Thrombogenicity of Vascular Cells
Comparison between Endothelial Cells Isolated from Different Sources and Smooth Muscle Cells and Fibroblasts


When the endothelial cell layer is damaged, a thrombotic reaction starts on the cells' subendothelium and on the connective tissue deposited by smooth muscle cells in the deeper layers. When more severe vascular damage occurs, hemostasis will involve the vessel adventitia in which fibroblasts are found. In this article, the influence of in vitro cultured endothelial cells, smooth muscle cells, and fibroblasts on the hemostatic balance was studied. To do so, perfusions were performed with low molecular weight heparin anticoagulated blood over the extracellular matrix of the cells. This method allowed the study of tissue factor-dependent thrombin generation and its influence on formation of fibrin and platelet aggregates. The experiments described in this article show that endothelial cells isolated from different human organs interfere differently in the hemostatic response. Endothelial cells isolated from umbilical veins are nonthrombogenic; they do not synthesize tissue factor under unstimulated conditions. On their extracellular matrix, only adherent platelets are found, but no aggregates and no fibrin. Endothelial cells isolated from omentum and atrium contain tissue factor activity under unstimulated conditions. As a consequence, thrombin is generated on their surfaces, and platelet aggregates and fibrin deposition are found on the extracellular matrices after perfusions with whole blood. The matrix of smooth muscle cells and fibroblasts behaved similarly. Increase in shear rate and perfusion time resulted in an increase in platelet aggregate formation. Polymerized fibrin deposition decreased when perfusions were performed at higher shear. Both platelet aggregation and fibrin deposition were tissue factor dependent and could be blocked more than 70% by an antibody against tissue factor. Based on these results, we conclude that endothelial cells isolated from umbilical veins form the best nonthrombogenic surface in vitro. Moreover, coagulation-dependent hemostasis should be included when thrombogenicity of subendothelium is discussed, especially when it concerns matrix derived from cells present in the deeper layer of the vessel wall.

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The participation of human endothelial cells isolated from umbilical cord vein in the regulation of the hemostatic balance has been extensively studied. In contrast, little is known about the hemostatic response of human endothelial cells isolated from large vessels or microvascular endothelial cells. Also, little attention has been paid to the role of adhesive proteins and thromboplastin synthesized by SMC and Fb in the formation of a hemostatic plug.

In this article, we have studied the contribution of cultured endothelial cells, SMC, and Fb in the hemostatic balance. Our main interest was in the vascular cells' matrix. In vascular injury, this connective tissue is exposed to flowing blood. Therefore, these matrices were perfused with anticoagulated whole blood because the interaction of platelets with reactive surfaces strongly depends on the shear rate of the blood. Low molecular weight heparin (LMWH) was used as an anticoagulant in the perfusate blood, and this set-up allowed the study of tissue factor-dependent thrombin formation and its effect on thrombogenicity of the perfused surface. In this way, all components influencing the hemostatic response (blood platelets, red blood cells, plasma, shear rate, and vessel...
wall components) were included in the study. As a source of endothelial cells, umbilical vein was compared with human atrium and omental capillaries. It was found that the vessel wall cells and their matrices interfere differently with the hemostatic balance; the endothelial cells isolated from the umbilical cord were the least thrombogenic. Tissue factor-dependent thrombin formation proved to be a main factor in the hemostatic process, as became clear from perfusions over the different cell matrices.

Methods

Cell Culture

All cells were of human origin. The culture medium consisted of RPMI-1640 (GIBCO Biocult, Paisley, UK), 20% (vol/vol) normal human serum (pool of 20 healthy donors), 2 mM glutamine, and the antibiotics penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (5 μg/ml) (GIBCO). FB were grown in 10% pooled bovine serum instead of in 20% human serum. When serum was omitted from the medium, 2% (vol/vol) utroser (LKB, Rockville, MA), human transferrin (20 μg/ml, Sigma Chemical, St. Louis, MO), and insulin from bovine pancreas (10 μg/ml, Sigma) were added instead. Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords according to the method originally described by Jaffe et al. with some minor modifications. Microvascular endothelial cells were isolated from omental tissue (HOTMEC) of patients undergoing cholecystectomy. Adult atrial endothelial cells (Atr-EC) were derived from atrial samples obtained from heart transplantation procedures. The specimens were stored up to 6 hours in ice-cold phosphate-buffered saline (PBS) with 100 U/ml penicillin and with 100 μg/ml streptomycin. Endothelial cells were detached by scraping with a rubber policeman in 0.025% trypsin in RPMI medium. After addition of human serum, cells were pelleted by centrifugation and were resuspended in culture medium before seeding. Endothelial cells were identified by their typical cobblestonelike pattern and endothelial characteristics such as the presence of von Willebrand factor (vWF). Matrix Surface von Willebrand Factor Assay

