Induction of endothelial cell proliferation by recombinant and microparticle-tissue factor involves β1-integrin and ERK activation

Collier. Recombinant and MP-TF induce EC proliferation

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Abstract

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 MAPK signalling mechanisms was examined. Methods and Results: Incubation of human coronary artery endothelial cells (HCAEC) with lipidated recombinant full length TF, or TF-containing microparticles (50-200 pmol/L TF) increased the rate of cell proliferation and induced phosphorylation of ERK1 in a TF-dependent manner. Inhibition of ERK1/2 using PD98059 or ERK1/2 anti-sense oligonucleotides, or inhibition of JNK 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 pre-incubation of cells with an inhibitory anti-FVIIa antibody had no additional influence on TF-mediated cell proliferation. However, pre-incubation of exogenous TF with a β1-integrin peptide (amino acids 579-799) reduced TF-mediated proliferation. Conclusions: High concentrations of recombinant or microparticle-associated TF stimulate endothelial cell proliferation through activation of the ERK1/2 pathway, mediated through a novel mechanism requiring the interaction of exogenous TF with cell surface β1-integrin and independent of FVIIa.

Key words: Tissue factor, microparticles, ERK1/2, endothelial cells, β1-integrin

Condensed Abstract

Incubation of endothelial cells with lipidated recombinant tissue factor or tissue factor-containing microparticles resulted in increases in cell proliferation *in vitro*. This was dependent on the activation of the ERK1/2 pathway and involved the interaction between tissue factor and β1-integrin but did not require FVIIa.

In addition to its role in haemostasis, tissue factor (TF) has non-haemostatic functions which arise from its ability to activate various intracellular signalling pathways [1-3], leading to changes in cell proliferation [4], cell migration [5], and gene expression [6]. It has previously been shown that TF:FVIIa-mediated activation of the ERK1/2 pathway results in smooth muscle cell proliferation [4]. Furthermore, it has been shown that incubation of endothelial cells with recombinant TF alone induces cellular proliferation, together with an associated upregulation of cyclin D1 expression in these cells [7]. Recently, a number of reports have demonstrated the ability of TF to interact with cell surface integrins [8-11], resulting in diverse cellular outcomes including the activation of cell signalling pathways, cell migration and capillary tube formation [8,9,11]. The binding of cell surface TF to β1, α3 and α6-integrins on endothelial cells was shown to require FVIIa [9], whereas binding of alternatively spliced TF (asTF) to αvβ3 and α6β1-integrins was shown to be independent of FVIIa [11]. We have previously demonstrated the interaction of lipidated recombinant TF with the β1-integrin subunit and shown that this was independent of FVIIa [10]. 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 [12]. TF-containing microparticles may be released from various cells including monocytes/macrophages [13], endothelial cells [13] and smooth muscle cells [14]. These microparticles are highly procoagulant due to 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 [15-18]. Furthermore, it has been demonstrated that microparticles can transfer TF between different cell types [19,20]. It has previously been shown that the incubation of human umbilical vein endothelial cells with exogenous recombinant TF results in the induction of a number of cell signalling pathways [21], despite the lack of PAR2 in these cells. Moreover, it was demonstrated that this form of TF interacts with the cell surface [7,10,21]. This study is an attempt to further elucidate the mechanisms by which exogenous TF induces endothelial cell proliferation. Throughout this investigation, two forms of "exogenous" TF; lipidated recombinant TF and TF-containing cell-derived microparticles, have been used at concentrations of TF corresponding to those found in healthy individuals (5-10 pmol/L) or in plasma of patients with cardiovascular disease (50-200 pmol/L) [22,23]. 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 β1-integrin, but is independent of FVIIa.

Methods

Materials

Polyclonal anti-human TF antibody, lipidated recombinant full length TF, non-lipidated recombinant TF, recombinant FVIIa and recombinant FXa were purchased from American Diagnostica Inc. The recombinant β1-integrin peptide (amino acids 579-799) was obtained from ProSpec-Tany TechnoGene Ltd. Please see http://atvb.ahajournals.org for a complete list of reagents.

Cell culture

Human coronary artery endothelial cells (HCAEC) (PromoCell) were cultured under 5% CO_2 at 37ºC in endothelial cell growth medium (PromoCell) containing 5% (v/v) foetal calf serum 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 EGF (10 ng/ml) and bFGF (20 ng/ml).

