

Activation of Factor X and Its Regulation by Tissue Factor Pathway Inhibitor in Small-Diameter Capillaries Lined With Human Endothelial Cells

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The activation of factor X at the surface of endothelial cells was investigated under controlled flow conditions. A method is described for preparing polyethylene capillaries whose inner walls are covered with a confluent layer of human umbilical vein endothelial cells. To obtain a stable and unperturbed layer of endothelial cells it was essential to pre-perfuse the endothelialized capillaries with medium for about 18 hours. At this stage no tissue factor activity could be detected, but when the seeded cells were perfused with medium containing tumor necrosis factor (TNF) a maximum steady-state rate of factor Xa production (16 fmol factor Xa/min/cm²) was observed within 8 hours. Further experiments were performed with endothelial cells incubated for 4 hours with TNF. Factor Xa was produced at a rate of 7 fmol

factor Xa/min/cm² on perfusion of the capillaries with factor X (100 nmol/L) and factor VII (0.1 U/mL) at a shear rate of 34 s⁻¹. The extracellular matrix preparations of these cells produced factor Xa at a 20-fold higher rate (150 fmol factor Xa/min/cm²). In both cases factor Xa formation was dependent on the presence of factor VII and was completely inhibited when the perfusate also contained 5 nmol/L recombinant tissue factor pathway inhibitor (rTFPI). Pre-perfusion with factor Xa-TFPI complex in the absence of factor VIIa caused a much lesser inhibitory effect, suggesting that TFPI-mediated neutralization of endothelial cell and matrix tissue factor activity requires the presence of factor VIIa in addition to the presence of factor Xa.

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ENDOTHELIAL CELLS have been studied extensively for their anticoagulant and procoagulant activities.¹ Resting cells actively express a number of procoagulant activities. They synthesize and express factor V and support blood coagulation by providing binding sites for a number of (activated) blood coagulation factors.^{2,3} The presence of anticoagulant components, eg, thrombomodulin and proteoglycans, counterbalances the procoagulant activities, and only when blood coagulation is initiated, resting cells propagate and amplify the process of blood coagulation.

Whereas intact human umbilical vein endothelial cells (HUVEC) do not initiate blood coagulation, treatment with agents such as endotoxin⁴ and tumor necrosis factor (TNF)⁵ are reported to increase the synthesis of the transmembrane protein tissue factor. Tissue factor present at the surface of endothelial cells forms, together with circulating factor VII(a), a highly efficient enzymatic activator of factor X and factor IX.⁶

Blood coagulation reactions at the surface of endothelial cells are at present studied almost exclusively in closed and static systems. However, these systems have a disadvantage in that they do not provide information about rheologic effects such as shear rate and shear stress on the reactivity of surface-bound catalytic complexes of blood coagulation factors. In addition, depletion of reactants from the fluid phase, as it easily may occur under static conditions, will never be seen in so-called open systems. So, open flow systems were introduced to investigate factor X and prothrombin activation at model surfaces, eg, phospholipid membranes⁷⁻¹¹ and the surface of perturbed endothelial cells and their extracellular matrix.¹² This opens the possibility to study the kinetics of the formation of surface-bound catalytic complexes and their reactions with substrate or inhibitor under conditions that are relevant to the in vivo situation. In this respect, much has still to be learned about the assembly and activity of (perturbed) endothelium-bound tissue factor-factor VIIa complex under conditions of continuous flow.

Here we report on factor X activation at endothelial cells attached to the inner wall of small-diameter tubes. We examined the stability and integrity of monolayers of unperturbed HUVEC and tumor necrosis factor (TNF)-

stimulated HUVEC, their potency to produce factor Xa on perfusion with factor VII and factor X, and some aspects of the inhibitory action of recombinant tissue factor pathway inhibitor (rTFPI) on factor Xa generation.

MATERIALS AND METHODS

Materials. The chromogenic substrate for factor Xa, S2337, and the chromogenic substrate for thrombin, S2238, were purchased from AB Kabi Diagnostica (Stockholm, Sweden). Recombinant TNF- α was a kind gift of Dr G. Adolf (Vienna, Austria). Bovine serum albumin (fatty acid free) was obtained from Sigma Chemical Co (St Louis, MO). Partial purified human fibronectin (coproduct obtained during the preparation of human factor VIII concentrate from cryoprecipitate) and a concentrate of vitamin K-dependent human coagulation factors were kindly provided by Dr Jan van Mourik (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service [CLB], Amsterdam, The Netherlands). rTFPI¹³ was from Novo-Nordisk A/S (Bagsvaerd, Denmark). Culture medium, RPMI-1640, was purchased from Flow Laboratories (Irvine, Scotland). The capillaries used in this study were medical grade high pressure polyethylene tubes (Talas, Ommen, The Netherlands) of 0.38-mm and 0.5-mm inner diameter.

Coagulation factors. Bovine factor V and the human coagulation factors factor X, activated factor X, and prothrombin were purified to homogeneity essentially as described.¹⁰ Human factor VII (specific activity of 1,500 U/mg protein) was partially purified according to Bom et al.¹⁴

Cell culture. Endothelial cells were isolated from veins of freshly obtained human umbilical cords and cultured essentially

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according to the method of Jaffe et al.¹⁵ Briefly, endothelial cells were grown to confluence at 37°C and 5% CO₂ in RPMI-1640 medium supplemented with 20% human serum, L-glutamine (2 mmol/L), streptomycin (100 µg/mL), and penicillin (100 IU/mL) in tissue culture flasks (25 cm²) coated with human fibronectin. Cells were passed using 0.25% trypsin and again grown to confluence in tissue culture flasks (25 cm²). At confluency the cell density varied from 4 to 5 × 10⁴ cells/cm². All experiments were performed with cells of the second passage.

