

## Stimulation of Tissue Factor Expression in Human Microvascular and Macrovascular Endothelial Cells by Cultured Vascular Smooth Muscle Cells *in vitro*

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### Key Words

Endothelial cells · Smooth muscle cells · Tissue factor

### Abstract

The effect of conditioned media obtained from different smooth muscle cells (SMC) on tissue factor (TF) expression in endothelial cells (EC) *in vitro* was investigated. We could show that conditioned media from cultured human aortic SMC, human umbilical artery SMC or human umbilical vein SMC all resembling the synthetic phenotype of SMC induced TF activity in human umbilical vein EC and human skin microvascular EC in a dose- and time-dependent fashion. This induction was also seen at the level of specific TF mRNA as evidenced by Northern blotting. The TF inducing activity was heat-labile and acid-stable and had an approximate molecular mass of 38 kD. This activity was found to be distinct from known inducers of TF expression in EC such as interleukin-1, tumor necrosis factor- $\alpha$ , bacterial lipopolysaccharide or vascular endothelial growth factor. Such a factor, if released by SMC *in vivo*, could contribute to the activation of EC under conditions such as when EC are in close contact with SMC of the synthetic (nondifferentiated) phenotype seen in processes like vessel development or neo-intima formation.

### Introduction

Tissue factor (TF) is a membrane-bound glycoprotein which forms complexes with factor VII and factor VIIa. Factor VIIa subsequently activates factor IX and X. Thus TF plays a key role in the activation of the extrinsic pathway of coagulation and indirectly via factor VII-dependent activation of factor IX also leads to activation of the intrinsic system [1, 2]. TF-producing cells have been identified in human vessels by *in situ* hybridization and immunocytochemistry. While present in adventitial fibroblasts, in atherosclerotic plaques and in cells in the tunica media, endothelial cells (EC) in normal arteries, veins and capillaries do not express TF mRNA or protein [3, 4]. Thus the functional expression of TF in intact endothelium *in vivo* is in doubt. *In vitro* unperturbed EC express only minute quantities of TF whereas the expression of TF in these cells is dramatically increased by cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) or interleukin-1 (IL-1) and by bacterial lipopolysaccharide (LPS) [5-7]. *In vivo* these inflammatory mediators can convert a resting, anticoagulatory endothelium into an activated, TF-expressing, procoagulatory one.

We have shown previously that the activation of EC cells could also be modulated by smooth muscle cells (SMC) – a cell type, which *in vivo* is in close contact with

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the endothelium – depending on the vascular source and cell doublings of these cells [8–10]. In one of these studies we identified a heat-labile, acid-stable factor produced by SMC which stimulated in EC the expression of plasminogen activator inhibitor-1 (PAI-1) which is – like TF – a marker of EC activation [8]. It was therefore the aim of this study to investigate whether SMC could also induce the expression of another marker of activated endothelium, namely TF in EC, thereby modulating the coagulation pathway.

## Methods

### Cell Culture

Human vascular SMC were isolated by the explant technique from pieces of human pulmonary artery and aorta after surgery, and from the umbilical artery and vein of cords obtained after vaginal delivery [11]. Briefly, tissue specimens were cut into 10–20 pieces 1–2 mm in diameter, placed in Petri dishes (Costar, Cambridge, Mass.) coated with 1% gelatine (Biorad, Richmond, Calif.), and covered with a drop of Medium 199 (M199, Sigma, St. Louis, Mo.) containing 20% supplemented calf serum (SCS, HyClone, Logan, Utah), 50 IU/ml penicillin, 50 µg/ml streptomycin, 250 ng/ml amphotericin B (all JRH Biosciences, Lenexa, Kans.) in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37 °C. After 3–5 days the explants became adherent, and the Petri dish was then filled with M199 containing 20% SCS. SMC that grew out from explants were grown to confluence and subcultured using a split ratio of 1:3. Cells were confirmed as SMC by their typical 'hill-and-valley' morphology and positive immunofluorescence staining with a monoclonal antibody against  $\alpha$ -smooth muscle actin (Boehringer Mannheim). All SMC used in this study were between passages 3 and 5. Human umbilical vein endothelial cells (HUVEC) were isolated by mild collagenase treatment as described [12], and human skin microvascular endothelial cells (HSMEC) were isolated from normal skin biopsies obtained after surgical operations according to the method of Jackson et al. [13] using Dynabeads<sup>®</sup> coated with *Ulex europaeus* agglutinin (Dyna, Norway). The EC were seeded into gelatine-coated Petri dishes, grown to confluence as described above in M199 containing 20% SCS, 50 µg/ml EC growth supplement (Technoclone, Vienna, Austria) and 5 U/ml heparin (Hoffman-La Roche, Basel, Switzerland) in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37 °C. After 5–8 days EC reached confluence and were subcultured using a split ratio of 1:3. Cells were confirmed to be EC by their typical cobblestone morphology [14], by positive immunofluorescence using anti-von-Willebrand factor antibodies (Cappel, Cochranville, Pa.) [15] and by the uptake of acetylated low density lipoprotein (LDL) [16] whereby >95% of the cells stained positive with anti-von-Willebrand antibodies and showed an uptake of acetylated LDL. All human EC used in this study were between passage 2 and 3.

