofold to 14-fold. Recombinant TFPI-2 bound to dermal microvascular endothelial cell monolayers and its ECM in a specific, dose-dependent, and saturable manner with K_d values of 21 and 24 nmol/L, respectively. TFPI-2 interacted with 4.5 × 10^{10} sites/cm^2 (3 × 10^3 sites/cell) and 2.3 × 10^9 sites/cm^2 on endothelial cells and ECM, respectively. In the presence of rabbit anti–TFPI-2 IgG, but not preimmune IgG, endothelial cells dissociated from the culture flask in a time- and IgG concentration–dependent manner. Our findings provide evidence that endothelial cell–derived TFPI-2 is primarily secreted into the abluminal space and presumably plays an important role in maintaining the integrity of the ECM essential for cell attachment. (Arterioscler Thromb Vasc Biol. 1998;18:40–46.)

Key Words: tissue factor pathway inhibitor-2 ■ endothelial cell ■ extracellular matrix ■ proteoglycan

Human TFPI-2 is a 32-kD serine protease inhibitor with an overall Kunitz inhibitory domain organization similar to TFPI, a major factor Xa–and factor VIIa–tissue factor inhibitory factor inhibitor found in human plasma. Recombinant human TFPI-2 has recently been shown to be a strong inhibitor of trypsin, plasmin, plasma kallikrein, and factor Xa amidolytic activity. TFPI-2 also weakly inhibited the amidolytic and proteolytic activities of chymotrypsin, factor VIIa–tissue factor, factor IXa–polysine, and cathepsin G, but failed to significantly inhibit the amidolytic activities of glandular kallikrein, urinary plasminogen activator, tissue plasminogen activator, activated protein C, factor Xa, thrombin, and leukocyte elastase.

Recent studies revealed that TFPI-2 is identical to an inhibitory protein isolated from human placenta designated as placent protein 5, or PP5. PP5 is synthesized in endothelial cells and circulates in blood of normal men and nonpregnant women in extremely low concentrations (0.43 to 0.49 ng/mL). The level of PP5 increases 40-fold to 70-fold in these individuals after injection of small doses of heparin, which provides suggestive evidence that a significant amount of PP5 is bound to the endothelium and is released from this site by heparin infusion. More recently, Rao et al. reported that three serine protease inhibitors (33, 31, and 27 kD), obtained from the extracellular matrices of either HUVECs or dermal fibroblasts, are immunochemically identical to TFPI-2 and represent differentially glycosylated forms of this inhibitor. Earlier work by Rao and coworkers, using casein reverse zymography, revealed that TFPI-2 was found primarily in the extracellular matrix (ECM), but not in the conditioned media, of unstimulated HUVECs. Treatment of these cells with phorbol 12-myristate,13-acetate resulted in increased levels of TFPI-2 in the extracellular matrix and cell lysates and secretion into the conditioned media.

The role of ECM–associated TFPI-2 is unclear but presumably may be important in the regulation of matrix turnover by serine proteases, such as plasmin. In addition, it is unknown whether endothelial cells derived from different blood vessels are equivalent in secreting TFPI-2 into their ECM. To address these issues, we have developed a simple and quantitative enzyme-linked immunoassay for human TFPI-2, and assessed the synthesis of TFPI-2 by a variety of cultured human endothelial cells. Our results provide corroborating evidence that the majority of TFPI-2 synthesized by endothelial cells is deposited into the ECMs of these cells, and that matrix–associated TFPI-2 inhibitory activity plays an important role in the integrity of the endothelial cell/ECM interaction.
Selected Abbreviations and Acronyms

DMEC = endothelial cell derived from dermal capillary
ECCGS = endothelial cell growth supplement
ECM = extracellular matrix
FBS = fetal bovine serum
HAEC = human aorta endothelial cell
HAT = human antithrombin III
HSVEC = human saphenous vein endothelial cell
HUVEC = human umbilical vein endothelial cell
125I-TFPI-2 = 125I-labeled TFPI-2
TFN-α = human tumor necrosis factor-α
TFPI = tissue factor pathway inhibitor
TFPI-2 = tissue factor pathway inhibitor-2

