Subendothelial Proteins and Platelet Adhesion

von Willebrand Factor and Fibronectin, Not Thrombospondin, Are Involved in Platelet Adhesion to Extracellular Matrix of Human Vascular Endothelial Cells

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Endothelial cell matrix contained von Willebrand factor (VWF), fibronectin, and thrombospondin. The role of these proteins in the adhesion of platelets was investigated by preincubation of the matrix with specific antibodies and subsequent perfusion with human blood. When perfusions were performed with platelets in a human albumin solution (HAS) platelet adhesion was similar to that with normal plasma, indicating that proteins in the matrix can fully support adhesion. Preincubation of the matrix with a monoclonal antibody to VWF and perfusion with HAS showed a nearly complete inhibition of platelet adhesion at 1300 s⁻¹, indicating a role for matrix-bound VWF at high shear rates and no requirement for VWF in plasma. Preincubation of the matrix with antihuman fibronectin F(ab)² showed a slight inhibition of adhesion. The same result was obtained with perfusions with fibronectin-free plasma, and an untreated matrix. Preincubation with antifibronectin F(ab)² and perfusion with fibronectin-free plasma showed a significant inhibition of platelet adhesion at all shear rates. These results indicate that fibronectin is required for adhesion at all shear rates. Preincubation of the matrix with different antibodies against human platelet thrombospondin showed no inhibition of platelet adhesion at all wall shear rates. Thrombospondin in the matrix is evidently not required for platelet adhesion.

(Arteriosclerosis 6:24–33, January/February 1986)

When endothelial cells lining the inner surface of blood vessels are damaged, the subendothelium becomes exposed to the blood flow resulting in a hemostatic response.¹ Platelets adhere to the subendothelium, then there is release of the content of their granules and aggregation.² The thrombogenic surface of the subendothelium is thus important to stop bleeding and is also involved in thrombosis and atherosclerosis.³,⁴

To study the interaction of platelets with subendothelium, animal models have been used.⁵,⁶ In vitro perfusion studies²,⁷,⁸ in which everted deendothelialized vascular segments were exposed to recirculating blood have been performed. A more recent approach⁹–¹² is to study platelet interaction with the isolated extracellular matrix of cultured vascular endothelial cells. This extracellular matrix resembles subendothelium in vivo in its organization and macromolecular constituents¹³ but it is devoid of components produced by other vessel wall cells and thus constitutes a relevant model for the study of platelet vessel wall interaction.

Endothelial cells synthesize and secrete von Willebrand factor (VWF),¹⁴,¹⁵ fibronectin,¹⁶ and thrombospondin;¹⁷,¹⁸ all are components of the pericellular matrix.¹⁶,¹⁵,²⁰ Factor VIII-VWF is required for platelets to adhere to the subendothelium of rabbit and human vessels.⁸,²¹,²² Plasma FVIII-VWF binds to the subendothelium and subsequently mediates platelet adhesion.²³ Subendothelium itself contains VWF,²⁴ which also plays a role in platelet adhesion.²⁵,²⁶ Fi-
bronecin promotes the spreading of platelets on collagenous surfaces and subendothelial matrices.\textsuperscript{27-30} We recently reported that fibronectin was required for platelet adhesion to subendothelial or purified collagen in flowing blood.\textsuperscript{31,32} Thrombospondin is released from the alpha granules of platelets after activation\textsuperscript{33} and reportedly\textsuperscript{34} acts as an endogenous lectin. Thrombospondin thus may be involved in aggregation, possibly by binding to fibrinogen.\textsuperscript{35} Thrombospondin could also have a role in platelet adhesion.\textsuperscript{36} Here we report on von Willebrand factor, fibronectin, and thrombospondin, that are present in the extracellular matrix of cultured human vascular endothelial cells and their role in the interaction of platelets with this matrix.

