Activated Factor X and Thrombin Formation Triggered by Tissue Factor on Endothelial Cell Matrix in a Flow Model: Effect of the Tissue Factor Pathway Inhibitor

By Cornelis van 't Veer, Tilman M. Hackeng, Cecile Delahaye, Jan J. Sixma, and Bonno N. Bouma

The procoagulant subcellular matrix of stimulated endothelial cells that contains tissue factor (TF) was used to investigate the mechanism by which TF pathway inhibitor (TFPI) inhibits thrombin formation initiated by TF/factor VIIa (FVIIa) under flow conditions. Purified coagulation factors VII, X, and V and prothrombin were perfused at a wall shear rate of 100 s⁻¹ through a flow chamber containing a coverslip coated with matrix of cultured human umbilical vein endothelial cells. This resulted in a TF- and FVIIa-dependent FXa and thrombin generation as measured in the effluent at the outlet of the system. Inhibition of this TF/FVIIa-triggered thrombin formation by TFPI purified from plasma was dependent on the amount of TF present on the endothelial cell matrix. The rate of prothrombinase assembly and steady-state levels of thrombin formation were decreased by TFPI. Because persistent albeit decreased steady-state levels of thrombin formation occurred in the presence of TFPI, we conclude that plasma-TFPI does not inhibit FXa present in the prothrombinase complex. The addition of FIX and FVIII to perfusates containing FVII and FX increased the FXa generation on endothelial matrices, and counteracted the inhibition of thrombin formation on endothelial cell matrices by TFPI. Our data provide further evidence for the hypothesis that the rapid inactivation of TF/FVIIa by TFPI in combination with the absence of either FVIII or FIX causes the bleeding tendency of patients with hemophilia A or B.

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of the clotting factors IX and VIII on FXa and thrombin generation under flow conditions on endothelial cell matrix in the absence and presence of TFPI.

**MATERIALS AND METHODS**

Freshly frozen citrated plasma and human serum were obtained from the local blood bank. Diethyl aminoethyl (DEAE)-Sepharose CL-6B, Q-Sepharose Fast Flow, CNBr-activated Sepharose, and protein-G Sepharose were from Pharmacia (Uppsala, Sweden). Bovine serum albumin (BSA), phenol-myristic acid (PMA), and soybean trypsin inhibitor (SBTI) were obtained from Sigma (St Louis, MO). S2222 and S2238 were purchased from Chromogenex (Möln达尔, Sweden). Thermanox coverslips were from Flow Laboratories Inc (Woodcock Hill, UK). Purified recombinant tumor necrosis factor (TNF; 10^6 U/mg) was generously provided by Dr P. Lomedico (Hoffman-La Roche, Nutley, NJ). Tissue culture supplies were obtained from GIBCO Biocult (Paisley, UK). Recombinant desulfated hirudin (12,000 U/mg) was kindly provided by Dr R. Wallis (Ciba Geigy, Horsham, UK). All other reagents were of the highest grade available.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting Techniques**

Proteins were subjected to SDS-PAGE (4% to 15% gradient gels) using the Phastsystem from Pharmacia. Gels were silver-stained according to the instructions of the manufacturer. For immunoblotting, proteins were transferred to Immobilon-P membranes (Millipore Corp, Bedford, MA). Membranes were blocked with 50 mM/L Tris, 150 mM/L NaCl, 5% nonfat dry milk, 0.5% Tween, pH 7.4, and then incubated in this solution with specific IgG followed by an incubation with peroxidase-conjugated anti-IgG antibodies (Dako, Glostrup, Denmark).

**Monoclonal Antibody (MoAb) Production**

Murine MoAbs were raised against FVII according to standard procedures using Balb/c mice and the myeloma cell line Ag8653.