### Preparation of SMC Conditioned Media

Human aortic smooth muscle cells (HASMC), human pulmonary artery SMC (HPASMC), human umbilical artery SMC (HUASMC) and human umbilical vein SMC (HUVSMC) were seeded into Petri dishes coated with 1% gelatine and cultured with M199 containing

20% SCS. Confluent cultures were washed with Hanks' balanced salt solution (Sigma) and incubated in M199 without serum for 24 h at 37 °C. Such serum-free conditioned media (CM) of SMC were harvested, centrifuged at 1,000 g for 5 min to remove cell debris and stored at –70 °C until used.

### Assays for IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ and Vascular Endothelial Growth Factor

Serum-free CM obtained from SMC as described above were assayed for the presence of various cytokines using commercially available enzyme-linked immunosorbent assays (ELISAs) specific for human IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$  and vascular endothelial growth factor (VEGF; R&D Systems, Minneapolis, Minn.). The lower limits of detection for these assays were 0.5, 1.0, 4.4 and 5.0 pg/ml, respectively.

### Assay for TF Activity in EC Treated with SMC CM

EC, grown to confluence in 24-well plates (Costar), were incubated for the time periods indicated with different concentrations of the respective CM obtained from SMC or with M199 as a control. To all the media SCS was added to give a final concentration of 4%. At the end of the incubation period the cells were washed 3 times with 2 ml PBS per well. Thereafter the plates with the cells were frozen at –70 °C. The lysed cells were scraped into 400 µl per well of clotting buffer containing 130 mM NaCl, 8 mM Na-barbital and 12 mM Na-acetate, pH 7.4. 100 µl of these lysates were added to 100 µl of human normal plasma and 100 µl of 20 mM CaCl<sub>2</sub>. Clotting time was determined using a coagulometer and compared to a standard curve constructed with different concentrations of rabbit brain thromboplastin (Sigma) calibrated against human TF. The coagulant activity observed in this assay reflects TF activity because no procoagulant activity was detected from EC when factor X-deficient plasma was used instead of normal plasma. Furthermore, the procoagulant activity could be completely blocked by a rabbit antihuman TF antibody (American Diagnostica, Greenwich, Conn.) at a concentration of 100 µg/ml whereas a nonimmune rabbit antibody had no effect when used at the same concentration.

### Assay for PAI-1 Antigen

PAI-1 antigen was determined by a specific ELISA using specific monoclonal antibodies for PAI-1 (Technoclone, Vienna, Austria). The PAI-1 ELISA measures active, complexed and latent PAI-1.

### Northern Blot Analysis

Total mRNA was prepared by the guanidinium thiocyanate-phenol-chloroform extraction from confluent EC treated for 4 h with the respective CM obtained from SMC or with M199 as a control [17]. SCS was added to all media to give a final concentration of 4%. RNA was electrophoretically separated on 1.2% agarose gels containing 6% formaldehyde, transferred to nitrocellulose (BioRad) and hybridized [10<sup>6</sup> cpm (Cerenkov/ml)] for 16 h at 42 °C with random primed  $\alpha$ -(<sup>32</sup>P)dCTP probes (Boehringer Mannheim, Mannheim, Germany) for either porcine TF (a 0.64-kb *EcoRI-EcoRI* fragment) or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH, a 1.3-kb *PstI* cDNA fragment, kindly provided by Dr. Busslinger, Vienna).

