Activation of Endothelial Cells Induces Platelet Thrombus Formation on Their Matrix
Studies of New In Vitro Thrombosis Model with Low Molecular Weight Heparin as Anticoagulant

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Previous studies have indicated that activation of endothelial cells may lead to the production of tissue factor. We have studied the effect of endothelial cell activation and subsequent tissue factor synthesis on thrombus formation on the extracellular matrix in flowing blood. Endothelial cells were stimulated with tumor necrosis factor, endotoxin, or phorbol ester. Coverslips with activated cells or their extracellular matrix were introduced into a perfusion system and exposed to blood anticoagulated with 20 U/ml low molecular weight heparin. This concentration allowed manipulation of blood without activation of the coagulation cascade. Platelet deposition and fibrin formation were evaluated by morphometry, and fibrinopeptide A formation was assayed as a measure of thrombin generation. Activation of endothelial cells caused fibrinopeptide A generation in the perfusate and some deposition of fibrin on endothelial cells; however, platelets were not deposited. The matrix of the stimulated endothelium also caused enhanced fibrinopeptide A generation, and platelet aggregates and fibrin were deposited on the matrix. Maximal effects were observed with stimulation periods between 4 and 10 hours and were still clearly present after 18 hours. Increase in shear rate, perfusion time, and platelet number resulted in an increase in platelet adhesion, but platelet aggregate formation as a percentage of adhesion remained constant. Platelet aggregate formation and fibrinopeptide A generation were inhibited with antibodies against tissue factor or factor VII. Platelet aggregate formation alone was inhibited by antibodies against glycoprotein IIb/IIIa. Polymerization of fibrin on the matrix was best supported in perfusions at a low shear rate. The new in vitro thrombosis model presented here provides a powerful tool for study of the regulation of thrombogenicity by the vessel wall in response to various stimuli. (Arteriosclerosis 10:49-61, January/February 1990)

Endothelial cells (EC) lining blood vessels play an active role in hemostasis. Resting intact endothelium is mainly antithrombotic, but activation of the EC by stimuli present in various pathological conditions may change this phenotype. Stern et al. have demonstrated the presence of receptors for coagulation factors on the intact endothelial surface. Activation of the endothelium induced tissue factor on the cell surface and made actual formation of thrombin on the endothelial surface possible. In vitro studies have identified tumor necrosis factor (TNF), endotoxin, interleukin-1, thrombin, and 12-phorbol 12-myristate 13-acetate (PMA) as agents able to activate endothelial cells. Changes caused by activation and relevant in the endothelial regulation of hemostasis are the synthesis of platelet activating factor, the release of von Willebrand factor, the changed protein composition of the matrix, the increased production of prostacyclin, the down-regulation of thrombomodulin expression, and the increased production of tissue factor. Thor-
anticoagulated blood, which does not allow the formation of thrombin. A native blood system in which blood was directly drawn from a blood vessel through a perfusion chamber was used, but this system has two drawbacks. First, thrombin formation in this system is not local. Perhaps because of this, fibrin formation on the vessel is much more pronounced than has been observed under similar conditions in vivo. Second, direct drawing of the blood through the perfusion chamber allows no opportunity to change the composition of the perfusion fluid, e.g., by adding antibodies or by removing clotting factors. For that reason, we have developed a system that was anticoagulated to such an extent that thrombin formation only occurred when perfusion over a vessel wall was performed, but which allowed in vitro handling of the blood without activation of the coagulation system.

Methods

Chambers and Surfaces

Perfusions with steady flow were carried out with an annular perfusion chamber and with a rectangular perfusion chamber. In the annular chamber, inverted artery segments isolated from the umbilical cord were used. The human umbilical cords were obtained, on the average, within 6 hours after birth. Artery segments were isolated, stored, inverted, and mounted on the central rod in the perfusion chamber as earlier described. Most perfusion experiments, however, were performed with the rectangular perfusion chamber, which has been characterized and described elsewhere. This perfusion chamber contains two circular knobs on which glass or Thermanox coverslips (Lab-Tek Division, Miles Laboratories, Naperville, IL) can be mounted. The coverslip surface in our experiments was covered with EC or with their extracellular matrix. These surfaces (3.24 cm²) were exposed to the flowing blood in the chamber.

