

## Endothelium and Vascular Development

# Tissue factor pathway inhibitor (TFPI) interferes with endothelial cell migration by inhibition of both the Erk pathway and focal adhesion proteins

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### Summary

Tissue factor pathway inhibitor (TFPI) is a plasma Kunitz-type serine protease inhibitor that is mainly known for its inhibition of tissue factor-mediated coagulation. In addition to its anti-coagulant properties, emerging data show that TFPI may also regulate endothelial cell functions via a non-haemostatic pathway. In this work we demonstrate that at concentrations within the physiological range, TFPI inhibits both endothelial cell migration and their differentiation into capillary-like structures *in vitro*. These effects were specific to endothelial cells since no inhibitory effect was observed on the migration of tumor (glio-

blastoma) cells. Inhibition of endothelial cell migration was correlated with a concomitant loss in cell adhesion, suggesting an alteration of focal adhesion complex integrity. Accordingly, we observed that TFPI inhibited the phosphorylation of focal adhesion kinase and paxillin, two key proteins involved in the scaffolding of these complexes, and that this effect was specific to endothelial cells. These results suggest that TFPI influences the angiogenic process via a non-haemostatic pathway, by downregulating the migratory mechanisms of endothelial cells.

### Keywords

Angiogenesis, TFPI, endothelial cell migration, focal adhesion complexes, extracellular signal-regulated kinases

**Thromb Haemost 2008; 99: 576–585**

### Introduction

Angiogenesis, the formation of new blood vessels from pre-existing ones, is a critical component of a large number of pathological states such as rheumatoid arthritis, diabetic retinopathies, inflammation, atherosclerosis, tumor growth and metastasis (1). Formation of the new vessel network is a multi-step process that requires endothelial cell activation by growth factor or cytokines, cell detachment and migration towards a chemotactic stimulus accompanied by degradation of the surrounding extracellular matrix (ECM) (2). Further elucidation of the basic mechanisms regulating of each of the steps involved in the angiogenic process is of crucial importance for the development of efficient antiangiogenic therapies (3).

Many reports have shown that several proteins involved in the coagulation cascade also modulate tumor angiogenesis (4). In most cases, these haemostatic proteins, as well as cryptic domains released from them by proteolysis, can influence their environment by their classical haemostatic function as well as by less studied, non-haemostatic activities (5). Indeed, it has been shown that tissue factor (TF), the main initiator of the blood coagulation cascade, is able to regulate angiogenesis via signalling originating from the cytoplasmic domain of the protein (6). Also, it has been reported that aberrant expression of TF in tumors contributes to the angiogenic phenotype (7). Sphingosine-1-phosphate (S1P), which is a bioactive sphingolipid metabolite stored in platelets and released upon their activation during the coagulation process, is another coagulation-related molecule that regulates the tumoral angiogenic phenotype. S1P is a potent ac-

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Financial support:  
This work was supported by a grant from the Canadian Institutes for Health Research to R.B. and D.G.

Received October 19, 2007  
Accepted after minor revision January 21, 2008

Prepublished online February 14, 2008  
doi:10.1160/TH07-10-0623

tivator of endothelial cell migration (8–10), further supporting a strong link between cancer and coagulation processes. The link between angiogenesis and components of the haemostatic pathway is consistent with the fact that coagulopathies and neovascularization are among the most relevant host responses associated with cancer (11). Recent studies showed that 50% of all cancer patients and up to 90% of those with metastatic lesions demonstrate clinically observable abnormalities in haemostatic parameters (11) even in the early stages of the disease (12). Therefore, tumour-induced coagulation is intrinsically involved with tumour growth, angiogenesis and metastasis.

Tissue factor pathway inhibitor is a plasma Kunitz-type serine protease inhibitor that acts as a specific inhibitor of TF-mediated coagulation. TFPI, a 35-kDa protein, consists of three Kunitz-type domains with an acidic N-terminal and a basic carboxyl-terminal end. The first Kunitz-type domain binds to FVIIa and the second domain binds to factor Xa (FXa). The third domain has no known function but may bind to plasma lipoproteins such as low-density lipoprotein (LDL) (13). TFPI is mainly produced by vascular endothelial cells under normal physiological conditions and, to a lesser extent, by monocytes, macrophages, lung fibroblasts and vascular endothelial cells, laryngeal squamous epithelial cells, astrocytes and mesothelial cells (14). In the body, the physiological concentrations of TFPI vary between 2.5 and 5 nM (14) and the half-life of the protein is rather short, between 60 and 120 minutes (min) (13). TFPI is distributed in three distinct pools; 80–85% is associated with endothelial cell-surface proteins, mostly glycosaminoglycans and proteoglycans, 10% circulates in plasma (principally bound to the lipoproteins) and 3% is found in platelets (14).

Besides its normal functions in coagulation, TFPI is important for the development of new blood vessels during embryogenesis (15). Inactivation of the TFPI gene results in complete intrauterine lethality and histologic analysis of TFPI<sup>-/-</sup> embryos revealed abnormalities in yolk sac and placental vasculature development. Furthermore, signs of fibrin deposition in the liver, intravascular thrombosis and hemorrhages in these embryos suggested that unregulated TF/VIIa activity leads to overt coagulopathy (15). These coagulation disorders are similar to those observed clinically in cancer patients, thus suggesting a role for TFPI in cancer. Moreover, relevant to an involvement in cancer, TFPI also exhibits antitumor and antimetastatic properties in rodent models (16, 17).

In this work, we demonstrate that TFPI is able to regulate angiogenesis via a non-haemostatic mechanism, through regulation of endothelial cell migratory and adhesion properties.

## Materials and methods

### Materials

All materials used in the production of TFPI (bacteria and plasmids) were obtained from Invitrogen (Carlsbad, CA, USA). Iso-propyl- $\beta$ -D-thiogalactoside (IPTG) was from Roche (Laval, QC, Canada). Chromozym-X was purchased from Roche and FXa was from Calbiochem (La Jolla, CA, USA). Cell culture media were obtained from Life Technologies (Burlington, Ontario, Canada) and serum was purchased from Hyclone Laboratories (Logan, UT, USA). Anti-p-ERK, anti-ERK, anti-p-p38,

anti-p38, anti-p-p70 and p-70 polyclonal antibodies were all obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-pTyr (pY99) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-FAK was from Upstate Cell Signalling (Lake Placid, NY, USA) and anti-PAX was from BD Transduction Laboratories (Mississauga, ON, Canada). Anti-mouse and anti-rabbit horseradish peroxidase (HRP)-linked secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Electrophoresis apparatus and reagents were purchased from Bio-Rad (Mississauga, ON, Canada). Polyvinylidene difluoride transfer membranes (PVDF) and Western Lightning Chemiluminescence Reagent Plus were obtained from PerkinElmer Life Sciences (Boston, MA, USA).