### Gel Filtration

Serum-free CM (450 ml) obtained from confluent HUASMC was lyophilized, resuspended in 20 ml distilled water, and dialyzed against PBS, pH 7.4, at 4 °C overnight. Gel filtration of this material

was performed using a Sephacryl S-200 HP column (2.6 × 90 cm) equilibrated in PBS, pH 7.4, at 4°C and at a flow rate of 13 ml/h. Fractions of 2.6 ml were collected and screened for protein and for their ability to induce TF activity in HUVEC as described above.

#### High-Performance Liquid Chromatography

Fractions containing TF-stimulating activity obtained after gel filtration were pooled and concentrated using ultrathimbles with a cutoff of 10 kD (UH 100/10; Schleicher & Schuell, Dassel, Germany) prior to purification with high-performance liquid chromatography (HPLC). 25 µl of the concentrated sample were applied to a C4 column (Beckman, Fullerton, Calif.) and HPLC was performed at a flow rate of 0.5 ml/min. Fractions of 0.3 ml were collected, screened for protein, dialyzed against PBS, pH 7.4 at 4°C and screened for their ability to induce TF activity in HUVEC as described above.

#### Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

The fractions containing TF-stimulating activity after HPLC were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining using the Phast-Gel® system (Pharmacia, Uppsala, Sweden).

#### Endotoxin Assay

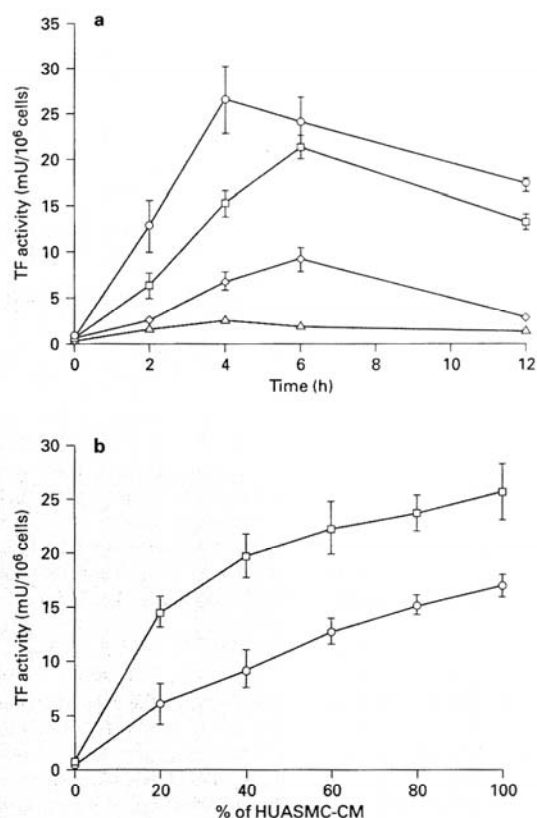
All solutions that came in contact with EC or SMC were assayed at <0.05 ng/ml endotoxin using the Coatest® Endotoxin Kit (Kabi Diagnostica, Sweden).

#### Statistical Analysis

A Student t test for unpaired observations was performed. Values of  $p < 0.05$  were considered significant.

