Induction of Endothelial Cell Proliferation by Recombinant and Microparticle-Tissue Factor Involves β1-Integrin and Extracellular Signal Regulated Kinase Activation

Mary E.W. Collier, Camille Ettelaie

Objective—Increased levels of circulating tissue factor (TF) in the form of microparticles increase the risk of thrombosis. However, any direct influence of microparticle-associated TF on vascular endothelial cell proliferation is not known. In this study, the influence of recombinant and microparticle-associated TF on endothelial cell proliferation and mitogen-activated protein kinase signaling mechanisms was examined.

Methods and Results—Incubation of human coronary artery endothelial cells with lipidated recombinant full-length TF, or TF-containing microparticles (50 to 200 pmol/L TF), increased the rate of cell proliferation and induced phosphorylation of extracellular signal regulated kinase 1 in a TF-dependent manner. Inhibition of extracellular signal regulated kinase 1/2 using PD98059 or extracellular signal regulated kinase 1/2 antisense oligonucleotides or inhibition of c-Jun N-terminal kinase reduced recombinant TF-mediated cell proliferation. PD98059 also reduced cell proliferation in response to TF-containing microparticles. Inclusion of FVIIa (5 nmol/L) and FXa (10 nmol/L) or preincubation of cells with an inhibitory anti-FVIIa antibody had no additional influence on TF-mediated cell proliferation. However, preincubation of exogenous TF with a β1-integrin peptide (amino acids 579 to 799) reduced TF-mediated proliferation.

Conclusion—High concentrations of recombinant or microparticle-associated TF stimulate endothelial cell proliferation through activation of the extracellular signal regulated kinase 1/2 pathway, mediated through a novel mechanism requiring the interaction of exogenous TF with cell surface β1-integrin and independent of FVIIa. (Arterioscler Thromb Vasc Biol. 2010;30:1810-1817.)

Key Words: tissue factor • microparticles • extracellular signal regulated kinase 1/2 • endothelial cells • β1-integrin

In addition to its role in hemostasis, tissue factor (TF) has nonhemostatic functions that arise from its ability to activate various intracellular signaling pathways, leading to changes in cell proliferation, cell migration, and gene expression. It has previously been shown that TF:FVIIa-mediated activation of the extracellular signal regulated kinase (ERK)1/2 pathway results in smooth muscle cell proliferation. Furthermore, it has been shown that incubation of endothelial cells (EC) with recombinant TF alone induces cellular proliferation, together with an associated upregulation of cyclin D1 expression in these cells. Recently, a number of reports have demonstrated the ability of TF to interact with cell surface integrins, resulting in diverse cellular outcomes, including the activation of cell signaling pathways, cell migration, and capillary tube formation. The binding of cell surface TF to β1-integrin, α3-integrin, and αβ6-integrin on endothelial cells was shown to require FVIIa, whereas binding of alternatively spliced TF (asTF) to αvβ3-integrin and αβ6β1-integrin was shown to be independent of FVIIa. We have previously demonstrated the interaction of lipidated recombinant TF with the β1-integrin subunit and shown that this was independent of FVIIa. However, the mechanism by which TF interacts with integrins remains unclear.

TF can be released from cells in the form of microparticles, which constitute a proportion of circulating TF. TF-containing microparticles may be released from various cells, including monocytes/macrophages, endothelial cells, and smooth muscle cells. These microparticles are highly procoagulant because of the presence of TF and phosphatidylserine, and elevations in the level of circulating microparticle-associated TF have been linked to an increased risk of thrombosis in a number of vascular conditions. Furthermore, it has been demonstrated that microparticles can transfer TF between different cell types. It has previously been shown that the incubation of human umbilical vein endothelial cells (HUVEC) with exogenous recombinant TF results in the induction of a number of cell signaling pathways, despite the lack of protease-activated receptor-2 (PAR2) in these cells. Moreover, it was demonstrated that
this form of TF interacts with the cell surface. This study is an attempt to further elucidate the mechanisms by which exogenous TF induces endothelial cell proliferation. Throughout this investigation, 2 forms of “exogenous” TF, lipiddated recombinant TF and TF-containing cell-derived microparticles, have been used at concentrations of TF corresponding to those found in healthy individuals (5 to 10 pmol/L) or in plasma of patients with cardiovascular disease (50 to 200 pmol/L). We provide evidence that both recombinant TF and microparticle-associated TF increase endothelial cell proliferation through a mechanism that involves ERK1/2 activation and requires cell surface \( \beta_1 \)-integrin but is independent of FVIIa.

**Methods**

**Materials**

Polyclonal antihuman TF antibody, lipiddated recombinant full-length TF, nonlipiddated recombinant TF, recombinant FVIIa, and recombinant FXa were purchased from American Diagnostica, Inc. The recombinant \( \beta_1 \)-integrin peptide (amino acids 579 to 799) was obtained from ProSpec-Tany TechnoGene Ltd. For a complete list of reagents, please see supplemental material, available online at [http://atvb.ahajournals.org](http://atvb.ahajournals.org).

