Relationship Between Tissue Factor Expression and Deposition of Fibrin, Platelets, and Leukocytes on Cultured Endothelial Cells Under Venous Blood Flow Conditions

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Endothelial cell-mediated coagulation and leukocyte adhesion are processes that might be connected by the generation of thrombin. To examine the interaction of procoagulant and proadhesive activity, cultures of endothelial cells were stimulated with tumor necrosis factor-α, which result in the surface expression of tissue factor. Subsequent exposure to human nonanticoagulated blood at a shear rate of 100 s⁻¹ in a parallel plate perfusion device led to the deposition of polymerized fibrin, which covered 63% of the endothelial surface. In addition, numerous platelet aggregates (71 ± 10 mm cross-section) and leukocytes (53 ± 6 mm²) were deposited on stimulated endothelial cells, whereas no fibrin and only a few platelet aggregates (4 ± 1 per 10 mm cross-section) and leukocytes (6 ± 1 mm²) were detected on control cells. A significant portion of the adherent leukocytes bind to fibrin and platelets, however, when the deposition of fibrin and platelet aggregates was inhibited with the anti-tissue factor antibody HTFI-7BB by 100% and 86%, respectively, leukocyte adherence remained unchanged (68 ± 6/mm²). This indicated that leukocytes could efficiently adhere to endothelial cells through direct cell-cell contact independent of both thrombin and deposited fibrin. Moreover, this direct adhesion of leukocytes to the endothelial surface was reduced twofold to threefold when fibrin deposition occurred. These data suggest a relationship between endothelial procoagulant and proadhesive properties in that tissue factor-initiated coagulation may contribute to leukocyte adhesion through the formation of an adhesive fibrin/platelet meshwork but concurrently prevents the adhesive endothelial surface to bind leukocytes at its full capacity.

Most studies on the biologic activity of tissue factor were conducted under static conditions. However, wall shear forces that prevail under physiologic conditions can strongly influence coagulation, as well as platelet thrombus formation and leukocyte adhesion. Only recently was the function of tissue factor in hemostasis studied under flow conditions using subendothelium, stimulated endothelial cells and their extracellular matrix, and purified tissue factor in a flow reactor system. We also showed previously that the surface of stimulated endothelial cells elicits a hemostatic response when exposed to flowing nonanticoagulated human blood. In extending these studies, we examined the relationship of endothelial cell surface-expressed tissue factor and the deposition of three main constituents of thrombi, ie, fibrin, platelets, and leukocytes, by using nonanticoagulated human blood at venous flow conditions. Experiments were performed by a parallel plate perfusion chamber developed for studying thrombogenesis ex vivo on collagen-coated surfaces. In place of collagen, we used monolayers of TNF-α-stimulated cultured endothelial cells that were exposed to nonanticoagulated human blood at a shear rate of 100 s⁻¹, simulating flow conditions in large veins. We found that deposition of fibrin and platelets on the endothelial surface was dependent on tissue factor, which was expressed on the endothelial surface upon TNF-α stimulation. Most of the adherent platelets were in aggregated form and bound to the fibrin layer, indicating a central role of thrombin in this process. The fibrin/platelet meshwork supported leukocyte adhesion, although leukocytes also adhered to the surface of endothelial cells by a mechanism that was independent of the tissue factor pathway and apparently involved direct cell-cell interaction. Moreover, this adhesion mechanism, probably mediated by endothelial cell adhesion receptors, was significantly inhibited when fibrin deposition occurred, indicating that endothelial procoagulant activity may affect endothelial proadhesive properties.

MATERIALS AND METHODS

Cell culture. Human endothelial cells were isolated from umbilical veins using a 0.1% collagenase solution (CLE; Worthington,
Cells were cultured in gelatine-coated flasks in medium M199 (Sigma, St Louis, MO) supplemented with 20% newborn calf serum (NGS; Gibco, Basel, Switzerland), penicillin, streptomycin, glutamine (GIBCO), 50 µg/mL endothelial cell growth factor (ECGF; Collaborative Research, Bedford, MA), and 100 µg/mL heparin (Sigma). Confluent cultures acquired the typical cobblestone morphology and examination by immunofluorescence showed that the cells were expressing von Willebrand factor antigen (polyclonal antisera directed against factor VIII-related antigen; Dakopatts, Glostrup, Denmark). The monoclonal antifibrin antibody was from American Diagnostica (Greenwich, CT). The specificity was verified in an enzyme-linked immunosorbent assay (ELISA) showing that this antibody did not recognize fibrinogen, but reacted strongly with fibrin monomers after fibrinogen was proteolytically cleaved by thrombin.