**Proteins**

**FVII.** FVII was purified from freshly frozen plasma (4 L) essentially as described by Bajaj et al. All steps were performed at 4°C. The procedure included barium citrate precipitation, ammonium sulfate fractionation, and DEAE-Sepharose chromatography (5 x 40 cm column). FVII activity was measured using a one-stage clotting assay. FVII-containing fractions eluting in the NaCl gradient of the DEAE-Sepharose column were pooled and diluted with 1 vol distilled water and applied to a Q-Sepharose column of 1.5 x 10 cm previously equilibrated in 20 mM/L Tris, 50 mM/L NaCl, pH 7.4. FVII activity was eluted with 20 mM/L CaCl_2 in the same buffer. FVII-containing fractions were pooled; dialyzed against 50 mM/L Tris, 150 mM/L NaCl, pH 7.4 (TBS); and stored at -70°C. A purified FVII preparation was obtained that appeared homogeneous on SDS-PAGE as a 50-kD band under reducing conditions and as a 48-kD band under nonreducing conditions. The FVII preparation was essentially free of activated (two-chain) FVII as seen by immunoblotting of reduced FVII with specific polyclonal rabbit anti-FVII antibodies, kindly provided by Dr K. Mertens (Central Laboratory of the Netherlands Red Cross Blood Transfusion Services, Amsterdam, The Netherlands).

An immunopurification method was developed using an MoAb, 2G3-2c, that was raised against FVII as described above. The antibody was purified from antibody-rich ascites fluid by protein-G Sepharose chromatography. This antibody was coupled to CNBr-activated Sepharose (2 mg/mL) according to the manufacturer's instructions. Citrated plasma containing 10 mmol/L benzamidine was passed over the antibody column. The column was subsequently washed with TBS containing 5 mmol/L EDTA and 1 mmol/L benzamidine. FVII was eluted with 3 mol/L KSCN in TBS and FVII-containing fractions were dialyzed against TBS. FVII was further purified and concentrated using Q-Sepharose chromatography as described above. The immunoaffinity-purified FVII was homogeneous on SDS-PAGE and essentially free of two-chain FVIIa. FVII preparations purified by the multistep or the immuno-affinity procedure had specific activities of approximately 2,000 U/mg protein.

**FX.** FX was purified as described. Traces of activated FX were removed by passing the preparation in TBS over SBTI-Sepharose (150 mg SBTI coupled to 10 mL CNBr-Sepharose) at 4°C. FX was stored at -70°C. FX activity was assayed using a one-stage clotting assay. The specific activity of the purified FX was approximately 100 U/mg protein and it appeared as a single band of 65 kD on SDS-PAGE. FXa was prepared as described.