Matrices on glass coverslips (3.24 cm²) of the various cells were put into new six-well (9.5 cm²) plates and were washed twice with ELISA buffer (Michaelis buffer: 28.5 mM Na-acetate. 28.5 mM Na-barbital, pH 7.35, and 116 mM NaCl) containing 0.1% (wt/vol) bovine serum albumin (BSA) and 0.1% (vol/vol) Tween-20 (E. Merck AG). The coverslips with the matrix were incubated for 1 hour at room temperature with a mixture of two monoclonal antibodies against vWF (CLB-RAg:50 and CLB-RAg:210, 1:1000 in EUSA-3% BSA buffer, 75 μl/cover slip). The monoclonal antibodies were generous gifts of Jan A. van Mourik, CLB, Amsterdam. Subsequently, the coverslips were washed twice with ELISA buffer and were incubated with 75 μl/cover slip peroxidase conjugated rabbit-antimouse IgG (Dako, Glostrup, Denmark), diluted 2500-fold with ELISA-3% BSA buffer. After 1 hour at room temperature, the coverslips were washed and developed for peroxidase activity with 1,2-phenylenediamine (0.5 mg/ml in 50 mM citric acid, 100 mM Na₂HPO₄, pH 5.0, 0.015% H₂O₂, 1 ml/well). The enzyme reaction was stopped with 4 N H₂SO₄ (250 μl/well), and the absorbance at 492 nm was read. Coverslips on which no cells were seeded were used as controls. In all surface vWF assays, coverslips with a matrix of HUVEC of one single batch were also measured and served as the references. Absorbance in the surface vWF assay was related to an absolute amount vWF by the use of this reference as follows. HUVEC of this cell batch were seeded and grown on duplicate pieces of culture flasks and wells of various sizes. After reaching confluence, the matrix was isolated by cell removal. One-half of the matrix was measured with the surface vWF assay. The absorbance was linear with surface area. The matrix from the other surfaces was scraped off with a rubber policeman in
2 ml 6 M urea, 1% Triton X-100. After dialysis against PBS, the absolute vWF content was measured with an ELISA. The vWF content was linear with the surface area of matrix. By combining both assays, we derived a linear relationship between absorbance and vWF content for this reference HUVEC matrix.

**Surface Procoagulant Assay**

Procoagulant activity associated with the cell surface or matrix was determined by a one-stage clotting assay performed in 24-well culture plates (2.0 cm²). The cells or matrices were washed three times with PBS at 37°C. Subsequently, the surface was incubated for 1 minute with 150 μl of Ca²⁺-Michaelis buffer (28.5 mM Na-acetate, 28.5 mM Na-barbital, pH 7.35, 50 mM NaCl, and 33 mM CaCl₂). Coagulation was started by the addition of 150 μl control plasma (derived from 20 healthy volunteers, anticoagulated with 20 mM of trisodium citrate). The time needed for the formation of a fibrin thread was measured. In separate experiments, the control plasma was replaced by commercially available factor VII and factor XII deficient plasma (George King Bio-Medical Inc., Overland Park, KS). The times measured in the surface procoagulant assay were related to standard thromboplastin dilutions in the following way: 150 μl of different dilutions of a standard human thromboplastin suspension [Thromborel S with an International Sensitivity Index (ISI) of 1.08 as compared to the British Comparative Thromboplastin Standard, Hoench-Penzelen, Marburg, FRG] was used for the clotting times. The anti-tissue factor serum had no effect on activated thromboplastin clotting time. The coagulation times in this assay showed an inverse linear relation with the log concentration of the thromboplastin standard starting with concentrations as low as 0.01%. Factor XII dependent coagulation became increasingly important at thromboplastin concentrations below 0.1%. Less than 5% of the thromboplastin activity was detected when factor VII deficient plasma was used. The addition of phospholipids such as cephalin (from a commercial APTT kit, Boehringer Mannheim GmbH, FRG) to the thromboplastin dilutions (in percentage of the standard suspension) was used as a calibration curve for the expression of procoagulant activity of the cells or the matrix.