Cell seeding of capillaries. The attachment protocol was essentially that described by Anderson et al.¹⁶ Briefly, cells were washed and treated with trypsin (0.25%) to detach the cells. Medium containing 20% human serum was then added to inactivate residual trypsin. After a centrifugation step, cells were resuspended to a concentration of 1.0 × 10⁷ cells/mL in Dulbecco's phosphate-buffered saline solution (DPBS). Cells were then placed in polyethylene capillaries (15 cm long, inner diameter 0.38 or 0.5 mm) whose inner surfaces were precoated with fibronectin (1 hour at room temperature). The cell suspension-filled capillaries were placed horizontally and rotated at 0.5 rpm during 60 minutes. Unattached endothelial cells were removed by rinsing with DPBS. The capillaries were then connected to a recirculating pumped-flow system filled with aerated (5% CO₂) complete medium. The flow rate was 30 µL/min. The whole procedure was performed under sterile conditions at 37°C. Unless stated otherwise, the perfusion time was 18 hours.

Preparation of extracellular matrix. Endothelial cells attached to the inner wall of the capillary were exposed to 0.1 mol/L NH₄OH for 15 minutes at room temperature. The remaining extracellular matrix was washed by perfusion of a solution containing 10 mmol/L HEPES, pH 7.45, 135 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L MgCl₂, 4 mmol/L CaCl₂, 11 mmol/L D-glucose, and 2 mg/mL bovine serum albumin, hereafter indicated as HEPES-buffer, at a shear rate of 200 s⁻¹ for 10 minutes.

Morphologic procedures. For scanning electron microscopy the cells attached to the inner wall of capillaries were fixed with glutaraldehyde. Longitudinally sliced sections of the capillaries were then dehydrated with ethanol and subjected to critical-point drying. They were subsequently mounted on aluminium stubs, sputter-coated with gold, and viewed in a scanning electron microscope (Stereoscan 180; Cambridge Instruments, Cambridge, UK). For transmission electron microscopy, the dehydrated samples were embedded in resin. Ultrathin sections were cut and stained with uranyl acetate and lead citrate. These samples were examined with a Philips EM 400 electron microscope (Philips, Eindhoven, The Netherlands).

Perfusion experiments. The perfusions were performed using the capillary flow system according to Gemmell et al.¹⁷ with minor modifications as previously described.¹¹ The whole system was placed in a hood thermostated at 37°C. All reactants were in HEPES-buffer. Product formation was measured by taking timed samples (10 µL) at the outlet of the capillary. The samples were immediately diluted (100-fold) with ice-cold Tris buffer (50 mmol/L), pH 7.9, containing 175 mmol/L NaCl and 0.5 mg/mL bovine serum albumin and assayed for factor Xa as previously described.¹⁷

RESULTS

Establishment of confluent endothelium. The formation of a confluent layer of endothelial cells in the capillaries was investigated 1 hour and 4 hours after seeding by means of scanning electron microscopy. For this purpose the capillaries were perfused with DPBS and subsequently with 2.5% glutaraldehyde. After fixation and dehydration the

capillaries were sliced longitudinally. Figure 1A shows that after 1 hour the cells had attached to the capillary in a patchy pattern, typical of endothelial cells. Some of the cells were already spread but most cells appeared spherically (Fig 1B).

After 4 hours all attached cells were spread, forming a continuous layer, which showed no defects within the analysed areas (Fig 1, C and 1D). Transmission electron microscopy showed a monolayer of flattened endothelial cells with cell-cell contacts (Fig 2).

Initially, numbers of endothelial cells were evaluated after each perfusion experiment. The cells in the capillaries were detached by trypsin treatment, collected in a trypan blue solution (0.04% in DPBS), counted, and evaluated for viability. The capillaries (15 cm long and inner diameter of 0.5 mm) contained 1.5 ± 0.3 × 10⁵ cells/cm², of which 95% ± 3% excluded trypan blue (±SD, n = 10). This cell density is slightly higher than that obtained in culture flasks where cells were grown to confluency.

Factor Xa generation by unperturbed endothelial cells. After the initial attachment period of 1 hour, capillaries with endothelial cells were maintained in a recirculating pumped-flow system filled with complete medium. At timed intervals capillaries were removed from the system, rinsed with HEPES-buffer at a shear rate of 34 s⁻¹ for 10 minutes, and perfused with a solution containing factor X (100 nmol/L) and factor VII (0.5 U/mL) in the HEPES-buffer solution. Figure 3 shows the factor Xa production determined at the outlet as a function of perfusion time, for the different capillaries whose endothelial cells had been pre-perfused with medium for different time intervals. It is seen that the steady-state production of factor Xa of the unperturbed cells, attached to the inner wall of the capillaries, decreased with prolonged pre-perfusion with medium. A pre-perfusion time of 18 hours greatly reduced the ability of the nonstimulated endothelial cells to generate activated factor X. Examination of the capillary after 24 hours of pre-perfusion by scanning electron microscopy showed an intact layer of endothelial cells (data not shown). For further experimentation, capillaries that had been maintained for at least 18 hours in the recirculating medium after the initial cell attachment period of 1 hour were used.