## Results

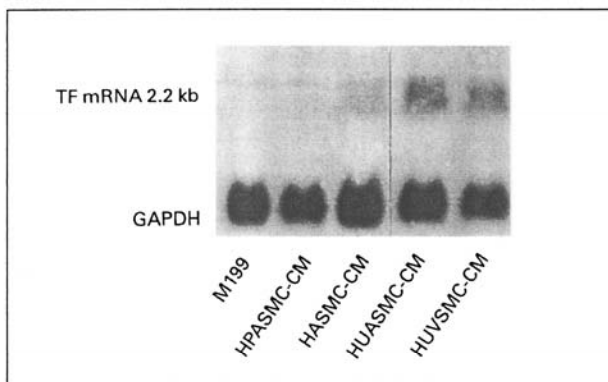
As can be seen from figure 1a CM obtained from HASMC, HUASMC or HUVSMC induced TF activity in HUVEC in a time-dependent way up to 14-, 38- and 31-fold over control, respectively, whereas CM obtained from HPASMC did not induce TF activity in HUVEC. HUVEC incubated with M199 and used as a control expressed  $0.68 \pm 0.19$  mU/10<sup>6</sup> cells of TF activity. TF activity was only found to be cell-associated and it was detected neither in CM derived from the respective vascular SMC nor in the supernatants obtained from HUVEC treated with the respective SMC-conditioned media (results not shown). As can be seen from figure 1 TF activity peaked in HUVEC after 4 h incubation with HUASMC-CM and after 6 h incubation with HUVSMC-CM or HASMC-CM, respectively, whereas no stimulation was seen at either of the time points when HUVEC were treated with HPASMC-CM. When the response of HUVEC towards the CM of HUASMC was compared with that of HSMEC, in both cases a dose-dependent response was seen, resulting in a 38- and 39-fold increase



**Fig. 1.** Induction of TF activity by SMC CM. **a** Confluent monolayers of HUVEC were incubated with CM harvested from HPASMC (△), HASMC (◇), HUVSMC (□) and HUASMC (○) for the indicated time periods. **b** Confluent monolayers of HUVEC (□) or HSMEC (◇) grown in 24-well plates were incubated with increasing concentrations of serum-free CM harvested from confluent HUASMC for 4 h. To all CM and the control medium used in these experiments SCS was added to give a final concentration of 4%. TF activity in cell lysates of HUVEC or HSMEC was determined as described in Methods. The data shown represent mean values ± SD of three independent experiments.

in TF activity over the respective control (fig. 1b). Similar to HUVEC unstimulated HSMEC expressed only little TF activity ( $0.43 \pm 0.02$  mU/10<sup>6</sup> cells).

As evidenced by Northern blotting CM obtained from HASMC, HUASMC and HUVSMC significantly increased the expression of TF mRNA in HUVEC, whereas CM from HPASMC had no effect on TF mRNA expression (fig. 2).



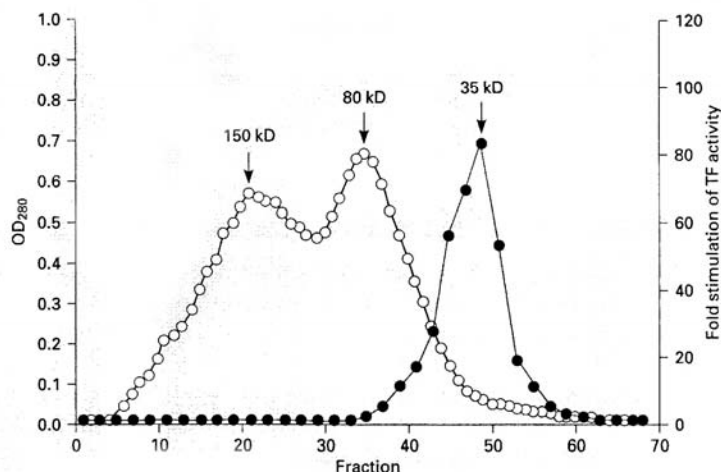
**Fig. 2.** TF mRNA levels in HUVEC after incubation with SMC CM. Confluent monolayers of HUVEC were incubated with M199 as a control (first lane), CM from HPASMC (second lane), CM from HASMC (third lane), CM from HUASMC (fourth lane) and CM from HUVSMC (fifth lane), for 2 h. Northern blots of RNA from these cells were hybridized to cDNA probes for TF and GAPDH as described in Methods. The size of the specific TF mRNA is indicated in kilobases.