**Methods**

**Culture of Endothelial Cells and Isolation of Extracellular Matrix**

Human vascular endothelial cells derived from umbilical veins were isolated and cultured according to the method of Jaffe et al.\textsuperscript{37} with some modifications.\textsuperscript{38} Briefly, the endothelial cells were isolated with 0.05\% trypsin and 0.02\% EDTA and cultured in plastic flasks precoated with fibronectin in a medium consisting of RPMI-1640 (Gibco, Paisley, UK) and 20\% pooled serum. After confluence, the cells were subcultured (2 \( \times \) 10\(^4\) cells/cm\(^2\)) on gelatin-coated glass coverslips. After 5 to 7 days when the cells reached confluence (8-10 \( \times \) 10\(^4\) cells/cm\(^2\)), the cultures were exposed to 0.1 M NH\(_4\)OH for 30 minutes at room temperature and were gently shaken. The cell layer was completely removed by this procedure leaving the underlying extracellular matrix intact, homogeneously and firmly attached to the glass. The matrix-coated coverslips were stored in phosphate-buffered saline (PBS) at 4°C and used for experiments the same day.

**Antibodies**

Rabbit antiserum against human plasma fibronectin, the corresponding IgG fraction and F(ab\(^\prime\))\(_2\) fragment were purchased lyophilized (Cappel Laboratories, Cochranville, Pennsylvania) and reconstituted in 0.02 M of PBS (pH 7.3). Protein concentrations were 40.0, 20.0 and 15.5 mg/ml respectively. Ouchterlony double diffusion analysis against normal plasma, fibronectin-free plasma and purified fibronectin, FVIII-VWF, and fibrinogen showed only one line of identity with normal plasma and purified fibronectin. CLB-RAg 35, a monoclonal antibody directed against the platelet binding domain of the human VWF molecule, was kindly provided by Jan A. van Mourik (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam). The properties of this monoclonal antibody have been described.\textsuperscript{39} Rabbit antithrombospondin antiserum, hereafter designated serum A, was kindly provided by Geir Gogstad (Oslo, Norway). This antiserum has been described.\textsuperscript{40} It did not cross-react with fibronectin, platelet factor 4, \( \beta \)-thromboglobulin, or fibrinogen.

A second antiserum against thrombospondin, serum B, was prepared by Karel Nieuwenhuis (University Hospital Utrecht, Utrecht). Thrombospondin was purified from the supernatant of thrombin-treated human platelets by affinity chromatography on a heparin-Sepharose CL-6B column. The protein was further purified by fast protein liquid chromatography on a Mono Q column (Pharmacia, Uppsala, Sweden). This thrombospondin preparation was more than 95\% homogeneous, as assessed by SDS polyacrylamide gel electrophoresis. Antiserum against this purified thrombospondin was raised in rabbits. Possible contaminating antibodies against fibrinogen, fibronectin, or FVIII-VWF were removed by absorption with cryoprecipitate prepared from normal human plasma. The antiserum did not cross-react with purified human fibrinogen, fibronectin, albumin, FVIII-VWF, or \( \beta \)-thromboglobulin as determined by Ouchterlony analysis and ELISA. The antiserum cross-reacted weakly with platelet factor 4 in an ELISA.

Two monoclonal antibodies against thrombospondin (TSPI-1 and TSPI-2) were kindly provided by Mark Grinsberg and Ed Plow (Scripps Clinic and Research Foundation, La Jolla, California). All monoclonal antibodies were used as ascites. Extensive previous experiments with control ascites have shown that this does not influence platelet adherence.\textsuperscript{39}