Factor Xa production of endothelial cells seeded in capillaries and stimulated with TNF. Capillaries seeded with endothelial cells were maintained for 18 hours in the recirculating medium before the addition of TNF (10 nmol/L) to the medium. The capillaries were removed from the system at timed intervals, rinsed for 10 minutes with HEPES-buffer at a flow rate of 25 µL/min (shear rate of 34 s⁻¹), followed by a perfusion with a reaction mixture containing factor X (100 nmol/L) and factor VII (0.5 U/mL) in HEPES-buffer. A typical time course of factor Xa production by perturbed cells is shown in Fig 4. In this case endothelial cells were incubated for 4 hours with TNF in the recirculating system. Factor Xa production was found to be completely dependent on the presence of factor VII in the perfusion solution. The steady-state production of factor Xa was stable for at least 30 minutes. Inspection of

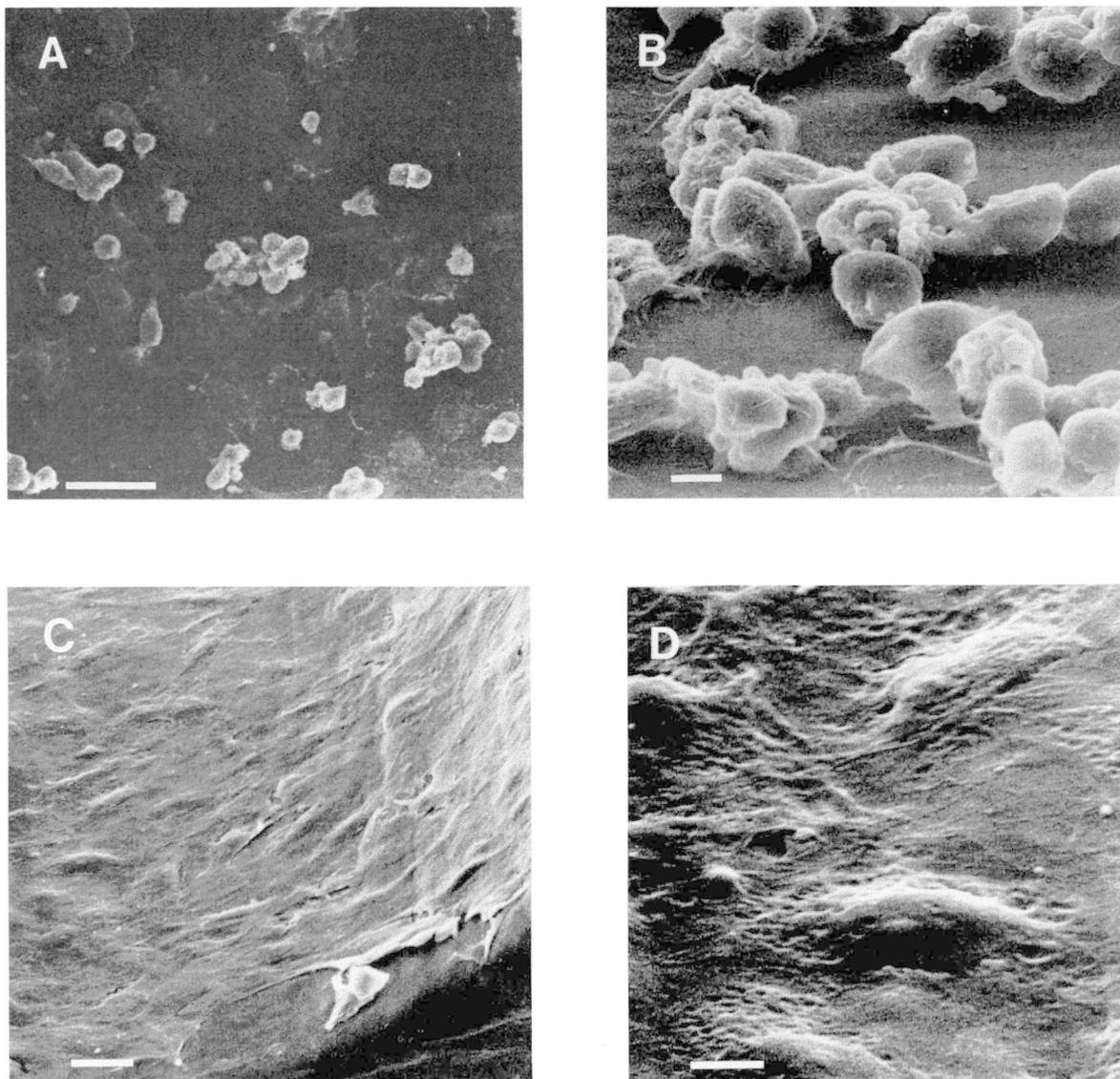


Fig 1. Scanning electron micrographs of human endothelial cells seeded in small-diameter capillaries. (A and B) After an initial attachment period of 1 hour. (C and D) Cells maintained for an additional 3-hour period in the perfusion system. Bar indicates 50 μm (A and C) or 10 μm (B and D).

the capillaries at the end of the experiment by scanning electron microscopy showed an intact layer of endothelial cells firmly attached to the inner wall of the capillary.