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. The cells were identified by their typical characteristics. For the experiments described here, EC of the second passage were subcultured on gelatin-coated Thermanox coverslips. Before the cells were seeded, gelatin was fixed with 0.5% glutaraldehyde. Cell monolayers were grown to confluence in 5 to 7 days (50 000 cells/cm²). Subsequently, at various times before use of the cells in perfusions or isolation of their matrix, PMA (Sigma, St. Louis, MO), human TNF (kindly provided by David M. Stern, NY), or lipopolysaccharide (LPS, a kind gift of Gert Muller-Berghaus, Max Planck Institute, Giessen, FRG) were added to the cell culture medium. PMA was dissolved in dimethylsulfoxide (DMSO) and was subsequently diluted 1:1000 (vol/vol) in the culture medium, resulting in 20 ng/ml PMA. Addition of DMSO alone in the same concentration had no influence on the cells. TNF stock was diluted in the culture medium to a 200 U/ml concentration. LPS was diluted in the culture medium to a concentration of 1 µg/ml. In this way, confluent cells were activated for varying periods with three different stimuli. On the day of the perfusion studies, the matrix was obtained by removal of the EC by exposure to 0.1 M NH₄OH at room temperature for 30 minutes and gentle shaking. In other experiments, the following isolation methods for endothelial cell matrix (ECM) were used: 1) exposure of EC to 0.5% Triton X-100 for 30 minutes at room temperature with gentle shaking, or exposure to EC to 2 M urea for 30 minutes at room temperature, and 3) application and gentle pressure of nitrocellulose-acetate paper moistened with phosphate-buffered saline (PBS) on the EC monolayer; removal of the nitrocellulose-acetate paper resulted in removal of the EC. Isolated matrices were washed three times with PBS (1:10, vol/vol, 0.1 M sodium phosphate buffer, pH 7.4, in 150 mM NaCl) and were used the same day for perfusion studies. In some experiments, two half coverslips (9×18 mm) were mounted on each knob with their long sides perpendicular to the flow. The first half coverslip in the flow direction contained a matrix of PMA-stimulated cells. The second half contained a matrix of unstimulated cells.

Blood Collection and Anticoagulation

Low molecular weight heparin (Fragmin, LMWH) with a mean molecular weight of 4500 and an anti-Xa/anti-thrombin ratio of 4 (165 U/mg anti-factor Xa activity and 40 U/mg anticoagulant activity as measured by kaolin cephalin clotting time) or normal standard heparin (both from Kabi Vitrum, Stockholm, Sweden) were diluted in saline to concentrations 10 times higher than concentrations desired in whole blood. Blood was collected by clean venepuncture from healthy human donors into this heparin-saline solution (1:10, vol/vol). Anticoagulation with citrate was obtained by collecting blood in 110 mM trisodium citrate (1:10, vol/vol).

Perfusates

Blood was kept at room temperature before use in perfusions. It was either used for whole-blood perfusions or further processed toward reconstituted perfusates. Reconstituted perfusates were prepared from platelet-rich plasma (PRP) obtained by centrifugation of whole blood (10 minutes at 190 g, 22°C) and platelet-poor plasma (PPP) obtained by centrifugation of whole blood (10 minutes at 3000 g, 22°C). To obtain varying platelet counts in the perfusates, PRP and PPP were mixed in varying ratios and were combined with red blood cells (RBC), which were prepared as follows. After centrifugation and collection of PRP and PPP, the remaining RBC were washed three times by sequential resuspension in saline containing 5 mM α-D-glucose followed by centrifugation (5 minutes at 3000 g, 22°C). Before use in reconstituted perfusates, RBC were “packed” by centrifugation (10 minutes at 4000 g, 22°C). Washed RBC were added to PRP/PPP mixtures just before the start of perfusions. The hematocrit (Ht) was standardized at 40%. All perfusates had a final volume of 15 ml. For counting platelets, a Platelet Analyzer 810 (Baker Instruments, Allentown, PA) was used. To measure the single platelet disappearance due to microaggregate formation, a modification of Frojmovic’s method as described by Verhoeven et al. was used. Briefly, 50 µl samples were added to 450 µl of
Antibodies cross-reactivity with plasma proteins was observed in the antihuman idiotype was used in the same concentration. The IgG was resuspended in PBS to a concentration of 15 ml perfusates. As control, a mouse monoclonal IgG (6D1) was performed with a specially constructed eyepiece micrometer in the ocular (Leitz). In sections derived from the coverslip of each knob, at least 1000 intersection points between 3.2 μm and 16 μm as described.

Washed platelets were prepared from PRP as previously described and were used in experiments designed to follow fibrinopeptide A (FPA) generation during all the above described blood manipulations. Washed platelets were also prepared for experiments with prothrombin (factor II)-deficient perfusates. Washed platelets were resuspended in either factor II-deficient or normal plasma, which had been adjusted to pH 7.4. Factor II-deficient plasma was prepared by immunoabsorption of PPP and contained less than 1% factor II as determined by a one-stage clotting assay.

No decrease in other clotting factors was found. For control perfusions, purified prothrombin was added to the factor II-deficient perfusates. Prothrombin was titrated to a concentration of 2.0 U/ml. Factor II-deficient plasma and purified factor II were generous gifts of Joost Meijers and Bonno Bouma from our laboratory. RBC of blood group O, rhesus positive, were used in preparation of whole blood (2 U/ml of plasma) just before the start of transfusion, Amsterdam. A1:50 (vol/vol) dilution in plasma contained less than 1% factor II as determined by a one-stage clotting assay. No cross-reactivity with other clotting factors was observed. Just before prewarming, 180 μl of the serum was added to 15 ml of perfusate (Ht=40%). An addition of 1:200 dilution of this serum inhibited 95% of the activity of 0.1 International Sensitivity Index (ISI) (as compared to British Comparative Thromboplastin) human thromboplastin suspension (Thromborel S, Hoechst-Behring, Marburg, FRG) in a standard prothrombin time assay. A 1:8 (vol/vol) dilution of the antiserum in PBS was incubated with the ECM for 60 minutes at 37°C. The incubated coverslips were rinsed and used subsequently for perfusion studies. In control perfusions, the matrices were incubated with 1:8 diluted normal rabbit serum.