**Gel Electrophoresis and Immunoblotting**

SDS gel electrophoresis was performed on 7\% polyacrylamide gel essentially according to the procedure of Laemmlli.\textsuperscript{41} The gels were run at 200 V (constant voltage) for 20 hours. After Western blotting, the gel was stained with Coomassie Brilliant Blue R250 and dried. After gel electrophoresis, the proteins were transferred electrophoretically to nitrocellulose filters (Schlierich and Schultz, Darsel, FRG), essentially as described by Towbin et al.\textsuperscript{42} The blots were washed in a buffer containing 10 mM Tris-HCl, 150 mM NaCl, 3\% bovine serum albumine, and 0.2\% Tween (pH 7.4), and then incubated overnight with antiserum (1:100 dilution). After the washing, the filters were incubated with peroxidase-labeled goat anti-rabbit IgG (Nordic, Tilburg, The Netherlands) and diluted 1:1000 in washing buffer. The proteins were visualized with diaminobenzidine-tetrahydrochloride (0.5 g/liter in 50 mM Tris-HCl buffer (pH 7.6) containing 0.006\% H\(_2\)O\(_2\)).

**Immunofluorescence Microscopy**

Matrices on glass coverslips were incubated with specific mouse or rabbit antibodies for 1 hour at room temperature and subsequently perfused with human...
blood. As a control, the matrices were incubated with normal rabbit serum. After perfusion, the matrices were fixed in 0.5% glutaraldehyde. Nonperfused matrices that had been preincubated with antibodies were also fixed. The presence of specific antibodies on the matrix before and after perfusion was then visualized by immunofluorescence. The matrices were immersed in methanol for 5 minutes, washed three times with phosphate-buffered saline (PBS) and subsequently incubated with a fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (1:50 dilution, Nordic, Tilburg, The Netherlands) or a FITC-conjugated goat antirabbit IgG (F(ab')2 fragment-specific, diluted 1:50 from Cappel Laboratories, Cochraneville, Pennsylvania). Incubation with these second antibodies was for 30 minutes at room temperature. The coverslips were washed with PBS, embedded in mounting fluid: 80% (vol/vol) glycerol, 1 mg/ml p-phenylene diamine, 1 mM sodium phosphate buffer (pH 8.6) and finally examined with a fluorescence microscope (Leitz, Wetzlar, FRG).

**Fibronectin-Free Plasma**

Fibronectin-depleted plasma was prepared by passing 200 ml of normal human plasma over a column of gelatin-Sepharose 4B (2.6 × 15 cm, equilibrated with 0.05 M Tris-HCl, pH 7.4). Gelatin-Sepharose 4B was prepared by coupling gelatin (A.G. E. Merck, Darmstadt, FRG) to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden), according to the manufacturer's procedure. The plasma eluting from the column was collected, and the first and last fractions were discarded to minimize dilution. This fibronectin-depleted plasma contained no detectable (less than 3 ng/ml) fibronectin in an electroimmunoassay with rabbit antihuman fibronectin serum and purified fibronectin as a standard. There was no change in the level of FVIII:Ag and FVIII:RCF after passage over the gelatin column. The plasma was frozen and stored at −70°C.

**Preparation of Perfusates**

Blood from normal human donors was anticoagulated with 1:10 volume 110 mM trisodium citrate. This whole blood was used for perfusion experiments. Perfusates were also performed with fibronectin-free blood. In the latter experiments, platelets were first isolated and washed by centrifugation as previously described and then resuspended in fibronectin-free plasma. For some experiments, the washed platelets were resuspended in a human albumin solution (HAS): 4% (wt/vol) human albumin (Bahringswerke AG, Marburg, FRG) in Krebs-Ringer buffer (pH 7.35) containing 19 mM citrate, 2.5 mM CaCl₂, and 5 mM glucose. Washed platelets were also resuspended in normal plasma.

Red blood cells were washed three times by centrifugation (10 minutes, 3000 g, 20°C) in isotonic saline, containing 5 mM glucose. Prior to each perfusion run, the perfusate (15 ml) was reconstituted by adding washed and packed red cells to the resuspended platelets (hematocrit 0.4; platelet count 1.14 × 10⁹/ml, final concentration).