It is shown in Fig 5 that the steady-state rate of factor Xa production increased with increasing incubation time with TNF (10 nmol/L) until a maximum value was obtained at 8 hours. Beyond that timepoint the factor X activating activity disappeared. Because the experiments were performed with different primary cell cultures and thus variation in the steady-state levels of factor Xa production was inevitable, factor Xa concentration at the outlet is expressed as a percentage of the maximum activity found in three series of experiments using overlapping time intervals around 8 hours. The maximum rate of factor Xa production reached with endothelial cells incubated for 8 hours with 10

nmol/L TNF in a recirculating pumped-flow system was 16 fmol factor Xa/min/cm².

Figure 5 also shows that when the TNF-containing medium was replaced with fresh medium after 4 hours, the factor Xa-producing capacity of the endothelial cells did not further increase. This seems to be consistent with the observation shown in Fig 4, where despite a short incubation time with TNF, and thus a less than maximum tissue factor activity, a constant steady-state level of factor Xa production was obtained. As a practical consequence capillaries seeded with endothelial cells could be simultaneously stimulated for 4 hours with TNF and kept in recirculating medium until used.

In conclusion, endothelial cells seeded in small-diameter capillaries behave similarly with respect to the TNF-

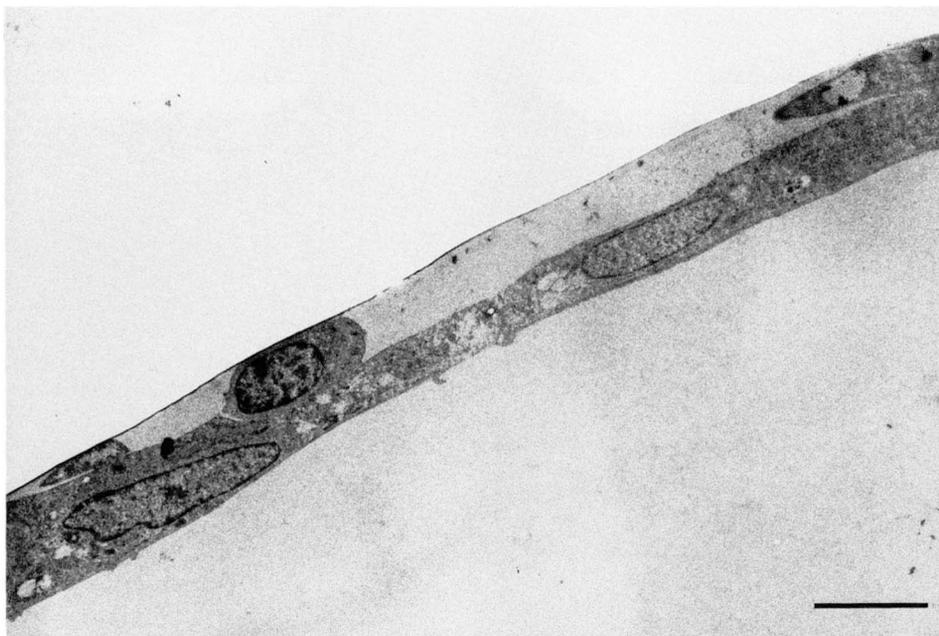


Fig 2. Transmission electron micrograph of seeded endothelial cells perfused for 3 hours with medium. Bar indicates 10 μm .

induced production of factor X converting catalytic activity, ie, tissue factor, at the cell surface as reported for endothelial cells cultured until confluency under static conditions.⁵

Relationship between rate of factor Xa production and surface-bound factor X converting catalytic activity. The rate of factor Xa production at the inner wall of the capillary as calculated from the factor Xa concentration at the outlet is not necessarily proportional with the surface enzyme density. That is, the rate of factor Xa production could be limited by the rate of transport of factor X from the bulk solution to the surface-bound enzyme.¹⁸

To establish whether we were dealing with a kinetically controlled regime, we set out to find a relationship between the rate of factor Xa production and the amount of catalytic complex bound to the endothelial surface. Factor VIIa was found to bind firmly to the endothelial cells because the

factor Xa-producing activity, as determined from a perfusion with factor X in the absence of factor VII, was not washed out during a 30-minute perfusion with the calcium-containing HEPES-buffer at a shear rate of 200 s^{-1} . Perfusion of the capillary with two capillary volumes (50 μL) of HEPES-buffer containing 5 mmol/L EDTA, though, resulted in a complete loss of the factor X converting activity. Thus, the amount of factor VIIa in the EDTA-effluent could be a measure for the amount of tissue factor at the inner wall of the capillary. Unfortunately, attempts to determine the factor VIIa activity in the EDTA-eluate failed because the factor VIIa activity was below the detection limit (0.1 mU/mL or 1 fmol VII/mL) of our factor VIIa assay. The factor VIIa assay used was a modification of the assay as described by Seligsohn et al.¹⁹