**TFPI.** TFPI was purified from plasma using an immunopurified polyclonal antibody raised in rabbits against a synthetic peptide consisting of residues 3 through 25 of human TFPI, as described. Fifteen milligrams of immunopurified anti-TFPI IgG was coupled to CNBr-activated Sepharose according to the instructions of the manufacturer. Freshly frozen plasma was mixed in a 1:1 ratio with 50 mM/L Tris, 12 mM/L sodium citrate, 100 mM/L benzamidine, 400 mM/L NaCl, 1% Triton X-100, pH 7.4, and passed over the antibody column. All purification steps were performed at 4°C. After washing the column with the same buffer and buffer without Triton X-100, bound proteins were eluted with 6 M/L glycerine, pH 2.7. Fractions were collected in 0.1 vol of 1 M/L Tris, pH 9, 10 immediately adjust the pH to 7.4. TFPI activity was assayed as described by Sandset et al. TFPI-containing fractions were subjected to mono-Q chromatography in 0.02 M/L Tris, 6 M/L urea, 0.05% Lubrol PX, pH 8.3. TFPI was eluted with 0.5 M/L Tris, 6 M/L urea, 0.05% Lubrol PX, pH 8.3. TFPI-containing fractions were dialyzed against TBS and subjected to FXa-Sepharose chromatography as described by Novotny et al. TFPI was eluted from the FXa column with TBS containing 0.5 M/L benzamidine and TFPI-containing fractions were pooled. After extensive dialysis against TBS, aliquots were stored at -70°C. SDS-PAGE and immunoblotting using rabbit polyclonal anti-TFPI IgG (kindly provided by Dr W. Kiel, University of New Mexico, Albuquerque, NM) or the polyclonal antibody directed against residues 3 through 25 of TFPI showed that this TFPI preparation contained TFPI antigen at 34, 40, and 60 kD and at minor high molecular weight bands. This is in agreement with the TFPI purified from plasma by Novotny et al. Bands stained with the anti-TFPI IgG also reacted with radiolabeled FXa. Ligand blotting with 125I-FXa was performed as described. Some TFPI preparations contained 110- and 150-kD proteins that reacted with peroxidase-conjugated rabbit IgG directed against anti-human IgG (Dakopatts). The TFPI preparations had specific activities of approximately 2,000 to 5,000 U/mg depending on the amount of IgG contamination. The heterogeneity of TFPI in plasma was recently studied by Broze et al. The main conclusion was that TFPI in plasma consists of more than 70% of forms that lack the C-terminal tail or lack the C-terminal tail and a part of the third Kunitz domain. Other forms of TFPI lacked only a small part of the C-terminal tail and forms were present that had an intact C-terminus. The C-terminus of TFPI is involved in the association of TFPI with FXa and heparin and is essential for the anticoagulant action of TFPI in a diluted prothrombin time (PT) clotting assay. To determine the amount of truncated and full-length TFPI in our purified plasma-TFPI preparation, we separated the different forms using heparin-Sepharose chromatography.
TFPI was loaded on a 2.5 mL heparin-sepharose column (Pharmacia) in 50 mmol/L Tris, 10 mmol/L trisodium citrate, pH 8.0, 0.3% BSA. According to Lindahl et al,23 the column was washed with buffer and then sequential step elutions were performed with 0.3, 0.55, and 0.9 mol/L NaCl in Tris buffer. Eighty-seven percent of the TFPI flowed directly through the column, indicating that 87% of the TFPI lacks the C-terminus. Only 13% of the preparation had affinity for heparin-Sepharose, of which only 5% eluted at 0.9 mol/L NaCl, indicating that only 5% of the TFPI in our TFPI preparation contains a totally intact C-terminus. This is consistent with the observation that only a fraction of TFPI in plasma is full-length TFPI.24 In agreement with this, no significant prolongation of the clotting time in a diluted PT coagulation assay25 was observed on addition of 1 U/mL purified TFPI of our preparation.

FV. FV was purified from freshly frozen plasma as described by Kane and Majerus.25 Specific activity was approximately 100 U/mg protein.

Prothrombin. Prothrombin was purified as described.4 It appeared as an 80-kD protein on SDS-PAGE and had a specific activity of 12.5 U/mg protein.

FIX. Purified human FIX was generously provided by Dr K. Mertens. FIX appeared homogeneous on SDS-PAGE as a 55-kD band and had a specific activity of 280 U/mg protein. FIXa was prepared from the zymogen by activation with FXIII as described.5

FVIII. Purified recombinant FVIII was a generous gift of Dr R. J. Kaufman (Genetics Institute Inc, Boston, MA).

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described by Jaffe et al26 in RPMI 1940 supplemented with 20% human serum, penicillin/streptomycin, and fungizone. HUVEC at the second passage after isolation were trypsinized and seeded in culture medium on gelatin-coated, 0.5% glutaraldehyde-fixed Thermanox coverslips of 2 μm high and 5-mm wide, creating a surface of 3 cm² in contact with endothelial cells. Coverslips of 2 μm high and 5-mm wide, creating a surface of 3 cm² in contact with endothelial cells stimulated with 20 ng/mL PMA (PMA-matrix). The culture medium was refreshed a day before the cells were stimulated. Cells were stimulated for 4 hours with the indicated agents in culture medium. After stimulation of the cells, the TF-containing endothelial cell matrix was isolated by removing the cells with 0.1 mol/L NH₄OH as described.27