**Cell Culture**

Human coronary artery endothelial cells (HCAEC) (PromoCell) were cultured under 5% CO\(_2\) at 37°C in endothelial cell growth medium (PromoCell) containing 5% (vol/vol) FCS and growth supplements. For serum-free experiments, the FCS concentration was gradually reduced, and finally the cells washed with PBS and placed in serum-free medium supplemented with epidermal growth factor (10 ng/mL) and basic fibroblast growth factor (20 ng/mL).

**Proliferation and Apoptosis Assays**

Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium-based assay and cell numbers confirmed by staining with crystal violet. Apoptosis was measured using the DeadEnd Fluorimetric TUNEL assay (Promega). For details, please see supplemental material.

**SDS-PAGE and Western Blot Analysis**

Whole-cell lysates (10 \( \mu \)g protein) were separated by SDS-PAGE and analyzed by Western blotting using antibodies against total or phosphorylated forms of ERK1/2, c-Jun N-terminal kinase-1/2, or p38. For details, please see supplemental material.

**Microparticle Preparation**

Cell-derived microparticles were prepared by ultracentrifugation as described before. The concentrations of microparticles were determined using the Zymuphen microparticle determination kit (Hyphen BioMed). TF antigen and activity levels were measured using a TF-ELISA kit (Affinity Biologica) and 2-stage chromatographic assay, respectively. For details, please see supplemental material.

**ERK1/2 Inhibition Using Antisense Oligodeoxynucleotides (ODN)**

Sequences for ERK1/2 antisense (5'-GCCGCCGCCGCAGCAT-3'), sense (5'-ATGGGCCGCCGCCCGC-3'), and scrambled (5'-GCCGCCGCCGCCGC-3') phosphorothioate-modified ODN were according to a published procedure. The scrambled ODN was synthesized with a 3'-fluorescein isothiocyanate group to allow transfection efficiency to be determined by flow cytometry. HCAEC (12.5\( \times \)10\(^6\)) were adapted to OptiMEM reduced serum medium and transfected with the ERK1/2 ODN (0.1 \( \mu \)mol/L) using Lipofectin according to the manufacturer’s instructions. The cells were incubated at 37°C for 5 hours and then placed in medium containing 1% (vol/vol) FCS. The cells were incubated at 37°C for 48 hours, adapted to serum-free medium, and used in experiments.

**Statistical Analysis**

Data are expressed as mean±SEM. The data were analyzed using the statistical package for the social sciences (SPSS). Significance was determined using 1-way ANOVA, and values of \( P<0.05 \) were considered to be significant.

**Results**

**Lipiddated Recombinant TF and Microparticle-TF Induce Proliferation in HCAEC**

To ensure consistency between the microparticle preparations, the concentration of TF antigen and levels of TF activity of the microparticles isolated from TF-transfected and nontransfected ECV304 and HCAEC cells were determined before use (supplemental Table I). Moreover, the concentration of total released microparticles was consistent between different preparations (0.32±0.06 nmol/L), and the phosphatidylserine:phosphatidylcholine ratio was consistently 33±2.4:67±2.4 as determined by thin-layer chromatography. The ratio of microparticle concentration to number of cells was 1.5±0.2 pmol microparticles per 10\(^6\) cells, and the total concentrations of microparticles from TF-transfected and nontransfected cells were similar. Furthermore, the absence of the exosome marker Tsg101 in the microparticle preparations from both ECV304 and HCAEC suggests that the microparticles were ectosomes derived from the cell surface rather than exosomes (supplemental Figure IA). Incubation of HCAEC with TF-containing microparticles (200 pmol/L) resulted in rapid depletion of TF from the culture media (supplemental Figure IB), indicating microparticle uptake by the cells. Incubation of HCAEC with high concentrations of lipiddated recombinant TF (50 to 200 pmol/L) resulted in increases in cell proliferation of up to 82% above that of the untreated control (Figure 1A). Addition of ECV304-derived microparticles (Figure 1B) or HCAEC-derived microparticles (Figure 1C) containing 100 to 200 pmol/L of TF resulted in increases in cell proliferation of up to 37%, whereas corresponding concentrations of control-microparticles had little influence. Preincubation of recombinant TF or TF-microparticles (100 pmol/L) with an anti-TF polyclonal antibody (100 \( \mu \)g/mL) reduced cell proliferation in response to all forms of exogenous TF. In contrast, nonlipiddated recombinant TF or a mixture of phosphatidylserine:phosphatidylcholine (30:70) alone had no measurable influence on cell proliferation (supplemental Figure IIA). TF-containing microparticles (100 pmol/L) isolated from the plasma of patients with cardiovascular disease (Innoven Medical, Inc.) also induced proliferation of endothelial cells, which was reduced by differing amounts in the different samples by preincubation of the microparticles with an anti-TF polyclonal antibody (supplemental Figure IIB). Incubation of HCAEC with lipiddated recombinant TF (100 pmol/L) or cell-derived microparticles did not induce cellular apoptosis (supplemental Figure III).
Lipidated Recombinant TF and Microparticle-TF Induce Activation of Mitogen-Activated Protein Kinase Cell Signaling Pathways