**Perfusons**

Platelet interaction with the extracellular matrix in flowing blood was studied at 37°C in a rectangular perfusion chamber. The matrices were preincubated for 1 hour at room temperature with specific antibodies, diluted in PBS. As a control, the matrices were incubated with buffer alone or with buffer containing normal rabbit serum or control ascites. The coverslip then was inserted in the perfusion chamber and rinsed with 25 ml 10 mM HEPES-buffered saline (pH 7.4). The perfusate (15 ml) was prewarmed for 5 minutes at 37°C and recirculated through the perfusion chamber under a nonpulsatile steady flow. The perfusates were performed for 5 minutes at wall shear rates ranging from 100 to 1300 s⁻¹. After each perfusion, the system was rinsed with 25 ml HEPES-buffered saline. The coverslip was removed, washed with HEPES-saline, and fixed in glutaraldehyde as previously described.

**Morphology**

Platelet adhesion on the extracellular matrix was evaluated by light microscopy after fixation with glutaraldehyde and staining with May-Grünwald-Giemsa, as previously described. Platelet adhesion is expressed as the percentage of the surface covered by platelets. This was evaluated by en face light microscopy at a magnification of 1000. The light microscope was interfaced with an image analyzer (Quantimet 720, L'manco, Royston, UK). For every coverslip, 10 fields, each consisting of 500,000 image points (0.028 mm²), were selected at random and evaluated.

**Statistics**

Statistical significance of differences between means was evaluated with Student’s t test for non-paired samples.

**Results**

**Platelet Interaction with Endothelial Cell Extracellular Matrix**

The presence and distribution of VWF, fibronectin, and thrombospondin in the isolated extracellular matrix of confluent cultures of endothelial cells was studied by indirect immunofluorescence. With specific antibodies against these three proteins, there was a fibrillar fluorescence pattern of the matrix homogeneously distributed over the glass coverslip (Figure 1). In double fluorescence experiments, a codistribution of all three proteins was found throughout the substratum (not shown).
Previous results obtained in our laboratory\textsuperscript{11,12} showed that platelet adhesion to the extracellular matrix increased with the duration of the perfusion. After 5 minutes, maximal adhesion was reached, but there was still no platelet aggregate formation. Therefore, in all experiments a standard perfusion time of 5 minutes was used. An example of the morphology of platelets adhering to the matrix is shown in Figure 2 in which platelets are evenly distributed over the surface without aggregates.

Platelet adhesion increased at higher wall shear rates. The results in Table 1 show that platelet adhesion increased with increasing wall shear rate between 100 to 800 s\textsuperscript{-1}, reaching a plateau at 800 s\textsuperscript{-1}. The effect of VWF on platelet adhesion to subendothelium is dependent on the wall shear rate.\textsuperscript{41} To investigate the role of matrix proteins in the adhesion of platelets to the extracellular matrix, we therefore performed perfusions at different shear rates.

**Preincubation with Anti-VWF**

To investigate the role of VWF in platelet adhesion to the extracellular matrix, we preincubated the matrix with mouse monoclonal antibody CLB-RAg 35. This antibody is directed against the platelet-binding domain on VWF\textsuperscript{39} and inhibits platelet adhesion to human subendothelium at high shear rates.\textsuperscript{39} Preincubation of the matrix with CLB-RAg 35 showed similar fluorescence both before and after perfusion (not shown).

Perfusion with whole blood after preincubation of the matrix with CLB-RAg 35 did not result in inhibition of platelet adhesion (Table 1). The blocking of VWF
Figure 2. Morphology (en face) of platelet adhesion on the extracellular matrix. Perusions were performed for 5 minutes at 300 s\(^{-1}\) (A) and 800 s\(^{-1}\) (B). After the perusions, the coverslips were fixed and stained as described in methods (× 1000).

in the matrix by the antibody could be overcome by VWF from plasma as was previously demonstrated for subendothelium. However, when the perfusate was preincubated with CLB-RAg 35, platelet adhesion was markedly diminished at high shear rates (800 and 1300 s\(^{-1}\)) but not at low shear rates (100 and 300 s\(^{-1}\)). The same result was obtained when both the matrix and the perfusate were preincubated with CLB-RAg 35.