Perfusion Studies

A rectangular flow chamber was designed based on the flow chamber of Sakariassen et al28 to perform single pass perfusion studies over cellular surfaces. Coverslips of 2 × 6 cm covered with endothelial cell matrix were placed in the flow chamber on top of a groove 0.2 mm high and 5-mm wide, creating a surface of 3 cm² in contact with 60 μL fluid. The flow chamber was placed in a water bath of 37°C and connected at the inlet to a syringe pump and at the outlet to a fraction collector via polyethylene tubes of 0.75 mm internal diameter. The flow profile in the reactor was determined with a laser-doppler technique and was found to be laminar throughout the flow area. The wall shear rate deduced from the parabolic flow profile was in agreement with the formula Q = 1/6h²wy(w), in which Q is the flow rate in microliters per second, h the slit height, w the slit width, and y(w) is the wall shear rate at a given flow rate.29 The laser-doppler technique was adapted to measure velocity flow profiles in small dimension flow chambers (<0.2 mm²).30 After placing the coverslips in the flow chamber, the matrix surface was first washed at a shear rate of 1,000 s⁻¹ (2 mL/min) for 10 minutes with buffer containing 25 mmol/L HEPES, 135 mmol/L NaCl, 4 mmol/L KCl, 3 mmol/L CaCl₂, 0.3% BSA, pH 7.4, before perfusion with coagulation factors in the same buffer. Fractions were collected at the outlet into excess EDTA (final concentration, 50 mmol/L) to prevent further activation of coagulation factors. Concentrations of FXa were determined using the chromogenic substrate S2222. Hydrolysis of the substrate was measured as the change in absorbance at 405 nm using a V-max ELISA-reader (Molecular Devices, Menlo Park, CA). Standard curves were prepared with purified FXa. Thrombin was assayed with S2238; standard curves were obtained with purified human thrombin (generous gift of Dr J.W. Fenton, Albany, NY). For adequate FXa measurement in thrombin-containing fractions, thrombin activity was inhibited by excess desulfato hirudin.

RESULTS

TF Activity on Endothelial Cell Matrix: FVII Dependency

TF activity present on endothelial cell matrix was studied in our flow model using purified FVII and FX. TF activity was estimated by the flux of FXa formation on the matrix (flux is defined as the amount of product formed per time unit per surface area). The activation of FX on the matrix of endothelial cells stimulated with 20 ng/mL PMA (PMA-matrix) at a wall shear rate of 100 s⁻¹ was shown to be FVII-dependent (Fig 1). The saturation rate of TF activity by FVII was the same with 1 or 10 nmol/L FVII present in the perfusate. Saturation of TF activity with 0.1 mmol/L FXII in the perfusate was approached at a decreased rate.

Assembly of Prothrombinase Complex on Endothelial Cell Matrix: FXa Dependency

The assembly of prothrombinase on the endothelial cell matrix was studied by perfusion of already activated FX (FXa) through the flow system in the presence of 1 nmol/L FXV and 1 μmol/L prothrombin. At a concentration of 1 nmol/L FXa, a steady-state flux was reached within ±10 minutes of approximately 10 pmol thrombin/min/cm² on PMA-ma-
FXa and thrombin generation in a flow system

The rate of prothrombinase assembly was dependent on the FXa concentration present in the perfusate (Fig 2). At a concentration of 0.12 nmol/L FXa, the same steady-state level of 10 pmol thrombin/min/cm² was reached after a delay of 20 minutes. Thrombin generation by perfusion of FXa, FV, and prothrombin was the same on matrices of unstimulated, TNF, or PMA stimulated endothelial cells (results not shown).

Thrombin generation on endothelial matrix: FV dependency

The prothrombinase assembly on PMA-matrices by perfusion of 1 nmol/L FVII, 160 nmol/L FX, and 1 μmol/L prothrombin at a shear rate of 100 s⁻¹ was studied in the presence of increasing concentrations of FV. The steady-state levels and time to reach steady-state levels of thrombin formation were independent of the FV concentration from 1 to 4 nmol/L (Fig 3). When FV was omitted from the perfusates, thrombin formation was totally absent. A FV concentration of 1 nmol/L was chosen for further experiments. The thrombin generation at steady-state level probably reflects the maximal thrombin formation on the endothelial matrix limited by the amount of phospholipid surface. An increase in prothrombin concentration did not result in increased levels of thrombin formation.