Perfusions

Before the start of the perfusions, the artery segments on the central rod in the annular perfusion chamber or the coverslips in the rectangular chamber were rinsed with 25 ml of prewarmed (37°C) 10 mM HEPES-buffered saline (pH 7.4). Perfusates prewarmed for 5 minutes at 37°C were then circulated for 1, 3, 5, or 10 minutes. Different wall shear rates were obtained by varying the flow rate, the chamber width, or both. After perfusion, the systems were thoroughly rinsed with 30 ml of HEPES saline, and the artery segments or coverslips were removed from the annular or the rectangular chambers.

The perfused and Heps saline-rinsed Thermox coverslips were fixed with 0.5% glutaraldehyde in PBS as previously described. Coverslips with matrix were subsequently exposed to osmium tetroxide (2.0%) for postfixation and were dehydrated by washing with increasing concentrations of ethanol. The perfused surface of the Thermox coverslips was covered with epon. Epon was prepared according to methods described earlier. After heat polymerization, the epon with the embedded matrix and the adhering platelets was separated from the coverslip by thermoshock (a fast change of epon-covered Thermox from a 70°C heating plate to liquid nitrogen).

For photomicrographs of the en face morphology, the coverslips were stained after perfusion and fixation with May-Grunwald/Giemsa. After the perfusions, the artery segments were fixed in 2.5% glutaraldehyde in PBS, followed by 2.0% osmium tetroxide postfixation, ethanol dehydration, and subsequent embedding in epon.

Evaluation

One μm-thick sections of the epon-embedded matrix and vessel wall segments were prepared and stained with methylene blue and basic fuchsin and were evaluated by light microscopy (Diapix 20 EB.E, Leitz GmbH, Wetzlar, FRG) at a 1000× magnification. Evaluation was performed with a specially constructed eyepiece micrometer in the ocular (Leitz). In sections derived from the coverslip of each knob, at least 1000 intersection points at 10 μm intervals evenly distributed between 1 and 7 mm from the upstream coverslip border were evaluated.

Total adhesion to the matrix was defined as the percentage of the surface covered by platelets. Platelet adhesion was subdivided into contact platelets, spread platelets, and spread platelets with an aggregate formation on top. The last category, aggregate formation with a minimum height of 2 μm, was further divided into aggregates less than 5 μm in height, those between
5 and 10 μm, and those higher than 10 μm. As a measure of platelet-platelet interaction, the percentage of spread platelets covered with aggregated platelets was calculated, and this was again divided into aggregates less than 5 μm in height and those higher than 5 μm.

**Fibrin Deposition and Fibrinopeptide A Assay**

Fibrin deposition on the perfused matrices was determined morphologically by evaluating cross sections and was expressed as a percentage of the total number of observations. A radioimmunoassay kit (Mallinckrodt, St. Louis, MO) was used for FPA measurements. Samples (900 μl) were collected during blood manipulations or before, during, and after perfusion from the perfusion container. Samples were added to 100 μl of the anticoagulant mixture provided in the kit. The manufacturer’s instructions were subsequently followed. The FPA values are given in nanograms/milliliter of plasma. FPA generation was calculated from the increase in FPA level compared to the initial values just before blood collection or just before perfusion.

### Results

**Anticoagulation, Thrombin Formation, and Platelet Disappearance during Collection and Manipulation of Blood**

An anticoagulation, which allowed thrombin formation locally at the vessel wall during perfusion, but not during in vitro handling of the blood beforehand, had to be developed. For this purpose, we compared the effects of various doses of low molecular weight heparin (LMWH) with standard heparin and with citrate. FPA generation was used as an indicator of thrombin formation. Table 1 depicts the FPA generation in PRP that was left standing at room temperature. FPA generation was observed at 5 and 10 U/ml LMWH, but not at 20 U/ml. We also studied the tendency of platelets to form small aggregates. LMWH concentrations of 10 and 5 U/ml gave a single platelet disappearance of 21% and 38%, respectively. The lowest single platelet disappearance was found with 20 U/ml of LMWH (13%), while with citrate, the value was 14%, and with standard heparin, 16%.

FPA generation in PPP and during perfusate preparation with this PPP was measured. Considerable amounts of FPA were generated in blood and PPP anticoagulated with 5 U/ml of LMWH. Small amounts of FPA were still formed in 10 U/ml of LMWH. The lowest levels were obtained with citrate. The values with 5 U/ml of standard heparin and with 20 U/ml of LMWH were intermediate.

**Effect of Anticoagulation on Platelet Deposition on Umbilical Artery Segments**

The influence of the presence of umbilical artery segments in the annular perfusion chamber on FPA generation is shown in Table 2. Without vessels, no appreciable FPA was generated in 20 U/ml of LMWH- and citrate-anticoagulated blood. The presence of an artery segment caused significantly higher FPA generation in perfusates anticoagulated with 20 U/ml of LMWH but not in perfusates anticoagulated with citrate. Lower LMWH concentrations (up to 7.5 U/ml) gave higher values of FPA generation. These results with LMWH-anticoagulated perfusates indicate that the thrombin required for generation of FPA is vessel wall-dependent.