### Production of a human recombinant TFPI expression vector

Recombinant human TFPI was obtained by cloning the full-length human TFPI cDNA for subsequent expression in *Escherichia coli* as described previously (18). Briefly, the TFPI gene was amplified from HUVEC mRNA by reverse-transcription-PCR using primers annealing to TFPI (sense, 5'-CATCAGAGATTTACTTAGATGA-3' and antisense, 5'-CATTGCTATAACAAATTCACA-3') and the PCR product was then ligated in pTrcHis B (Invitrogen) and used to transform in *E. coli* BL-21 strain.

### Purification of TFPI

Freshly grown colonies of bacteria were incubated overnight in 100 ml of LB-media and transferred to a 1 l flask. Bacteria were then incubated for 4 hours (h) and log-phase cultures were stimulated with 0.1 mM IPTG. Bacteria were then pelleted by centrifugation (6,500 rpm, 15 min, 4°C), subjected to lysis and TFPI was purified as described previously (18). For renaturation, the protein concentration was adjusted to 0.3 mg/ml in buffer (6 M urea, 0.5 M NaCl, 0.05 M NaHPO<sub>4</sub>, pH 10.5); L-cysteine was added to 0.1 mM and the pH was adjusted to 10.5 if necessary. After mixing for 10 min, the solution was incubated without stirring at 10°C for 42 h.

### TFPI activity

TFPI activity was monitored by its inhibitory effect on FXa activity. Briefly, different concentrations of TFPI were preincubated with FXa (50 nM) for 5 min at 37°C, and the reaction was initiated by addition of the chromogenic substrate Chromozym-X. The cleavage of Chromozym-X by active FXa was quantified by spectrophotometry at 405 nm.

### Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics, and human glioblastoma cells (U-87) were obtained from the American Type Culture Collection (Manassas, VA, USA). All cells were cultured at 37°C under a humidified atmosphere containing 5% CO<sub>2</sub>. HUVECs were maintained in the endothelial cell growth medium BulletKit (EGM-2) supplemented with 10% fetal bovine serum (FBS), human EGF (hEGF), hydrocortisone, VEGF, human basic-FGF (hFGF-B), IGF-1, ascorbic acid, heparin, gentamycin, and amphotericin-B. U-87 were grown in minimum essential medium (MEM) supple-

mented with 10% FBS and 1 mM pyruvate. For experimental purposes, cells were plated in 0.15% gelatin-PBS coated 100-mm plastic dishes at 12,000 cells/cm<sup>2</sup> and were grown to confluence before overnight serum starvation. Cells were treated with TFPI diluted in serum-free medium and stimulated with 1 μM S1P, as described below.

#### Matrigel endothelial cell tube formation assays

Matrigel™ from BD Biosciences (Mississauga, ON, Canada) (12.5 mg/ml) was thawed at 4°C, and aliquots of 50 μl were quickly added to each well of a 96-well plate and allowed to solidify for 30 min at 37°C. Cells (HUVEC 20,000 cells/well) pre-treated with TFPI (1 to 20 nM) were added on top of the Matrigel™ and incubated for 6 h at 37°C. The formation of capillary-like structures was examined microscopically and pictures (50X magnification) were taken using a Retiga 1300 camera and a Zeiss Axiovert S100 microscope. The extent to which capillary-like structures formed in the gel was quantified by analysis of digitized images to determine the thread length of the capillary-like network, using a commercially available image analysis program (Northern Eclipse), as previously described (19).

#### Migration assays

Transwells inserts (8 μm pore size; Costar, Cambridge, MA, USA) were pre-coated with 0.15% gelatin-PBS by adding 600/100 μl in the lower/upper chambers for 24 h at 4°C. The Transwells were then washed with PBS and assembled in 24-well plates. The upper chamber of each Transwell was filled with 50 μl of cells (100,000 cells/chamber) and the cells were allowed to adhere for 30 min. Cells were then treated for 2 h by adding 50 μl of two-fold concentrated TFPI solution prepared in serum-free medium into the upper chamber and 600 μl of the TFPI solution into the lower chamber. Migration was initiated by adding S1P (1 μM) to the lower chamber (HUVEC and U-87). The plate was placed at 37°C in 5% CO<sub>2</sub> – 95% air for 3 h. Cells that had migrated to the lower surface of the filters were fixed with 10% formalin phosphate and stained with 0.1% Crystal Violet–20% methanol (v/v). The migration was quantified using computer-assisted imaging and data are expressed as the average density of migrated cells per four fields (50X magnification).

#### Adhesion assays

Overnight, 96-well plates were pre-coated with gelatin (0.15%). Wells were saturated with 0.5% BSA/PBS for 1 h at room temperature and then washed with 100 μl of adhesion buffer EBM-2 (Bullectkit) serum-free media (0.2% BSA, 15 mM HEPES and 0.12% NaHCO<sub>3</sub>). Meanwhile, HUVEC and U-87 were pre-treated with TFPI (20 nM) for 0–60 min, and cells were harvested by incubation with EDTA (0.53 mM) for 10–15 min at 37°C. EDTA was neutralized by the addition of adhesion buffer and cells were centrifuged for 3 min at 1,000 g. 5 x 10<sup>4</sup> cells were added per well and allowed to adhere on gelatin or plastic for 30 min at 37°C. Cells were then fixed with 3.7% formaldehyde for 10 min at 4°C, washed twice with 100 μl water and stained with 0.1% Crystal Violet–20% methanol (v/v) for 30 min at room temperature as previously described (20). Results were quantified by spectrophotometry at 600 nm.

#### TFPI treatment and stimulation of HUVEC and U-87 with S1P

HUVECs and U-87 cells grown to 90% confluence were serum-starved overnight in medium containing 1% serum. Cells were then pre-incubated for 30 min at 37°C with TFPI (1 nM to 20 nM), followed by incubation for 5 min with 1 μM S1P. Cells were then washed with PBS containing 1 mM sodium orthovanadate and 1 mM sodium fluoride. Cells were solubilized in SDS lysis buffer (10 mM Tris-HCl pH 7.4, 1% SDS, 1 mM sodium orthovanadate, 1 mM sodium fluoride), followed by boiling for 5 min at 100°C and homogenization using a 26-gauge needle. Protein concentrations were determined by the bicinchoninic acid method (Pierce).