Quite unexpectedly we observed that capillaries, whose

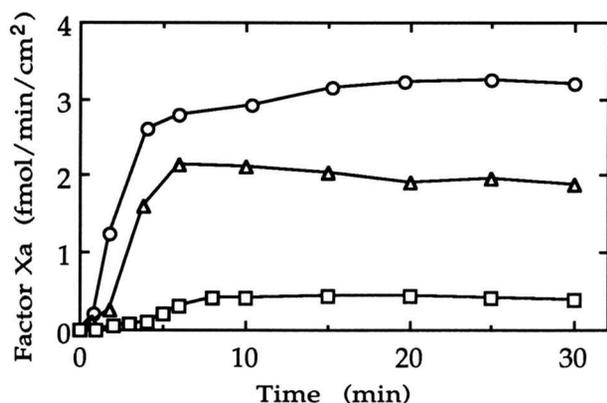


Fig 3. Factor Xa generation at the surface of nonstimulated HUVEC. Cell seeded capillaries were after a 1-hour attachment period maintained in a perfusion system with medium for 4 hours (○), 8 hours (△), and 18 hours (□). Capillaries were then perfused with factor VII (0.5 U/mL) and factor X (100 nmol/L). Wall shear rate was 34 s^{-1} .

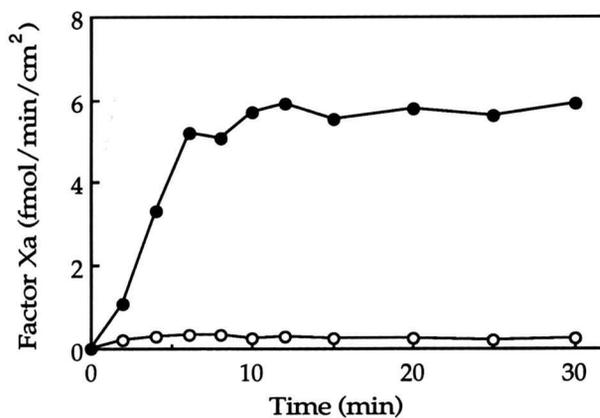


Fig 4. Factor Xa generation at surface of TNF-treated HUVEC. Rate of factor Xa production is shown as a function of the perfusion time for nonstimulated HUVEC (○) and for HUVEC stimulated during 4 hours with 10 nmol/L TNF (●). Perfusate contained 0.5 U/mL factor VII and 100 nmol/L factor X. Wall shear rate was 34 s^{-1} .

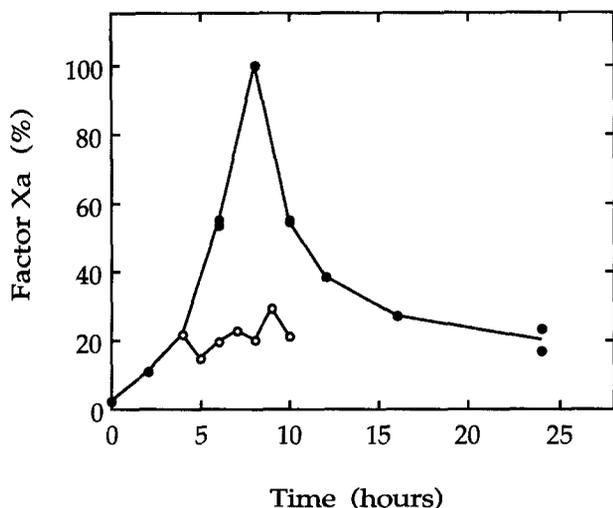


Fig 5. Time course of tissue factor activity induced by 10 nmol/L TNF (●). The time course of tissue factor activity when TNF was removed from the medium after a 4-hour pre-perfusion with TNF containing medium (○). Wall shear rate was 34 s^{-1} .

inner walls were coated with TNF-stimulated endothelial cells, after a 5-minute perfusion with EDTA (5 mmol/L) in HEPES-buffer, produced factor Xa on perfusion (shear rate of 50 s^{-1}) with factor X (100 nmol/L) and factor VII (0.5 U/mL) at a higher rate than before the perfusion with EDTA. Inspection of the capillaries by scanning electron microscopy showed that the endothelial cell layer had lost its integrity showing gaps between the cells. On removal of all cells by perfusion with a 0.1 mol/L NH_4OH solution the rate of factor Xa production further increased. Rates of factor Xa production were 7.1 ± 3.9 (SD, $n = 32$) fmol factor Xa/cm²/min, 27.3 ± 8.2 (SD, $n = 4$) fmol factor Xa/cm²/min, and 135.8 ± 50.9 (SD, $n = 17$) fmol factor Xa/cm²/min for a nontreated, EDTA-treated, and NH_4OH -treated confluent layer of TNF-stimulated HUVEC, respectively. Our findings can readily be explained by the recent observation that TNF-induced endothelial cell tissue factor activity is predominantly associated with the subendothelial matrix.²⁰

Because the capillaries could produce factor Xa at a 20-fold higher rate, it is clear that the system is largely, if not fully, reaction controlled. Further evidence that this was indeed the case, also for the highly reactive extracellular matrix, was obtained as follows. Capillaries (15 cm long and inner diameter of 0.38 mm) were seeded with endothelial cells and stimulated for 4 hours with TNF (10 nmol/L), after which the extracellular matrix was prepared as described. The steady-state rate of factor Xa production at the extracellular matrix was determined after a perfusion with factor VII (0.5 U/mL) and factor X (100 nmol/L) at a shear rate of 50 s^{-1} . Then, while the perfusion was continued, the capillaries were halved and perfusion was continued for the 7.5 cm part that remained connected to the perfusion apparatus. The rate of factor Xa production was 138.8 ± 34.0 (SD, $n = 3$) fmol/cm²/min and 143.9 ± 31.9 (SD, $n = 3$) fmol/cm²/min before and after removing half of the capillary, respectively. These results indicate

that factor Xa production is not limited by the transfer of factor X to the active surface, because in that case the steady-state rate of factor Xa production in the first 7.5 cm of the capillary would be 1.26-fold higher than that of a 15-cm capillary.¹⁸