The effects of anticoagulation on platelet adhesion and aggregation on vessel wall segments are also shown in Table 2. Perfluents with blood anticoagulated with 20 U/ml of LMWH over artery segments showed adhesion and aggregation values similar to those observed with citrated blood. Increased platelet adhesion and aggregate formation were found at 7.5 U/ml LMWH and increased aggregation, at 12.5 U/ml LMWH.

**Effect of Endothelial Cell Activation on Fibrinopeptide A Generation and Platelet Deposition on Matrix**

FPA generation in perfusors over cultured human umbilical vein EC and their matrix is shown in Table 3. Similar to umbilical artery segments (Table 2), nonactivated EC and matrix gave a small, but significant, increase in FPA generation above FPA generation when no reactive surface was present. PMA activation gave a strong increase in FPA generation, with a maximum after 4 hours for both cells and their matrix. TNF activation also increased FPA generation, but its effect was weaker. A separately performed experiment with LPS as a stimulus showed FPA generation at matrix and cells; the cell surface only expressed an increase in activity at 4 hours of stimulation. The much larger increase generated by
Table 2. Fibrinopeptide A Generation and Platelet Adhesion in Whole-Blood Perusions over Inverted Vessel Wall Segments

<table>
<thead>
<tr>
<th></th>
<th>Low molecular weight heparin</th>
<th>Citrate</th>
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<tbody>
<tr>
<td></td>
<td>7.5 U/ml</td>
<td>12.5 U/ml</td>
</tr>
<tr>
<td>FPA generation (ng/ml±SD, n=3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>With vessel wall</td>
<td>70.2±18.1</td>
<td>20.8±6.9</td>
</tr>
<tr>
<td>Without vessel wall</td>
<td>nd</td>
<td>nd</td>
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</tbody>
</table>

Platelet adhesion (% surface coverage±SD, n=6)

<table>
<thead>
<tr>
<th></th>
<th>Contact platelets</th>
<th>Spread platelets</th>
<th>Aggregates &lt;5 µm</th>
<th>Aggregates 5-10 µm</th>
<th>Total adhesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>With vessel wall</td>
<td>1.7±0.4</td>
<td>4.2±1.9</td>
<td>2.1±1.4</td>
<td>3.4±0.7</td>
<td></td>
</tr>
<tr>
<td>Without vessel wall</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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</tbody>
</table>

Perfusions for 5 minutes at 1300 s⁻¹ with whole blood anticoagulated with low molecular weight heparin or with trisodium citrate were performed with an annular perfusion chamber with or without inverted umbilical artery segments.

FPA=fibrinopeptide A, nd=not determined.

Table 3. Fibrinopeptide A Generation In Perusions over Stimulated Endothelial Cells or over Their Matrix and Platelet Adhesion at Matrix of Stimulated Cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>FPA generation</th>
<th>Platelet adhesion</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>EC</td>
<td>ECM</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No coverslips</td>
<td>3.5±2.0</td>
<td></td>
</tr>
<tr>
<td>No stimulation</td>
<td>10.5±0.8</td>
<td>17.4±0.7</td>
</tr>
<tr>
<td>Stimulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 hr</td>
<td>44.5</td>
<td></td>
</tr>
<tr>
<td>4 hrs</td>
<td>186.0</td>
<td>196.5</td>
</tr>
<tr>
<td>18 hrs</td>
<td>49.2</td>
<td>57.0</td>
</tr>
<tr>
<td>24 hrs</td>
<td>nd</td>
<td>44.0</td>
</tr>
<tr>
<td>TNF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 hr</td>
<td>18.1</td>
<td></td>
</tr>
<tr>
<td>4 hrs</td>
<td>26.5</td>
<td>35.6±0.5</td>
</tr>
<tr>
<td>18 hrs</td>
<td>12.4</td>
<td>20.8±5.5</td>
</tr>
<tr>
<td>24 hrs</td>
<td>nd</td>
<td>17.8±2.3</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No stimulation</td>
<td>18.7±4.1</td>
<td>15.8±6.4</td>
</tr>
<tr>
<td>LPS stimulation</td>
<td>4 hrs</td>
<td>778±88</td>
</tr>
<tr>
<td>18 hrs</td>
<td>16.7</td>
<td>512±63</td>
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All values are ±SD. For adhesion values in % surface coverage, n=6; for fibrinopeptide A (FPA) values in ng/ml, n=3; other values represent a typical mean (n=2) in one of three independent experiments.

Whole blood (20 U low molecular weight heparin/ml) was perfused for 5 minutes at 1300 s⁻¹ over endothelial cells (EC), over their matrix (ECM), or through an empty chamber. Before perfusion, EC were stimulated for varying periods with tumor necrosis factor (TNF), 4β-phorbol12-myristate13-acetate (PMA), and lipopolysaccharide (LPS) in another experiment.

FPA=fibrinopeptide A.