Methods

Materials

TPCK-trypsin was purchased from Worthington. N-α-Benzoyloxy-carbonyl-d-arginyl-l-glucyl-l-arginine-p-nitroanilide-dihydrochloride (S-2765) was from Helena Laboratories. O-phenylenediamine, MITT, soybean trypsin inhibitor, protamine sulfate, PMA, penicillin-streptomyein solution, trypsin-EDTA solution (1X), porcine intestinal mucosal heparin sodium salt (175 USP U/mg), and lipopolysacharide from Esherichia coli (O26:B6, LPS) were from Sigma. Medium 199 was from Mediatech. Fetal bovine serum was obtained from Novo Nordisk. Recombinant human TNF-α were from Boehringer Mannheim. Biotin-N-hydroxysuccinimide conjugated avidin and fatty acid–free bovine serum albumin (BSA) were of the highest quality commercially available.

Proteins

Recombinant TFPI-2 and TFPI were expressed in baby hamster kidney cells and purified as described.1,10 HAT11 and protein A-Sepharose–purified rabbit IgG monospecific for HAT12,13 TFPI,14 TFPI-2,5 and bovine AT14 were prepared according to published methods.

General Methods

The concentrations of trypsin and rabbit IgG were determined according to Bradford15 using BSA and rabbit IgG as reference proteins, respectively. The concentration of standard TFPI-2 was determined by an amino acid analysis.12 125I-TFPI-2 was prepared to a concentration of 106 cpm/μg as described previously.12 Briefly, 20 μL of 25 mg/mL bovine-N-hydroxy succinimid e ester in dimethylformamide was added to 1 mL of trypsin (500 μg/mL) in 50 mMol/L HEPES, pH 8.3. After 1-hour incubation at room temperature, the solution was dialyzed against 50 mmol/L HEPES, pH 6.0 containing 1 mmol/L benzamidine at 4°C overnight. After this treatment, the biotin-labeled trypsin retained 96% of its amidolytic activity toward S-2765 compared with that of untreated trypsin.

Measurement of TFPI-2 Concentration

The concentration of TFPI-2 was determined by ELISA using biotin-labeled trypsin and 96-well plates coated with rabbit anti—TFPI-2 IgG. In this procedure, 100 μL rabbit IgG against TFPI-2 (10 μg/mL in 50 mmol/L sodium carbonate, pH 9.5) was added to wells of 96-well microtiter plates (MaxiSorp ImmunoPlate, Nunc) incubated overnight at 4°C. After washing the plate three times with TBS–0.1% Tween 20, each well was blocked with 200 μL of TBS–2% BSA at 37°C for 2 hours; the plate was then washed three times with TBS–TWEEN 20. Subsequently, 100 μL of sample diluted with TBS–1% BSA–50 μg/mL heparin was added to each well and allowed to incubate at 37°C for 2 hours. The plate was then washed three times with TBS–TWEEN 20 and 100 μL of biotin-labeled trypsin (0.5 μg/mL in 1% BSA–TBS) was added to each well. After a 2-hour incubation at 37°C, the plate was washed three times with TBS–TWEEN 20 and subsequently treated with 100 μL peroxidase-conjugated avidin (diluted 5000-fold with TBS–1% BSA) for 2 hours. After the plate was washed six times with TBS/TWEEN 20, 100 μL 0.1% phenylenediamine (1 mg/mL in 0.1 mol/L sodium citrate, pH 4.5, and 0.5% hydrogen peroxide) was added to each well. After the plate was incubated 3 to 5 minutes at room temperature, 100 μL 2.5 mol/L sulfuric acid was added to each well and the A490 measured. The concentration of TFPI-2 in test samples was interpolated from standard curves of A490 versus recombinant TFPI-2 concentration.