Because of the high reactivity and stability of the tissue factor activity present in the extracellular matrix, we could also perform a perfusion study at different shear rates. The steady-state rate of factor Xa production was $199 \text{ fmol/cm}^2/\text{min}$ at a shear rate of 400 s^{-1} , and when the same capillary was perfused at a shear rate of 50 s^{-1} the rate of factor Xa production was $170 \text{ fmol/cm}^2/\text{min}$. If the system was solely diffusion controlled a eightfold increase in shear rate would have resulted in a twofold increase in the steady-state rate of factor Xa production.¹⁸ This was not the case. Therefore, in summary, all these results point in the same direction, namely that under the flow conditions of our experiments and with these tissue factor densities at the different surfaces the system is reaction controlled.

Effect of TFPI on the rate of factor Xa production by TNF-stimulated endothelial cells and their extracellular matrix. We next set out to examine the efficacy of rTFPI as an inhibitor of the TF/factor VIIa-catalyzed activation of factor X at the surface of perturbed endothelial cells and their extracellular matrix under controlled flow conditions. Because the system is reaction controlled, we assumed that the rate of factor Xa production could be taken as a measure for tissue factor/factor VIIa activity.

Endothelial cells in the capillary were incubated for 4 hours with TNF (10 nmol/L), rinsed with HEPES-buffer, and then perfused with factor VII (0.5 U/mL) and factor X (100 nmol/L). Twelve minutes after the start of the perfusion a steady-state rate of factor Xa production was reached (Fig 6A). The capillary was then perfused for 10 minutes

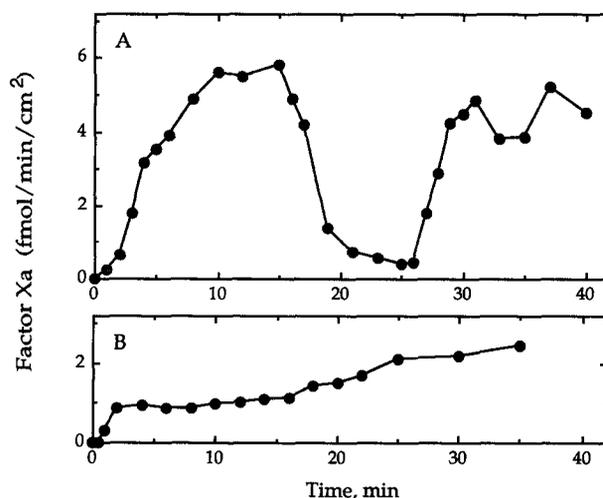


Fig 6. Effect of recombinant extrinsic pathway inhibitor on factor Xa production by TNF-stimulated HUVEC. (A) A capillary was perfused for 15 minutes with 100 nmol/L factor X, 0.5 U/mL factor VII and then with rTFPI (2 nmol/L) for 10 minutes and finally with 100 nmol/L factor X, 0.5 U/mL factor VII. (B) A capillary with TNF-stimulated endothelial cells was perfused for 10 minutes with 100 nmol/L factor X, 0.5 U/mL factor VII, 2 nmol/L rTFPI and then with 100 nmol/L factor X, 0.5 U/mL factor VII. Wall shear rate was 77 s^{-1} .

with a solution containing only rTFPI (2 nmol/L), and when the capillary was again perfused with the factor VII/factor X solution a slight reduction (~10%) of the factor Xa-producing capacity of the perturbed endothelium was observed. However, the addition of 2 nmol/L rTFPI to the perfusate containing 100 nmol/L factor X and 0.5 U/mL factor VII resulted in a greatly reduced factor Xa activity at the outlet of the capillary lined with TNF-stimulated endothelial cells (Fig 6B).

Two possible explanations for the effect of rTFPI on factor Xa generation have to be considered. Firstly, factor Xa generation is blocked because of the formation of an inactive quaternary complex between factor Xa, factor VIIa, TF, and rTFPI or alternatively, rTFPI neutralizes in a direct way the factor Xa activity by forming a noncovalent complex.²¹ The latter possibility, though, is not very likely because before samples were assayed for free factor Xa, they were diluted 100-fold, so that the factor Xa and TFPI concentrations were far below the inhibitory constant of the direct rTFPI-factor Xa reaction ($K_i \sim 0.2$ nmol/L).¹³

After the 10-minute perfusion with 100 nmol/L factor X, 0.5 U/mL factor VII, and 2 nmol/L rTFPI, the capillary was perfused with a solution containing 100 nmol/L factor X and 0.5 U/mL factor VII. The steady-state rate of factor Xa production increased slowly with perfusion time, but remained far below the level seen in the absence of rTFPI. This experiment indicates that at the surface of stimulated endothelial cells, at least under the conditions of the experiment, the rTFPI-factor Xa-tissue factor-factor VIIa complex is a rather stable one.