The change induced by EC activation on platelet deposition on their matrix is shown in Figures 1A and 1B. Large aggregates oriented parallel to the stream appeared on the matrix of PMA-activated cells in contrast to the adhesion of single platelets found on the matrix of non-activated cells. Platelet adhesion and aggregate formation were quantified in cross-sections of epon-embedded endothelial matrix. The results are summarized in Table 3. A small increase in adhesion observed after 4 hours of PMA or LPS activation was in accordance with earlier observations. TNF stimulation did not show this. A decrease in adhesion after a longer stimulation with PMA and TNF was observed; a similar phenomenon was reported earlier for PMA stimulation. Aggregate formation increased almost three times by PMA stimulation. Most aggregates were small (<5 µm), but larger aggregates were also observed. TNF and LPS activation resulted in a doubling of the aggregate formation. A clear revers-
Figure 1. En face morphological appearance of endothelial cell matrix that was perfused for 5 minutes at 1300 s⁻¹. A. Matrix of nonstimulated cells. B. Matrix of cells that were stimulated for 18 hours with 4 β-phorbol12-myristate13-acetate. × 1000

ability of this increase was seen after 18 hours of stimulation. The maximal effects were observed with all agonists after 4 hours of activation, coinciding with the maximal FPA generation.

At a shear of 1300 s⁻¹, less than 15% of the cell and matrix surface was covered with visible fibrin. No platelets were found associated with the EC surface. Coverslips with matrix obtained from the different knobs in the chamber had comparable adhesion and aggregation values.

The fact that maximal procoagulant activity at the EC surface was only expressed after 4 hours (Table 3) suggested the necessity of de novo protein synthesis by the EC for the observed phenomena. Cycloheximide (1 μg/ml) in the cell culture medium during 4 hours of PMA activation indeed prevented induction of procoagulant activity at cell surface and matrix. FPA generation levels were comparable to those found in the absence of PMA (not shown).

The effects that various methods of cell removal had on the procoagulant activity in the matrix of PMA-stimulated endothelium were also studied. The highest FPA generation and platelet aggregate formation were found at matrices obtained by ammonia treatment or by nitrocellulose stripping of cells. Matrix isolated with urea was intermediate. Triton X-100 destroyed all procoagulant activity and resulted in aggregate formation and FPA generation values as observed with the matrix of nonactivated EC (results not shown).

Variations in Perfusion Conditions

Perfusion parameters were varied in perfusions over matrix of PMA-activated cells. Changes in shear rate had no influence on FPA generation (Figure 2). At all shear rates tested, FPA was formed rapidly between 1 and 5 minutes and more slowly between 5 and 10 minutes. Platelet adhesion (Figure 3) increased with the shear rate, leveling off at 1300 sec⁻¹, whereas aggregate formation increased more slowly above shear rates of 800 s⁻¹. The percentage of platelet aggregates on the total spread was constant (between 57% and 64%) in the range of investigated shear rates. At shear rates above 800 s⁻¹, less than 15% of the surface was covered with fibrin (Figure 4). Significantly more matrix surface was covered with fibrin when perfused at low shear rates. This is illustrated in Figure 5B, which shows the presence of a dense fibrin network on the surface often located beneath the aggregates at a shear rate of 100 s⁻¹. At higher shear rates (Figure 5A), fibrin was found predominantly in association with aggregates, from which fibrin fibers were often sprouting.

The time dependence of platelet adhesion at 1300 sec⁻¹ is shown in Figure 6. Total platelet adhesion slowed down after 5 minutes, whereas total aggregate formation still increased with time. This was illustrated by an increasing percentage of aggregates on the total spread.

The effect of the platelet count in the perfusate on platelet deposition and thrombin formation is shown in Table 4. Platelet adhesion and aggregation increased when more platelets were present in the perfusates. FPA generation did not increase consistently with platelet
posttranslational modification on shear rate on rate and amount of FPA generation in perfusions over matrix of endothelial cells treated for 18 hours with 4 μ-phorbol12-myristate13-acetate.

Figure 3. Platelet adhesion at matrix of endothelial cells treated for 18 hours with 4 μ-phorbol12-myristate13-acetate and perfused with whole blood for 5 minutes (20 U low molecular weight heparin/ml) at varying shear rates. Values are in percent surface coverage ± SEM, n=3. The percent aggregates ± SD as the platelet-platelet interaction parameter are noted.

counts from 10 to 300 x 10^3/μl. Addition of a monoclonal antibody against platelet glycoprotein Ib blocked virtually all adhesion and gave no decrease in FPA generation.

Whether the time of contact between the EC matrix and the perfusate was essential for the observed FPA generation was tested, and this is illustrated in Figure 7. FPA generation was followed for 10 minutes in whole blood perfused for varying times. FPA generation was dependent on the actual perfusion time, with a steep increase in FPA generation in the first 3 minutes and a leveling off after 5 minutes of interaction. When perfusion times were shorter, FPA generation stayed fixed at the level reached immediately after circulation stopped.