### Microdensitometry of immunogold/silver-stained fibrin.

The coverslips were incubated for 30 minutes at 37°C with 2.5 µg/mL of monoclonal antifibrin antibody in PBS. The secondary antibody was a 5 nm gold-labeled antimouse antibody (Auro Probe LM; Amersham, Amersham, UK; 1:50 dilution in PBS). After fixation with 2% glutaraldehyde in PBS, the coverslips were washed with H2O and treated for 10 minutes with silver enhancer IntenseM (Amersham), washed with H2O, fixed (Rapidfix; Kodak, Rochester, NY), and air dried. After embedding in Merckoglass (Merck, Darmstadt, Germany), the relative optical density of the silver-stained fibrin meshwork was measured with a computerized image analysis system consisting of a Zeiss Axioshot microscope equipped with a camera connected to a PC using the MCID program (Imaging Research Inc, Ontario, Canada). The system provided a resolution of 512 x 512 pixels and 256 grey level numbers. For each coverslip, an individual background level was determined and subtracted from the actual value obtained from two measurements of areas of 1 mm² in the center of the coverslip.

### Immunohistochemical detection of tissue factor on endothelial cells.

Human endothelial cells were grown in 96-well plates (Falcon Microtest III; Becton Dickinson, Lincoln Park, NJ) precoated with 1% gelatine. After reaching confluency, the cells were incubated for increasing time periods with 600 µmol/L TNNf-a in M199 containing 10% NCS, 50 µg/mL ECGF, and 100 µg/mL heparin. The coverslips were washed with M199-0.25% BSA and positioned in a parallel plate perfusion chamber filled with M199 prewarmed at 37°C. Blood was drawn with M199-0.25% BSA and positioned in a parallel plate perfusion buffer for 40 seconds (for immunochemical staining of tissue factor). The blood flow rate was adjusted to 10 mL/min, which resulted in a wall shear rate of 100 s⁻¹ at the endothelial cell layer on the coverslip. This shear rate reflects flow conditions in veins and favors fibrin-rich clot formation. After a 3-minute perfusion period, the coverslips were washed and fixed in the chamber by perfusing with prewarmed M199 for 20 seconds, followed by 2.5% paraformaldehyde in phosphate-buffered saline (PBS) for 40 seconds (for immunohistochemical staining of fibrin and leukocytes). For morphometrical examinations, the coverslips were fixed for 40 seconds with 2.5% glutaraldehyde in 0.1% cacodylate buffer, pH 7.4, containing 2.5 mmol/L CaCl₂ and 0.9 mmol/L MgCl₂. The coverslips were removed from the chamber and incubated in fresh fixative for an additional 30 minutes and stored in PBS until further processing for morphometrical determinations and microdensitometry.