To elucidate the signaling pathways involved in the induction of endothelial cell proliferation by exogenous TF, the activation of the mitogen-activated protein kinase pathways was examined. Incubation of HCAEC with lipidated recombinant TF (100 pmol/L) resulted in ERK1 phosphorylation, which peaked at 30 minutes after treatment (Figure 2A). Furthermore, incubation of cells with lipidated recombinant TF (100 pmol/L) resulted in JNK1 phosphorylation, which peaked at the later time point of 50 minutes (Figure 2B). No phosphorylation of p38 was detected in any of the samples (data not shown). HCAEC-derived microparticles containing 200 pmol/L of TF induced ERK1 phosphorylation, whereas control microparticles at similar microparticle densities had little influence (Figure 2C).

Inhibition of ERK1/2 suppresses lipidated recombinant TF and microparticle-TF-induced cell proliferation

Inhibition of ERK1/2 phosphorylation before incubation of cells with lipidated recombinant TF (100 pmol/L) was achieved by preincubation of HCAEC with the mitogen-activated protein kinase inhibitor PD98059 (15 to 50 μmol/L) for 30 minutes, and inhibition of the phosphorylation of the JNK substrate c-Jun was achieved by preincubation with SP600125 (60 nmol/L) for 30 minutes (supplemental Figure IVA). Incubation of HCAEC with PD98059 (15 to 50 μmol/L) completely inhibited cell proliferation in response to lipidated recombinant TF (100 pmol/L) (Figure 3A), whereas inclusion of SP600125 (20 to 60 nmol/L) showed a dose-dependent decrease in cell proliferation, inhibiting cell proliferation at 60 nmol/L compared with cells treated with TF (100 pmol/L) alone (Figure 3B). Preincubation of HCAEC with PD98059 (50 μmol/L) also reduced cell proliferation in response to HCAEC-derived microparticles containing TF (200 pmol/L), whereas inclusion of SP600125 (60 nmol/L) had a marginal effect (supplemental Figure IVB). Furthermore, inclusion of PD98059 alone (50 μmol/L) modestly reduced cell numbers compared with the untreated sample, which was attributed to a small increase in cellular apoptosis observable in the presence of high concentrations of this inhibitor (supplemental Figure III).

To confirm the data obtained using PD98059, HCAEC were transfected with ERK1/2 antisense ODN (optimized to 0.1 μmol/L) (supplemental Figure VA) to inhibit the expression of ERK1/2. Optimal suppression of ERK1/2 expression following transfection of HCAEC with ERK1/2 antisense ODN (0.1 μmol/L) was achieved at 48 hours compared with nontransfected cells, whereas JNK expression remained unaffected (Figure 3C). Furthermore, sense and scrambled ODN (0.1 μmol/L) modestly reduced cell numbers compared with the untreated sample, which was attributed to a small increase in cellular apoptosis observable in the presence of high concentrations of this inhibitor (supplemental Figure III).

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lipidated recombinant TF (100 pmol/L) (Figure 3D). In contrast, transfection of cells with ERK1/2 sense ODN had no measurable influence on TF-mediated cell proliferation.

**Induction of HCAEC Proliferation by Recombinant TF and Microparticle-TF Does Not Require FVIIa**

Examination of HCAEC revealed no detectable surface TF expression or activity as determined by flow cytometry and the 2-stage chromogenic assay, respectively (data not shown). In addition, the expression of PAR1 and PAR2 in HCAEC was confirmed using RT-PCR (supplemental Figure VIA). Incubation of HCAEC with recombinant FVIIa (5 nmol/L) and FXa (10 nmol/L) together with lipidated recombinant TF (100 pmol/L) had no additional influence on TF-mediated proliferation (Figure 4A). The enzymatic activities of recombinant FVIIa and FXa were confirmed using chromogenic substrates. Furthermore, preincubation of cells with an inhibitory anti-FVIIa antibody (30 μg/mL) (Figure 4A), or the inhibitory anti-PAR2 antibody SAM11 (25 μg/mL) (supplemental Figure VIB), did not reduce TF-mediated cell proliferation. The addition of recombinant FVIIa (5 nmol/L) alone or recombinant FXa (10 nmol/L) alone had no effect on cell proliferation (Figure 4A). Preincubation of TF-containing microparticles with an inhibitory anti-FVIIa antibody also did not reduce cell proliferation in response to TF-containing microparticles (Figure 4B). These results were mirrored in ERK1 phosphorylation, because the incubation of HCAEC or HCAEC-derived microparticles with the anti-FVIIa antibody did not reduce the level of ERK1 phosphorylation in response to either lipidated recombinant TF (Figure 4C) or microparticle-associated TF (Figure 4D).