Whether procoagulant activity associated with the matrix of activated endothelium had only a local effect or whether it influenced downstream platelet vessel wall interaction was investigated as follows (Table 5). The normal coverslip (3.24 cm²) in the perfusion chamber was replaced by two half coverslips. The upstream half coverslip contained the matrix of activated endothelial cells, the downstream half had matrix from nonactivated cells. In the control perfusions, both coverslips were covered with matrices from either activated or nonactivated cells. When perfused with 20 U/ml of LMWH blood, the activated upstream matrix did not increase aggregate formation on the nonactivated downstream matrix. At the latter, aggregation values were comparable with those obtained in perfusions with nonactivated upstream matrix. No significant differences in adhesion and aggregate height were observed between the two half coverslips on one knob. These results confirm that matrix procoagulant has only a local effect on platelet thrombi formation under the perfusion conditions of our experiments. The same experiments, however, with 5 U/ml of LMWH showed an increase in large aggregate formation at the downstream coverslip with a matrix of nonactivated endothelial cells. When LMWH concentration is too low, locally formed thrombin is less efficiently inhibited and more likely to induce the formation of downstream platelet aggregate on the matrix of nonactivated cells.

**Effect of Clotting Factors and Glycoprotein Ib/Illa Complex**

In a special series of experiments, we tested whether the increase in aggregate formation after EC activation could be attributed to matrix-associated procoagulant activity alone. For this purpose, perfusates were reconstituted with plasma that was made deficient in prothrom-
Figure 5. An electron microscopic en face cross-section of a platelet thrombus on endothelial cell matrix (M). A. Matrix of endothelial cells that was stimulated for 18 hours with 4β-phorbol12-myristate 13-acetate was perfused at 1300 s−1. The arrows point to fibrin strands. Bar=1 μm. Inset. A detail of fibrin showing characteristic cross-striping. Bar=200 μm. B. Matrix of stimulated cells that was perfused at 100 s−1. F=fibrin deposition at matrix.

bin. The prothrombin-deficient perfusates were circulated over the matrix of stimulated cells. Prothrombin was added back into these perfusates in control perfusions. Adhesion was not affected by the absence of prothrombin. However, prothrombin-deficient perfusates decreased the aggregate formation and the FPA generation even below the levels obtained with the matrix of unstimulated cells (Table 6). The addition of prothrombin reversed this decrease to levels seen with normal plasma. These observations confirm that the increase in aggregate formation seen upon the activation of EC was due to thrombin formation.

Subsequently, a polyclonal antiserum against tissue factor was incubated with the stimulated matrix, or a polyclonal antiserum against factor VIIa was added to the perfusate and compared to control perfusions with non-specific rabbit serum incubated with matrix and added to the perfusate. In both experiments, FPA generation was reduced to the same levels as observed with the matrix of nonactivated endothelial cells. Also, aggregate formation was significantly inhibited and comparable to values found at nonactivated matrices.

A monoclonal antibody against glycoprotein IIb/IIa, which was able to inhibit fibrinogen binding and platelet aggregation in the aggregometer, also blocked aggregate formation in our perfusion system. This antibody directed against glycoprotein IIb/IIa on platelet membranes had no influence on FPA generation.

An interesting question was whether the tissue factor and factor VII-mediated thrombin formation went directly via the activation of factor Xa, or whether it involved factor
Anticoagulated blood thus had to be used to prevent coagulation during collection and storage, manipulation, and perfusion of blood in a system without vessel segments. Moreover, this concentration of LMWH permitted FPA generation when artery segments, known to possess procoagulant activity, were perfused. Total adhesion and aggregate formation were not altered when compared with citrated blood perfusions. Lower LMWH concentrations caused increased adhesion and thrombus formation. However, these concentrations were insufficient to prevent coagulation during manipulation of blood. To avoid this nonspecific, nonvessel-wall-dependent thrombin generation, we decided to use 20 U LMWH/ml in succeeding experiments.

Although tissue factor has recently been reported in the subendothelium, its localization and possible association with smooth muscle cells and fibroblasts add an uncertainty to the use of blood vessel segments. The risk of variable mechanical damage to the subendothelium, and therefore variable expression of procoagulant activity and other adhesive proteins, is hard to avoid. A more important drawback is that vessel segments do not allow the study of the variability of the subendothelium as a function of the exposure of endothelium to various physiological conditions, agonists, or both. These limitations are not encountered when the extracellular matrix of cultured vessel wall cells is used as a model for the vessel wall in platelet-vessel wall interaction. Although several reports have stressed the importance of the endothelial surface in regulating activation of the coagulation cascade. EC stimulated with TNF, endotoxin (LPS), or PMA synthesize and express tissue factor on their surface, which leads to thrombin generation. We were interested in whether increased procoagulant activity of the EC surface could be detected in perfusions with LMWH-anticoagulated blood, whether increased procoagulant activity was also expressed in the EC matrix, and how this would influence platelet interaction with this matrix. For this purpose, we circulated LMWH-anticoagulated blood through a rectangular perfusion chamber with resting EC or their extracellular matrix.