### Morphometrical determination of fibrin, platelet aggregates, and leukocytes on semi-thin sections.

The coverslips were embedded in Epon (Fluka Chemie, Buchs, Switzerland). Semi-thin sections perpendicular to the blood flow were prepared as described previously. After staining with 0.1% toluidine blue and 0.01% fuchsin, the semi-thin sections were analyzed for fibrin deposition on the endothelial surface by determining the presence or absence of fibrin at 10 µm intervals along the length of the section. The adhesion of single platelets and platelet aggregates was measured by counting their number along the length (8 mm) of the cross-section. Platelet aggregates were defined as two or more cohesive platelets that were in contact with fibrin, the endothelial surface, and leukocytes. Leukocyte adhesion was separated into two categories: (1) leukocytes adhering directly to the endothelial surface in the absence of fibrin and platelets (up to a distance of 5 µm from the endothelial surface) and (2) leukocytes adhering to fibrin and platelets. The total number of leukocytes in each category was counted along the length of the semi-thin section (8 mm).
Fibrinopeptide A levels were measured at the perfusion chamber exit (distal). After flowing through the chamber containing the differently treated endothelial cell monolayers or uncoated plastic coverslips, the blood was collected into a mixture of anticoagulants (32 mg/mL Tris/citrate, 1,000 IU/mL heparin, 1 TIU/mL aprotinin) to prevent further generation of fibrinopeptide A. To mix the flowing blood with the anticoagulants, we used a mixing device consisting of a plexiglas chamber in which blood and anticoagulants were mixed (10:1) by a magnetic stirrer. After discarding the first 9 mL, the blood was continuously collected over the whole perfusion period (3 minutes). The flow rate of the blood in the perfusion chamber (10 mL/min) was controlled by a roller pump located distally to the mixing device. The anticoagulant mixture was infused into the mixing device at a flow rate of 1 mL/min by a roller pump. The flow conditions and shear rates on the endothelial cells were identical to those described for the perfusion experiments. The fibrinopeptide A concentrations in the collected blood were determined according to the manufacturer's instructions (ELISA FPA; Boehringer Mannheim GmbH, Mannheim, Germany).

RESULTS

Fibrin deposition on endothelial cells. Exposure of TNF-α-stimulated endothelial cells to nonanticoagulated blood at a shear rate of 100 s⁻¹ resulted in the deposition of significant amounts of fibrin on the cell surface as determined by micromethods of immunogold/silver-stained fibrin (Fig 1). Inspection of semi-thin sections demonstrated that the cell layer remained intact under all experimental conditions (Table 1), suggesting that the procoagulant activity was cell surface mediated. Unstimulated cells were virtually noncoagulant, as shown by the absence of any detectable fibrin. In accordance, tissue factor was not detectable on the surface of the noncoagulant control cells using immunochmical detection with anti-tissue factor antibody and the biotin-streptavidin system. However, TNF-α treatment time-dependently induced surface expression of tissue factor, reaching maximal values after 4 to 5 hours when perfusion experiments were performed (data not shown).

To evaluate the importance of tissue factor for fibrin generation, TNF-α-stimulated cells were treated with the monoclonal anti-tissue factor antibody HTFI-7B8 that inhibits the complex formation with factor VIIa. As shown in Fig 1, HTFI-7B8 at 5 μg/mL completely prevented fibrin deposition, whereas incubation with a control antibody had no significant inhibitory effect as compared with TNF-α-treated cells (Student's t-test). In a different set of experiments, semi-thin sections of the coverslips were morphometrically examined after toluidine blue/fuchsin staining. In agreement with the immunogold/silver-staining method, we found that HTFI-7B8 at 5 μg/mL reduced the fibrin coverage of stimulated endothelial cells from over 63% to less than 1% (Table 1). In the presence of control antibody, the fibrin deposited on the cells was not reduced but was even slightly increased, although statistically not significant, as compared with TNF-α-stimulated endothelial cells without antibody treatment (Student's t-test). In addition, the levels of fibrinopeptide A were measured in the blood leaving the perfusion chamber that was collected over the whole perfusion period. TNF-α stimulation dramatically increased the fibrinopeptide A levels from 5.0 ± 0.2 ng/mL (n = 8) of controls to 80.9 ± 7.5 ng/mL (n = 9). HTFI-7B8 at 5 μg/mL reduced fibrinopeptide A levels to 16.7 ± 6.4 ng/mL (n = 4), whereas the value obtained with the control antibody, ie, 63.3 ± 17 ng/mL (n = 3), was similar to the TNF-α value (Student's t-test; not significant from TNF-α value). Perfusion over uncoated plastic coverslips resulted in a fibrinopeptide A level of 5.1 ± 0.5 ng/mL (n = 4).

Figure 2A shows the appearance of the immunogold/silver-stained fibrin network overlaying the monolayer of TNF-α-stimulated endothelial cells. The shear force strongly affected the structure of the fibrin network in that most of the fibers were aligned along the direction of the blood flow (left to right). Numerous accumulations of fibrin were found throughout the coverslip and were also seen in cross-sections. Figure 2B illustrates the complex cellular interactions within the fibrin network in that platelets interacted with fibrin fibers.

