Platelet Adhesion to Laminin: Role of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} Ions, Shear Rate, and Platelet Membrane Glycoproteins

By Georg Hindriks, Martin J.W. IJsseldijk, Arnoud Sonnenberg, Jan J. Sixma, and Philip G. de Groot

The adhesion of platelets to purified laminin under flow conditions was investigated. Adhesion to laminin was strongly dependent on the presence of divalent cations. In the absence of cations platelet adhesion (8% coverage in 5 minutes) was maximal at a shear rate of 100/s and no adhesion could be detected at shear rates above 800/s. In the presence of 0.8 mmol/L Mg\textsuperscript{2+} and 2 mmol/L Ca\textsuperscript{2+} platelet adhesion reached its maximum (30% coverage) around 800/s. Antibodies against the E8 domain of laminin and antibodies against the \( \alpha \delta \) and \( \beta 1 \) chains of platelet membrane glycoprotein very late activation antigen-6 (VLA-6), completely inhibited adhesion. No inhibition was found with antibodies against glycoprotein IIB:IIA, against the \( \alpha 2 \) chain of VLA-2, and against the \( \alpha 5 \) chain of VLA-5. Fibronectin and von Willebrand factor were not involved in laminin-dependent adhesion. Anti-VLA-6 partly inhibited platelet adhesion to the extracellular matrix of endothelial cells at shear rates below 800/s. Preincubation of the matrices with antilaminin E8 antibodies did not influence the adhesion. These results show that purified laminin supports platelet adhesion and that the presence of VLA-6 is important for platelet adhesion under flow conditions. The protein in the matrix with which VLA-6 interacts is currently unknown.

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A DHESION OF PLATELETS to an injured vessel wall is essential for thrombus formation.\textsuperscript{1,2} When the nonthrombogenic endothelial cells are removed, platelets rapidly adhere to the underlying subendothelium. This process depends on adhesive proteins present in the vessel wall.\textsuperscript{3} Identification and characterization of the adhesive proteins present in the vessel wall and the platelet membrane receptors involved in this process is important for understanding the process of thrombosis.

Subendothelium contains a number of proteins known to be adhesive for platelets. Adhesion of platelets to purified von Willebrand factor (vWF),\textsuperscript{4} fibronectin,\textsuperscript{5} different types of collagen,\textsuperscript{6,7} laminin,\textsuperscript{8,9} and thrombospondin\textsuperscript{10} has been shown. Receptors for all these adhesive proteins are present on the platelet outer membrane.\textsuperscript{11} Potentially, platelets can thus adhere to all these proteins. However, platelet adhesion not only depends on the presence of adhesive proteins and the matching receptor on the platelet, but also on the shear forces acting on the platelets.\textsuperscript{12} The potential importance of a certain protein in the interaction with platelets has to be analyzed under different shear conditions.

Laminin is a ubiquitous basement membrane component.\textsuperscript{13-15} The molecule, as visualized by rotary shadowing, is a cross-shaped molecule with a molecular weight of 850 Kd. It consists of three distinct chains (A = 400 Kd, B1 = 215 Kd, and B2 = 205 Kd) and it interacts with collagen type IV and heparan sulfate proteoglycans to form the structural part of the basement membrane.\textsuperscript{16} Cell adhesion to laminin is mediated by specific cell surface receptors.\textsuperscript{13,17} These receptors belong to the very late activation antigen (VLA)-proteins that are members of the integrin superfamily.\textsuperscript{18} Four of the six known VLA-proteins function as laminin receptors on various cells.\textsuperscript{20,21} The specificity of an integrin for laminin is determined by the cell type that expresses the receptor. Platelets express different VLA-proteins with the potential to bind to laminin, but only VLA-6 functions as laminin receptor under static conditions.\textsuperscript{9}

A question unanswered at present is whether VLA-6 on platelets can support platelet adhesion to purified laminin under flow conditions. We show here that platelets adhere to purified laminin at shear rates up to 1,800/s. The adhesion is completely dependent on VLA-6 on the platelet and the E8-domain on the long arm of the laminin molecule. There is an absolute requirement for physiologic concentrations of Mg\textsuperscript{2+} and Ca\textsuperscript{2+} ions. The contribution of laminin to platelet adhesion to endothelial cell extracellular matrix is limited.