**Perfusate Blood Collection and Anticoagulation**

LMWH (Fragmin kindly provided by Kabi Vitrum, Stockholm, Sweden) with a mean molecular weight of 4500 and an anti-Xa/antithrombin ratio of 4 (165 U/mg antifactor Xa activity and 40 U/mg anticoagulant activity as measured by kaolin cephalin clotting time) was diluted in saline (200 anti-Xa-units/ml). Blood was collected by clean venipuncture from healthy human donors in 1:10 (vol/vol) of this heparin-saline. Anticoagulation with citrate was obtained by collecting blood in 1:10 (vol/vol) 110 mM trisodium citrate. To compare blood anticoagulated with LMWH to that anticoagulated with citrate, we used perfusates in which hematocrit and platelet count were identical; by adding autologous platelet-poor plasma or erythrocytes to whole blood of the controls, equal hematocrit and platelet counts were obtained for both controls.

Whole blood was kept at room temperature before use in perfusions. All perfusates had a final volume of 15 ml. A platelet analyzer 810 (Baker Instruments, Allentown, PA) was used for counting platelets.

**Perfusion Chambers and Surfaces**

Perfusions with steady flow were carried out with a rectangular perfusion chamber, which was described and characterized extensively elsewhere. This perfusion chamber contains two knobs. On each knob two Thermony (2×1.62 cm²) coverslips can be mounted. The extracellular matrix on these coverslips obtained after removal of the cultured cells was in this way exposed to the flowing blood. To evaluate the influence of matrix associated tissue factor activity, we incubated matrices for 1 hour at room temperature with an anti-tissue factor hybridoma supernatant (a kind gift of Thomas S. Edgington, Scripps Clinic, La Jolla, CA) 1:50 (vol/vol) in PBS. This concentration was able to inhibit 95% of the activity of a 0.1 ISI/ml (as compared to the British Comparative Thromboplastin human thromboplastin suspension (Thromborel S, Hoechst-Behring) in a standard prothrombin time assay. In control perfusions, we incubated the matrices with an aspecific hybridoma supernatant in the same dilution. The anti-tissue factor serum had no effect on activated thromboplastin clotting time. The incubated coverslips were rinsed and used subsequently for perfusion studies.

**Perfusions**

The coverslips in the rectangular chamber were rinsed before the start of the perfusions with 25 ml prewarmed (37°C) 10 mM HEPES-buffered-saline (pH 7.4). Perfusates prewarmed for 5 minutes at 37°C were then circulated for 3, 5, or 10 minutes. Different wall shear rates were obtained by varying flow rate and/or chamber width. After each perfusion run, the system was thoroughly rinsed with 30 ml of HEPES-saline. The coverslips with matrix were then removed from the rectangular chamber. The coverslips were subsequently rinsed with 1 ml of HEPES saline and were fixed with 0.5% glutaraldehyde in PBS as previously described. Coverslips with matrix were subsequently exposed to osmium tetroxide (2.0%) as postfixation and were dehydrated and embedded in Epon as described earlier. The Epon with the embedded matrix and the adhering platelets were separated from the coverslip by thermoshock. For en face evaluation and microphotographs of platelet adhesion, coverslips were stained after perfusion and fixation with May-Grunwald/Giemsa.

**Evaluation**

Sections of the Epon-embedded matrix (1 μm thick) were prepared and stained with methylene blue and basic fuchsin and were evaluated by light microscopy (Dialux 20 EB, E, Leitz GmbH, Wetzlar, FRG) at a 1000×...
Table 1. Cell and Matrix Characteristics of Umbilical Vein Endothelial Cells, Atrial Endothelial Cells, Omental Capillary Endothelial Cells, Smooth Muscle Cells, and Fibroblasts

<table>
<thead>
<tr>
<th>Cells</th>
<th>HUVEC</th>
<th>Atr-EC</th>
<th>HOTMEC</th>
<th>SMC</th>
<th>Fb</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGI₂ release (ng/24 hrs)</td>
<td>9.6±0.6</td>
<td>2.4 (n=2)</td>
<td>30.3 (n=2)</td>
<td>3.9±0.6</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>vWF release (µg/24 hrs)</td>
<td>1.8±0.3</td>
<td>3.9 (n=2)</td>
<td>0.1 (n=2)</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Procoagulant act. (% t.f.)</td>
<td>0.014±0.12</td>
<td>6.7±4.3</td>
<td>29.3±5.3</td>
<td>6.7±2.9</td>
<td>1.5±1.4</td>
</tr>
</tbody>
</table>