**Table 1.** Characterization of TF-inducing activity present in CM obtained from HUASMC

	TF activity % of control
Control	100 ± 10.7
HUASMC-CM, untreated	3,632.3 ± 339.9*
HUASMC-CM, boiled	105.8 ± 12.2
HUASMC-CM, acid-treated	3,687.3 ± 168.3*
HUASMC-CM, treated with heparin Sepharose	3,969.6 ± 463.2*

Serum-free CM obtained from HUASMC as described in Methods was boiled for 5 min, acidified to pH 3.0 for 1 h and readjusted back to pH 7.4, and incubated with heparin-Sepharose for 24 h at 4°C. To all these CM and control medium, SCS was added to give a final concentration of 4%. Confluent monolayers of HUVEC were incubated for 4 h with such treated or untreated HUASMC CM or with fresh medium as a control. TF activity was determined in the lysates of these cells as described in Methods. Data are expressed as percent of the mean of a triplicate control and represent mean ± SD of three independent experiments. \*  $p < 0.001$  as compared to control.

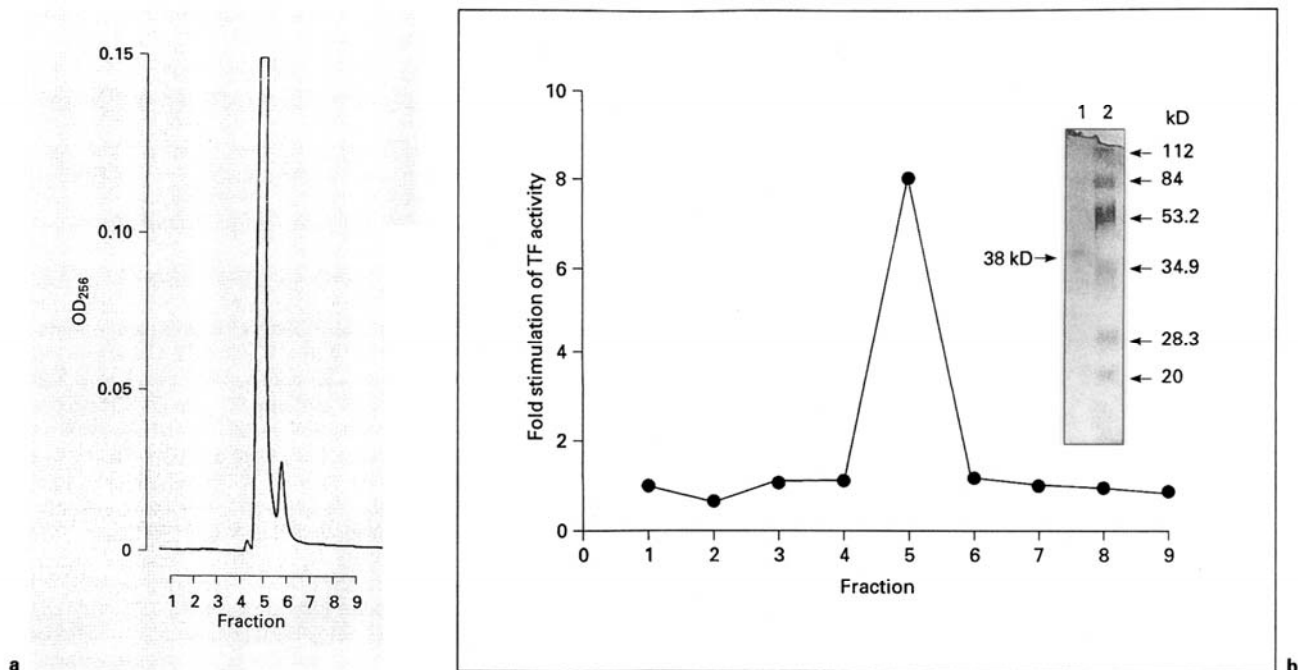
**Fig. 3.** Gel filtration profile of SMC-derived CM on Sephacryl S-200. Serum-free CM (450 ml) obtained from HUASMC was lyophilized, resuspended in distilled water, and dialyzed against PBS. The sample was then separated by gel filtration on a Sephacryl S-200 HP column and fractions were collected as described in Methods. The protein content of the respective fractions is shown as absorbance at 280 nm (○). The fractions were diluted 1:4 in M199 and SCS was added to give a final concentration of 4%. Confluent monolayers of HUVEC were incubated for 4 h with the respective fractions or – as a control – with PBS diluted 1:4 in M199 containing a final concentration of 4% SCS. TF activity in the lysates of these cells was determined as described in Methods and expressed as fold stimulation over control (●). Arrows indicate molecular masses of respective peaks.