Compared to perfusions through an empty system, nonactivated cells or their matrix caused only a small amount of FPA generation. Stimulation of EC with TNF, LPS, or PMA largely increased thrombin generation as reflected by the increased FPA levels in the perfusate (Table 3). The procoagulant activity associated with the matrix was expressed by increased FPA generation in the perfusate and was associated with an increase in platelet aggregate formation. The matrix of stimulated EC, however, also seems to be associated with lower adhesion as compared to unstimulated matrices (Table 3). This...
thrombus formation on the matrix was found. The aggregation was seen in adhesion values between stimulated and depleted plasma with purified prothrombin brought of up to 20% of surface coverage, and no differences were seen in adhesion values between stimulated and unstimulated matrices (Tables 5 and 6). Prothrombin-deficient perfusates showed that thrombin formation was essential for the observed phenomena. Supplementing the depleted plasma with purified prothrombin brought back the platelet thrombi and FPA generation (Table 6). No indication of an alternative reason for the increased thrombus formation on the matrix was found. The aggregates were directed along the stream and reached heights of up to 20 μm. This relatively low aggregate height is probably the reason that in this system no axial dependency was observed for aggregate height. Electron microscopy showed fibrin strands mainly oriented around the aggregates. Because of its stability and powerful action, PMA stimulation was used in further experiments to characterize the system. An 18-hour stimulation was most frequently used.

Thrombin induced by matrix-associated procoagulant activity was only locally active and did not circulate in perfusates anticoagulated with 20 U/ml LMWH. This was deduced from the observation that contact of the perfusate with the matrix was essential for FPA generation. After the perfusion was stopped, no further FPA was generated in the perfusate (Figure 4). Moreover, with 20 U LMWH/ml, the procoagulant activity associated with the stimulated matrix did not increase aggregate formation on the matrix of the nonactivated cells present on a half coverslip in the same perfusion chamber. We detected no axial dependence for adhesion and aggregation at one coverslip, as was recently reported in citrated blood; neither did we detect significant differences between the two half coverslips with identical matrices on one knob. Perfusates at a shear of 1300 s⁻¹ and with most aggregates lower than <5 μm should not be very sensitive for axial dependence.

Perfusates anticoagulated with 5 U LMWH/ml did allow upstream procoagulant activity to increase aggregate formation on an unstimulated matrix. We found that 5 U LMWH/ml, a concentration which gave FPA generation by a mere manipulation of the blood (Table 1), is less effective in inhibiting thrombin generated at the tissue factor-rich matrix. This thrombin might pre-activate platelets in the boundary layer or bind to the unstimulated matrix downstream. The thrombin formed at the tissue factor-rich matrix would in this way cause the increased adhesion and aggregate formation at the unstimulated matrix. Translocation of thrombi from the upstream matrix to the downstream matrix cannot be excluded as a cause for increased aggregate formation downstream with 5 U LMWH/ml. The fact that no indication for such a mechanism was seen in perfusions with 20 U/ml made this mechanism less likely. LMWH at 5 U/ml increased platelet adhesion and aggregation compared with 20 U/ml at both stimulated and unstimulated matrix when compared to 20 U/ml LMWH (Table 5). This phenomenon was also seen at umbilical artery segments (Table 2) and again

<table>
<thead>
<tr>
<th>Platelet count (x10⁷/μl)</th>
<th>FPA (ng/ml, n=3)</th>
<th>Platelet adhesion (% surface coverage, n=6)</th>
<th>Aggr &lt;5 μm</th>
<th>Aggr 5–10 μm</th>
<th>Total adhesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>198±31</td>
<td>1.4±0.9 (22.1%)</td>
<td>0.0±0.0 (0.0%)</td>
<td>6.4±1.5</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>102±40</td>
<td>20.6±3.7 (56.4%)</td>
<td>0.8±0.7 (2.1%)</td>
<td>37.0±3.0</td>
<td></td>
</tr>
<tr>
<td>160</td>
<td>169±55</td>
<td>21.8±2.2 (54.2%)</td>
<td>0.7±0.6 (1.8%)</td>
<td>40.8±3.5</td>
<td></td>
</tr>
<tr>
<td>160 + antiGP Ib IgG</td>
<td>145±31</td>
<td>0.4±0.4 (18.6%)</td>
<td>0.0±0.0 (0.0%)</td>
<td>1.6±1.2</td>
<td></td>
</tr>
<tr>
<td>3.00</td>
<td>214±69</td>
<td>25.2±3.2 (56.7%)</td>
<td>3.9±1.7 (8.6%)</td>
<td>45.4±2.5</td>
<td></td>
</tr>
</tbody>
</table>

All values are ±SD.

Reconstituted perfusates (low molecular weight heparin 20 units/ml with varying platelet counts were circulated for 5 minutes at 1300 s⁻¹ over matrix of 18-hour, PMA-stimulated endothelial cells. The percentage aggregate coverage on the total spread coverage is given in parentheses. Purified antiglycoprotein Ib IgG was added to the perfusate (0.5 μg/ml); a control aspecific immunoglobulin IgG, in the same concentration had no effect.

FPA=fibrinopeptide A, Aggr=aggregate, PMA=4β-phorbol12-myristate13-acetate, antiGP=antiglycoprotein.
The tissue factor was dependent on de novo protein.

The tissue factor activity in our system would agree with the idea that less efficient coagulation allows locally

suggests that less efficient coagulation allows locally formed thrombin to systematically increase platelet-vessel wall interaction.