TF-triggered prothrombinase complex assembly on endothelial cell matrix

To study TF-triggered thrombin formation, we performed perfusions at 100 s⁻¹ with perfusates containing FVII, FX, FV, and prothrombin. In these experiments, FX is activated on the endothelial matrix by the TF/FVIIa complex. Because FV is included in the perfusate, the in situ-formed FXa is able to form the prothrombinase complex with FVa on the phospholipids of the matrix. Endothelial cell matrices with increasing TF activities were obtained by stimulation of endothelial cells with increasing TNF concentrations for 4 hours. A maximal induction of TF deposition in the matrix was obtained by stimulation of the endothelial cells with 20 ng/mL PMA. Perfusion of 1 nmol/L FVII, 160 nmol/L FX, 1 nmoVL FV, and 1 pmol/L prothrombin resulted in FXa steady-state levels of 3, 14, 80, and 435 fmol/min/cm² on matrices of, respectively, control, 2.5 pmol/L TNF, 10 pmol/L TNF, and 20 ng/mL PMA stimulated endothelial cells (Fig 4A). The in situ generation of 3 fmol FXa/min/cm² caused a prothrombinase assembly on control matrices after 6 minutes of perfusion, resulting in a steady-state thrombin generation of 10 pmol thrombin/min/cm² after 13 minutes of perfusion (Fig 4B). This thrombin generation was dependent on TF/FVIIa activity because no thrombin generation was observed when FVII was omitted from the perfusate (Fig 4B). The rate of prothrombinase assembly was increased on 2.5 pmol/L TNF matrices compared with control matrices, and the same steady state of thrombin generation was reached within a shorter time period. A further decrease of the time to steady-state thrombin formation was found on 10 pmol/L TNF matrices. No further increase in the rate of prothrombinase assembly was found on PMA-matrices, indicating that the TF activity on 10 pmol/L TNF is not rate-limiting for the rate of prothrombinase assembly in the flow system under the chosen conditions.

Effect of TFPI on TF-dependent thrombin formation

The effect of TFPI on thrombin formation was studied by perfusion of 1 nmol/L FVII, 160 nmol/L FX, 1 nmol/L FV, and 1 μmol/L prothrombin over endothelial matrices with
different TF activities in the presence of 1 U/mL TFPI. Compared with the situation without TFPI in the perfusate (Fig 4A), FXa generation was inhibited when measured in the effluent at the outlet with chromogenic substrate S2222. Small amounts of FXa generation in the presence of TFPI were detected on PMA-matrices. No FXa generation was detected after perfusion over control and 0.5, 2.5, and 10 pmol/L TNF matrices. Assembly of prothrombinase on these endothelial matrices indicated that some FXa was generated on these matrices in the presence of TFPI. However, the rate of prothrombinase assembly and the steady-state level of thrombin generation was both inhibited. The extent of inhibition is dependent on the TF activity present on the matrices (Fig 5B). No inhibition of the rate of prothrombinase assembly or steady-state level of thrombin generation was found on PMA-matrices (Figs 4B and 5B). Rate of assembly and steady-state level of prothrombinase activity was decreased on 10 pmol/L TNF-matrices. A further decrease was found on 2.5 and 0.5 pmol/L TNF-matrices and prothrombinase activity was totally abolished by TFPI on matrices of unstimulated endothelial cells (Fig 5B). In the presence of TFPI, the level of thrombin generation at steady state is thus dependent on the TF activity present on the matrices. This suggests that after an initial burst of FXa generation, further FXa generation is blocked by TFPI. In the presence of TFPI, it could be possible that a steady-state thrombin generation is the result of a balance between the inhibition of prothrombinase complexes by TFPI and the formation of new prothrombinase complexes. However, this is very unlikely because steady-state thrombin generation in the presence of TFPI is observed independently of the TF activity present on the matrices. Furthermore, TFPI inhibition of FXa in the prothrombinase complex would result in a decrease of prothrombinase activity after prolonged perfusion over matrices with lower TF activities. This was not observed (Fig 5B).