Matrix

<table>
<thead>
<tr>
<th>Matrix</th>
<th>HUVEC</th>
<th>Atr-EC</th>
<th>HOTMEC</th>
<th>SMC</th>
<th>Fb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrix bound vWF (in µg)</td>
<td>34.8±14.1</td>
<td>2.4±5.4</td>
<td>17.7 (n=2)</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Procoagulant act. (% t.f.)</td>
<td>0.005±0.04</td>
<td>1.5±1.1</td>
<td>0.5±0.3</td>
<td>0.9±0.3</td>
<td>0.6±0.7</td>
</tr>
</tbody>
</table>

Cells were grown to confluence on glass coverslips (3.24 cm²) as used for perfusion experiments. Matrix was obtained by cell removal with 0.1 M NH₄OH. Prostacyclin and von Willebrand factor release of cells was measured over 24 hours after reaching confluence. Procoagulant activity is expressed as a percentage of a thromboplastin standard with comparable clotting activity. All values are calculated per 10 cm² and are means of measurements±SD with three different cell batches. Measurements in a cell batch were performed in triplicate.

HUVEC=human umbilical vein endothelial cells, Atr-EC=atrial endothelial cells, HOTMEC=human omental capillary endothelial cells, SMC=smooth muscle cells, Fb=fibroblasts, PGI₂=prostacyclin, vWF=von Willebrand factor, act.=activity, t.f.=thromboplastin.

Fibrin Deposition and Fibrinopeptide A Generation

Fibrin deposition on the perfused matrices was determined morphologically by evaluating cross-sections and was expressed as a percentage of the total number of observed intersections. A radioimmunoassay kit (Mal- linckrodt, St. Louis, MO) was used for fibrinopeptide A (FPA) measurements. Samples (900 µl) were collected before and after perfusion and were added to the 100 µl anticoagulant mixture provided in the kit. The instructions of the manufacturer were subsequently followed. FPA generation was calculated from the increase in FPA level compared to the initial values just before perfusion and are given in ng/ml plasma.

Results

Cell and Matrix Characteristics

A confluent monolayer of HUVEC, Atr-EC, and HOTMEC contained 50±5x10³ cells/cm²; a confluent monolayer of SMC and Fb consisted of 30±5x10³ cells/cm².

The release of vWF and prostacyclin was measured over a period of 24 hours (Table 1). HUVEC, Atr-EC, and HOTMEC showed the characteristic release of vWF and prostacyclin. HOTMECs distinguished themselves by very low vWF release with a high prostacyclin release. Atr-EC deposited seven to 15 times less vWF in their matrix as compared to HOTMEC and HUVEC. Neither SMC nor Fb released vWF to the medium or deposited it on their matrix. SMC, however, were able to produce prostacyclin in amounts comparable to Atr-EC.

Procoagulant activity of cells was measured with an assay which leaves the cells intact. Procoagulant activity present at the surface of the cells was always more pronounced than the activity associated with their respective matrices. For endothelial cells, there was a remarkably high procoagulant activity present at the Atr-EC and HOTMEC surfaces. The extracellular matrix of these cells also contained procoagulant activity. This matrix procoagulant activity was comparable for all cells tested, with the exception of HUVEC matrix, which showed, in agreement with the data of intact cells, very low activity. When factor VII deficient plasma or matrices isolated by Triton X-100 were used in the assay, procoagulant activity was blocked more than 95%. The use of factor XII deficient plasma did not influence expression of procoagulant activities (results not shown).

Table 2. Platelet Adhesion on Matrices of Various Vascular Cells. Perfusions with Perfusates Anticoagulated with Citrate or Low Molecular Weight Heparin

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Citrate</th>
<th>LMWH</th>
</tr>
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<tbody>
<tr>
<td>HUVEC</td>
<td>33.9±4.1</td>
<td>29.2±3.4</td>
</tr>
<tr>
<td>Atr-EC</td>
<td>12.0±2.7</td>
<td>12.2±3.9</td>
</tr>
<tr>
<td>HOTMEC</td>
<td>23.8±3.6</td>
<td>16.2±3.1*</td>
</tr>
<tr>
<td>SMC</td>
<td>15.7±2.3</td>
<td>14.8±3.5</td>
</tr>
<tr>
<td>Fb</td>
<td>15.5±4.8</td>
<td>18.9±4.0</td>
</tr>
</tbody>
</table>

Whole blood of two healthy donors was anticoagulated with 19 mM citrate or with 20 U/ml low molecular weight heparin (LMWH) and was normalized to comparable hematocrit (38% to 41%) and platelet number (180 000 to 210 000/µl). Perfusions were performed for 5 minutes at a shear of 1300 s⁻¹. Platelet adhesion is given in percent surface coverage±SD (n=6). *p<0.001 (Student's t test) as compared to citrated perfusates.