**Induction of HCAEC Proliferation by Recombinant TF and Microparticle-TF Involves an Interaction With β1-Integrin**

Because TF has been shown to interact with integrins, the possibility that exogenous TF activates signaling through integrins was examined. The expression of β1-integrin and β3-integrin on HCAEC was examined by flow cytometry, and HCAEC were found to express high levels of β1-integrin (90%) and lower levels of β3-integrin (32%) (data not shown). A peptide corresponding to amino acids 579 to 799 of β1-integrin was used to competitively inhibit the binding of exogenous TF to cell surface β1-integrin. Preincubation of lipidated recombinant TF (100 pmol/L) with the β1-integrin...
peptide (0.02 to 1 nmol/L) reduced TF-mediated cell proliferation in a dose-dependent manner (Figure 4E). Concurrent with the reduced rate of cell proliferation, incubation of HCAEC with recombinant TF (100 pmol/L) preincubated with the β1-integrin peptide (1 nmol/L) reduced TF-mediated ERK1 phosphorylation (Figure 4C), and a similar result was observed with TF-containing microparticles (200 pmol/L TF) (Figure 4D). An inhibitory anti-α3-integrin antibody also reduced recombinant TF-mediated HCAEC proliferation, whereas blocking β3-integrin or α6-integrin had no significant influence on recombinant TF-mediated increases in cell proliferation (supplemental Figure VIC). In addition, ERK1 phosphorylation in response to lipidated recombinant TF (100 pmol/L) was unaltered by preincubation of HCAEC with antibodies against β3-integrin, α3-integrin, or α6-integrin (data not shown). The β1-integrin peptide (1 nmol/L) also significantly reduced cell proliferation (Figure 4B) and ERK1 phosphorylation (Figure 4D) in response to TF-containing microparticles derived from HCAEC. Preincubation of the β1-integrin peptide (1 nmol/L) with plasma-derived microparticles from patients with cardiovascular disease reduced cell proliferation in response to the microparticles in 2 of the samples (supplemental Figure IIB).

**Influence of TF-Containing Microparticles From Other Vascular and Blood Cells on HCAEC Proliferation**

Microparticles were isolated from activated THP-1 cells (monocytes), isolated peripheral blood mononuclear cells, and human coronary artery smooth muscle cells. TF-containing microparticles (100 pmol/L TF) from these cells induced HCAEC proliferation to a similar extent as HCAEC-derived TF-containing microparticles and was partly reduced by preincubation of the microparticles with an anti-TF antibody (supplemental Figure VII).

Discussion

Throughout this study, lipidated recombinant full-length TF was used to eliminate the influence of other proteins. In addition, cell-derived microparticles from primary endothelial cells and patient plasma-derived microparticles were used as a model of TF-containing microparticles. TF-containing microparticles from the ECV304 cell line were also used in initial experiments (Figure 1B) and showed a similar pattern of inducing cell proliferation compared with microparticles derived from primary endothelial cells (Figure 1C), which were used thereafter.

In this investigation both lipidated recombinant TF and TF-containing microparticles induced proliferation in coronary artery endothelial cells through activation of the ERK1/2 pathway. A previous study has demonstrated that TF:FVIIa activation of the ERK1/2 pathway is capable of inducing proliferation in smooth muscle cells, whereas our studies have shown that recombinant TF induces the proliferation of HUVEC, as well as the activation of various cell signaling pathways. In an extension to these studies, here, we have shown the ability of microparticle-associated TF and recombinant TF to induce signaling in endothelial cells, and the data presented further support the role of exogenous TF in promoting endothelial cell proliferation. Lipidated recombinant TF showed a greater ability to induce cell proliferation than the same concentrations of TF in cell-derived microparticles. This may be due to a proportion of the TF in the microparticles already being associated with integrins or the presence of other proteins within the microparticles that may modulate these processes. Furthermore, the rate of cell proliferation varied between the different patient plasma-derived microparticle samples, even though the TF concentration was the same, which may be explained by the heterogeneous nature of these microparticles. In addition, TF-containing microparticles isolated from other vascular and blood cells (THP-1 cells, isolated peripheral blood...
mononuclear cells, and human coronary artery smooth muscle cells) induced proliferation in HCAEC, which was reduced by preincubation of the microparticles with anti-TF antibody, further supporting the role of TF in the induction of endothelial cell proliferation. In contrast, nonlipidated recombinant TF did not induce cell proliferation (supplemental Figure IIA), indicating the requirement for exogenous TF to be in phospholipids. It is known that TF may be transferred between cell types by microparticles.19,20 Furthermore, lipidated recombinant TF has been shown to be incorporated into the cell membrane.7,10 It is therefore likely that the phospholipid component assists the fusion of microparticles with the cell membrane, resulting in the incorporation of TF into the cell membrane. The uptake of microparticles by HUVEC has previously been demonstrated.27 Moreover, the rapid depletion of TF from the media (supplemental Figure IB) suggests uptake of TF-containing microparticles by HCAEC. However, a role for the ligand-mediated interaction of TF within microparticles and cell surface integrins in incorporating TF into the cell membrane cannot be ruled out.