Apparently the antibody in plasma was also able to block VWF in the matrix. Another possibility is that VWF in the matrix has no role in platelet adhesion. To test this, we performed perusions with platelets resuspended in a human albumin solution (HAS). Platelet adhesion to the matrix was similar whether the perusions were performed with normal plasma or with HAS (Table 2). However, when VWF in the matrix was blocked by preincubation with CLB-RAg 35, platelet adhesion at high shear rate was completely abolished when the perfusion was performed with HAS. At low shear rate, preincubation with the antibody had no effect.

Preincubation with Antifibronectin

To see whether fibronectin plays a role in platelet adhesion to the endothelial cell matrix, we preincubated the latter with a rabbit polyclonal antibody against human plasma fibronectin. When the matrix was preincubated with antifibronectin serum or IgG, the surface coverage was diminished but extensive platelet aggregate formation was observed (not

<table>
<thead>
<tr>
<th>Preincubation with CLB-RAg 35</th>
<th>Platelet adhesion (% surface coverage)</th>
<th>Wall shear rate, s(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECM</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>−</td>
<td></td>
<td>11.0±2.1</td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>12.9±2.0</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>8.4±1.0</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>11.0±1.3</td>
</tr>
</tbody>
</table>

The extracellular matrix (ECM) of human vascular endothelial cells was preincubated for 60 minutes at room temperature with CLB-RAg 35, a monoclonal antibody against the platelet-binding domain of the von Willebrand Factor (VWF) molecule (see reference 39). Ascites fluid was diluted 1:500 in phosphate-buffered saline. The perfusate was preincubated with CLB-RAg 35 (ascites) for 10 minutes at 37°C at a 1:500 dilution in plasma. Perusions were performed for 5 minutes with anticoagulated whole blood at the indicated wall shear rates. After perfusion, the matrices were fixed, stained, and the percentage of surface coverage was determined by morphometrical evaluation (see methods). Values are means ± SEM; n = 3. + = preincubation with antibody; − = no preincubation.

*P < 0.001, compared with perfusion without CLB-RAg 35 preincubation.
shown). To overcome this problem, we used the antifibronectin F(ab')2 fragment. Fibronectin in the matrix was visualized by indirect immunofluorescence with antifibronectin F(ab')2 as the first antibody. Similar fluorescence was observed before and after perfusion (Figure 1A and B).

When perfusion was performed with fibronectin-free plasma, platelet adhesion was slightly, but significantly, diminished at 150 and 1000 s⁻¹, but not at 400 s⁻¹ (Table 3). The same results were obtained when fibronectin in the matrix was blocked by preincubation with antibody, but was normally present in plasma. However, when the matrix was preincubated with antifibronectin F(ab')2 and fibronectin was omitted from plasma, the inhibition of platelet adhesion was highly significant at all shear rates.

**Preincubation with Antithrombospondin**

To investigate the possibility that matrix-bound thrombospondin is involved in platelet adhesion, the matrix was preincubated with two different rabbit antisera against thrombospondin and two antithrombospondin monoclonal antibodies (Table 4). The presence of thrombospondin in the matrix was dem-

### Table 2. Platelet Adhesion to the Endothelial Cell Matrix: Role of von Willebrand Factor

<table>
<thead>
<tr>
<th>ECM preincubation</th>
<th>Perfusate</th>
<th>Platelet adhesion (% surface coverage)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wall shear rate, s⁻¹</td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>Normal plasma</td>
<td>14.9 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>HAS</td>
<td>18.9 ± 1.8</td>
</tr>
<tr>
<td>CLB-Rag 35</td>
<td>HAS</td>
<td>17.8 ± 4.7</td>
</tr>
</tbody>
</table>

The extracellular matrix (ECM) was preincubated for 1 hour at room temperature with a 1:500 dilution of CLB-Rag 35 or buffer alone. Subsequently, perfusions were performed with platelets in either normal plasma or a human albumin solution (HAS) for 5 minutes at wall shear rates of 300 and 1300 s⁻¹. Values are means ± SEM; n = 4.