#### Immunoprecipitation and Western Blotting

For the immunoprecipitation studies, identical amounts of proteins from each sample were incubated in SDS lysis buffer overnight at 4°C in the presence of 1 μg of a specific antibody. The immune complexes were collected by incubating the mixtures with 20 μl of a 50% suspension of protein A (rabbit primary antibody) or protein G (mouse primary antibody)-Sepharose beads. Nonspecifically bound proteins were removed by washing the beads three times in 1 ml of lysis buffer, and bound material was solubilized in 20 μl of two-fold concentrated Laemmli sample buffer, boiled for 5 min and resolved by SDS-PAGE. The proteins were transferred onto polyvinylidene difluoride membranes (PVDF) and blocked overnight at 4°C with TBST buffer (147 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.1% Tween 20) containing 3% bovine serum albumin (BSA). Membranes were incubated with the specific primary antibody for 1 h at room temperature. Immunoreactive bands were revealed following 1 h incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies, and the signals were visualized using an ECL detection system.

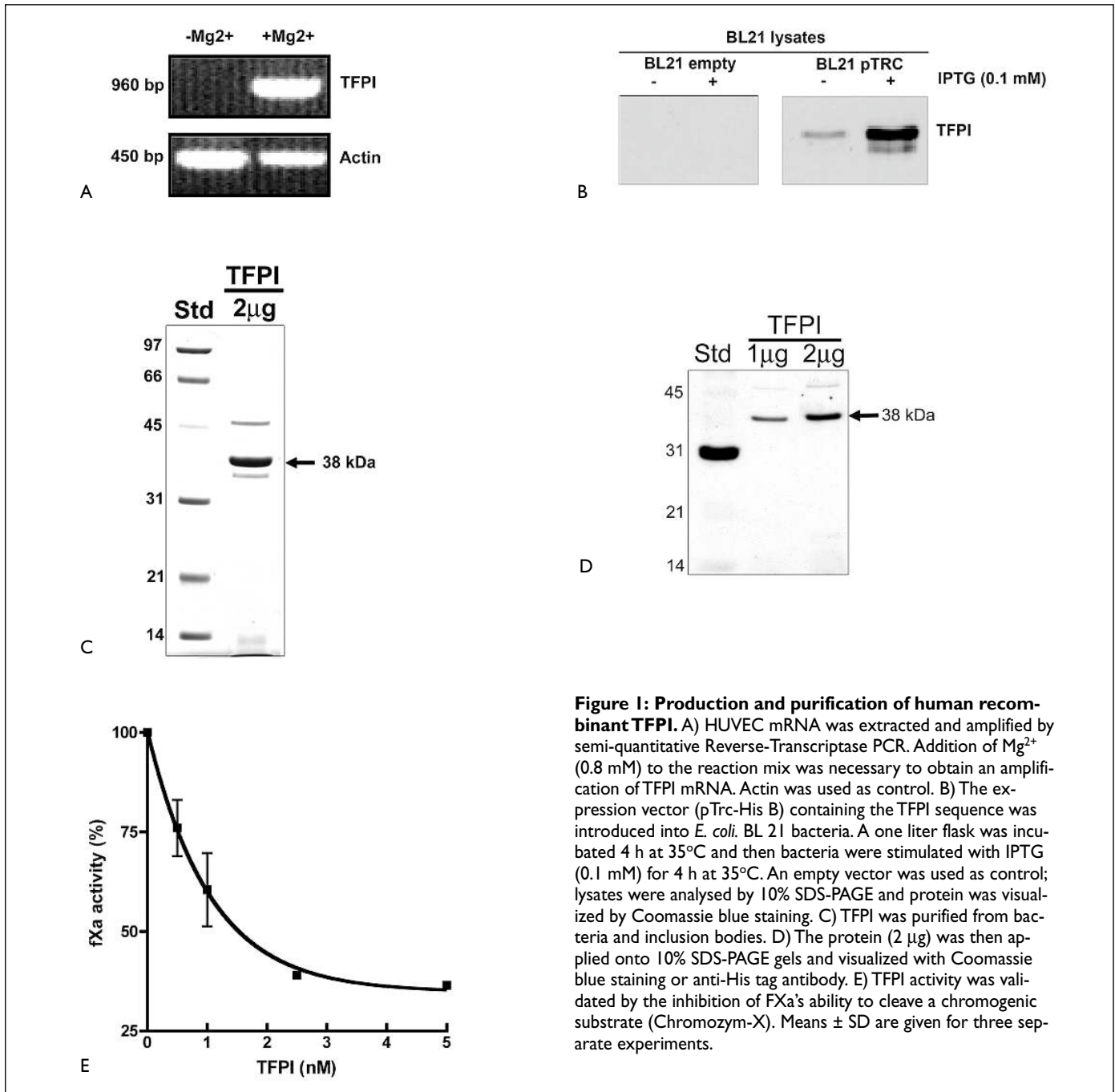
#### Immunofluorescence and confocal microscopy

HUVECs or U-87 seeded onto coverslips coated with 0.15% gelatin-PBS in a 24-well plate were serum-starved overnight in media containing 1% FBS. The cells were then treated for 2 h with TFPI at the indicated concentrations. After this treatment, HUVECs were stimulated with or without 1 μM S1P for 5 min. Cells were fixed in 3.7% formaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 5 min and stained with a 1/2,500 dilution of Texas Red phalloidin (Sigma) for 30 min. Non-specific sites were blocked with 1% BSA in buffered Saline containing 0.1% Tween 20 for 30 min and cells were stained with primary antibodies against Focal Adhesion Kinase (FAK) or Paxillin (PAX) (1/100 dilution) for 30 min. Cells were then incubated with Alexa488-conjugated secondary antibody, and slides were mounted with ImmunoFluor Mounting Medium (MP Biomedicals). Immunostaining was visualized and photographed using a Zeiss LSM 510 Meta confocal microscope.