Concentration-Dependent Inhibition by TFPI of TF-Triggered Prothrombinase Assembly on Endothelial Cell Matrix

Perfusion of a mixture of 1 nmol/L FVII, 160 nmol/L FX, 1 nmol/L FV, and 1 μmol/L prothrombin at 100 s⁻¹ over matrices obtained from endothelial cells stimulated with 10 pmol/L TNF (TNF-matrix) resulted in a FXa generation of 30 fmol/min/cm² (Fig 6A) and a thrombin formation of 15 pmol/min/cm² (Fig 6C). The addition of 0.2 U/mL of TFPI to the perfusate abolished the FXa generation almost completely, whereas thrombin formation was not affected. The addition of 1 U/mL of TFPI to the perfusate blocked FXa generation completely and decreased the rate of prothrombinase assembly to a level of 60% of thrombin generation at steady state. A concentration of 2 U/mL of TFPI resulted in a more pronounced decrease of prothrombinase assembly and a decrease of thrombin formation to 25% at steady-state level. The decreased steady-state levels of thrombin formation at 1 and 2 U/mL TFPI indicate that FXa generation by TF/FVIIa is rapidly blocked, otherwise the prothrombinase formation would continue and the maximal steady-state level of thrombin generation would be reached (see Fig 2). The effect of TFPI on thrombin formation by perfusion of FVII, FX, FV, and prothrombin as described above was not observed on PMA-matrices that contain approximately 10 times more TF activity as 10 pmol/L TNF-matrices (Fig 6A and B). The rates of prothrombinase assembly or the steady-state levels of thrombin generation on PMA-matrices were...
only slightly affected by TFPI (Fig 6D), and no concentration dependency was observed. The FXa generation on PMA-matrices in the presence of 0.2 U/mL TFPI was decreased by 85%, reaching the level observed on 10 pmol/L TNF matrices in the absence of TFPI (Fig 6A and B). At 1 and 2 U/mL TFPI, the FXa generation was completely blocked on PMA-matrices, whereas prothrombinase activity was hardly affected at these TFPI concentrations. This indicates that the amount of TF present on PMA-matrices generates enough FXa in the presence of TFPI to saturate prothrombinase activity on the endothelial cell matrix.

**FIXa-Dependent FXa and Thrombin Generation on Endothelial Cell Matrix**

To investigate the generation of FXa by FIXa/FVIIIa activity under flow conditions on endothelial cell matrix, we perfused 160 nmol/L FX, 0.4 nmol/L FVIII, 1 nmol/L FV, and 1 µmol/L prothrombin with different concentrations of FIXa over endothelial cell matrices. The steady state of FXa generation was dependent on the FIXa concentration in the perfusate (Fig 7A). Unlike the TF/FVIIa activity or prothrombinase activity, the FIXa/FVIIIa activity did not accumulate on the matrix surface (Fig 7A). The relatively high dissociation constant of the FIXa/FVIIIa complex on this phospholipid surface results in a balance of the association and dissociation of FIXa/VIIIa complexes and therefore the FXa steady-state generation is dependent on the FIXa concentration in the perfusate. A prolonged lag-phase of the FXa generation occurred when the FIXa concentration was decreased in the perfusate. This probably reflects the activation of FVIII that is necessary for effective FXa generation by FIXa. Thrombin generation occurred at 5, 8, and 13 minutes of perfusion with, respectively, 0.3, 0.03, and 0.003 nmol/L FIXa in the perfusate (Fig 7B). This thrombin generation coincides with the onset of FXa generation (Fig 7A and B). After the onset of FXa generation, the potential sites for prothrombinase activity on the matrix become rapidly saturated. The experiment shown was performed on matrices of endothelial cells that were stimulated with 2.5 pmol/L TNF; the same results were obtained with matrices of unstimulated or 10 pmol/L TNF endothelial cells.