MATERIALS AND METHODS

Laminin

Mouse laminin (A\textsubscript{1}, B\textsubscript{1}, B\textsubscript{2}) (GIBCO, Grand Island, NY) was analyzed on a 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). It yielded the characteristic single band of about 800 Kd on unreduced gels and three bands of 400, 215, and 205 Kd under reducing conditions. Glass coverslips (18 x 18 mm; Menzel, Braunschweig, Germany) were cleaned by soaking overnight in 80% ethanol and rinsed thoroughly with distilled water. They were coated for 1 hour with laminin (100 \( \mu \)g/mL in 50 mmol/L Tris-HCl and 0.15 mol/L NaCl, pH 7.2). After coating, the glass coverslips were incubated with 1% human albumin solution in phosphate-buffered saline (PBS; 10 mmol/L phosphate buffer, pH 7.4, and 0.15 mol/L NaCl) for 2 hours to block aspecific adhesion to glass. No adhesion was found with glass coverslips coated with albumin alone.

Laminin was labeled with \( ^{125} \text{I} \) by the Iodo-Gen method (Pierce Chemical Co, Rockford, IL). Free \( ^{125} \text{I} \) was removed by extensive dialysis against PBS (overnight with three times exchange of buffer). The specific activity of \( ^{125} \text{I} \)-laminin preparation was 440 kCi/mg; the amount of free \( ^{125} \text{I} \) was less than 1%.

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Monoclonal Antibodies (MoAbs)

The MoAb GoH3, directed against the α6-chain of VLA-6, and the MoAb C17, directed against the Illa-chain (β3) of the glycoprotein (GP) IIb-IIIa complex, have been described elsewhere.2,3 Ten micrograms of GoH3 IgG per milliliter of plasma was used in the experiments. C17 (4 μg/mL) completely inhibits platelet aggregation induced by ADP. Anti-vWF (CLB-RAG 35) was provided by Dr J.A. van Mourik (CLB, Amsterdam, NL), 3 μg/mL IgG completely inhibited ristocetin induced platelet agglutination; anti-α2 chain of VLA-2 was provided by Dr Granlik (Bethesda, MD), 5 μg/mL completely inhibited platelet aggregation induced by collagen; anti-α5 (BIIG2) and anti-β1 (AHIB2) were provided by Dr C. Damsky (University of California, San Francisco, CA).9 1:500 dilution of the hybridoma culture medium inhibits the adhesion of platelets to fibronectin under static conditions. Rabbit antilaminin fragment E8 serum was provided by Dr R. Timpl (Martinsried, BRD).10 IgG was purified from the ascites with protein G-Sepharose 4FF (Pharmacia, Uppsala, Sweden) according to the manufacturers instructions. The RGD-containing peptide was a generous gift of Dr G. Marguerie (Grenoble, France).

Perfusion Studies

Perfusion studies were performed in a parallel plate perfusion chamber with well-defined rheologic characteristics devised to accommodate duplicate protein-coated glass microscope cover-slips.11 Whole blood obtained by venipuncture from healthy, volunteer donors was anticoagulated with 1/10 vol 110 mmol/L trisodium citrate (citrate blood) or with 1/10 vol 200 U/mL low molecular weight heparin (LMWH-blood; Fragmin, Kabi Vitrum, Stockholm, Sweden). Whole blood (15 mL) was prewarmed at 37°C for 5 minutes and then recirculated through the perfusion chamber for 5 minutes at wall shear rates ranging from 30/s to 1,800/s. The coverslips were removed, rinsed with 10 mmol/L HEPES-buffer, pH 7.4, containing 150 mmol/L NaCl, then fixed in 0.5% glutardialdehyde, dehydrated in methanol, and stained with May-Grünwald-Giemsa as previously described.12 Platelet adhesion was evaluated with a light microscope and the coverage was measured with an image analyser (AMS 40-10; Saffron Walden, UK). Platelet adhesion was expressed as the percentage of the surface covered with platelets.