Figure 7A shows that during the initial stage of the perfusion with factor X/factor VII/rTFPI, low rTFPI concentrations (< 1 nmol/L) are not very effective in inhibiting free factor Xa generation. However, with increasing perfusion time a significant reduction in the rate of factor Xa production is seen. At higher rTFPI concentrations optimal rates of factor Xa generation are followed by a markedly reduced steady-state rate of factor Xa production, which decreased with increasing rTFPI concentrations.

Although the extracellular matrix of TNF-stimulated endothelial cells contained a much higher tissue factor activity, the time courses of neutralization of tissue factor/factor VIIa in both cases were similar for the same amounts of rTFPI (Fig 7B).

Function of factor VIIa in the formation of the endothelial rTFPI-tissue factor complex. It has recently been published that factor VIIa contributes to the stability of the tissue factor-factor Xa-TFPI complex when the surface does not contain a substantial amount of negatively charged phospholipid, like phosphatidylserine.⁹ Under the conditions of our experimental set-up we found that in the absence of factor VII(a) the tissue factor activity at the surface of perturbed endothelial cells could hardly be inhibited by the rTFPI-factor Xa complex. A 10-minute pre-perfusion with a solution containing equimolar amounts of rTFPI and factor Xa (2 nmol/L of each) was not sufficient to neutralize the tissue factor activity at the surface of perturbed cells, as a second perfusion with factor X/factor VII solution showed

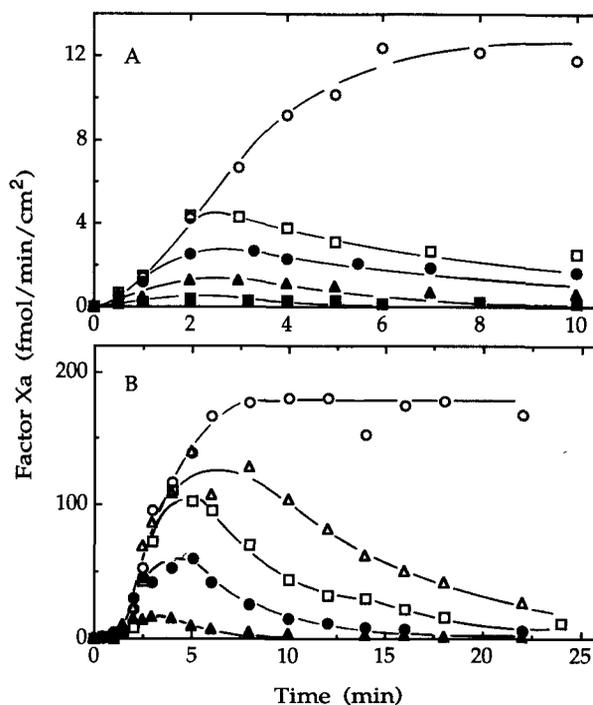


Fig 7. Effect of rTFPI concentration on the rate of factor Xa production at the surface of TNF-stimulated endothelial cells (A) and their extracellular matrix (B) during perfusion with 0.5 U/mL factor VII and 100 nmol/L factor X. The rTFPI concentrations were: none (○), 0.05 nmol/L (△), 0.1 nmol/L (□), 0.5 nmol/L (●), 2 nmol/L (▲), and 5 nmol/L (■). Wall shear rate was 77 s^{-1} .

only a 10% neutralization of tissue factor activity. Pre-perfusion of perturbed endothelial cells with factor X/factor VII followed by the perfusion of an equimolar rTFPI-factor Xa complex (2 nmol/L) did inhibit the tissue factor activity by nearly 100%. Finally, pre-perfusion with factor VII (0.5 U/mL), factor Xa (2 nmol/L), and rTFPI (2 nmol/L) also resulted in a complete neutralization of the tissue factor activity. Altogether these results indicate that a stable tissue factor-rTFPI complex on the endothelial cell surface can be formed only when both factor Xa and factor VIIa are present.

In the case of extracellular matrix, pre-perfusion of the extracellular matrix with a pre-formed factor Xa-TFPI complex (2 nmol/L) did not cause a reduction of the steady-state rate of factor Xa production. However, when the matrix was first perfused with factor VII and then with the preformed factor Xa-TFPI complex, the tissue factor activity was totally blocked: no factor Xa was produced after a perfusion with the calcium containing HEPES-buffer and a solution containing factor VII-factor X. These observations are not different from those seen with endothelial cells.

DISCUSSION

We have used a recently developed⁷ in vitro capillary perfusion system for studying blood coagulation reactions at the surface of endothelial cells. The clear advantage of

flow systems is the possibility of studying blood coagulation reactions at surfaces while there is a continuous supply of reactants under laminar flow and at physiologic wall shear rates. Another, more practical, advantage of this system is its relatively low volume/surface ratio of $13.5 \mu\text{L}/\text{cm}^2$.

We first established the conditions for achieving a confluent and stable endothelial cell coverage of the flow surface of the polyethylene capillary tubes. In accordance with a previous report,¹⁶ satisfying results could only be obtained when the cells were seeded at a high density. Cell concentrations of 1.0×10^7 cells/mL DPBS gave an immediate saturation attachment density of 1.5×10^5 cells/cm². Examination of the surface by scanning electron microscopy showed that it took more than 1 hour to achieve complete spreading of the cells. An additional period of about 3 hours of perfusion with complete medium was required to achieve a stable confluent layer of endothelial cells.