Proliferation and apoptosis assays

Cell proliferation was measured using an MTS-based assay and cell numbers confirmed by staining with crystal violet. Apoptosis was measured using the DeadEnd Fluorimetric TUNEL assay (Promega). Please see http://atvb.ahajournals.org for further details.

SDS-PAGE and western blot analysis

Whole cell lysates (10 μg protein) were separated by SDS-PAGE and analysed by western blot using antibodies against total or phosphorylated forms of ERK1/2, JNK1/2 or p38. Please see http://atvb.ahajournals.org for further details.

Microparticle preparation

Cell-derived microparticles were prepared by ultracentrifugation as described before [10]. 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 Biologicals) and two-stage chromogenic assay respectively [24,25]. Please see http://atvb.ahajournals.org for further details.

ERK1/2 inhibition using anti-sense oligodeoxynucleotides

Sequences for ERK1/2 anti-sense (5'-GCCGCCGCCGCCGCCAT-3'), sense (5'-ATGGCGGCGGCGGCGGC-3') and scrambled (5'-CGCGCGCTCGCGCACCC-3') phosphorothioate-modified oligodeoxynucleotides (ODN) were according to a published procedure [26]. The scrambled ODN was synthesised with a 3'-FITC group to allow transfection efficiency to be determined by flow cytometry. HCAEC (12.5×10^4) were

adapted to OptiMEM reduced serum medium and transfected with the ERK1/2 ODN (0.1 μmol/L) using Lipofectin according to the manufacturers instructions. The cells were incubated at 37ºC for 5 h and then placed in medium containing 1% (v/v) FCS. The cells were incubated at 37ºC for 48 h, adapted to serum-free medium and used in experiments.

Statistical analysis

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

Results

Lipidated 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 non-transfected ECV304 and HCAEC cells were determined prior to 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 (PS:PC) 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 non-transfected 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 I-A) [27]. Incubation of HCAEC with TF-containing microparticles (200 pmol/L) resulted in rapid depletion of TF from the culture media (Supplemental Figure I-B), indicating microparticle uptake by the cells. Incubation of HCAEC with high concentrations of

lipidated recombinant TF (50-200 pmol/L) resulted in increases in cell proliferation of up to 82% above that of the untreated control (Fig 1A). Addition of ECV304-derived microparticles (Fig 1B) or HCAEC-derived microparticles (Fig 1C) containing 100-200 pmol/L of TF resulted in increases in cell proliferation of up to 37%, whereas corresponding concentrations of control-microparticles had little influence. Pre-incubation of recombinant TF or TF-microparticles (100 pmol/L) with an anti-TF polyclonal antibody (100 μg/ml) reduced cell proliferation in response to all forms of exogenous TF. In contrast, non-lipidated recombinant TF, or a mixture of PS:PC (30:70) alone had no measurable influence on cell proliferation (Supplemental Figure II-A). TF-containing microparticles (100 pmol/L TF) isolated from the plasma of patients with cardiovascular disease (Innovative Research Inc.) also induced proliferation of endothelial cells which was reduced by differing amounts in the different samples by pre-incubation of the microparticles with an anti-TF polyclonal antibody (Supplemental Figure II-B). Incubation of HCAEC with lipidated 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 MAPK cell signalling pathways

To elucidate the signalling pathways involved in the induction of endothelial cell proliferation by exogenous TF, the activation of the mitogen activated protein kinase (MAPK) pathways was examined. Incubation of HCAEC with lipidated recombinant TF (100 pmol/L) resulted in ERK1 phosphorylation, which peaked at 30 min post-treatment (Fig 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 min (Fig 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 (Fig 2C).

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

Inhibition of ERK1/2 phosphorylation prior to incubation of cells with lipidated recombinant TF (100 pmol/L), was achieved by pre-incubation of HCAEC with the MEK inhibitor PD98059 (15-50 μmol/L) for 30 min, and inhibition of the phosphorylation of the JNK substrate c-Jun was achieved by pre-incubation with SP600125 (60 nmol/L) for 30 min (Supplemental Figure IV-A). Incubation of HCAEC with PD98059 (15-50 μmol/L) completely inhibited cell proliferation in response to lipidated recombinant TF (100 pmol/L) (Fig 3A), while inclusion of SP600125 (20-60 nmol/L) showed a dose-dependent decrease in cell proliferation, inhibiting cell proliferation at 60 nmol/L compared to cells treated with TF (100 pmol/L) alone (Fig 3B). Pre-incubation of HCAEC with PD98059 (50 μmol/L) also reduced cell proliferation in response to HCAEC-derived microparticles containing TF (200 pmol/L), while 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 to 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 anti-sense ODN (optimised to 0.1 μmol/L, Supplemental Figure V-A) to inhibit the expression of ERK1/2. Optimal suppression of ERK1/2 expression following transfection of HCAEC with ERK1/2 anti-sense ODN (0.1 μmol/L) was achieved at 48 h compared to non-transfected