**TF-Dependent FXa and Thrombin Generation in the Presence of FIX and FVIII**

To investigate the effect of FIX and FVIII on thrombin generation initiated by TF/FVIIa activity, the FIX preparation was first tested for the presence of activated FIX. We therefore perfused FIX and FVIII at their plasma concentration, defined as 1 U/mL, which is 80 nmol/L for FIX and 0.4 nmol/L for FVIII, together with 160 nmol/L FX, 1 nmol/L FV, and 1 µmol/L prothrombin over endothelial cell matrices in the presence or absence of FVII. The experiment shown in Fig 8 was performed on 2.5 pmol/L TNF-matrices. FXa and thrombin generation occurred after 5 minutes of perfusion in the presence of 1 nmol/L FVII and a steady-state thrombin generation of 10 pmoL/min/cm² was reached after 10 minutes of perfusion. In the absence of FVII, thrombin generation was detectable after 15 minutes of perfusion. The major part of this thrombin generation is caused by traces of FVII in one of the protein preparations because this thrombin generation was inhibited by pretreatment of the matrix with an MoAb against human TF (3 µg/mL, kindly provided by Dr Y. Nemerson, New York, NY) in combination with the addition of immunopurified polyclonal rabbit IgG directed against human FVII (5 µg/mL, generous gift of Dr W. Kisiel, University of New Mexico, Albuquerque,
Fig 6. FXa generation at 100 s⁻¹ on 10 pmol/L TNF-matrix (A) and PMA-matrix (B) with 0 (⌀), 0.2 (■), 1 (▲), and 2 (●) U/ml TFPI added to perfusates containing 1 nmol/L FVIII, 160 nmol/L FX, 1 nmol/L FV, and 1 μmol/l prothrombin. Thrombin generation was detected in the same experiment on 10 pmol/L TNF-matrix (C) and PMA-matrix (D) in the presence of 0 (⌀), 0.2 (■), 1 (▲), and 2 (●) U/ml TFPI.

Fig 7. FXa-triggered FXa and thrombin generation on endothelial cell matrix. (A) FXa generation after perfusion of 160 nmol/L FX, 1 nmol/L FV, and 1 μmol/L prothrombin in the presence of varying FXa concentrations (indicated in the figure). (B) Thrombin generation in the same experiment shown in (A).
increase in thrombin generation. However, the inhibition of thrombin generation on 10 pmol/L TNF-matrix by 1 U/mL TFPI was completely abolished in the presence of FVIII and FIX (Fig 10).

Although this is not detectable in the effluent at the outlet (Fig 9), these results suggest that in the presence of 1 U/mL TFPI additional FXa is generated on the endothelial cell matrix by FIXa/FVIIa activity.

**DISCUSSION**

To study the activation of coagulation factors under flow conditions, a rectangular flow chamber was used to perform single pass perfusions with purified coagulation factors over procoagulant endothelial cell matrices. Clotting factors were perfused over the matrices, fractions were collected, and FXa or thrombin was determined in the effluent. The constant supply and removal of reactants by the fluid flow allows estimations of regulation of coagulation under more physiologic conditions.

TF activity on the endothelial cell matrices was saturated in time by perfusion with 0.1 nmol/L FVII and FX. The low dissociation constant of the TF/FVII(a) complex (10^-7 mol/L) results in the saturation of TF activity by the constant delivery of FVII by the flow. These results are in agreement with the results obtained with recombinant TF inserted into model membranes in glass capillaries. TF/FVIIa activity on the endothelial cell matrices was inhibited by TFPI purified from plasma to the same extent as recombinant TFPI or TFPI purified from Hep-G2 cell culture supernatant in similar flow systems.

The concentration of FXa in the perfusate that eventually saturated the prothrombinase activity on the matrix was in the subnanomolar range (±0.1 nmol/L; Fig 2), indicating a very low dissociation constant of the prothrombinase complex on the phospholipids present in the endothelial cell matrix. This is in agreement with the k_d in the picomolar range found for FXa on negatively charged phospholipid surfaces in the presence of FVIII.

By perfusion of a mixture of the clotting factors FVII, FX, FV, and prothrombin over endothelial cell matrices, a TF-dependent thrombin generation by in situ-generated FXa was obtained (Fig 4B). The ability of TFPI to inhibit the formation of TF-triggered prothrombinase activity on endothelial cell matrix was dependent on the TF activity on the matrix. Because persistent albeit decreased steady-state levels of thrombin formation occurred in the presence of TFPI, we conclude that plasma-TFPI does not inhibit FXa present in the prothrombinase complex. Studying thrombin formation in a flow system, Schoen et al.18 found that once the prothrombinase complex is formed it hardly dissociates in a purified system and withstands low shear forces. This explains the steady-state levels of thrombin generation observed in the presence of TFPI. Our experiments were performed with TFPI purified from plasma that predominantly contains the form that lacks the C-terminal region that is essential for the anticoagulant function of TFPI in a diluted PT clotting assay30 and for the fast inactivation of FXa.29,41 Full-length TFPI is able to inhibit prothrombinase activity in a purified model in which thrombin generation is started...
by addition of FXa to a mixture of phospholipid vesicles, FVa, prothrombin, and TFPI. This does not necessarily mean that full-length TFPI inhibits already formed prothrombinase because the observed inhibition of prothrombinase activity may be caused by the inhibition of assembly of prothrombinase complexes by TFPI. Interestingly, however, phospholipids and FVa were found to accelerate the initial collision of FXa and full-length TFPI. Our results suggest that plasma TFPI, which is truncated at its C-terminus, is unable to inhibit or dissociate prothrombinase complexes. It would be of interest to compare full-length TFPI and plasma TFPI in our model.