## Results

#### Purification of human recombinant TFPI

In order to examine the inhibitory action of TFPI on angiogenesis, a cDNA encoding the protein was obtained by RT-PCR and



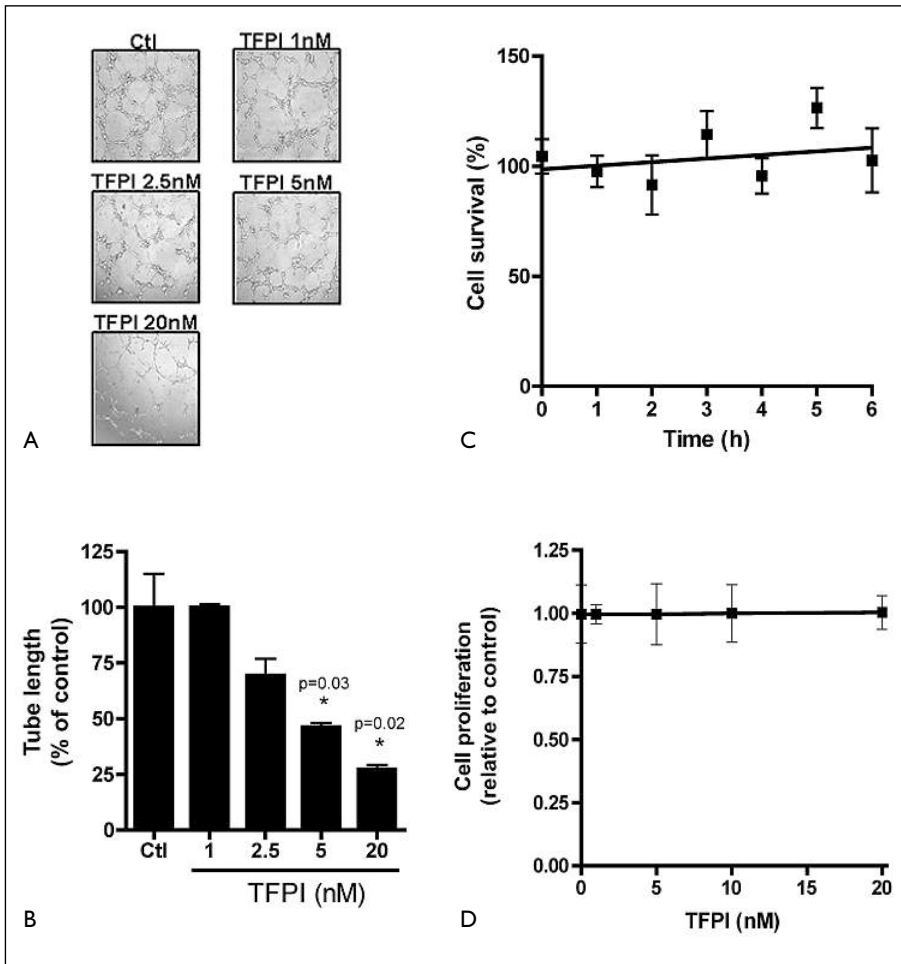
**Figure 1: Production and purification of human recombinant TFPI.** A) HUVEC mRNA was extracted and amplified by semi-quantitative Reverse-Transcriptase PCR. Addition of  $Mg^{2+}$  (0.8 mM) to the reaction mix was necessary to obtain an amplification of TFPI mRNA. Actin was used as control. B) The expression vector (pTrc-His B) containing the TFPI sequence was introduced into *E. coli* BL 21 bacteria. A one liter flask was incubated 4 h at 35°C and then bacteria were stimulated with IPTG (0.1 mM) for 4 h at 35°C. An empty vector was used as control; lysates were analysed by 10% SDS-PAGE and protein was visualized by Coomassie blue staining. C) TFPI was purified from bacteria and inclusion bodies. D) The protein (2 µg) was then applied onto 10% SDS-PAGE gels and visualized with Coomassie blue staining or anti-His tag antibody. E) TFPI activity was validated by the inhibition of FXa's ability to cleave a chromogenic substrate (Chromozym-X). Means  $\pm$  SD are given for three separate experiments.

the protein was purified following its overexpression in bacteria (Fig. 1A and B). As shown in Figure 1C and D, purified TFPI was present as a major band at 38 kDa as revealed by Coomassie blue staining and by immunoblotting with an anti-histidine tag antibody. A minor degradation product of 36 kDa was also observed, possibly originating from the harsh steps necessary for the extraction of the protein from the inclusion bodies. At concentrations within the physiological range, the purified protein inhibited FXa activity by 70%, suggesting some loss of activity during the purification (Fig. 1E). Since TFPI is a complex protein, requiring nine disulfide bonds in order to obtain full activ-

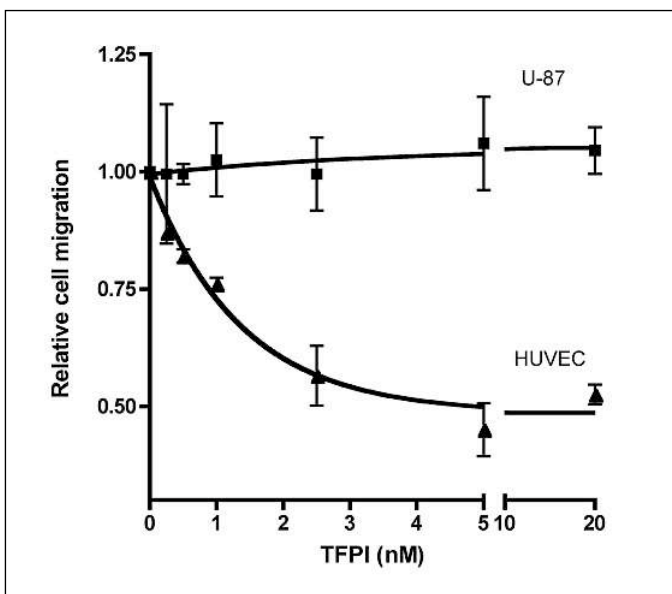
ity, this loss of activity is likely due to the denaturation-renaturation steps performed during purification.