After the initial attachment period of 1 hour and a subsequent 3-hour period of perfusion, the endothelial cells are still in a somewhat perturbed state as judged by their ability to produce factor Xa when perfused with a mixture of factor VII and factor X. The endothelial cell surface almost completely lost this ability to initiate blood coagulation when the capillary was continuously perfused with medium for at least 18 hours. It is interesting to note that the decay of tissue factor activity is fairly rapid; indeed, a half-life time of about 9 hours has been reported for tissue factor induced in HUVEC by endotoxin.²² Apparently, cell harvesting, attachment and/or spreading induced the exposure of some tissue factor at the surface of the cells lining the capillary. That the factor Xa producing ability of the cells was caused by tissue factor activity was concluded from the observations that factor VII was an essential component and that factor Xa production was abrogated when the capillary was perfused with a mixture of factor VII, factor X, and rTFPI.

We demonstrated that the ability to respond to TNF by synthesis of tissue factor is retained in endothelial cells attached to the inner wall of polyethylene capillary tubes. As in previous studies^{4,5,23} under static conditions, the presence of TNF in the perfusate induced a transient increase in tissue factor activity at the flow surface of the endothelial cells. As yet, it remains to be established what causes the decay of tissue factor activity. Andoh et al²² concluded from their work that the synthesis of a protein with a rather short half-life influences the decay of tissue factor activity.

Of particular importance is the observation that when the cells are stimulated with TNF they are able to produce factor Xa at a constant rate for at least 30 minutes. When TNF is removed from the perfusion medium no further increase or decrease in tissue factor activity was observed. As a matter of fact, the endothelial cells retained their factor Xa producing activity for more than 6 hours. This observation is in an apparent disaccordance with a reported half-life of 9 hours.²² We note that in the latter study HUVEC was grown for several passages in the presence of

endothelial cell growth factor and heparin, whereas we used second passage cells grown in the absence of heparin and ECGF.

Interestingly, the factor Xa production at the surface of TNF-stimulated endothelial cells is rather small when compared with that at the corresponding surface extracellular matrix. Because no tissue factor activity could be detected at the extracellular matrix of control endothelial cells, our finding confirms the notion that the TNF-induced tissue factor activity in endothelial cells is predominantly associated with their extracellular matrix.²⁰ Obviously, our conclusion is based on the assumption that there is a relationship between the steady-state rate of factor Xa production and the surface tissue factor density. We were unable to provide direct proof for the existence of a linear relationship. However, we could demonstrate that the steady-state rate was dependent neither on the length of the capillary nor on the shear rate. Thus, apparently the rate of factor Xa production is not limited by the rate of diffusion of factor X to the surface, but by the surface tissue factor density.

At low shear rates, typical of large veins, and factor VII and factor X concentrations typical of plasma, factor Xa production seems to be proportional with tissue factor activity at the surface of perturbed endothelial cells. Therefore, neutralization of tissue factor activity should be an efficient way to regulate factor Xa. Our experiments with rTFPI, as reported here, showed an almost complete neutralization of tissue factor activity at TFPI concentrations typical of human plasma, ie, $2.2 \text{ nmol}/\text{L}$.²⁴ But it is also demonstrated that during the initial stage of factor Xa generation at the surface of perturbed endothelial cells, rTFPI is a rather inefficient inhibitor, considering that factor Xa could still be produced. In accordance with previous findings^{9,25} we demonstrated that neutralization of tissue factor activity by TFPI requires the presence of factor Xa. Therefore, a significant inhibition of factor Xa generation by rTFPI is seen only after a certain amount of factor Xa has been generated (Fig 7). It was recently suggested by Gemmell et al⁹ that TFPI and factor Xa need not to associate in the fluid phase before their interaction with tissue factor:phosphatidylserine/phosphatidylcholine or tissue factor:phosphatidylcholine surfaces. That is, tissue factor is reported to serve as a high-affinity binding site for factor Xa. This complex then binds TFPI in the absence of factor VIIa when the surface contains phosphatidylserine. However, TFPI binding to factor Xa-tissue factor needs factor VIIa when the surface consists solely of phosphatidylcholine. We observed that when TNF-stimulated endothelial cells were perfused with factor X/factor VII a very stable complex between tissue factor and factor VIIa was formed: the factor X converting activity could not be washed out. However, when the capillary was subsequently perfused with a solution of rTFPI ($2 \text{ nmol}/\text{L}$), hardly any tissue factor activity was neutralized (Fig 6).

Our data also indicate that besides factor Xa, factor VIIa is essential to the formation of the inactive TFPI-tissue factor complex at the surface of endothelial cells stimulated

with TNF and their extracellular matrix. Pre-perfusion with a preformed factor Xa-rTFPI complex was less effective than when factor VII(a) was also present. It is noteworthy that according to the findings of Gemmell et al⁹ a stable complex between TF and TFPI could only be formed in the absence of factor VIIa when the phospholipid membrane contained the negatively charged phospholipid phosphatidylserine (30 mol%). Therefore, it is tempting to speculate that negatively charged phospholipids are not available in

the immediate surrounding of tissue factor exposed on the surface of endothelial cells or extracellular matrix after stimulation with recombinant TNF- α .

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