Cell Culture

HUVECs were prepared by the method of Jaffe et al.20 as modified by Thamann et al.21 HAT11,12 and DMECs were obtained from Cell Systems. These cells were grown to confluence in medium 199 supplemented with 20% FBS, 100 μg/mL heparin, 100 μg/mL ECGS, and 100 μg/mL penicillin-streptomycin (medium199–FBS) in 75-cm² tissue culture flasks (Corning) in a humidified atmosphere at 33°C and 6% CO2,22 and subcultured using 0.5 g/L trypsin and 0.2 g/L EDTA. Experiments were performed using cells between passages 2 and 6 after cells were grown to confluence in either 2 cm² wells of 24-well plates (Corning) or in 75-cm² flasks. In PMA, LPS, and TNF-α stimulation experiments, HUVECs were cultured in the absence of heparin at 37°C in medium 199 and 20% FBS containing either 100 ng/mL PMA, 5 μg/mL LPS, or 20 ng/mL TNF-α.

Preparation of Endothelial Cell Culture Media, ECM, and Cell Lysate Samples

Endothelial cells were seeded in 75-cm² dishes at 5.0 to 7.0×10⁴ cells/cm², and after 2 to 3 days reached confluence (1.5 to 2.0×10⁶ cells/cm²). The culture medium, medium 199–FBS, was removed and the flask washed three times with medium 199 containing 0.5% BSA (serum-free medium 199). Next, 10 mL fresh medium 199–FBS without heparin [medium 199–FBS(-)] was added to the flasks. After cells were cultured at 37°C for 4 days, medium 199–FBS–H(−) was harvested and assayed for TFPI-2. The flasks were then rinsed three times with Tris-buffered saline (50 mmol/L Tris-HCl, pH 7.5, containing 100 mmol/L NaCl and TBS). Subsequently, 2 mL of 10 mmol/L EDTA–TBS was added to the flasks. After a 10–20-minute incubation at 37°C, cells dissociated from the surface of the flask. The floating cells were removed from the flask and washed three times with TBS by centrifugation (600×g for 5 minutes). After the third centrifugation, the supernatant was removed and 500 μL of 1% SDS–TBS was added. The cell lysates were centrifuged (11 000×g for 60 minutes) and the supernatants stored at −80°C. To prepare ECM samples, 2 mL of 1% SDS in TBS was added to the flasks after removal of the cells and the flasks were agitated at 25°C for 2 hours. After this process, the SDS solutions were collected and stored at −80°C.

Binding Assays

The methodology used for measuring the association of 125I-TFPI-2 to DMEC or its ECM was a slight modification of that described by Stern et al.23 Twenty-four-well plates were initially seeded with 5.0×10⁴ cells/cm², and the cells were grown to confluence. To prepare DMEC, DMEC was removed with 10 mmol/L EDTA–TBS and the wells were incubated with 1 mL/well of 1 mol/L NaCl-TBS for 1 hour at 37°C to remove endogenous TFPI-2.24 These wells were washed three times with serum-free medium 199–H(−), and subsequently incubated with 200 μL of 125I-TFPI-2 in serum-free medium 199–H(−) for 2 hours at 37°C with constant oscillation (50 rpm) on an orbit shaker (Laboratory-Line). The plates...
were then rapidly washed six times with ice-cold buffer A (10 mmol/L HEPES, pH 7.4 containing 137 mmol/L NaCl, 4 mmol/L KCl, 2 mmol/L CaCl₂, and 0.5% BSA). The amount of well-bound ¹²⁵I-TFPI-2 was determined after incubation with 500 μL/well of 200 mmol/L NaOH, 1% SDS, and 10 mmol/L EDTA for 1 hour at 37°C and counting in gamma counter (Minaxi γ, Packard). Bound radioactivity was expressed as an average value of radioactivity observed in duplicate wells. The amount of specific well-associated ¹²⁵I-TFPI-2 was determined by subtracting the amount of ¹²⁵I-TFPI-2 associated with wells in the presence of a 50-fold molar excess of unlabeled TFPI-2 from the total amount of well-bound ¹²⁵I-TFPI-2.

To measure the time dependency of TFPI-2 binding, 200 μL of 2 nmol/L ¹²⁵I-TFPI-2 and various concentrations of competitors in serum-free medium 199–H(-) were added. After being incubated for 2 hours at 37°C, the wells were washed six times with ice-cold buffer A and the ECM-bound TFPI-2 were added. After being incubated for 2 hours at 37°C, the wells were washed and bound ¹²⁵I-TFPI-2 was measured as described above.