**TFPI inhibits endothelial cell tube formation**

Previous reports have suggested that the antiangiogenic activity of low-molecular-weight heparin (LMWH) may be associated with the release of endothelial TFPI in the vascular milieu (21, 22). To explore this possibility, we examined the effect of TFPI on the ability of HUVEC to spontaneously form capillary-like structures on Matrigel™. As shown in Figure 2, pre-treatment of HUVECs with increasing concentrations of TFPI disrupted the



**Figure 2: TFPI inhibits endothelial cell tube formation *in vitro* and induced no cytotoxicity or inhibitory effects on proliferation.** Confluent HUVECs seeded on gelatin-coated (0.15% gelatin) 60-mm dishes were serum-starved overnight in media containing 1% FBS. Cells were pre-treated with various TFPI concentrations (1 nM to 20 nM) for 2 h at 37°C. HUVECs ( $2 \times 10^4$  cells) were subjected to tube formation assays as described in *Materials and methods*, using a 96-well Matrigel™-coated plate. A) Capillary-like structures were photographed (50X) with Retiga 1300 camera and a Zeiss Axiovert S100 microscope. B) Results were quantified by analysis of digitized images using a commercially available image analysis program (Northern Eclipse). To further confirm that TFPI induces no cytotoxicity, confluent HUVEC plated on gelatin-coated (0.15% gelatin) 60 mm dishes were serum-starved overnight in media containing 1% FBS. C) Cells were then treated with 20 nM TFPI for 0 to 6 h at 37°C and the cytotoxicity of the treatment was measured by cleavage of WST-1 by spectrophotometry. D) HUVECs (8,000 cells/well) were plated on a 96-well plate and incubated overnight at 37°C. Different concentrations of TFPI (0 to 20 nM) were added for 48 h at 37°C. The effect on cell proliferation was quantified by cleavage of WST-1 in formazan by spectrophotometry. Means  $\pm$  SD are given for three separate experiments ( $p = 0.03$  and  $p = 0.02$  for 5 and 20 nM respectively).

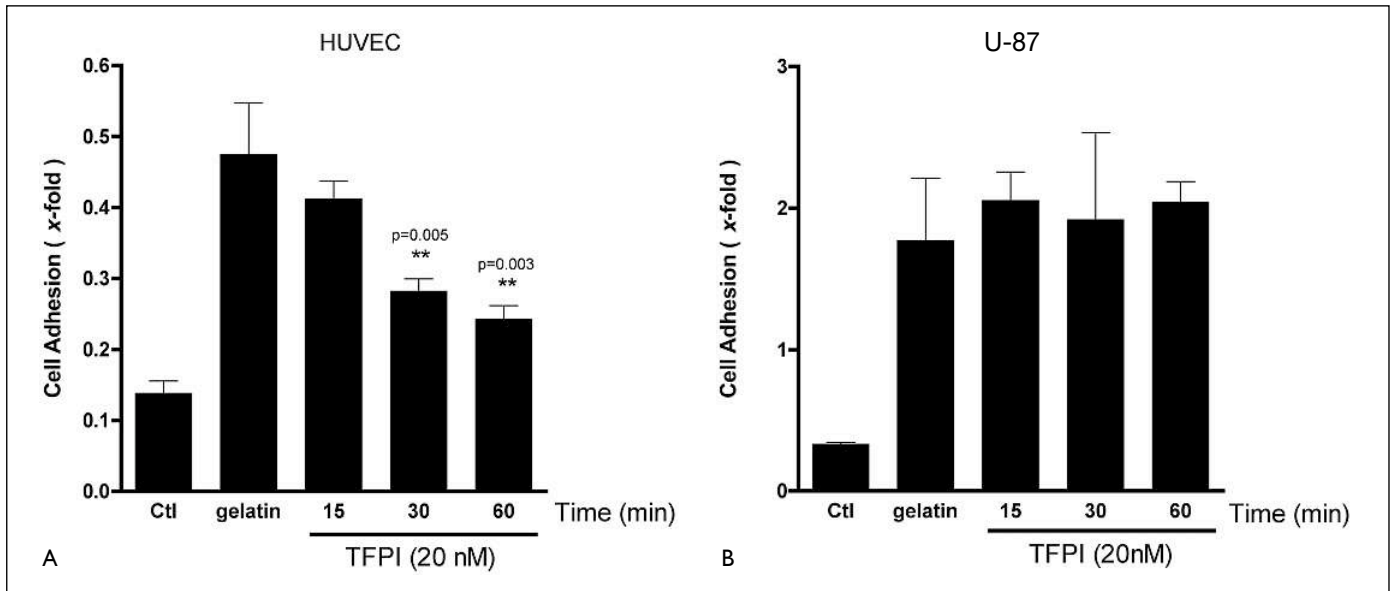


**Figure 3: TFPI specifically inhibits endothelial cell migration.** HUVECs and U-87 cells ( $1 \times 10^5$  cells) were subjected to migration assays as described in *Materials and methods*, using Transwells pre-coated with 0.15% gelatin and  $1 \mu\text{M}$  sphingosine-1-phosphate (S1P) as the chemoattractant. Values are expressed relative to untreated control cells ( $n=3$  for U-87 and  $n=4$  for HUVEC).

capillary-like structure network in a dose-dependent manner (Fig. 2A and B). TFPI inhibits endothelial cell tube formation by 50% at 5 nM, which is within its normal physiologic concentration in blood, and this inhibitory effect was maximal at 20 nM. Under these conditions (20 nM TFPI, 6 h), HUVECs showed no signs of cytotoxicity (Fig. 2C). In addition, TFPI used at these concentrations did not influence cell proliferation over a 48 h period (Fig. 2D). Overall, these data suggest that TFPI, at concentrations within the physiological range, is an inhibitor of endothelial cell tube formation *in vitro*.

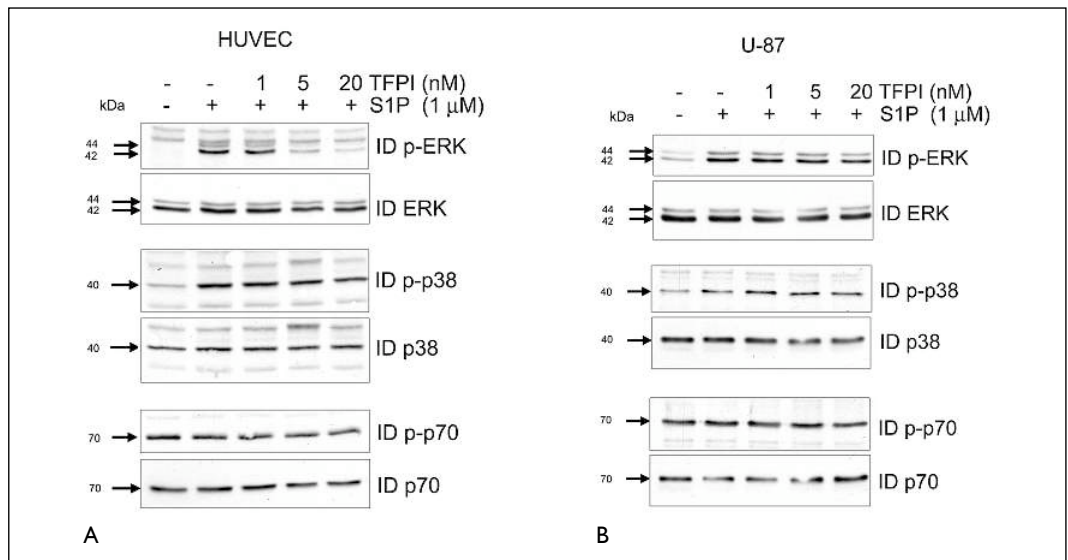
**TFPI specifically inhibits endothelial cell migration**