See the legend to Table 1 for the explanations of abbreviations.
### Adhesion Studies

Perfusion experiments were performed with blood of donors anticoagulated with citrate or with LMWH. Perfusates with standardized hematocrits and platelet counts were perfused for 5 minutes at 1300 s\(^{-1}\) over the matrices from the various vascular cells. Platelet adhesion (Table 2) was independent of anticoagulation except for a significant decrease in adhesion seen at HOTMEC matrix, when it was perfused with LMWH anticoagulated blood. Platelet adhesion to matrices of Atr-EC, of SMC, and of Fb was significantly lower than adhesion observed with HUVEC and HOTMEC matrix. The distribution of adhered platelets was homogeneous in perfusions with citrated blood and consisted for more than 80% of the spread or contact platelets. The surface covered with small platelet aggregates (<5 \(\mu\)m) was always less than 5%. No aggregates above 5 \(\mu\)m were present. Fibrin deposition and FPA generation with citrated blood perfusions. Aggregate formation on Atr-EC, HOTMEC, SMC, and Fb matrices was strongly increased above the values obtained in the citrated blood perfusions. Aggregate formation on HUVEC matrix after LMWH perfusions was as low as in citrate perfusions. Aggregate formation on Atr-EC, HOTMEC, SMC, and Fb matrices was significantly increased above the aggregate formation on HUVEC matrix (expressed as a percent of spread platelets covered with platelets). Fibrin deposition on and FPA generation by HUVEC matrix were negligible and comparable to values seen with citrated blood. In contrast again, perfusions over the other matrices with LMWH anticoagulated blood generated considerable amounts of FPA, and large parts of the matrix were covered with fibrin within 5 minutes (between 23% and 70% surface coverage).

### Thrombus and Fibrin Formation

Table 3 illustrates the platelet interaction with various matrices when perfused with LMWH anticoagulated blood. Fibrin deposition on the matrix and FPA generation in the perfusate were also determined. Aggregate formation on Atr-EC, HOTMEC, SMC, and Fb matrices was strongly increased above the values obtained in the citrated blood perfusions. Aggregate formation on HUVEC matrix after LMWH perfusions was as low as in citrate perfusions. Aggregate formation on Atr-EC, HOTMEC, SMC, and Fb matrices was significantly increased above the aggregate formation on HUVEC matrix (expressed as a percent of spread platelets covered with platelets). Fibrin deposition on and FPA generation by HUVEC matrix were negligible and comparable to values seen with citrated blood. In contrast again, perfusions over the other matrices with LMWH anticoagulated blood generated considerable amounts of FPA, and large parts of the matrix were covered with fibrin within 5 minutes (between 23% and 70% surface coverage).

### Time Dependence

In Figure 1 the time dependence for platelet adhesion to matrices of HUVEC, SMC, and Fb is shown. Adhesion, aggregate formation, fibrin coverage, and FPA generation increased predominantly in the first 2 minutes of the perfusion. Between 5 and 10 minutes, all parameters leveled off or showed only small increases.

### Effects of Shear Rate

To investigate the influence of shear on adhesion, aggregate formation, FPA generation, and fibrin deposition, LMWH anticoagulated perfusates were circulated for 5 minutes at various shear rates. Increased adhesion to SMC, Fb, and HUVEC matrices was observed when perfused at higher shear (Figure 2A). At all shear rates, HUVEC matrix had the highest adhesion; less than 20% of this adhesion consisted of small aggregates (Figure 2B). At 1800 s\(^{-1}\) only 10% of the SMC matrix surface was covered with platelets, but this coverage consisted primarily of aggregates (±40% small and ±45% large aggregates). On the fibroblast matrix, intermediate values were observed. Fibrin deposition decreased with increasing shear on Fb and HUVEC matrices (Figure 2C). FPA generation was not systematically influenced by shear (Figure 2D).