Inhibition of TFPI-2 Binding to ECM
The inhibition of TFPI-2 binding to dermal microvascular endothelial cell–derived ECM by various materials was investigated using 24-well plates. After achieving confluence, the ECM was prepared as described under “Methods.” Then 200 μL of 2 nmol/L ¹²⁵I-TFPI-2 and various concentrations of competitors in serum-free medium 199–H(-) were added. After being incubated for 2 hours at 37°C, the wells were washed six times with ice-cold buffer A and the ECM-bound ¹²⁵I-TFPI-2 was measured as described under “Binding Assays.”

Effect of Anti–TFPI-2 IgG on Endothelial Cell Monolayers
The effect of rabbit anti–TFPI-2 IgG on endothelial cells derived from various blood vessels was studied. After reaching confluence in wells of 24-well plates, the endothelial cells were cultured with medium 199–FCS-H(-) overnight. The wells were then washed twice with serum-free medium 199–H(-), and 200 μL of either rabbit anti–TFPI-2 IgG or preimmune rabbit IgG, both 1 mg/mL in serum-free medium 199–H(-), was added. At selected incubation times at 37°C, the cell monolayer was examined with an inverted microscope (Optron, Zeiss). In addition, the relative amount of viable cells adherent to well surfaces after anti–TFPI-2 IgG treatment was determined by the MTT assay.²⁵

Results
TFPI-2 Assay
A sensitive microtiter plate assay was developed to permit quantification of TFPI-2 antigen concentrations in the culture media, cell lysates and ECMs of a variety of human endothelial cells. This assay was based on the specific interaction of TFPI-2 with a monovalent polyclonal anti–TFPI-2 IgG and its ability to bind and neutralize trypsin.³ In this assay, the dose-response curve was linear over the range 1.0 to 10 ng/mL of TFPI-2 (Fig 1). This assay was specific for human TFPI-2, because bovine TFPI-2 was not detected in culture media containing 20% fetal bovine serum. In addition, our preparation of rabbit anti–TFPI-2 IgG failed to detect 500 ng of recombinant TFPI-2. Anti–TFPI-2 IgG failed to detect 500 ng of recombinant TFPI-2.

Synthesis of TFPI-2 by Various Cultured Endothelial Cells
TFPI-2 antigen levels were quantified in the cell lysates, culture media and ECMs of cultured human endothelial cells derived from umbilical vein, aorta, saphenous vein, and dermal capillaries. As seen in Table 1, all endothelial cells examined constitutively synthesized and secreted TFPI-2, with DMECs synthesizing threefold to sevenfold higher levels of TFPI-2 compared with other endothelial cell cultures. While all endothelial cells secreted a small percentage of TFPI-2-into the culture medium, the majority (60% to 90%) of the TFPI-2 secreted by these cells was deposited in their ECMs (Table 1). Treatment of washed confluent HUVEC monolayers with medium 199 containing 50 U/mL heparin for 1 to 2 hours at 33°C failed to release detectable TFPI-2-into the medium (data not shown). Consistent with earlier data by Rao et al,⁴ all TFPI-2–containing fractions obtained from HUVECs (cell lysates, culture medium and ECM) consisted of three differentially glycosylated molecular weight species (Mr 33, 31 and 27 kD) in approximately equal amounts as determined by immunoblotting (Fig 2). Similar results were obtained for TFPI-2–containing samples derived from HAEC, HSVEC and DMEC (data not shown).