Although HCAEC were found to express PAR2, the ability of lipidated recombinant TF and microparticle-associated TF to induce cell proliferation did not require FVIIa. Furthermore, inclusion of an inhibitory PAR2 antibody did not prevent TF-mediated cell proliferation. Together, these data indicate that exogenous TF is capable of inducing cell proliferation through a protease-independent mechanism. It has been reported that endothelial cells possess a cell-surface ligand for binding to TF.28 More recent studies have shown that cell surface TF can interact with α3β1-integrin and α6β1-integrin on endothelial cells.9 Additionally, it has been shown that in epithelial cells and HUVEC, the interaction between cell surface TF and β1-integrin is enhanced by the presence of FVIIa, independently of FVIIa activity and PAR2

Figure 4. β1-integrin is involved in TF-mediated HCAEC proliferation. A, HCAEC adapted to serum-free media were incubated with recombinant TF (100 pmol/L) together with combinations of FVIIa (6 nmol/L) and FXa (10 nmol/L), an inhibitory anti-FVIIa antibody (30 μg/mL), or FVIIa and FXa alone for 24 hours and cell proliferation measured (n=3, *P<0.05 vs untreated control). B, HCAEC were incubated with TF-containing microparticles (200 pmol/L TF) previously preincubated with the β1-integrin peptide (1 nmol/L) or FVIIa antibody (30 μg/mL) and proliferation measured after 24 hours (n=4, *P<0.05 vs untreated control; #P<0.05 vs TF-MP). HCAEC were incubated for 30 minutes with recombinant TF (100 pmol/L TF) (C) or TF-containing microparticles (200 pmol/L TF) (D) in the presence of the β1-integrin peptide (1 nmol/L) or anti-FVIIa antibody (30 μg/mL) and ERK1/2 phosphorylation examined (n=3, *P<0.05 vs untreated control). E, Recombinant TF (100 pmol/L) preincubated with the β1-integrin peptide (0.02 to 1 nmol/L) for 40 minutes was added to HCAEC for 24 hours and cell proliferation measured (n=5, *P<0.05 vs TF). GADPH indicates glyceraldehyde 3-phosphate dehydrogenase; Ab, antibody; C, control; MP, microparticles.
signaling, whereas in cancer cells, the interaction between cellular TF and integrins is constitutive. A recent study has also shown that αsTF can interact with αvβ3-integrin and αvβ6-integrin on HUVEC or an endothelial cell line, resulting in endothelial cell migration and capillary tube formation by a FVIIa-independent mechanism. These reports further support our findings that TF can interact with integrins independently of FVIIa. In fact, we have previously shown that recombinant TF binds to the surface of breast cancer cells through direct binding to the β1-integrin subunit, and that this interaction was reduced by preincubation of cells with a polyclonal antibody directed against amino acids 579 to 799 of β1-integrin. Therefore, in this study, a recombinant β1-integrin peptide corresponding to amino acids 579 to 799 of β1-integrin was used to competitively inhibit the binding of exogenous TF to cell surface β1-integrin. This β1-integrin peptide corresponds to the C terminus of β1-integrin and includes the 4th epidermal growth factor domain and β-tail domain of the extracellular domain of β1-integrin, as well as the transmembrane and cytoplasmic domains of β1-integrin. The reduction in cell proliferation and ERK1/2 phosphorylation in response to both recombinant and microparticle-TF preincubated with this peptide suggests the presence of a novel binding site for exogenous TF within amino acids 579 to 799 of β1-integrin and that this interaction is required for TF-mediated proliferation of endothelial cells. Moreover, preincubation of patient plasma-derived microparticles with the β1-integrin peptide reduced cell proliferation to differing extents in the 3 patient samples. As previously mentioned, these variations may be a result of the heterogeneous nature of these microparticles, which may also be responsible for different levels of β1-integrin within the patient-plasma microparticles. Furthermore, patient-plasma microparticles are likely to be derived from other cell types in addition to endothelial cells, such as macrophages, platelets, and smooth muscle cells, and therefore contain different combinations of integrin subunits with which TF may interact. In addition, endothelial cell proliferation and ERK1 phosphorylation in response to exogenous TF did not require β3-integrin or α6-integrin, whereas αv3-integrin was partially involved in cell proliferation. In contrast to our data using recombinant full-length TF or microparticle-associated TF, it has been shown that αsTF is not capable of inducing proliferation in endothelial cells and does not interact with α3β1-integrin. However, the interaction of αsTF with αvβ3-integrin and αvβ6-integrin has been shown to induce cell migration and tube formation, respectively. These differences may be due to the structure of αsTF, because αsTF lacks the cytoplasmic and transmembrane domains, as well as part of the extracellular domain of full-length TF, and therefore may interact with integrins in a different conformation and have different cellular outcomes compared with full-length exogenous TF. Furthermore, both αsTF and exogenous TF have been shown to induce capillary tube formation. Because endothelial cell proliferation is essential for angiogenesis, it would be interesting to examine the influence of microparticle-associated TF on angiogenesis in coronary artery endothelial cells. In conclusion, the binding of full-length exogenous TF to β1-integrin provides a potentially novel mechanism for FVIIa-independent induction of endothelial cell proliferation by recombinant or microparticle-associated TF.