cells, while JNK expression remained unaffected (Fig 3C). Furthermore, sense and scrambled ODN (0.1 μmol/L) had no effect on ERK1/2 expression (Supplemental Figure V-B). Transfection efficiency of the cells following transfection of HCAEC with the 3'-FITC-labelled scrambled ODN (0.1 μmol/L) was determined to be 85% using flow cytometry (data not shown). Transfection of HCAEC with ERK1/2 anti-sense ODN prevented the induction of cell proliferation in response to lipidated recombinant TF (100 pmol/L) (Fig 3D). In contrast, transfection of cells with ERK1/2 sense ODN had no measurable influence on TF-mediated cell proliferation.

The 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 two-stage chromogenic assay respectively (data not shown). In addition, the expression of PAR1 and PAR2 in HCAEC was confirmed using RT-PCR (Supplemental Figure VI-A). 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 (Fig 4A). The enzymatic activities of recombinant FVIIa and FXa were confirmed using chromogenic substrates. Furthermore, pre-incubation of cells with an inhibitory anti-FVIIa antibody (30 μg/ml) (Fig 4A), or the inhibitory anti-PAR2 antibody SAM11 (25 μg/ml) (Supplemental Figure VI-B) 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 (Fig 4A). Pre-incubation of TF-containing microparticles with an inhibitory anti-FVIIa antibody also did not reduce cell proliferation in response to TF-containing microparticles (Fig 4B). These results were mirrored in ERK1 phosphorylation, since 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 (Fig 4C), or microparticle-associated TF (Fig 4D).

The induction of HCAEC proliferation by recombinant TF and microparticle-TF involves an interaction with β1-integrin

Since TF has been shown to interact with integrins, the possibility that exogenous TF activates signalling 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 579-799 of β1-integrin was used to competitively inhibit the binding of exogenous TF to cell surface β1-integrin. Pre-incubation of lipidated recombinant TF (100 pmol/L) with the β1-integrin peptide (0.02-1 nmol/L) reduced TF-mediated cell proliferation in a dose-dependent manner (Fig 4E). Concurrent with the reduced rate of cell proliferation, incubation of HCAEC with recombinant TF (100 pmol/L) pre-incubated with the β1-integrin peptide (1 nmol/L) reduced TF-mediated ERK1 phosphorylation (Fig 4C), and a similar result was observed with TF-containing microparticles (200 pmol/L TF) (Fig 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 VI-C). In addition, ERK1 phosphorylation in response to lipidated recombinant TF (100 pmol/L) was unaltered by pre-incubation 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 (Fig 4B) and ERK1 phosphorylation (Fig 4D) in response to TF-containing microparticles derived from HCAEC.

Pre-incubation 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 two of the samples (Supplemental Figure II-B).

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 pre-incubation of the microparticles with an anti-TF antibody (Supplemental Figure VII).

Discussion

Throughout this study, lipidated recombinant full-length TF was used in order 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 (Fig 1B), and showed a similar pattern of inducing cell proliferation compared to microparticles derived from primary endothelial cells (Fig 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 [4], while our studies

have shown that recombinant TF induces the proliferation of human umbilical vein endothelial cells (HUVEC) [7], as well as the activation of various cell signalling pathways [21]. In an extension to these studies, here we have shown the ability of microparticle-associated TF and recombinant TF to induce signalling in endothelial cells and the data presented further supports 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 PBMC and HCASMC) induced proliferation in HCAEC, which was reduced by pre-incubation of the microparticles with anti-TF antibody, further supporting the role of TF in the induction of endothelial cell proliferation. In contrast, non-lipidated recombinant TF did not induce cell proliferation (Supplemental Fig 2A), 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 I-B) 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 and α6β1-integrins 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 signalling, whereas in cancer cells the interaction between cellular TF and integrins is constitutive [9]. A recent study has also shown that asTF can interact with αvβ3 and α6β1-integrins on HUVEC or an endothelial cell line, resulting in endothelial cell migration and capillary tube formation by a FVIIa-independent mechanism [11]. 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 pre-incubation of cells with a polyclonal antibody directed against amino acids 579-799 of β1-integrin [10]. Therefore, in this study a recombinant β1-integrin peptide corresponding to amino acids 579-799 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-terminal of β1-integrin and includes the fourth EGF domain and βTD domain of the extracellular domain of β1-integrin, as well as the transmembrane and cytoplasmic domains