One of the critical steps occurring during the angiogenic process is endothelial cell migration (2) induced by tumor or host-derived chemoattractants. Among these chemoattractants, the release of S1P into the tumor environment upon activation of platelets during the coagulation process has been shown to be a very potent inducer of endothelial cell migration (23). As shown in Figure 3, the addition of increasing concentrations of TFPI (0.25 to 20 nM) induced a dose-dependent inhibition of S1P-dependent endothelial cell migration. Interestingly, this inhibitory effect was restricted to the migration of endothelial cells, as it was observed for HUVECs and BAECs (bovine aortic endothelial cells) (data not shown), whereas TFPI failed to inhibit glioblastoma cell migration stimulated by S1P.



**Figure 4: TFPI specifically inhibits endothelial cell adhesion.** Confluent HUVECs and U-87 cells were serum-starved overnight in media containing 1% FBS, then pre-treated with 20 nM TFPI for 0–60 min at 37°C. Cells ( $5 \times 10^4$  cells) were subjected to adhesion assays as described in *Materials and methods*. Values are expressed as X-fold compared to cell adhesion on plastic. Results are means  $\pm$  SD of three distinct experiments ( $p = 0.005$  and  $p = 0.003$  for 30 and 60 min, respectively).

**Figure 5: TFPI inhibits the MAP kinase ERK-1/2 pathway in endothelial cells.** Confluent HUVECs (A) or U-87 (B) were seeded on gelatin-coated 100 mm dishes, serum-starved overnight in media containing 1% FBS and treated with 1  $\mu$ M S1P. Equal amounts of protein from cell lysates were subjected to electrophoresis and Western blotting. Levels of phospho-ERK, phospho-p38 and phospho-p70 were detected using specific polyclonal antibodies. Total levels of protein in whole lysates were also detected as a control.

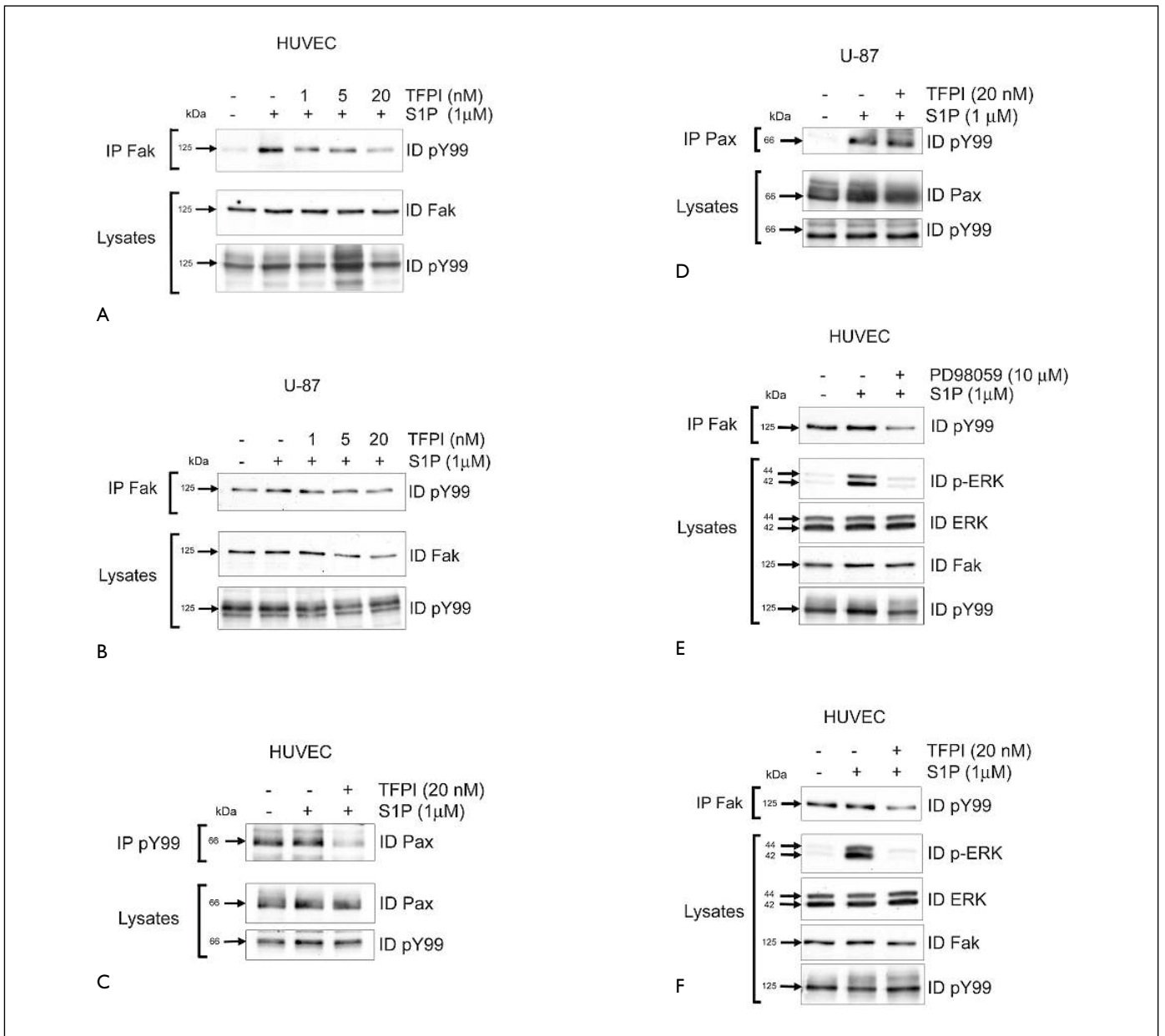


**Treatment of HUVECs with TFPI inhibits cell adhesion**

Because dynamic integrin activation is required during cell migration (24, 25), we hypothesized that TFPI could interfere with cell adhesion. Both cell lines tested (HUVEC and U-87) adhere efficiently to gelatin-coated wells after 30 min incubation at 37°C compared to cells plated onto non-coated wells (Fig. 4 A and B). Vascular endothelial cells pre-treated with 20 nM TFPI for 0–60 min showed a 50% reduction in cell adhesion to gelatin. However, the same treatment applied to glioblastoma cells did not modify the extent of adhesion (Fig. 4 A and B). These results suggest that the activity of molecules involved in cell adhesion, such as components of focal adhesion complexes, may be affected by TFPI.