Reconstituted perfusates were prepared as follows. Platelet-rich plasma (PRP) was obtained from whole blood by centrifugation (10 minutes at 200g, 20°C). One volume of Krebs-Ringer buffer (4 mmol/L KCl, 107 mmol/L NaCl, 20 mmol/L NaHCO3, and 2 mmol/L Na2SO4, containing 19 mmol/L citrate, pH 5.0) was added to 1 volume of PRP. The final pH was adjusted to 6. A platelet pellet was obtained by centrifugation (10 minutes at 500g, 20°C). The platelet pellet was resuspended in Krebs-Ringer buffer pH 6.0 and washed twice by centrifugation (10 minutes at 500g). After the second wash, platelets were resuspended to a platelet count of 190,000/μL in plasma or in a human albumin solution (HAS; 4% human albumin in Krebs-Ringer buffer without citrate, pH 7.35, containing 20 U/mL LMWH, 5 mmol/L glucose, and Ca2+ and Mg2+ as indicated). Red cells were washed three times with PBS containing 5 mmol/L glucose (2,900g at 20°C, twice for 5 minutes, the last time for 15 minutes). Washed red cells were added to obtain a hematocrit of 0.4±0.15 minutes before perfusion.

Preparation of Manipulated Plasmas

vWF-deficient plasma was obtained by passing normal citrated plasma over a Sepharose column to which an MoAb against vWF was coupled. The amount of vWF was measured with an enzyme-linked immunosorbent assay (ELISA).35 Plasma with vWF-antigen levels below 0.05 U/mL was accepted as vWF-depleted plasma. No effect of the procedure was found on the plasma level of fibronectin.

Fibronectin-deficient plasma was obtained by passing normal citrated plasma over a gelatin-Sepharose column. The level of fibronectin in the fibronectin-free plasma was less than 10 μg/mL. No influence was found on the level of vWF.

To study the role of the cations Mg2+ and Ca2+, citrate-plasma (normal, vWF-deficient or fibronectin-deficient) was dialyzed against Krebs-Ringer buffer without citrate containing the desired concentration of the Mg2+ and Ca2+ ions. Before dialysis, 20 U/mL desulphatohirudin (a generous gift of Dr R.B. Wallis, Ciba-Geigy Pharmaceuticals, Horsham, UK) and 20 U/mL LMWH were added to the citrated blood. During dialysis, buffers were changed at least three times. After dialysis, the total concentrations of Ca2+ and Mg2+ were determined with a Ektachem Analyser 700 XR (Kodak, Rochester, NY).

Purification of Proteins

vWF was purified from control cryoprecipitates by gel filtration on Sepharose 4B (Pharmacia AB) as described earlier.36 vWF in the void volume was precipitated by dialysis against 1.9 mol/L ammonium sulphate, pH 7.0, at 4°C and stored as ammonium sulphate suspension at 4°C until use. Precipitated protein was collected by centrifugation (2 minutes at 10,000g). The pellet was dissolved in 0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, pH 7.4, and dialyzed against the same buffer.

Fibronectin was isolated from human plasma by affinity chromatography on gelatin-Sepharose as previously described.37

Preparation of Endothelial Cell Matrices

Human endothelial cells derived from umbilical veins were isolated and cultured as described before.38 For the experiments described, endothelial cells were cultured on glass coverslips previously coated with gelatin. To isolate the extracellular matrix, cells grown to confluence were exposed to 0.1 mol/L NH3OH for 30 minutes at room temperature. The cell layer was completely removed by this procedure, leaving the extracellular matrix intact.39 The extracellular matrix was washed three times with PBS before use.

RESULTS

Characteristics of Platelet Adhesion to Laminin-Coated Coverslips

Coating of laminin. Purified laminin was coated for 1 hour onto glass coverslips by adsorption. The amount of adsorbed protein was quantified with 125I-labeled laminin (Table 1). Maximal coating of the coverslip was reached at a concentration of 100 μg/mL laminin. At this concentration, approximately 350 ng/cm2 laminin was adsorbed to the coverslip. Prolonging the time of coating did not increase the amount of laminin on the surface. There was no difference in binding between labeled and nonlabeled laminin to the glass coverslip, as was deduced from the linear correlation between the percentage of labeled laminin added to the coverslip and the amount of label bound (results not shown).