When CM obtained from HUASMC was boiled for 5 min such treated CM did not induce TF activity in HUVEC whereas acid treatment and incubation with heparin Sepharose did not affect its TF-stimulating activity (table 1). No detectable levels of either IL-1 $\alpha$  or IL-1 $\beta$  or TNF- $\alpha$  or VEGF were found in CM from HUASMC

using specific ELISAs as described in Methods (data not shown).

When CM from HUASMC was subjected to gel filtration the TF-stimulating activity eluted in a single peak corresponding to a molecular mass of 35 kD (fig. 3). When HUVEC were incubated with the respective fractions



**Fig. 4.** HPLC purification of TF-stimulating activity. **a** Fractions found after gel filtration to stimulate TF activity were pooled and concentrated, the sample was subsequently separated by HPLC on a C4 column and fractions were collected as described in Methods. The protein content of the respective fractions is shown as absorbance at 256 nm. **b** The fractions were dialyzed against PBS, pH 7.4. Thereafter the samples were diluted 1:4 in M199 and SCS was added to give a final concentration of 4%. Confluent monolayers of HUVEC were

incubated for 4 h with the respective fractions or – as a control – with PBS diluted 1:4 in M199 containing a final concentration of 4% SCS. TF activity in the lysates of these cells was determined as described in Methods and expressed as fold stimulation over control. Inset: The fraction containing TF-stimulating activity was subjected to SDS-PAGE under reducing conditions using the PhastGel system and protein was visualized by silver staining (lane 1). A molecular weight standard is shown in lane 2.

containing the TF-stimulating activity for 24 h an up to 3-fold increase in PAI-1 antigen in CM of such treated cells was also seen (data not shown).

When the fractions containing TF-stimulating activity after gel filtration were pooled, concentrated and subjected to HPLC, two minor and one major protein peaks were obtained. When HUVEC were incubated with the respective fractions for 4 h it could be shown that the TF-stimulating activity eluted in the major protein peak. Subsequent SDS-PAGE of this peak revealed a single protein band corresponding to an approximate molecular mass of 38 kD (fig. 4). When HUVEC were incubated with this fraction for 24 h an up to 1.5-fold increase in PAI-1 antigen in CM of such treated cells was also seen (data not shown).

## Discussion

Unperturbed EC in vitro express only minute amounts of TF and only when activated by cytokines such as TNF- $\alpha$  or IL-1 or by LPS do these cells change to a procoagulatory phenotype by expressing TF activity [5–7]. Normal vessels have been shown by in situ hybridization and immunocytochemistry to be negative for TF mRNA and TF protein. Thus the expression of TF in the intact endothelium in vivo does not seem likely. Based on these findings TF, like IL-6, E-selectin or PAI-1, is considered to be an activation marker in endothelial cells [18–20]. We recently showed that SMC depending on their vascular source and the number of cell doublings could either up- or downregulate PAI-1 production in EC [8–10]. In one of these studies we characterized a heat-labile, acid-stable factor with an approximate molecular mass of 23 kD which increased PAI-1 expression in EC in vitro [8]. Here

we present evidence that SMC can also modulate TF expression in EC. We showed that CM obtained from HUASMC, HUVSMC or HASMC stimulated TF expression in human microvascular as well as in human macrovascular EC in a dose- and time-dependent manner. When CM from HPASMC were used no induction of TF activity in EC was seen. These results were also reflected on the level of specific TF mRNA as evidenced by Northern blotting. Corresponding to the activity data, treatment of EC with CM from HUASMC, HUVSMC resulted in a dramatic increase in the expression of TF mRNA in HUVEC and treatment of EC with CM from HASMC resulted in a slighter increase in TF mRNA in these cells; CM from HPASMC had no effect. In this respect it is noteworthy that – as we showed recently – CM from HUASMC, HUVSMC and HASMC stimulated the expression of PAI-1 in EC whereas CM from HPASMC induced a downregulation of PAI-1 protein and PAI-1 mRNA in EC [8, 9]. This would suggest that similar to the effects on the fibrinolytic system the effect of SMC on TF expression depends on the source of the SMC.