Disclosures
None.

References


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Materials and Methods

MAPK inhibitors PD98059 and SP600125 were obtained from Tocris Bioscience. Antibodies against total and phosphorylated forms of ERK1/2, JNK1/2, p38 and c-Jun were purchased from Cell Signalling Technologies. The anti-human GAPDH HRP-conjugated antibody, anti-human PAR2 antibody (SAM11), anti-human α3-integrin antibody (P1B5) and anti-human α6-integrin antibody (GOH3) were obtained from Santa Cruz Biotechnology. The inhibitory anti-human FVIIa antibody was from Abcam and the polyclonal anti-human β3-integrin antibody was purchased from R&D Systems. Patient plasma samples were obtained from Innovative Research Inc. All oligonucleotides were synthesised by Eurofins MWG Operon.

Proliferation assay

HCAEC (2 × 10^4) in 48-well plates were treated as described in the results section and then incubated at 37°C for 24 h. In order to determine the number of metabolically active cells, CellTitre 96 AQueous One Reagent (Promega) (20 µl) was added to each well and incubated at 37°C, and the absorptions were measured at 490 nm. To confirm cell numbers, the cells were fixed using 3 % (v/v) glutaraldehyde and stained with Crystal Violet (100 µl). Following several washes with PBS, the dye was solubilised by incubation with 1 % (w/v) SDS for 1 h and the absorptions were measured at 595 nm using a plate reader. Standard curves of cell numbers against absorption at 490 nm or 595 nm were prepared in order to determine cell numbers.

Apoptosis assay
Apoptosis was measured using the DeadEnd Fluorimetric TUNEL assay (Promega). HCAEC (2 \times 10^4) in 8-well culture slides were treated for 24 h as described in the results section and then fixed with 3 % (v/v) glutaraldehyde. The cells were washed with PBS and incubated with Triton X-100 (0.2 % (v/v)) (200 µl) for 5 min. Cells were incubated with 100 µl of equilibration buffer (200 mmol/L potassium cacodylate, 25 mmol/L Tris-HCl pH 6.0, 0.2 mmol/L DTT, 0.025 % (w/v) BSA, 2.5 mmol/L CoCl₂) for 5 min followed by incubation with fluorescein 12-dUTP (5 µmol/L) and TdT enzyme (30 units) at 37°C for 1 h. The cells were then incubated with 2× SSC (0.3 mol/L NaCl, 0.03 mol/L sodium citrate) for 10 min and washed three times with PBS. Images were obtained using a Coolsnap Pro colour camera attached to a Leitz Laborlux S fluorescence microscope. Ten fields of view were taken for each sample and images were analysed using Image-Pro Plus.

**SDS-PAGE and western blot analysis**

Whole cell lysates (10 µg protein) were separated by 12% (w/v) SDS-PAGE and proteins transferred onto nitrocellulose membranes. The membranes were blocked with TBST (20 mmol/L Tris-HCl pH 8.0, 150 mmol/L NaCl, 0.05% (v/v) Tween 20) for 2 h and probed with antibodies against total or phosphorylated forms of ERK1/2, JNK1/2 or p38 diluted 1:1000 in TBST, followed by HRP or AP-conjugated secondary antibodies (Santa Cruz) and developed using 3,3',5,5 tetramethylbenzidine (TMB)-stabilised substrate (Promega) or Western Blue stabilised substrate for alkaline phosphatase (Promega). Images were taken using the GeneSnap imaging program (Syngene) and phosphorylated proteins compared to total protein using the GeneTool program (Syngene).

**Microparticle preparation**
ECV304 or HCAEC cells (2 × 10^5) were transfected with the pCMV-XL5-TF plasmid (Origene) encoding full length TF, using Lipofectin (Invitrogen) and the cells were incubated at 37°C for 48 h to allow expression of TF. Both transfected and non-transfected (control) cells were adapted to serum free media for 1 h and then incubated with a PAR2 agonist peptide (SLIGRL, 20 µmol/L) (Sigma) for 90 min to stimulate microparticle release. The media were centrifuged at 9,000 g for 10 min on a microcentrifuge to pellet cell debris. Microparticles were then collected from the media by ultracentrifugation at 100,000 g for 1 h on a Beckman TL-100 ultracentrifuge using a TLA-100.2 rotor (Beckman Coulter Ltd) and washed with PBS. The TF concentrations of the microparticles were measured using a TF ELISA and compared to a standard curve prepared with non-lipidated TF. TF activity was measured using the two stage chromogenic assay and compared to a standard curve prepared using lipidated recombinant TF (Dade Behring) with the stock solution taken to contain 1000 U/ml. Total microparticle densities were measured using the Zymuphen microparticle determination kit.
Supplemental Table I. TF antigen concentrations and activities of cell-derived and patient plasma-derived microparticles. ECV304 or HCAEC cells were transfected with pCMV-XL5-TF or not transfected as a negative control. In addition, cells were transfected with pCMV-Luc to express luciferase instead of TF as a negative control. Cells were stimulated with PAR2 agonist peptide for 90 min and microparticles were isolated from the media (1 ml) as described in the methods section. TF antigen was measured using a TF ELISA, and TF activity measured using a two-stage chromogenic assay. Data for the cell-derived microparticles are the mean ± SD of four separate microparticle preparations, each measured in duplicate. Data for the patient plasma-derived microparticles are the mean ± SD of each sample measured in duplicate.