The addition of the antihemophilic FIX and FVIII to the perfusate totally counteracted the inhibition of thrombin formation by 1 U/mL TFPI on 10 pmol/L TNF-matrices. This indicates effective FX conversion in the presence of 1 U/mL TFPI on the endothelial cell matrix by FXa/FVIIIa activity, which results in maximal prothrombinase activity on the matrix (Fig 10). Our data provide further evidence for the hypothesis that the failure of the hemostatic response on injury in hemophilia A or B patients is caused by the rapid inactivation of low concentrations of TF by TFPI. This hypothesis was supported by the flow studies of Repke et al., which were performed in glass capillaries with high concentrations of recombinant TF inserted in model membranes containing 30% phosphatidylserine, an artificial highly procoagulant surface. We used endothelial cell matrices for our experiments, and although these matrices were isolated by removal of the cells with 0.1 M NH4OH, it is clear that TF is inserted in these matrices in a spectrum of phospholipids on which the procoagulant pathway can be triggered in vivo.

A disturbed fibrin formation and platelet activation in the center of hemostatic plugs at the end of transected small venules and arterioles of skin wounds was observed in hemophilia A patients. The hemostatic plug in hemophilia A patients consisted of a peripheral layer of fibrin fibers interspersed with empty platelets surrounding a central area consisting of red blood cells and leukocytes. The central area of hemostatic plugs in normal subjects consisted of empty platelets with thick fibrin fibers between them. The experiments performed in the present study show that thrombin formation on a physiologic procoagulant surface containing relatively low concentrations of TF becomes limited by the
action of TFPI in the absence of either FIX or FVIII. The fibrin formation at the peripheral area of hemostatic plugs in hemophilia A patients is probably generated by the little burst of FXa formed directly by TF/FVIIa activity before TF/FVIIa is quenched by TFPI. Of interest is also that platelets release TFPI upon stimulation, which may result in a threefold increase of the local concentration of TFPI at the site of aggregating platelets. The mass of fibrin formed in the center of the hemostatic plug in normal subjects seems to be generated via FXa produced by FIXa/VIIa activity. According to this hypothesis, treatment of patients suffering from hemophilia with agents that block the function of TFPI should be possible. In agreement with this, it was recently found that inhibition of TFPI shortens the bleeding in rabbits with antibody-induced hemophilia A.\(^5\) Our results suggest that TFPI is the inhibitor that controls prothrombinase formation in such a way that thrombin formation is related to the amount of TF exposed to the blood. After prothrombinase assembly has been limited by the action of TFPI, other inhibitors are needed to disrupt the prothrombinase complex. Because FXa is also protected in the prothrombinase complex against inactivation by antithrombin-III, this is probably the function of the combined action of activated protein C and protein S. Protein S serves in this mechanism as a cofactor that disrupts the protective effect of FXa for FVa for the inactivation by activated protein C.\(^46\)\(^47\) Upon degradation of FVa by activated protein C, FXa loses its high-affinity binding site on a phospholipid surface and may dissociate into solution. The fate of unbound FXa is then probably removal by the fluid flow and/or inactivation either by antithrombin-III, TFPI, or α2-macroglobulin.

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**REFERENCES**

Activated factor X and thrombin formation triggered by tissue factor on endothelial cell matrix in a flow model: effect of the tissue factor pathway inhibitor

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