![Fig 1](http://www.bloodjournal.org)
directly or, in some cases, indirectly via leukocytes. Leukocytes were found to be associated with fibrin (Fig 2B) or with large platelet aggregates (not shown). In contrast, on HTFI-7B8–treated endothelial cells no fibrin deposition or fibrin-dependent cell interactions were observed (Fig 2C).

Deposition of platelets on endothelial cells. Platelet deposition was measured by morphometry on toluidine blue/fuchsin-stained cross-section. The results showed that TNF-α activation of endothelial cells increased the number of platelet aggregates per 10 mm cross-section from 4 to more than 70. When cells were pretreated with HTFI-7B8, the deposition of platelet aggregates was inhibited by 86%, whereas a control antibody did not exert this inhibitory effect and even slightly increased the platelet deposition (Table 1). On TNF-α– and control antibody-treated endothelial cells, about 90% of the platelet aggregates were attached to the fibrin layer and about 10% to the cell surface. But on nonstimulated and HTFI-7B8–treated endothelial cells, all platelet aggregates were surface associated (data not shown). Furthermore, under all experimental conditions only a few single platelets were detected. The apparent correlation between fibrin and platelet deposition might explain the increased platelet deposition in the presence of control antibody because determinations on the same coverslips also showed a slight increase in fibrin deposition (Table 1).

Leukocyte adhesion to endothelial cells. The role of the fibrin layer and the endothelial surface in leukocyte adhesion was examined using anti-tissue factor antibody HTFI-7B8 as a tool to differentiate between leukocyte adhesion in the absence and presence of cell-bound fibrin. The total number of adherent leukocytes was determined in a defined area of the endothelial cell layer in the center of the coverslip. Figure 3 shows that 68 ± 6 leukocytes/mm² adhered to TNF-α–stimulated cells that were treated with HTFI-7B8 antibody to prevent fibrin deposition. We expected that the deposition of fibrin on the endothelial surface would provide an additional adhesive surface resulting in an increased number of adhering leukocytes. However, the number of leukocytes was about the same (53 ± 6/mm²). In contrast, only 6 ± 1 leukocytes/mm² adhered to nonstimulated endothelial cells. Further examination on semi-thin sections showed that leukocytes adhering to TNF-α–stimulated endothelial cells incubated with HTFI-7B8 interacted with the endothelial cells through direct cell-cell contact (Table 2). Figure 2C exemplifies the apparently direct leukocyte-endothelial cell interaction and additionally shows that some leukocytes were already in the process of spreading and transmigration. In contrast, only about 56% of all leukocytes found on TNF-α–stimulated endothelial cells were adhering through direct cell-cell contact (Table 2). A significant portion of leukocytes indirectly bound to the endothelial cell layer by adhering to deposited fibrin or platelet aggregates (Table 2). Thus, the adhesion of leukocytes to stimulated endothelial cells through cell-cell interaction was significantly less in the presence of deposited fibrin (10 per 10 mm cross-section) as compared with when fibrin was absent (26 per 10 mm cross-section). The few leukocytes that adhered to nonstimulated endothelial cells all appeared to be in direct cell-cell contact (Table 2).

To show that the leukocytes were strongly and irreversibly attached to the endothelial cells, the washing period after blood perfusion was extended from 20 to 60 seconds at the same flow rate (10 mL/min). Under these conditions, about 54 leukocytes/mm² (n = 3) adhered to stimulated and HTFI-7B8–treated (40 μg/mL) endothelial cells, suggesting that the leukocytes specifically attached to the endothelial cell layer. Furthermore, leukocytes expressing the neutrophil/monocyte surface marker CD15 were detected by immunohistochemical staining using an anti-CD15 antibody. The number of CD15+ leukocytes per mm² were 4.4 ± 2.5 (n = 6), 43.8 ± 9.8 (n = 7), and 59.7 ± 5.5 (n = 5) on nonstimulated endothelial cells, TNF-α–stimulated cells, and TNF-α–stimulated cells incubated with HTFI-7B8, respectively. Figure 4 illustrates the adhesion of CD15+ leukocytes to endothelial cells under different experimental conditions. Whereas leukocyte adhesion to nonstimulated endothelial cells was virtually absent, CD15+ leukocytes avidly adhered to stimulated endothelial cells. A significant proportion of the CD15+ leukocytes was contained in the thrombi, as depicted in Fig 4B.