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**TABLE 1. TFPI-2 Synthesis by Various Endothelial Cells**

<table>
<thead>
<tr>
<th>Cell</th>
<th>TFPI-2 Antigen, ng/10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture Medium</td>
</tr>
<tr>
<td>HUVEC</td>
<td>5.0±0.4</td>
</tr>
<tr>
<td>HAEC</td>
<td>97.5±5.6</td>
</tr>
<tr>
<td>HSVEC</td>
<td>66.4±3.2</td>
</tr>
<tr>
<td>DMEC</td>
<td>225.0±10.5</td>
</tr>
</tbody>
</table>

Values are mean±SD.
TNF-α, 5 to 20 ng/mL; and LPS, 0.5 to 5 μg/mL. In addition to increasing the total synthesis of TFPI-2, treatment of HUVECs individually with LPS, PMA, and TNF-α appeared to significantly increase the relative amounts of TFPI-2 secreted into the culture medium (Table 2), in agreement with earlier findings by Rao et al,9 who observed that TFPI-2 levels in HUVEC-conditioned medium was greatly increased after treatment of these cells with PMA.

Binding of TFPI-2 to Dermal Microvascular Endothelial Cells and its Extracellular Matrix

Because DMECs secreted the highest levels of TFPI-2, we next determined the isotherms of TFPI-2 binding to these cells as well as its derivative ECM. The binding of TFPI-2 to confluent DMEC monolayers and its ECM was time dependent, reaching a maximum after 2 hours of incubation at 37°C (Fig 3). As shown in Fig 4, radiolabeled TFPI-2 bound to DMEC monolayers (Fig 4A) and ECM (Fig 4B) in a concentration-dependent manner and approached saturation at ~75 nmol/L. The binding isotherms for each system exhibited a hyperbolic profile, and Scatchard plots of these equilibrium binding data at 37°C indicated a single class of binding sites for TFPI-2 on DMECs and ECM with K_d values of 21 nmol/L and 24 nmol/L, respectively (Fig 4C). The number of specific binding sites for TFPI-2 on DMECs and ECM were 4.5 ± 10^10 sites/cm^2 (3.5 ± 10^5 sites/cell) and 2.3 ± 10^11 sites/cm^2, respectively.

The reversibility of ECM or endothelial cell–bound 125I-TFPI-2 was demonstrated by assessing the time-dependent dissociation of the radioligand from the endothelial cell monolayer or ECM. In these studies, 125I-TFPI-2 was first incubated with endothelial cell monolayers or ECM for 2 hours at 37°C to establish equilibrium binding. The cells or ECM were then treated with 200 μL of medium 199–H(-) and the residual TFPI-2 radioligand bound to the surface was determined as a function of time. The results of these studies indicated that both ECM-bound and endothelial cell-bound 125I-TFPI-2 dissociated from the surface at a linear rate, and that 60% to 70% of bound TFPI-2 dissociated from each surface after a 2-hour incubation.

The specificity of radiolabeled TFPI-2 binding to DMEC-derived ECM was also demonstrated in competition studies. In these studies, ECM was incubated for 2 hours at 37°C, either with 125I-TFPI-2 (2 nmol/L) alone or with severalfold molar excesses of various unlabeled competitor proteins including TFPI-2, TFPI, HAT, aprotinin, and soybean trypsin inhibitor. Under these conditions, only unlabeled TFPI-2 and HAT strongly inhibited radioligand binding (Table 3). High concentrations (10 μg/mL) of other Kunitz-type protease inhibitors such as TFPI, soybean trypsin inhibitor, and aprotinin weakly inhibited TFPI-2 binding (5% to 26%), whereas HAT, at 10 μg/mL, inhibited 125I-TFPI-2 binding ~60%, presumably through its ability to bind to ECM heparan sulfate proteoglycans. In additional studies, heparin (at 50 μg/mL) and protamine (at 100 μg/mL) inhibited TFPI-2 binding to ECM ~95% (Table 3), providing further evidence that TFPI-2 was interacting with charged proteoglycans in the ECM.