The various endothelial cells were compared among each other in perfusions at shear rates of 300 and 1300 s\(^{-1}\) (Table 4). Adhesion to HUVEC matrix especially increased with shear. Adhesion at Atr-EC and HOTMEC matrices only showed a small increase at higher shear rates. Aggregate formation (>5 \(\mu\)m) on Atr-EC matrix and to a smaller degree on HOTMEC matrix increased when the surfaces were perfused at higher shear rate. Similar to the experiments described in Figures 2C and 2D for SMC and Fb matrices, fibrin deposition on Atr-EC, HOTMEC, and even on HUVEC matrices decreased at 1300 s\(^{-1}\). FPA generation in the perfusates was not influenced by shear.

### Morphology

The morphological appearance of platelet and fibrin deposition at the various matrices was shown en face and in cross-sections in Figure 3 (A to J) for perfusions at shear rate of 1300 s\(^{-1}\). In Figure 1 the time dependence for platelet adhesion to matrices of HUVEC, SMC, and Fb is shown. Adhesion, aggregate formation, fibrin coverage, and FPA generation increased predominantly in the first 2 minutes of the perfusion. Between 5 and 10 minutes, all parameters leveled off or showed only small increases.

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Figure 1. Low molecular weight heparin anticoagulated perfusates were circulated at 1300 s⁻¹ for various times (in minutes) over matrices of smooth muscle cells (Δ—Δ), fibroblasts (○—○), and umbilical endothelial cells (■—■). The influence of perfusion time on adhesion is given in percent surface coverage (A); aggregate formation is given in percent of spread platelets covered with aggregates; (B') aggregates <5 μm, (B") aggregates >5 μm; fibrin deposition is given in percent surface coverage (C), and fibrinopeptide A (FPA) generation in the perfusate in ng/ml (D). All values±SEM (n=4).

rates of 300 s⁻¹ and 1300 s⁻¹. HUVEC matrix (Figures A, B, E, and F) distinguished itself by evenly distributed homogeneous adhesion of contact and spread platelets with hardly any aggregate formation. The other matrices, however, showed large aggregates, which were more or less oriented parallel to the direction of flow. Matrices of Atr-EC, HOTMEC, SMC, and Fb also showed fibrin deposition associated with the matrix and the platelet aggregates. Less adhesion, lower aggregates, and more pronounced fibrin deposition (F in figure) was observed when these matrices were perfused at low shear rate. Single platelets and even red blood cells (R in figure) were trapped within and adhered to this pronounced fibrin network. Platelets and thrombi stuck to or within the fibrin network were not considered as adhesive to the matrix when cross-sections were evaluated.

**Effect of Tissue Factor**

To evaluate the influence of matrix-associated tissue factor activity, we incubated the matrices of HUVEC, SMC, and Fb with an anti-tissue factor monoclonal (Table 5). Incubation of HUVEC matrix with anti-tissue factor did not change platelet adhesion, aggregate formation, nor the already low FPA generation in 5-minute perfusions at 1300 s⁻¹. Incubation with anti-tissue factor inhibited 90% of the FPA generation in perfusions over SMC and Fb matrices. The aggregate formation on these matrices was concomitantly inhibited; 70% to 90% of the small aggregates and all aggregates between 5 and 10 μm disappeared when we blocked tissue factor activity in SMC and Fb matrices. Only adhesion at SMC matrix was influenced by the incubation. Anti-tissue factor blocked 74% to 80% of the fibrin deposition on all tested matrices. Especially at SMC matrix anti-tissue factor strongly increased the amount of matrix free of fibrin coverage and therefore available for platelets to interact. A higher adhesion at this matrix incubated with anti-tissue factor might be explained by this phenomenon.

Also, in a procoagulant assay, anti-tissue factor incubation of Atr-EC and HOTMEC matrices blocked more than 90% of their procoagulant activity (not shown).

**Discussion**

The endothelial cell layer is a dynamic interface between blood and the rest of the body. It is the main protection against unintentional activation of the hemostatic response and guarantees the maintenance of normal blood fluidity. To maintain this nonthrombogenic nature, the cells express a number of activities, including the synthesis of prostacyclin, nitric oxide, and plasminogen activators, and the expression of thrombomodulin and heparin-like molecules on their surface. In addition to these antithrombotic functions, the endothelial cells
appear capable of prothrombotic behavior. Not only do they synthesize adhesive proteins, such as vWF and fibronectin and procoagulant factors such as factor V and tissue factor, but they also provide a surface on which clotting factors can assemble and be activated. There must be a balance between the pro- and antithrombotic properties, which determines the status of the local hemostatic and thrombotic activity.