Similarly to the PAI-1 upregulating factor in CM of SMC the TF-upregulating activity present in SMC-conditioned media was heat-labile but acid-stable. Furthermore, it did not bind to heparin. The activity was eluted upon gel filtration as a single peak with a molecular mass of approximately 35 kD. Further purification of this material by HPLC yielded a single protein band as evidenced by SDS-PAGE and silver staining, which corresponded to an approximate molecular mass of 38 kD. However, this information does not allow us to distinguish it from other known inducers of TF expression in EC such as IL-1 with a molecular mass between 15 and 30 kD, TNF- $\alpha$  with a molecular mass of 17 kD, VEGF with a molecular mass between 15 and 24 kD or thrombin

with a molecular mass of 37 kD [5, 6, 21–24]. The involvement of these factors in the TF-stimulating effect is unlikely because we showed that under the conditions employed in our study CM obtained from SMC does neither contain IL-1 nor TNF- $\alpha$  nor VEGF. Furthermore, to our knowledge, SMC do not secrete thrombin. The respective CM were also shown to be free of LPS, another known inducer of TF expression in EC. Therefore we provide evidence that SMC isolated from aorta, umbilical artery and umbilical vein produce a factor which increases the expression of functionally active TF in EC. This factor seems to be distinct from known stimulators of TF expression in these cells such as IL-1, TNF- $\alpha$ , VEGF, thrombin or LPS. In this respect it is also of interest that in *in vitro* studies contaminating SMC have been shown to cause TF expression in EC cultures [25].

SMC in culture are considered to resemble rather the synthetic (nondifferentiated) phenotype found under *in vivo* conditions only in proliferating SMC [26, 27]. Provided that such a factor is also released by proliferating SMC *in vivo*, it would contribute to local fibrin formation in newly formed vessels, thereby limiting extravasation of blood. A critical role for TF in vasculogenesis has been suggested by recent studies using gene-deficient mice [28, 29]. On the other hand, this factor could also activate EC resulting in an increased thrombogenicity in areas of neointima formation.

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### References

- Nemerson Y: Tissue factor and haemostasis. *Blood* 1988;71:1–8.
- Bach R: Initiation of coagulation by tissue factor. *Crit Rev Biochem* 1988;23:339–351.
- Wilcox JN, Smith KM, Schwartz SM, Gordon D: Localization of tissue factor in the normal vessel wall in the atherosclerotic plaque. *Proc Natl Acad Sci USA* 1989;86:2839–2843.
- Fleck RA, Rao LVM, Rapaport SI: Localization of human tissue factor antigen by immunostaining with monospecific, polyclonal anti-human tissue factor antibody. *Thromb Res* 1990;57:765–781.
- Nawroth P, Stern D: Modulation of endothelial cells hemostatic properties by tumor necrosis factor. *J Exp Med* 1986;163:740–745.
- Bevilacqua MP, Pober JS, Majeau GR, Cotran RS, Gimbrone MA: Interleukin 1 (IL-1) induces biosynthesis and surface expression of procoagulant activity in human vascular endothelial cells. *J Exp Med* 1984;160:618–623.
- Colucci J, Balconi G, Lorenzet R, Pietra A, Locati D, Donati MB, Semararo N: Cultured human endothelial cells generate tissue factor in response to endotoxin. *J Clin Invest* 1983; 71:1893–1896.
- Galicchio M, Argyriou S, Ianches G, Filonzi EL, Zoellner H, Hamilton JA, McGrath K, Wojta J: Stimulation of PAI-1 expression in endothelial cells by cultured vascular smooth muscle cells. *Arteriosclero Thromb* 1994;14: 815–823.
- Christ G, Seiffert D, Hufnagl P, Gessel A, Wojta J, Binder BR: Type 1 plasminogen activator inhibitor synthesis of endothelial cells is down-regulated by smooth muscle cells. *Blood* 1993; 81:1277–1283.