Supplemental Figure I. A) Analysis of the exosome marker Tsg101 in microparticle preparations. Microparticles were isolated by ultracentrifugation from the medium of ECV304 and HCAEC cells stimulated with PAR2 agonist peptide (20 µmol/L). The microparticles and cells were lysed in Laemmli’s buffer and 10 µg of protein from each sample separated by 12 % (w/v) SDS-PAGE. Membranes were probed using an anti-human Tsg101 antibody followed by HRP-conjugated secondary antibody. As a loading control, gels were stained for total protein using Coomassie Blue. NS indicates a non-specific band. B) Examination of the uptake of TF-containing microparticles by HCAEC. HCAEC were incubated with HCAEC-derived microparticles containing 200 pmol/L of TF (final concentration) and samples of media collected at intervals up to 90 min. The concentration of TF in the samples of media was determined using a TF-ELISA (n=3).

Supplemental Figure II. The requirement for the phospholipid component for TF-mediated cell proliferation and the influence of patient plasma-derived microparticles on HCAEC
proliferation. A) HCAEC ($2 \times 10^4$) were incubated with lipidated recombinant TF (100 pmol/L), non-lipidated recombinant TF (100 pmol/L) or lipids alone (phosphatidylserine (PS): phosphatidylcholine (PC) at a ratio of 30:70) (50 and 100 µg/ml) and cell proliferation was measured following 24 h incubation (n=4). *=p<0.05 vs. untreated control. B) HCAEC were incubated with patient plasma-derived microparticles (MP) at a TF concentration of 100 pmol/L with or without pre-incubation with an anti-TF polyclonal antibody (100 µg/ml) or β1-integrin peptide (1 nmol/L) and cell proliferation was measured following 24 h incubation (n=4). *=p<0.05 vs. untreated control. # =p<0.05 vs. untreated microparticles.

Supplemental Figure III. Determination of cellular apoptosis in response to recombinant and microparticle-associated TF. HCAEC ($2 \times 10^4$) in 8-well culture slides were incubated with lipidated recombinant TF (100 pmol/L) or microparticles from TF-transfected (100 pmol/L TF) or non-transfected ECV304 and HCAEC for 24 h. H$_2$O$_2$ (100 µmol/L) treated cells were used as a positive control. The effect of PD98059 (50 µmol/L) on HCAEC apoptosis was also examined. The Dead-End Fluorimetric apoptosis kit was used to measure DNA fragmentation in the cells. The number of fluorescent events in 10 fields of view was counted for each sample. (n=3).

Supplemental Figure IV. Confirmation of the inhibition of ERK1/2 and JNK using PD98059 and SP600125, and the influence of ERK1/2 and JNK inhibition on HCAEC proliferation in response to TF-containing microparticles. A) HCAEC were pre-treated with PD98059 (15-50 µmol/L) or SP600125 (20-60 nmol/L) for 30 min and then incubated with lipidated recombinant TF (100 pmol/L). Following incubation at 37°C for 30 min and 50 min, the cells were lysed in Laemmli’s buffer and analysed for ERK1/2 and c-Jun phosphorylation respectively. B) HCAEC were treated with PD98059 (50 µmol/L) or SP600125 (60 nmol/L)
for 30 min and control-microparticles or TF-containing microparticles (200 pmol/L TF) were added. Cell proliferation was measured following incubation for 24 h. (n=3). *=p<0.05 vs. untreated control. #=p<0.05 vs. TF-MP.

Supplemental Figure V. Optimisation of ERK1/2 gene knockdown using ERK1/2 ODN. HCAEC were transfected with A) 0.025-0.2 µmol/L of ERK1/2 ODN or B) sense and scrambled ERK1/2 ODN (0.1 µmol/L). The cells were lysed after 48 h incubation and proteins separated by 12 % (w/v) SDS-PAGE. Membranes were probed for ERK1/2, JNK and GAPDH. Data are representative of three independent experiments from which the mean and SD were calculated.