Platelet adhesion to laminin. Glass coverslips coated with laminin were perfused with LMWH-anticoagulated
Table 1. Binding of Laminin to Glass Coverslips

<table>
<thead>
<tr>
<th>Laminin Added (µg/mL)</th>
<th>Laminin Bound (ng/cm²)</th>
<th>Platelet Adhesion (% coverage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>25</td>
<td>175 ± 16</td>
<td>27.8 ± 4.8</td>
</tr>
<tr>
<td>50</td>
<td>285 ± 9</td>
<td>39.7 ± 11.3</td>
</tr>
<tr>
<td>100</td>
<td>348 ± 22</td>
<td>40.3 ± 6.0</td>
</tr>
<tr>
<td>200</td>
<td>336 ± 16</td>
<td>41.8 ± 7.3</td>
</tr>
<tr>
<td>400</td>
<td>360 ± 30</td>
<td>ND</td>
</tr>
</tbody>
</table>

Purified ¹²⁵I-laminin was incubated for 1 hour with glass coverslips and the amount of laminin bound was determined by counting the bound radioactivity. Platelet adhesion to the bound laminin was measured separately by perfusing coated glass coverslips for 5 minutes with LMWH-anticoagulated blood at a shear rate of 300/s. Results are expressed as mean ± SD (n = 4).

Abbreviation: ND, not determined.

blood for 5 minutes at a wall shear rate of 300/s. Platelet coverage increased with increasing amounts of laminin bound to the coverslip, reaching maximal coverage at a coating concentration of 50 µg/mL laminin, corresponding to about 300 ng/cm² (Table 1). In all further experiments coverslips were coated with 100 µg/mL.

Scanning electron microscopy. Laminin coated coverslips were also examined with scanning electron microscopy (SEM) to make the interaction of platelets with laminin and the extent of platelet spreading visible. Figure 1 shows that when laminin is perfused with LMWH-anticoagulated blood, platelets spread on laminin-coated surfaces and formed pseudopods. When perfused with LMWH-anticoagulated blood, besides spread and contact platelets, sometimes small aggregates were observed. After perfusion with citrated blood, no aggregates were found (not shown).

Influence of Mg²⁺ and Ca²⁺. To study the potential role of cations in the adhesion of platelets to laminin, perfusions with LMWH-anticoagulated blood and citrate anticoagulated blood were compared (Fig 2). Maximal adhesion in citrated blood was reached at a shear rate of 100 to 300/s, and no adhesion was found above a shear rate of 800/s. Adhesion in LMWH blood was generally higher than in citrated blood, but its magnitude varies with the shear rate. Maximal adhesion was reached at a shear rates ranging from 300/s to 800/s. At a shear rate of 1,800/s there was still significant adhesion.

To test whether Ca²⁺ or Mg²⁺ ions were responsible for this difference in adhesion, citrated plasma to which hirudin and LMWH was added was dialyzed against Krebs-Ringer buffer with various concentrations of Ca²⁺ and/or Mg²⁺ ions. Whole blood was reconstituted from this dialysed plasma with washed platelets and red blood cells and perfusions were performed for 5 minutes at shear rates of 300/s. In the absence of Mg²⁺ ions, no adhesion was found to laminin-coated coverslips (Fig 3A). Increasing Mg²⁺ concentrations caused increased platelet adhesion. Maximal adhesion was reached at 2.0 mmol/L Mg²⁺. When laminin was perfused with a Ca²⁺-free perfusate, platelet coverage was about 10%. Increasing the Ca²⁺ concentration to 0.5 mmol/L increased platelet coverage to about 40% (Fig 3B). Platelet adhesion to laminin thus completely depends on the presence of both Mg²⁺ and Ca²⁺.