**DISCUSSION**

This study shows that under venous blood flow conditions the expression of tissue factor on the endothelial cell surface induced the deposition of fibrin and platelet aggregates. In addition, the platelet-containing fibrin layer supported leukocyte adhesion. Independent of tissue factor-initiated coagulation, leukocytes also adhered to the stimulated endothelial cells by direct cell-cell interaction. Inhibition studies with an anti-tissue factor antibody showed that this cell-cell interaction mechanism was significantly inhibited when tissue factor-mediated fibrin and platelet deposition occurred, indicating that endothelial procoagulant and proadhesive activities may be linked functionally.

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**Table 1. Fibrin and Platelet Aggregates on TNF-α–Stimulated Endothelial Cells After Exposure to Flowing Blood at a Shear Rate of 100 s⁻¹**

<table>
<thead>
<tr>
<th>No. of Experiments</th>
<th>% Coverage With Endothelial Cells</th>
<th>% Coverage With Fibrin</th>
<th>Platelet Aggregates per 10 mm Cross-Section</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>99.6 ± 0.2</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>6</td>
<td>99.7 ± 0.2</td>
<td>63.3 ± 12.3</td>
</tr>
<tr>
<td>TNF-α + HTFI-7B8*</td>
<td>6</td>
<td>98.6 ± 1.3</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>TNF-α + control Ab*</td>
<td>6</td>
<td>94.4 ± 3.1</td>
<td>78.3 ± 7.5</td>
</tr>
</tbody>
</table>

Values represent the mean ± SEM.

* TNF-α (600 pmol/L)-treated endothelial cell monolayers were incubated for 30 minutes with 5 μg/mL of anti-tissue factor antibody HTFI-7B8 or a control antibody before blood perfusion.
The experimental design allowed us to measure the endothelial procoagulant response to its full extent because no anticoagulants were present. We found that TNF-α treatment resulted in the deposition of polymerized fibrin, which covered 63% of the endothelial cell surface after 3 minutes of perfusion. Thrombin generation that might have occurred during the passage of the blood from the donor vein to the endothelial cells in the chamber was insignificant as neither

Fig 2. Photographs of endothelial cells and the fibrin meshwork after blood perfusion at a shear rate of 100 s⁻¹ for 3 minutes. (A) En face preparation of TNF-α-treated endothelial cells. Fibrin fibers are overlaying the endothelial monolayer, which is not visible. The fibers were visualized using a fibrin-specific monoclonal antibody in conjunction with a secondary, gold-labeled antimouse antibody and silver enhancement. (Original magnification X 1,200). (B) Two semi-thin sections of TNF-α-stimulated endothelial cells after staining with toluidine blue/fuchsin solution; (C) is the same as (B), but with prior treatment of anti-tissue factor antibody HTFI-78B. Bar = 10 μm. F, fibrin; P, single platelets; P’, platelet aggregates; L, leukocytes; L*, leukocyte in process of transmigration.
that platelet activation had occurred. In contrast, inhibition of tissue factor activity with HTFI-7B8 antibody resulted in a dramatic reduction of deposited platelet aggregates. These results indicated that tissue factor-dependent formation of thrombin, which can activate platelets as well as induce fibrin deposition, is important for the adhesion of platelets to TNF-α-stimulated endothelial cells. To further support this conclusion, about 90% of the platelet aggregates bound to fibrin and only a few nonaggregated single platelets were detected on stimulated endothelial cells. In agreement, Hantgan et al demonstrated that under similar low shear conditions platelets can adhere to polymerized fibrin, but only when they are activated. Moreover, by providing binding sites for coagulation factors, the adherent platelets might have further enhanced thrombin generation and subsequent fibrin deposition.