*p < 0.001 compared with perfusion with normal plasma.

### Table 3. Platelet Adhesion to the Endothelial Cell Matrix: Preincubation with Anti-Fibronectin

<table>
<thead>
<tr>
<th>ECM preincubation</th>
<th>Perfusate</th>
<th>Platelet adhesion (% surface coverage)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wall shear rate, s⁻¹</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Normal plasma</td>
<td>15.7 ± 1.0</td>
</tr>
<tr>
<td>Control</td>
<td>FN-free plasma</td>
<td>8.6 ± 0.6*</td>
</tr>
<tr>
<td>Anti-FN F(ab')2</td>
<td>Normal plasma</td>
<td>7.0 ± 0.7*</td>
</tr>
<tr>
<td>Anti-FN F(ab')2</td>
<td>FN-free plasma</td>
<td>3.9 ± 0.3†</td>
</tr>
</tbody>
</table>

The extracellular matrix (ECM) was preincubated for 60 minutes at room temperature with antifibronectin F(ab')2 fragment at a 1:20 dilution in phosphate-buffered saline (0.78 mg/ml). As a control, the matrix was preincubated with the same concentration of non-immune rabbit F(ab')2. The perfusions were performed for 5 minutes at the indicated shear rates with platelets in either normal plasma or fibronectin-free plasma. Values represent the means ± SEM; n = 3. The p values are compared with the control preincubation and perfusion with normal plasma.

* p < 0.005.
† p < 0.05.
‡ p < 0.001.

### Table 4. Platelet Adhesion to the Endothelial Cell Matrix: Preincubation with Anti-Thrombospondin

<table>
<thead>
<tr>
<th>ECM preincubation</th>
<th>Perfusate</th>
<th>Platelet adhesion (% surface coverage)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wall shear rate, s⁻¹</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>19.5 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>34.1 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>59.8 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>1300</td>
<td>54.7 ± 4.5</td>
</tr>
<tr>
<td>Anti-TSP serum A</td>
<td>17.5 ± 1.3</td>
<td>(16)</td>
</tr>
<tr>
<td></td>
<td>27.4 ± 2.2</td>
<td>(11)</td>
</tr>
<tr>
<td>Anti-TSP serum B</td>
<td>18.1 ± 2.2</td>
<td>(10)</td>
</tr>
<tr>
<td></td>
<td>37.3 ± 3.3</td>
<td>(2)</td>
</tr>
<tr>
<td>Anti-TSP ascites</td>
<td>41.2 ± 4.6</td>
<td>(8)</td>
</tr>
</tbody>
</table>

The extracellular matrix was preincubated for 60 minutes at room temperature with rabbit antihuman thrombospondin serum at a 1:100 dilution in phosphate-buffered saline. The matrix was also preincubated with several monoclonal antibodies against thrombospondin (TSP 1-1, TSP 1-2; mouse ascites, 1:50). As a control the matrix was preincubated with PBS or non-immune rabbit serum. Perfusions were performed for 5 minutes with anticoagulated whole blood at the indicated wall shear rates. Values are means ± SEM with the number of perfusions in parentheses.
We previously reported on platelet interaction with endothelial cell extracellular matrix in a perfusion chamber under well-defined flow conditions. Here we studied the role of von Willebrand factor, fibronectin, and thrombospondin in platelet adhesion to the endothelial cell matrix. The presence of these proteins in the matrix was visualized by indirect immunofluorescence with specific antibodies. Similar fluorescence was observed both before and after perfusion with blood, indicating that there was no substantial loss of substratum or of bound antibody during the perfusion run.