Effect of Anti-TFPI-2 IgG on Confluent Endothelial Cell Monolayers

In preliminary studies aimed at assessing the ability of endothelial cell–bound TFPI-2 to neutralize offered serine pro-

TABLE 2. The Effects of Inflammatory Mediators on TFPI-2 Expression by HUVECs

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Culture Medium</th>
<th>Cell Lysate</th>
<th>ECM</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.4±0.5</td>
<td>86.3±13.6</td>
<td>98.4±15.6</td>
<td>190.1±29.7</td>
</tr>
<tr>
<td>LPS</td>
<td>39.3±1.3</td>
<td>88.3±6.8</td>
<td>176.5±7.4</td>
<td>304.1±15.5</td>
</tr>
<tr>
<td>PMA</td>
<td>750.0±23.0</td>
<td>56.1±2.0</td>
<td>1855.5±131.2</td>
<td>2661.6±156.2</td>
</tr>
<tr>
<td>TNF</td>
<td>67.0±8.3</td>
<td>92.8±8.0</td>
<td>602.5±101.6</td>
<td>762.3±117.9</td>
</tr>
</tbody>
</table>

Values are mean±SD.
teases, we observed in control studies that treatment of endothelial cell monolayers with anti–TFPI-2 IgG but not preimmune rabbit IgG caused a progressive detachment of endothelial cells from the subendothelial matrix. To further investigate this process, various confluent endothelial cell monolayers were treated with either anti–TFPI-2 IgG (1 mg/mL) or preimmune IgG (1 mg/mL) for various times at 37°C, and at selected intervals, the percentage of viable cells attached to the wells was determined by the MTT assay. The results of these studies, shown in Figs 5 and 6, indicated that endothelial cells cultured in the presence of preimmune IgG remain virtually intact, whereas endothelial cells treated with anti–TFPI-2 IgG showed significant time-dependent changes in the percentage of viable cells attached to their ECM (Fig 5). As shown in photomicrographs for DMECs (Fig 6), in the initial phase of incubation with anti–TFPI-2 IgG, small, holelike regions appeared in the monolayers, which, after 8 hours of incubation, became enlarged with many reticular-like regions appearing. On further incubation (8–16 hours) with anti–TFPI-2 IgG, DMECs no longer attached to the surface of plastic wells, with most of the cells visible in the culture medium (Fig 6). PMA-treated HUVEC monolayers were most sensitive to anti–TFPI-2 IgG treatment, whereas quiescent HUVEC monolayers were most resistant to anti–TFPI-2 IgG treatment (Fig 5). Anti–TFPI-2 IgG-induced endothelial cell detachment was IgG dose–dependent (minimum 200 μg/mL IgG) and was inhibited by exogenous TFPI-2 (minimum 100 μg/mL of TFPI-2 for 1 mg/mL IgG). In addition to preimmune IgG, anti-HAT IgG, anti-human TFPI IgG, and anti-bovine ATIII IgG, all at 1 mg/mL, had no effect on the endothelial cell monolayers after 16 hours of incubation at 37°C. Heparin, at 5 μg/mL final concentration, completely prevented the endothelial cell detachment in the presence of anti–TFPI-2 IgG, whereas aprotinin (100 μg/mL), a plasmin inhibitor, and amiloride (500 μmol/L), a urokinase inhibitor, had no measurable effect on this process.

**Discussion**

The data presented in this paper demonstrate that TFPI-2 is synthesized and secreted by a variety of human endothelial cells and is primarily deposited in the ECM of these cells. In comparison to aortic, saphenous vein and umbilical vein endothelial cells, dermal microvascular endothelial cells syn-

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**TABLE 3. Inhibition of 125I-TFPI-2 Binding to ECM**

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Concentration, μg/mL</th>
<th>Total Binding of 125I-TFPI-2 to ECM, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100.0</td>
</tr>
<tr>
<td>TFPI</td>
<td>1</td>
<td>103.0</td>
</tr>
<tr>
<td>TFPI-2</td>
<td>10</td>
<td>74.1</td>
</tr>
<tr>
<td>HAT</td>
<td>1</td>
<td>11.3</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>10</td>
<td>39.1</td>
</tr>
<tr>
<td>SBTI</td>
<td>10</td>
<td>94.6</td>
</tr>
<tr>
<td>Protamine</td>
<td>10</td>
<td>95.8</td>
</tr>
<tr>
<td>Heparin</td>
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</tr>
<tr>
<td>5</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>4.5</td>
<td></td>
</tr>
</tbody>
</table>