Supplemental Figure VI. Expression of PAR1 and 2 in HCAEC and the effect of inhibition of PAR2, β3-, α3- and α6-integrins on TF-mediated cell proliferation. A) Total RNA was isolated from HCAEC (2.5×10^5) and used for RT-PCR analysis of PAR1, PAR2 and β-actin mRNA expression. RT-PCR amplification was carried out using Ready-To-Go RT-PCR beads (GE Healthcare), 0.1 µg of total RNA and specific primers. PCR was carried out with an annealing temperature of 56°C for 29 cycles for PAR1 and PAR2, or 58°C for 18 cycles for β-actin. DNA products were separated using 2 % (w/v) agarose gel electrophoresis. DNA product sizes were 850 bp for PAR1, 503 bp for PAR2 and 460 bp for β-actin. B) HCAEC were pre-incubated with the anti-human PAR2 inhibitory antibody SAM11 (25 µg/ml) for 45 min. Lipidated recombinant TF (100 pmol/L) was then added to the cells and cell proliferation measured following 24 h incubation. (n=3). *=p<0.05 vs. untreated control. C) HCAEC were pre-incubated with an anti-β3-integrin antibody (30 µg/ml), anti-α3-integrin antibody (50 µg/ml) or anti-α6-integrin antibody (50 µg/ml) for 45 min. Lipidated
recombinant TF (100 pmol/L) was then added and cell proliferation measured following 24 h incubation. (n=3). * = p<0.05 vs. untreated control. # = p<0.05 vs. TF.

Supplemental Figure VII. TF-containing microparticles from other vascular and blood cells induce HCAEC proliferation. THP-1 cells (monocytes) and isolated peripheral blood mononuclear cells (PBMC) were differentiated by the addition of GM-CSF (0.2 ng/ml) for 24 h. The leukocytes and human coronary artery smooth muscle cells (HCASMC) were then activated by incubation with PAR2 agonist peptide (20 µmol/L) for 90 min and microparticles isolated from the culture media as before. The concentration of TF antigen in the samples was measured using a TF-ELISA. HCAEC were incubated with cell-derived microparticles containing 100 pmol/L of TF. Samples of microparticles were also pre-incubated with a polyclonal anti-TF antibody (100 µg/ml). HCAEC were incubated for 24 h following which cell proliferation was measured. (n=3). * = p<0.05 vs. no MPs. # = p<0.05 vs. microparticles alone.
<table>
<thead>
<tr>
<th>Source of microparticles</th>
<th>TF antigen concentration (ng/ml)</th>
<th>TF activity (U/ml)</th>
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<tr>
<td>Non-transfected ECV304 cells</td>
<td>0.15 ± 0.71</td>
<td>1.2 ± 2.5</td>
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<tr>
<td>TF-transfected ECV304 cells</td>
<td>4.28 ± 0.83</td>
<td>38 ± 4.7</td>
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<tr>
<td>Non-transfected HCAEC cells</td>
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<td>1.1 ± 3.3</td>
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<tr>
<td>TF-transfected HCAEC cells</td>
<td>2.14 ± 0.4</td>
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<td>HCAEC cells transfected with pCMV-Luc</td>
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<tr>
<td>Patient A</td>
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<tr>
<td>Patient B</td>
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<td>Patient C</td>
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<tr>
<td>Healthy individual</td>
<td>1.94 ± 0.4</td>
<td>13.6 ± 1.1</td>
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</table>
Supplemental Figure I.

A.

B.
Supplemental Figure II.

A.

B.
Supplemental Figure III.
Supplemental Figure IV.

A.

![Image of Western blot showing p-ERK1/2 and Total ERK1/2](image1.png)

<table>
<thead>
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<th>TF (100 pmol/L)</th>
<th>PD98059 (µmol/L)</th>
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<tr>
<td>+</td>
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![Image of Western blot showing p-cJun and Total cJun](image2.png)

<table>
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<th>TF (100 pmol/L)</th>
<th>SP600125 (nmol/L)</th>
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<tr>
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<td>60</td>
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B.

![Graph showing number of cells (x1000)](image3.png)

<table>
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<tbody>
<tr>
<td>Control-MP</td>
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<td>TF-MP</td>
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<td>SP600125</td>
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</tbody>
</table>
Supplemental Figure V.

A.

B.

ERK1/2 ODN
(µmol/L)

0 0.025 0.05 0.1 0.2

Control S Scr

ERK1/2:JNK ratio

ERK1/2
JNK
GAPDH

ERK1/2
JNK
GAPDH

ERK1/2
JNK
GAPDH

Control  S  Scr
Supplemental Figure VI.

A.

![DNA gel image showing bands for PAR1, PAR2, and β-actin](image)

B.

![Bar graph showing number of cells (x1000) for Control, TF, and TF+PAR2 Ab](image)

C.

![Bar graph showing number of cells (x1000) for Control, TF, TF+β3 Ab, TF+α3 Ab, and TF+α6 Ab](image)
Supplemental Figure VII.

The figure shows a bar chart comparing the number of microparticles (MPs) and MPs+TF Ab from different cellular sources: No MPs, THP-1, PBMC, and HCASMC. The vertical axis represents the number of cells (x1000), and the horizontal axis indicates the cellular source of microparticles. The chart includes error bars for each category.