To study the balance between these pro- and antithrombotic functions, we have used a recently published perfusion set-up in which blood anticoagulated with LMWH was perfused over cultured endothelial cells or their extracellular matrix. Anticoagulation with this heparin allowed handling of blood without activation of the coagulation cascade. Cultured umbilical vein endothelial cells did not deposit tissue factor in their matrix, and perfusions over these matrices did not lead to activation of coagulation. However, stimulation of the endothelium resulted in a tissue factor-rich matrix, and when perfused with the LMWH anticoagulated blood, FPA generation in
Figure 3. En face morphology (top) and in cross-section (bottom), the appearance of platelet and fibrin deposition at matrices of the various vascular cells perfused for 5 minutes with low molecular weight heparin anticoagulated perfusates at 300 s⁻¹ (left figures: A, C, E, G, I) and 1300 s⁻¹ (right figures: B, D, F, H, J). A, B. Matrices of umbilical endothelial cells. C, D. Matrices of atrial endothelial cells. E, F. Matrices of microvascular omental endothelial cells. G, H. Matrices of fibroblasts. I, J. Matrices of smooth muscle cells. F=fibrin, a=aggregate, r=red blood cell. Bars=10 μm.
the perfusate was measured. Local thrombin formation was not only responsible for FPA generation and fibrin deposition on tissue factor-rich matrix, but also platelet aggregate deposition on this matrix was enhanced. This perfusion set-up, which is sensitive for tissue factor-dependent thrombogenicity of the perfused surface, was used in our present experiments.

Despite its general morphological homogeneity, there is enough evidence that the vascular endothelial lining exhibits regional variation, which may have important implications for the local hemostatic balance. For example, differences have been found for the synthesis of prostacyclin, vWF expression, fibrinolytic activity, synthesis of platelet activating factor, and the sensitivity for hormones. For this reason, we studied endothelial cells isolated from human large vessels, from umbilical vein, and from microcirculation and compared them with SMC and Fb. The important vascular cell types and all parameters involved in the hemostatic or thrombotic balance are thus included in these studies to gain more insight about factors contributing to localized vascular thrombosis in humans.

All the endothelial cells tested synthesized and released prostacyclin and vWF, but there were quantitative differences. Omentum endothelial cells secreted 15 times as much prostacyclin as atrium endothelial cells and three times as much as umbilical vein cells. In contrast, they released only a small amount of vWF compared to the other endothelial cells. Surprisingly, the omentum endothelial cells deposited almost all of their vWF in their extracellular matrix, while the other endothelial cells tested secreted a substantial amount of the newly synthesized vWF into the culture medium.

The most striking difference between the different types of endothelial cells was the presence of tissue factor. While endothelial cells isolated from the umbilical cord contained no significant amounts of cell-associated tissue factor, both the cells isolated from atrium and omentum expressed tissue factor activity. It is known that endothelial cells express tissue factor activity after stimulation of the cells with, for instance, interleukin-1 or endotoxin. In the experiments performed, the cells were not activated. The reason why unstimulated atrium and omentum cells express tissue factor activity is not known.

Figure 3 (continued).
The presence of endotoxin in the culture medium as cause for the observed differences was unlikely because all endothelial cells were cultured with the same batch of growth medium at least during 1 week before the experiments were performed. Moreover, the endothelial cells of different origin did not show different growth rates. However, we cannot exclude the possibility that an increased sensitivity of omental and atrial endothelium for tracer amounts of endotoxin is partly responsible for the observed results.