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**Figure 5.** The effect of anti–TFPI-2 IgG on endothelial cell monolayers. Monolayers of HUVEC (△, ○), PMA-treated HUVEC (●, □), HAEC (■, ▧), HSVEC (●, ○), or DMEC (▴, ▼) were incubated with anti–TFPI-2 IgG (△, ○, ▶, ▼) or nonspecific IgG (●, □, ○, ▸, ▼). At the indicated times, the percentages of cells attached on the wells were measured by the MTT assay.
thesized threefold, fivefold, and sevenfold higher levels of TFPI-2, respectively. In addition, several inflammatory agents, including phorbol esters, endotoxin and tumor necrosis factor-α, significantly upregulated synthesis and secretion of TFPI-2 by HUVECs, which provides suggestive evidence that the inhibitory properties of the subendothelium may be enhanced in an inflammatory or septic response through the increased synthesis of TFPI-2. TFPI-2, secreted by all endothelial cells examined in this study, migrated in SDS-PAGE/immunoblots as a triplet, exhibiting Mr values of 33, 31 and 27 kD, consistent with earlier data by Rao and coworkers that demonstrated differential glycosylation of TFPI-2 secreted by human endothelial cells and dermal fibroblasts.

Recombinant human TFPI-2 specifically bound to dermal microvascular endothelial cell monolayers and to its ECM in a time-dependent and saturable manner. By Scatchard analyses, TFPI-2 interacted with $4.5 \times 10^{10}$ sites/cm$^2$ and $2.3 \times 10^{11}$ sites/cm$^2$ on endothelial cell monolayers and ECM, respectively, with similar affinities (21 and 24 nmol/L, respectively). Binding of $^{125}$I-TFPI-2 to the ECM was inhibited $>90\%$ by unlabeled preparations of TFPI-2, whereas other Kunitz-type inhibitors, including human TFPI, aprotinin, and soybean trypsin inhibitor, had little effect on $^{125}$I-TFPI-2 binding to this surface. On the other hand, antithrombin III, heparin and protamine sulfate inhibited $^{125}$I-TFPI-2 binding to the ECM, suggesting that TFPI-2 was interacting with either heparan sulfate or chondroitin sulfate proteoglycans in the matrix.

As noted above, TFPI-2 is a structural and functional homologue to TFPI, a major plasma inhibitor of the extrinsic pathway of blood coagulation. While the endothelial cell has been shown to be the principal site of TFPI synthesis, the distribution of secreted TFPI antigen in the ECM and culture media has not been reported. In this regard, we have measured TFPI antigen in culture medium, cell lysates, and ECM of HUVECs using biotin-labeled trypsin and monospecific anti–TFPI IgG-coated microtiter plates. By this assay, the amount of TFPI antigen in HUVEC culture medium was $\sim 1.5 \mu g/10^6$ cells, whereas cell lysates and extracellular matrix contained 173 and 35 ng TFPI/10$^6$ derivative cells, respectively. Thus, on the basis of our findings, it would appear that endothelial cells secrete the majority of synthesized TFPI-2 into the extracellular matrix and secrete virtually all of the synthesized TFPI into the bloodstream.

While our studies clearly demonstrate synthesis of TFPI-2 by endothelial cells and the directional secretion of TFPI-2 into the ECM, they fail to provide significant insight into the physiological role of TFPI-2 in the subendothelium. Presumably, ECM-bound TFPI-2 exhibits inhibitory activity toward serine proteases involved in ECM remodeling, but this remains to be established. Furthermore, precisely how anti–TFPI-2 IgG treatment of endothelial cell monolayers reproducibly leads to detachment of endothelial cells from the subendothelial matrix in a plasmin- and urokinase-independent manner,
and how heparin blocks this process, will require further investigation. In this regard, one or more previously unrecognized serine proteases secreted by the endothelial cell, important for matrix turnover and cell attachment, may be inhibited by TFPI-2, and antibody neutralization of TFPI-2 permits its (their) unregulated expression. Alternatively, TFPI-2 may play an important role in the maintenance of the endothelium by mechanisms unrelated to its protease inhibitory activity, similar to that recently described for the matrix metalloprotease inhibitors, TIMP-1 and TIMP-2.29,30

Acknowledgments

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