The role of von Willebrand factor in platelet adhesion was studied by use of a monoclonal antibody (CLB-RAg 35) directed against the platelet-binding domain of this protein. When this antibody was added to the perfusate, platelet adhesion to the matrix was almost completely abolished at high shear rates (Table 1). Apparently not only VWF in plasma, but also VWF in the matrix, was blocked by CLB-RAg 35. However, platelet adhesion could not be inhibited when only VWF in the matrix was blocked by preincubation with antibody. This can be explained by the possibility that VWF from plasma binds to the matrix and subsequently supports platelet adhesion, as was previously demonstrated for subendothelium. Another explanation can be that matrix-VWF has no role in platelet adhesion.

To prove that VWF in the matrix is truly involved in platelet adhesion, we performed perfusions with washed platelets in a human albumin solution (HAS), with no other plasma proteins present. Under these conditions, platelet adhesion to the endothelial cell matrix was similar as with normal plasma (Table 2). At 1300 s⁻¹, platelet adhesion was somewhat lower in HAS than in normal plasma, although not statistically significantly. This is in agreement with previous data comparing platelet adhesion to the endothelial cell matrix in normal plasma and von Willebrand's disease plasma after different perfusion times; after 5 minutes platelet adhesion was lower in von Willebrand's disease plasma. Platelet adhesion with normal plasma at 1300 s⁻¹ was lower than generally obtained (compare Table 2 with Tables 1, 3, and 4). Occasionally this happens with certain batches of cultured cells. At high shear rate, however, platelet adhesion was completely abolished when the matrix was preincubated with CLB-RAg 35 and the perfusate was preincubated with CLB-RAg 35 at a 1:20 dilution, no inhibition was seen (not shown).

**Discussion**

The extracellular matrix of cultured endothelial cells may be used as a model for subendothelium in studies of blood platelet-substrate interaction. We demonstrated by indirect immunofluorescence; similar fluorescence was observed before and after perfusion (Figure 1C and D). In a separate experiment, endothelial cell matrix was scraped off the coverslip and applied to polyacrylamide gel electrophoresis and Western blotting to show that thrombospondin present in the matrix is intact (Figure 3). Preincubation of the matrix with the different antithrombospondins did not result in inhibition of platelet adhesion at all shear rates tested (Table 4). When the matrix was treated with antithrombospondin and the perfusate was preincubated with antithrombospondin at a 1:20 dilution, no inhibition was seen (not shown).
cal arteries. This is in accordance with reported data that in von Willebrand's disease platelet adhesion to subendothelium was reduced at high, but not at low, shear rates. We previously demonstrated that on purified collagen also platelet adhesion in von Willebrand's disease plasma was impaired only at high shear rates.

Several investigators have reported that fibronectin can promote the spreading of platelets on collagen-coated surfaces or on extracellular matrices. We previously demonstrated that fibronectin was required for platelet adhesion to purified collagen under flow conditions. In our laboratory, we also established, by preincubation with rabbit antibody against fibronectin, that fibronectin in the subendothelium of human umbilical arteries was involved in platelet adhesion. In this study, the same antibody was used to explore the role of fibronectin in the matrix.

When perfusions were performed after preincubation of the matrix with rabbit anti-human fibronectin serum or IgG fraction it was observed that, although the percentage surface coverage was diminished, platelet aggregates were present on the surface. This could be avoided by using the corresponding F(ab')_2 fragment. The Fc portion of the antifibronectin antibody apparently stimulated the release of the platelets.

When the matrix was preincubated with antifibronectin F(ab')_2, and the perfusions were performed with fibronectin-free plasma, platelet adhesion was significantly inhibited at all shear rates tested (Table 3). As indicated above, platelet adhesion to the endothelial cell matrix was similar whether the perfusions were performed with HAS or with normal plasma, indicating no requirement for plasma proteins. However, when perfusions were performed with fibronectin-free plasma, platelet adhesion was slightly diminished as 150 and 1000 s^-1, but not at 400 s^-1. Thus fibronectin from plasma was partially required here, perhaps because the synthesis of fibronectin and deposition in the matrix was less in this batch of cultured endothelial cells. The same result was obtained when the perfusions were performed with normal plasma after preincubation of the matrix with anti-fibronectin F(ab')_2. Apparently the inhibition by anti-fibronectin F(ab')_2 could be partly abolished by fibronectin from plasma.