Whether cells grown in vitro are representative for the cells in vivo remains an unsolved question. The in vitro culture conditions without doubt greatly determine the phenotypic expression of the cells. Although all experiments with different endothelial cells were performed at the same time during the cell cycle (namely, 1 day after the cells reached confluence), we cannot simply conclude from the results found here that endothelial cells present in the atrium or the omentum express tissue factor activity and are, therefore, more thrombogenic compared to umbilical vein endothelial cells. In a separate experiment (not shown), the endothelium of segments of atrium obtained after obduction was incubated with plasma, and a substantial amount of tissue factor activity was found associated with the endothelial layer. Although this experiment is not conclusive (obduction material is at least 1 day old, and no absolute proof can be given that the endothelial cell layer was intact), this experiment suggests that, indeed, some tissue factor activity is associated with atrial endothelium. Whether tissue factor expression on these cells is necessary for the optimal functioning of the protein C-dependent anticoagulant pathway has to be investigated.26 Whatever the in vitro extrapolation of our in vitro experiments might be, the fact that endothelial cells isolated from atrium and omentum respond differently on in vitro culture conditions than umbilical vein cells is an important finding. It is tempting to speculate that these differences in the response or adaption in vitro may be present in vivo when local conditions change. Also, the in vitro encountered characteristics of the studied cells may change when the cells are cultured beyond the passage numbers we used. Recently it was shown that matrix derived from Fb with a higher passage number was less able to support platelet adhesion.36 We, therefore, preferred passage numbers as low as possible attainable. Finally, it is interesting to note that the endothelial cells isolated from fetal tissue up to the sixth passage (results not shown) correspond best to the "classic" expectations, as a cell providing a nonthrombogenic surface.

As expected, SMC and Fb did not produce vWF. Fb released no detectable amounts of prostacyclin; SMC synthesized prostacyclin, as was shown before.37

When the endothelial monolayer is damaged, the endothelial cell matrix, Fb, and SMC embedded in their matrix are exposed to flowing blood. To investigate the hemostatic and thrombotic processes when vessel wall injury occurs, we perfused the matrix of vascular cells with flowing blood. Especially the Fb and SMC that are embedded in tissue in vivo deposit matrix to all sides of the intercellular space; therefore, we perfused the matrix of Fb and SMC. Both the cultured subendothelial cells and also their extracellular matrix expressed tissue factor activity (Table 1). The presence of tissue factor in the subendothelium of umbilical artery segments has recently been confirmed.6,30,38 However, the contribution of the various cells in the vessel wall to the presence of tissue factor in the vessel wall remained unclear.

When matrices isolated from the different cells were perfused for 5 minutes with citrated or LMWH blood, comparable amounts of platelets adhere to the different matrices. Only the adhesion to matrices of umbilical vein endothelial cells was about twice as high as to the other matrices, probably due to the high amount of vWF in the matrix of umbilical vein endothelial cells. Perfusions with LMWH anticoagulated blood allowed the study of tissue factor-associated thrombogenicity of these matrices. With these perfusates, platelet aggregates and fibrin deposition were found on the matrix of atrium and omentum endothelial cells and on the matrices of SMC and Fb. The appearance of aggregates and fibrin coverage did not show completely comparable patterns for the different matrices. A more quantitative measure of fibrin deposition may solve this discrepancy and is currently
being developed. Fibrin deposition decreased with increasing shear rate, similar to earlier findings at the matrix of stimulated umbilical endothelial cells. The thrombi formed on SMC matrix contained a striking amount of fibrin and fewer platelets than compared to the thrombi on the other matrices. Platelet aggregation by collagen and thrombin formation by intrinsic activation of the coagulation cascade seems to be less important in the technical set-up used. When matrices were incubated with antibodies against tissue factor, aggregate formation, fibrin deposition, and FPA generation were inhibited 70% to 90%.

Only endothelial cells isolated from umbilical veins are nonthrombogenic in vitro; they do not synthesize tissue factor under unstimulated conditions. On their abluminal side, they deposit an extracellular matrix on which only adherent platelets are found but no aggregates and no fibrin. Further studies are necessary to understand the differences between the different types of endothelial cells. Recently doubts were raised about the endothelial nature of the cells derived from the omental fat. Whether the current methods to isolate and culture HOTMEC allow the very similar mesothelial cells to remain in these cultures is still unclear. However, the thrombogenicity remains of great importance, particularly when these cells are used for seeding purposes of vascular grafts. Based on the data now available, we conclude that endothelial cells isolated from umbilical veins offer the best nonthrombogenic surface in vitro and that endothelial cells isolated from different human organs interfere differently in the hemostatic response. We cannot exclude that the in vitro differences between the endothelial cells are caused by mere differences in sensitivity towards in vitro culture conditions. Recently it was shown, in tissues obtained by obduction or biopsy, that tissue factor was only present in the vessel stone, 1986:1-13

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Index Terms: endothelial cells • von Willebrand factor • umbilical vein endothelial cells • omental tissue • atrial endothelial cells • smooth muscle cells • fibroblasts • tissue factors • platelet adhesion
Thrombogenicity of vascular cells. Comparison between endothelial cells isolated from different sources and smooth muscle cells and fibroblasts.

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