**TFPI specifically inhibits ERK-1/2 phosphorylation in vascular endothelial cells**

In order to determine how TFPI could influence endothelial cell migration and adhesion, we analyzed the phosphorylation pattern of different pathways of MAP kinases involved in cell migratory processes (26). Cells were treated with various concentrations of TFPI (1 to 20 nM) for 30 min at 37°C and cell lysates were analysed for phospho-ERK, phospho-p-38 and phospho-p-70 levels. As shown in Figure 5, TFPI-treated HUVECs showed a dose-dependent inhibition of ERK-1/2 phosphorylation, while phospho-p-38 and phospho-p-70 remained unaffected. TFPI had no specific effect on MAP kinase phosphorylation in U-87. Other pathways, such as phospho-AKT and



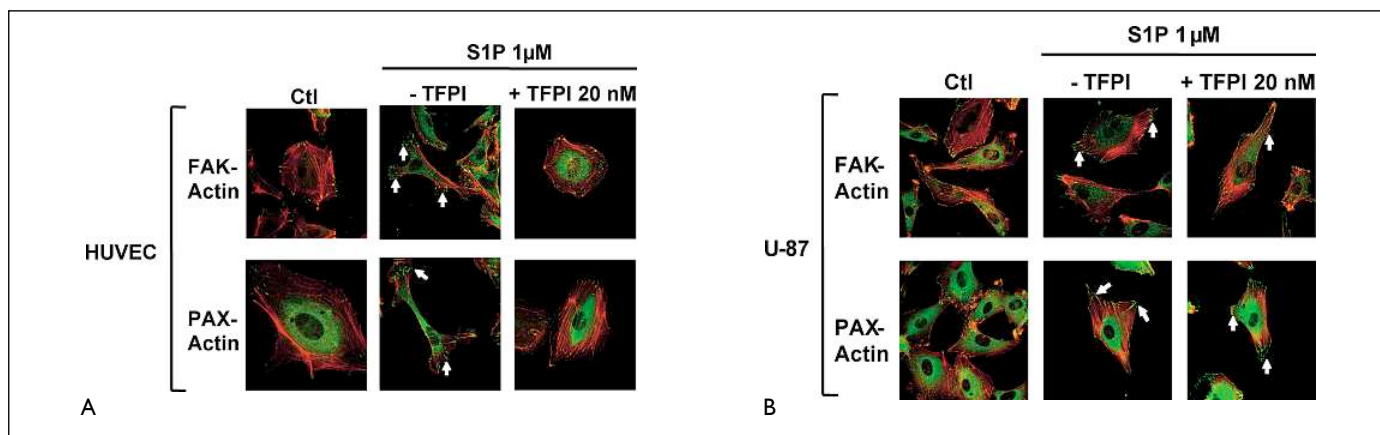
**Figure 6: TFPI inhibits FAK and PAX phosphorylation in vascular endothelial cells.** Confluent HUVECs (A) or U-87 cells (B) were seeded on gelatin-coated 100 mm dishes and serum-starved overnight in media containing 1% FBS. Cells were treated for 30 min with different concentrations of TFPI at 37°C, and stimulated with 1 μM S1P for 5 min. Equal amounts of protein from cell lysates were immunoprecipitated with a polyclonal antibody raised against focal adhesion kinase (FAK), and the phosphorylation level of FAK was detected with anti-phosphotyrosine (pY99) antibody and monitored by Western blotting. The level of total FAK and pY99 was detected in lysates as a control. HUVECs (C) or U-87 (D) lysates were immunoprecipitated with polyclonal antibodies raised against phosphotyrosine (pY99) or PAX and the associated paxil-

lin or pY99 was detected with a specific polyclonal antibody for the PAX protein or pY99, respectively. HUVEC were treated with PD98059 (E) or TFPI (F) and equal amount of proteins from cell lysates were immunoprecipitated with a polyclonal antibody raised against FAK; the phosphorylation level of FAK was detected with anti-phosphotyrosine (pY99) antibody and monitored by Western blotting. Also, equal amounts of proteins from cell lysates were subjected to electrophoresis and Western blotting. Levels of phospho-ERK were detected using specific polyclonal antibodies. Total levels of protein in whole lysates were also detected as a control. These results are representative of three distinct experiments.

phospho-JNK were also unaffected by TFPI (data not shown), suggesting that ERK-1/2 kinase activity may represent a key pathway by which TFPI interfere with endothelial cell migration.

**TFPI inhibits FAK and PAX phosphorylation in vascular endothelial cells**

Cell migration is a coordinated process that involves rapid changes in the dynamics of actin filaments as well as the formation and disassembly of cell adhesion sites, better known



**Figure 7: TFPI treatment prevents morphological changes induced by S1P.** HUVECs ( $3 \times 10^4$  cells/well) (A) or U-87 cells (B) were seeded onto gelatin-coated coverslips in a 24-well plate. Cells were serum-starved overnight in media containing 1% FBS. HUVECs were treated for 2 h with 20 nM TFPI at 37°C and stimulated with 1  $\mu$ M S1P for 5 min. Cells were fixed and double stained with Texas Red-con-

jugated phalloidin for actin and with specific antibodies against FAK and PAX, followed by incubation with Alexa488-secondary antibody. Representative cell images were obtained by confocal microscopy, as described in *Materials and methods*. White arrows indicate staining of focal adhesion kinase or paxillin present in membrane protrusions.

as focal adhesion complexes (27). We hypothesized that TFPI could modulate focal adhesion protein phosphorylation and thereby affect the migratory mechanics of these complexes, as shown for other endogenous angiogenesis inhibitors (28). In order to examine the possible involvement of focal adhesion proteins, HUVECs and U-87 cells were treated with different concentrations of TFPI, and the phosphorylation of two primary focal adhesion proteins, focal adhesion kinase and paxillin, was monitored. As shown in Figure 6 A and B, TFPI dose-dependently inhibited the phosphorylation of immunoprecipitated FAK and PAX in HUVECs but had no effect on FAK and PAX phosphorylation in U-87 cells (Fig. 6 C and D), again suggesting that effect of TFPI is specific to endothelial cells. In order to determine whether the effect of TFPI on ERK phosphorylation occurs upstream or downstream from FAK, we used the MEK1 inhibitor PD98059 to inhibit the phosphorylation of ERK. As shown in Figure 6 E and F, the phosphorylation level of immunoprecipitated FAK was reduced in both PD98059 and TFPI-treated cells, strongly suggesting that the inhibition of ERK phosphorylation by TFPI is essential to the inhibitory effect of the protein on FAK and PAX phosphorylation.