The data presented here clearly indicate that in addition to VWF, fibronectin in the endothelial cell matrix is also required for platelet adhesion. In contrast to VWF, fibronectin is required at both low and high shear rates. This is in accordance with the data previously reported for platelet adhesion to purified collagen which showed that fibronectin was also required at low and high shear rates.

We investigated thrombospondin as a third protein possibly involved in platelet adhesion. Thrombospondin is present in the a-granules of platelets and is released after activation with thrombin. The protein is also synthesized by endothelial cells and by other cell-types where it is present in the extracellular matrix. Thrombospondin binds to the platelet membrane in a Ca^{2+}-dependent manner after activation and has been proposed as the endogenous lectin. It probably has a function in platelet aggregation by binding to fibrinogen. By use of a radioactive cross-linking agent, it was observed that when platelets interact with fibronectin or collagen adsorbed to glass, thrombospondin was the major platelet protein cross-linked to the substratum. This indicates a possible role for thrombospondin in platelet-substratum interaction.

Incubation of the endothelial cell matrix with antithrombospondin antibodies and subsequent staining with fluorescein-conjugated antibody showed the presence of thrombospondin throughout the matrix (Figure 1 C and D). Western blots showed that thrombospondin in the matrix had the same molecular weight as platelet thrombospondin. However, preincubation of the matrix with monoclonal and polyclonal antithrombospondins did not result in any inhibition of platelet adhesion at either low and high shear rates (Table 4). This lack of inhibition by the monoclonal antibodies could be explained by assuming that they were not directed against the proper domain. On the other hand, the polyclonal antithrombospondin sera gave no inhibition of platelet adhesion either. We therefore think it most likely that matrix-thrombospondin has no role in platelet adhesion.

From our results presented in Table 2, one may conclude that no other plasma proteins have a role in platelet adhesion to the endothelial cell matrix. The plasma concentration of thrombospondin is very low, about 1% of the VWF concentration in plasma. Thus it is also unlikely that thrombospondin from plasma is involved in platelet adhesion. Although we do not think that matrix-associated thrombospondin is involved in platelet adhesion, we cannot exclude the possibility of a role for thrombospondin released from the platelets after activation, as previously suggested by Lahav et al. After activation of platelets, the thrombospondin concentration in plasma rises about 100 times and released thrombospondin binds to the platelet membrane. The high effective local concentration of thrombospondin may have an effect then. However, the addition of antithrombospondin has no inhibitory effect on the perfusate. Young et al. previously reported an increased platelet deposition on artificial surfaces precoated with purified platelet thrombospondin or platelet releasate.

Double-label immunofluorescence studies showed a codistribution of VWF in the extracellular matrix of endothelial cells with fibronectin and collagen types IV and V. ELISA studies showed an interaction of thrombospondin with collagen type V, VWF, and fibronectin. In the matrix, an interaction between these proteins may thus exist. Studying platelet adhesion to purified collagen, we previously reported that VWF and fibronectin act independently; preincu-
bation of collagen with VWF showed only a requirement for fibronectin in the perfusate or the reverse. The adhesion site in the endothelial cell matrix for platelets is probably a complex of VWF, fibronectin, thrombospondin and collagen molecules. In this complex, VWF and fibronectin, but probably not thrombospondin, are involved in a direct interaction with the platelet membrane.

Acknowledgment
We thank Mieke Ottenhof-Rovers for her technical assistance.

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Index Terms: endothelial cell • extracellular matrix • platelet adhesion • von Willebrand factor • fibronectin • thrombospondin • thrombosis

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Arterioscler Thromb Vasc Biol. 1986;6:24-33
doi: 10.1161/01.ATV.6.1.24
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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