The fact that 20 U LMWH/ml, and even concentrations as high as 40 U/ml (results not shown), allowed matrix-dependent FPA generation, suggested protection of the matrix-initiated coagulation against inhibition. Binding of coagulation factors to the matrix may be important in the protection against inhibition. Indeed, endogenous thrombin bound to ECM could hardly be inhibited by antithrombin III (Eldor A, Vlodavsky I, personal communication). The binding of coagulation factors to the matrix would agree with the observed local action of thrombin in our system.

Thrombin generation was tissue factor- and factor Vlla-dependent. Antibodies against both factors inhibited FPA generation and aggregate formation at the matrix. The tissue factor was dependent on de novo protein synthesis by the EC. The expression of procoagulant activity in EC matrix was independent of the cell removal procedure. Tissue factor remained in the matrix after cell lysis (NH4OH), mechanical removal of endothelium (nitrosoyldine), and should therefore be tightly anchored to the matrix. Triton X-100 abolished the matrix procoagulant activity, but is known to specifically solubilize tissue factor apoprotein from its lipid environment. The configuration of tissue factor in the matrix is yet to be investigated.

The lack of effect of factor IX removal suggests that the factor Vlla-X pathway was responsible for the thrombin formation under the experimental conditions used. This situation may be similar to that observed in hemostatic plug formation in animal and human skin wounds, in which peripheral fibrin formation is not affected by lack of factor IX or VIII.53,54 The reported factor Vlla activation of

Table 5. Fibrinopeptide A Generation and Platelet Adhesion at Matrices of Stimulated and Nonstimulated Endothelial Cells within One Perfusion Chamber

<table>
<thead>
<tr>
<th>Anticoagulation</th>
<th>Matrices on one knob</th>
<th>FPA (ng/ml, n=3)</th>
<th>Platelet adhesion (% surface coverage, n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Upstream</td>
<td>Downstream</td>
<td>Aggr &lt;5 μm</td>
</tr>
<tr>
<td>LMWH 5 U/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A.</td>
<td>ECM + PMA</td>
<td>ECM + PMA</td>
<td>217±25</td>
</tr>
<tr>
<td>B.</td>
<td>ECM + PMA</td>
<td>ECM</td>
<td>136±19</td>
</tr>
<tr>
<td>C.</td>
<td>ECM</td>
<td>ECM</td>
<td>31±2</td>
</tr>
<tr>
<td>LMWH 20 U/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A.</td>
<td>ECM + PMA</td>
<td>ECM + PMA</td>
<td>174±30</td>
</tr>
<tr>
<td>B.</td>
<td>ECM + PMA</td>
<td>ECM</td>
<td>84±10</td>
</tr>
<tr>
<td>C.</td>
<td>ECM</td>
<td>ECM</td>
<td>7±1</td>
</tr>
</tbody>
</table>

Table 6. Influence of Coagulation Factors and Antiglycoprotein IIb/IIIa IgG on Fibrinopeptide A Generation and Platelet Adhesion

<table>
<thead>
<tr>
<th>PMA</th>
<th>Incubation</th>
<th>Addition</th>
<th>FPA (ng/ml, n=3)</th>
<th>Platelet adhesion (% surface coverage, n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Aggr &lt;5 μm</td>
</tr>
<tr>
<td>A</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>129±29</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>α-tf</td>
<td>−</td>
<td>32±21</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>α-Vlla</td>
<td>29±11</td>
<td>5.2±3.9 (22.9%)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>nrs</td>
<td>nrs</td>
<td>81±20</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>−</td>
<td>Fil def</td>
<td>4±3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>−</td>
<td>Fil def + Fil</td>
<td>173±14</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>−</td>
<td>α-GP IIb IIIa</td>
<td>153±51</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>−</td>
<td>aspec IgG</td>
<td>170±56</td>
</tr>
</tbody>
</table>

All values are ±SD.

Whole blood (20 units LMWH/ml) was circulated for 5 minutes at 1300 s⁻¹ over matrix of 18-hour, PMA-stimulated endothelial cells. B. Upstream a coverslip with matrix of stimulated cells and downstream, a matrix of nonstimulated cells. C. Two coverslips with matrix of nonstimulated cells. Platelet adhesion was evaluated at matrix indicated in boldface. The percentage aggregate coverage on the total spread coverage is given, in parentheses.

FPA=fibrinopeptide A, LMWH=low molecular weight heparin, ECM=endothelial cell matrix, PMA=4β-phorbol12-myristate13-acetate.
factor IX, which in cooperation with factor VIII would activate factor X, probably becomes more important when smaller amounts of tissue factor are present.38

The role of platelets' phospholipids in supporting thrombin generation9,16 could not be demonstrated by FPA generation in our system. The amount (Table 4) and rate (not shown) of FPA generation was identical with varying platelet numbers. Inhibition of adhesion from 41% to 2% surface coverage (Table 4) and of thrombosis formation from 54% to 9% (Table 6) by antibodies against glycoproteins Ib and glycoproteins IIb/IIIa, had no effect on the FPA levels in the perfusate. This was in agreement with earlier reports in which the blood of thrombocytopenic or thrombasthenic donors come by using a vessel wall model with increased tissue factor activity might become possible with selective inhibition. Furthermore, only a limited portion of the potential amount of the blood of thrombocytopenic or thrombasthenic donors is present.95

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