of β1-integrin [29]. The reduction in cell proliferation and ERK1 phosphorylation in response to both recombinant and microparticle-TF pre-incubated with this peptide suggests the presence of a novel binding site for exogenous TF within amino acids 579-799 of β1-integrin, and that this interaction is required for TF-mediated proliferation of endothelial cells. Moreover, pre-incubation of patient plasma-derived microparticles with the β1-integrin peptide reduced cell proliferation to differing extents in the three 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 [13,14], 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 α3-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 asTF is not capable of inducing proliferation in endothelial cells and does not interact with α3β1-integrin [11]. However, the interaction of asTF with αvβ3 and α6β1-integrins has been shown to induce cell migration and tube formation respectively [11]. These differences may be due to the structure of asTF, since asTF lacks the cytoplasmic and transmembrane domains as well as part of the extracellular domain of full length TF [30], and therefore may interact with integrins in a different conformation [11] and have different cellular outcomes compared to full length exogenous TF. Furthermore, both asTF [11] and exogenous TF [7,24] have been shown to induce capillary tube formation. Since 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

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Figure 1. Recombinant and microparticle-TF induce proliferation in HCAEC. HCAEC were incubated with either lipidated recombinant TF (0-200 pmol/L) (n=8) (A), or microparticles (MP) derived from TF-transfected or non-transfected ECV304 cells (n=6) (B), or HCAEC (n=3) (C). Additional samples (100 pmol/L TF) were pre-incubated with an anti-human TF antibody (100 μg/ml) and added as above. HCAEC were incubated for 24 h following which cell proliferation was measured using the MTS-based assay and cell numbers confirmed by staining with crystal violet. *=$p<0.05$ vs. untreated control. #=$p<0.05$ vs. 100 pmol/L TF.

Figure 2. Recombinant and microparticle-TF induce MAPK activation. HCAEC were incubated with recombinant TF (100 pmol/L) for 0-90 min and phosphorylation of ERK1/2 (A) and JNK (B) analysed (n=3). C) HCAEC were incubated with microparticles derived from TF-transfected HCAEC at 50-200 pmol/L TF, or from non-transfected cells at equivalent microparticle densities. Additional samples (100 pmol/L TF) were pre-incubated with an anti-TF polyclonal antibody (100 μg/ml) and ERK1/2 phosphorylation analysed at 30 min (n=3). *=$p<0.05$ vs. untreated control. Presented micrographs are representative of three independent experiments.

Figure 3. Inhibition of ERK1/2 prevents recombinant and microparticle TF-mediated cell proliferation. HCAEC were pre-treated (30 min) with ERK1/2 inhibitor PD98059 (15-50 μmol/L) (A) or JNK inhibitor SP600125 (20-60 nmol/L) (B) and recombinant TF (100 pmol/L) added. Cells were incubated with TF and the inhibitors for 24 h following which cell proliferation was measured (n=6). *=$p<0.05$ vs. untreated control. #=$p<0.05$ vs. TF alone. C) The expression of ERK1/2, JNK and GAPDH in HCAEC was examined at 24, 48 and 72 h post-transfection with anti-sense ERK1/2 ODN (0.1 μmol/L). D) HCAEC (12.5×10^4) were transfected with anti-sense (AS), sense (S) and scrambled (Scr) ERK1/2

ODN (0.1 μmol/L) for 48 h. Cells were then incubated with recombinant TF (100 pmol/L) and cell proliferation measured following a further 24 h incubation (n=4). *=p<0.05 vs. S.

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 (5 nmol/L) and FXa (10 nmol/L), an inhibitory anti-FVIIa antibody (30 μg/ml), or FVIIa and FXa alone for 24 h 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 pre-incubated with the β1-integrin peptide (1 nmol/L) or FVIIa antibody (30 μg/ml), and proliferation measured after 24 h (n=4, *=p<0.05 vs. untreated control, #=p<0.05 vs. TF-MP). HCAEC were incubated for 30 min 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) pre-incubated with the β1-integrin peptide (0.02-1 nmol/L) for 40 min was added to HCAEC for 24 h and cell proliferation measured (n=5, *=p<0.05 vs. TF).