- 10 Christ G, Hufnagl P, Kaun C, Mundigler G, Laufer G, Huber K, Wojta J, Binder BR: Anti-fibrinolytic properties of the vascular wall. Dependence on the history of smooth muscle cell doublings in vitro and in vivo. *Arterioscler Thromb Vasc Biol* 1997;17:723-730.
- 11 Chamley-Campbell J, Campbell GR, Ross R: The smooth muscle cell in culture. *Physiol Rev* 1979;59:1-61.
- 12 Gimbrone MA, Cotran RS, Folkman J: Human vascular endothelial cells in culture: Growth and DNA synthesis. *J Cell Biol* 1974;60:673-684.
- 13 Jackson CL, Garbett PK, Nissen B, Schrieber L: Binding of human endothelium to *Ulex europaeus* I-coated Dynabeads: Application to the isolation of microvascular endothelium. *J Cell Sci* 1990;96:257-262.
- 14 Booyse FM, Sedlak BJ, Rafelson R: Culture of arterial endothelial cells. Characterization and growth of bovine aortic endothelial cells. *Thromb Diath Haemorrh* 1975;34:825-839.
- 15 Jaffe EA, Hoyer LW, Nachman R: Synthesis of antihemophilic factor antigen by cultured human endothelial cells. *J Clin Invest* 1973;52:2757-2764.
- 16 Stein O, Stein Y: Bovine aortic endothelial cells display macrophage-like properties towards acetylated <sup>125</sup>I-labelled low density lipoprotein. *Biochim Biophys Acta* 1980;620:631-635.
- 17 Chomczynski P, Sacchi N: Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156-159.
- 18 Sironi M, Brevario F, Proserpio P, Biondi A, Vecchi A, Van Damme J, Dejana E, Mantovani A: IL-1 stimulates IL-6 production in endothelial cells. *J Immunol* 1989;142:549-553.
- 19 Bevilacqua MP, Pober JS, Mendrick DL, Cotran RS, Gimbrone MA: Identification of an inducible endothelial-leukocyte adhesion molecule. *Proc Natl Acad Sci USA* 1988;84:9238-9242.
- 20 Schleef RR, Bevilacqua MP, Sawdey M, Gimbrone MA, Loskutoff DJ: Cytokine activation of vascular endothelium: Effects on tissue-type plasminogen activator inhibitor. *J Biol Chem* 1988;263:5797-5803.
- 21 Clauss M, Weich H, Breier G, Knies U, Rockl W, Waltenerberger J, Risau W: The vascular endothelial growth factor receptor Flt-1 mediates biological activities. Implications for a functional role of placenta growth factor in monocyte activation. *J Biol Chem* 1996;271:17629-17634.
- 22 Heldin CH, Westermark B: Growth factors as transforming proteins. *Eur J Biochem* 1989;184:487-496.
- 23 Shaw AR: Molecular biology of cytokines: An introduction; in Thomson A (ed): *The Cytokine Handbook*. London, Academic Press, 1991, pp 13-36.
- 24 Fenton JW: Thrombin. *Ann NY Acad Sci* 1985;485:5-11.
- 25 Mulder AB, Blom NR, Smit JW, Ruiters MH, van der Meer J, Halie MR, Bom VJ: Basal tissue factor expression in endothelial cell cultures is caused by contaminating smooth muscle cells. *Thromb Res* 1995;80:399-411.
- 26 Hedin U, Bottger BA, Forsberg E, Johansson S, Thyberg J: Diverse effects of fibronectin and laminin on phenotypic properties of cultured arterial smooth muscle cells. *J Cell Biol* 1988;107:307-319.
- 27 Thyberg J, Blomgren K, Hedin U, Dryjski M: Phenotypic modulation of smooth muscle cells during formation of neointimal thickening in the rat carotid artery after balloon injury: An electron-microscopic and stereological study. *Cell Tissue Res* 1995;281:421-433.
- 28 Carmeliet P, Mackman N, Moons L, Luther T, Gressens P, Van Vlanderen I, Demunck H, Kasper M, Breier G, Evrad P, Muller M, Risau W, Edgington T, Collen D: Role of tissue factor in embryonic blood vessel development. *Nature* 1996;383:73-75.
- 29 Bugge TH, Xiao Q, Kombrinck KW, Flick MJ, Holmback K, Danton MJ, Colbert MC, Witte DP, Fujikawa K, Davie EW, Degen JL: Fatal embryonic bleeding events in mice lacking tissue factor, the cell-associated initiator of blood coagulation. *Proc Natl Acad Sci USA* 1996;93:6258-6263.