#### TFPI prevents morphological changes of endothelial cells induced by S1P

We sought to corroborate our results obtained by immunoprecipitation by monitoring the effect of TFPI on cell morphology using confocal microscopy and immunofluorescent staining. HUVECs grown on gelatin-coated coverslips showed a relatively round shape with a low expression of focal adhesion proteins FAK and PAX, as visualized by both anti-FAK and anti-PAX antibodies (Fig. 7A). Stimulation of HUVECs with 1  $\mu$ M S1P for 5 min caused the appearance of migratory structures containing FAK and PAX, but the formation of these structures was completely absent in endothelial cells treated with TFPI (20 nM). In general, TFPI-treated HUVECs showed a round shape, similar to non-treated control cells. The same treatment and staining were

performed on U-87 cells and, as shown in Figure 7B, TFPI influenced neither the cell shape nor the localisation of the focal adhesion proteins in these cells. These data clearly demonstrate that TFPI prevents the morphological changes induced by S1P in the endothelial cell migratory process, and that this effect may be responsible for the inhibitory effect of the protein on vascular endothelial cell migration and adhesion.

## Discussion

The inhibitory effect of TFPI on TF-dependent activation of coagulation cascade has been extensively studied (29, 30). However, in addition to its anticoagulation properties, new TF-independent roles for TFPI have been reported in the last few years (17, 22, 31), indicating a possible non-haemostatic function for TFPI in normal and pathological physiology.

In this work, we observed that addition of low concentrations of TFPI to vascular endothelial cells led to the inhibition of endothelial cell tube formation and migration, two hallmarks of angiogenesis. Interestingly, these effects were not related to cytotoxicity or to inhibition of endothelial cell proliferation. TFPI was previously reported to inhibit proliferation of HUVEC, but this effect was observed only at high concentrations of the protein (350 nM) (31). At concentrations within the physiological range, however, our results suggest that the inhibitory effect of TFPI on angiogenesis is likely to involve distinct mechanisms.

The endothelial specificity of TFPI remains to be established. In resting conditions, HUVEC do not express TF (31), thereby suggesting that TFPI interacts with endothelial cells in a TF-fVIIa-independent manner. This TF-fVIIa-independent interaction is also supported by the fact that TFPI-treated HUVEC showed no effect on either TF mRNA or protein expression patterns (data not shown). Previous findings reported that TFPI binds to the VLDL receptor, a protein that is highly expressed at the endothelial cell surface (31), suggesting that this receptor may play a critical role in mediating the observed inhibitory ef-

fect of the protein. Such a TF-independent interaction would also explain why glioblastoma, in which TF is highly expressed (32, 33), were unaffected by TFPI. The specificity of the TFPI inhibitory action towards endothelial cells could also be explained by the localization of TFPI to endothelial cell caveolae which seems to regulate its cell surface exposure and activity (34). However, such a mechanism is unlikely to play a role in the antimigratory effect of TFPI, since the migration of glioblastoma cells is not inhibited by TFPI in spite of the presence of caveolae in these cells. Moreover, it was previously shown that exogenously added TFPI does not localize to caveolae (35).

We observed that TFPI specifically inhibits endothelial cell migration and that inhibitory effect is likely to play an essential role in its antiangiogenic properties. During cell adhesion, spreading and migration processes, focal contacts are formed at ECM-integrin junctions, bringing together cytoskeletal and signaling proteins (27). Treatment of endothelial cells with TFPI resulted in inhibition of the phosphorylation of two proteins (FAK and PAX) which are crucial components of focal adhesion complex assembly (27). This suggests that the complex interplay between the actin cytoskeleton and cell adhesion sites is lost in endothelial cells following treatment with TFPI and explains the absence of the membrane protrusions normally observed by confocal microscopy in cells stimulated with the chemoattractant S1P.

It is interesting to note that other antiangiogenic molecules, such as endostatin, are also known to modulate focal adhesion complexes (28). In fact, treatment of endothelial cells with endostatin induced the redistribution of focal adhesion proteins and actin. These reversible effects of endostatin could conceivably affect the adhesive, migratory and apoptotic behavior of endothelial cells. These effects are somewhat similar to the effects obtained in this work with TFPI.

The inhibitory effect of TFPI on FAK and PAX involved inhibition of the ERK-1/2 pathway in endothelial cells, while other pathways such as p-38, p-70, phospho-AKT and phospho-JNK were unaffected by the protein. MAP kinases are involved in the process of focal adhesion complex formation by modulating different phosphorylation patterns on FAK and PAX (26). By using the specific inhibitor of MEK1 (PD98059), we determined that this inhibition of ERK-1/2 phosphorylation occurs upstream from the effect of TFPI on FAK phosphorylation in endothelial cells. Conversely, glioblastoma cell intracellular pathways remained unaffected by TFPI, again supporting the endothelial cell specificity of the protein.

While the role of FAK in cancer cells has been widely studied (36), antiangiogenic molecules such as TFPI and endostatin specifically target endothelial cell focal adhesion proteins, which could result in new therapeutic opportunities. The fact that TFPI can precisely target specific properties of endothelial cells may significantly contribute to the antiangiogenic activity of the protein. These particular new and interesting roles for TFPI open the door to more complete and efficient antiangiogenic therapies.

### Acknowledgements

This work was supported by a grant of the Canadian Institutes of Health Research to R.B. and D.G. We thank Mr. Ryan Veitch and Mr. David Labbé for their careful reading of the manuscript.

### Abbreviations

TFPI, tissue factor pathway inhibitor; HUVEC, human umbilical vein endothelial cells; S1P, sphingosine-1-phosphate; FAK, focal adhesion kinase; PAX, paxillin.

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