Analysis of the different adhesion mechanisms on semithin sections showed that on stimulated endothelial cells about 44% of the leukocytes bound to deposited fibrin and platelets. This is in accordance with the ascribed role of fibrin as an adhesin substrate for leukocytes and with the reported receptor-mediated binding of neutrophils and monocytes to activated platelets. The results also agree well with earlier findings showing that the number of adherent leukocytes increased with increasing fibrin coverage in the same perfusion model, except that collagen was used as a thrombogenic surface.

The number of leukocytes (53/mm²) adhering to stimulated endothelial cells was the sum of leukocytes indirectly adhering to the endothelial surface via fibrin or platelets and directly adhering through cell-cell contact. Therefore, it was surprising to find that a similar number of leukocytes (68/mm²) adhered to stimulated endothelial cells when thrombogenic surface was created by tissue factor-dependent formation of thrombin, which can activate platelets as well as induce fibrin deposition.

Table 2. Leukocyte Adhesion to the Endothelial Cell Layer via Direct Cell-Cell Interaction or Indirectly via Fibrin/Platelet Interaction

<table>
<thead>
<tr>
<th>Cell-Cell Interaction (leukocytes per 10 mm² cross-section)</th>
<th>Fibrin/Platelet Interaction (leukocytes per 10 mm² cross-section)</th>
<th>Total (leukocytes per 10 mm² cross-section)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.8 ± 1.2</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>10.1 ± 3.4</td>
<td>7.8 ± 1.1</td>
</tr>
<tr>
<td>Control + HTFI-7B8</td>
<td>26.5 ± 3.5</td>
<td>0.1 ± 0.1</td>
</tr>
</tbody>
</table>

Values represent the mean ± SEM of 13 (control) and 12 (TNF-α and TNF-α + HTFI-7B8) coverslips.
As shown on semi-thin sections, the interaction of leukocytes with HTFI-7B8-treated endothelial cells apparently occurred through direct cell-cell contact most likely involving some of the recently described endothelial cell adhesion molecules,6,7 which are upregulated by TNF-α.

Immunochemical staining showed that most of the leukocytes that adhered to the endothelial cells were CD15+, indicative of the presence of neutrophils and/or monocytes, which is in agreement with the reported increase in neutrophil adhesion to cytokine-stimulated endothelium under static and flow conditions.23,24,58-60 This presumed adhesion receptor-mediated leukocyte adhesion was strongly influenced by the tissue factor-controlled procoagulant activity of endothelial cells, as suggested by the twofold to threefold reduced leukocyte adhesion in the presence of fibrin (Table 2). It remains unclear how fibrin deposition influenced the leukocyte-endothelium interaction, but it is possible that the rapid fibrin deposition on the endothelial cell layer simply reduced the access of leukocytes to the endothelial surface, thereby limiting surface-mediated leukocyte adhesion.

In this report, we demonstrated that inhibition of endothelial tissue factor activity dramatically reduced fibrin and platelet deposition in an ex vivo flow system with human nonanticoagulated blood. This agrees well with studies of
animal models of disseminated intravascular coagulation showing that fibrin formation could be inhibited by administering anti-tissue factor antibodies.\textsuperscript{61,62} Furthermore, our results suggested that whereas tissue factor-initiated generation of thrombin on TNF-\textalpha-stimulated endothelial cells was important for the deposition of fibrin and platelets, it was not a prerequisite for leukocyte adhesion to the endothelial cell surface. Therefore, TNF-\textalpha stimulation resulted in a persisting leukocyte adhesion despite prevention of endothelial cell procoagulant activity. Likewise, in a rabbit venous thrombosis model, leukocyte adhesion to endothelium continued after fibrin formation was inhibited by administering heparin as an anticoagulant.\textsuperscript{63} The results further showed that this presumable cell surface receptor-mediated leukocyte adhesion was not operative at its full capacity when fibrin deposition occurred. This indicated that under venous flow conditions the TNF-\textalpha-induced adhesive properties of the endothelial surface can be influenced by tissue factor-dependent coagulation.

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