Platelet Receptors and Plasma Components

Identification of receptors involved in platelet adhesion to laminin. The effects of MoAbs against different platelet membrane receptors on platelet adhesion to laminin at a shear rate of 300/s are summarized in Table 2. All the antibodies tested inhibited the functional activity of the receptor to which they were directed. Antibodies against the VLA α5- and α2-chain had no influence on platelet adhesion to laminin. Antibodies against the VLA α6-chain and against the β1-chain completely inhibited platelet adhesion to laminin. Platelet adhesion to laminin could also be completely inhibited by anti-VLA α6-chain antibody in citrated blood (not shown). Antibodies against GPIIb:IIIa slightly increased platelet adhesion. The addition of an RGD containing peptide to the perfusate (10 times the concentration that completely inhibited ADP induced platelet aggregation) did not influence platelet adhesion, while
pretreatment of the platelets with prostacyclin (20 nmol/L) completely inhibited platelet adhesion. The complete inhibition by anti-VLA-6-chain and the lack of inhibition by anti-GPIIb:IIIa was also observed in LMWH-blood at 1,300/s (Table 2).

Identification of plasma proteins involved in platelet adhesion to laminin. To analyze the participation of plasma components in the adhesion of platelets to laminin, perfusions were performed with vWF-free plasma or fibronectin-free plasma at shear rates of 300 and 1,300/s. Adhesion of washed platelets resuspended in these deficient plasmas was compared with the adhesion when purified vWF or fibronectin was added back at a physiologic concentration (Table 3). There was no difference in platelet adhesion when platelets were resuspended in fibronectin-free plasma or in fibronectin-free plasma to which 200 μg/mL purified fibronectin was added. Also, no difference in platelet adhesion was found between vWF-free plasma and vWF-free plasma to which 10 pg/mL vWF was added (Table 3).

When platelets were resuspended in a human albumin solution containing physiologic concentrations of Mg2+ and Ca2+, platelet adhesion was comparable with adhesion of platelets resuspended in plasma (not shown). The addition of 1 mg/mL fibrinogen to the albumin solution did not influence platelet adhesion (not shown).

Role of Laminin in Adhesion to Extracellular Matrix

Effect of anti-VLA-6. Extracellular matrices of cultured endothelial cells (ECM) were perfused with LMWH-

Table 2. Platelet Adhesion to Laminin in the Presence of Specific Antibodies

<table>
<thead>
<tr>
<th>Addition</th>
<th>300/s % Platelet Coverage</th>
<th>1,300/s % Platelet Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>28.3 ± 5.0</td>
<td>7.5 ± 0.7</td>
</tr>
<tr>
<td>Anti-VLA-2 (α2)</td>
<td>29.6 ± 4.2</td>
<td>ND</td>
</tr>
<tr>
<td>Anti-VLA-5 (α5)</td>
<td>22.3 ± 0.9</td>
<td>ND</td>
</tr>
<tr>
<td>Anti-VLA-6 (α6)</td>
<td>0.6 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Anti-β1</td>
<td>0.1 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td>Anti-GPIIb:IIIa</td>
<td>41.1 ± 1.5</td>
<td>10.9 ± 0.8</td>
</tr>
<tr>
<td>Anti-laminin E8</td>
<td>0.6 ± 0.1</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Anti-vWF</td>
<td>33.1 ± 6</td>
<td>ND</td>
</tr>
<tr>
<td>RGD-peptide</td>
<td>32.3 ± 1.1</td>
<td>ND</td>
</tr>
<tr>
<td>Prostacyclin</td>
<td>0.4 ± 0.1</td>
<td>1.5 ± 1.8</td>
</tr>
</tbody>
</table>

LMWH-anticoagulated blood was perfused for 5 minutes over purified laminin. Five minutes before the start of the perfusion, the MoAbs were added to the perfusate in a concentration five times that of the concentration required for maximal inhibition of their functional activity in the appropriate other test system (see Materials and Methods). The prostacyclin concentration was 20 ng/mL. The final concentration of RGD-containing peptide was 100 μmol/L. This concentration is five times the concentration required to block platelet adhesion to fibrinogen.

Abbreviation: ND, not determined.

Fig 2. Shear rate dependence of platelet adhesion to laminin. Laminin was perfused for 5 minutes with whole blood anticoagulated with citrate or anticoagulated with LMWH. Platelet adhesion is expressed as percentage of the surface covered with platelets. The error bars denote the SD of data obtained by four independent coverslips.

Fig 3. Influence of Ca2+ and Mg2+ ions on platelet adhesion to laminin. Citrated plasma was dialyzed against a buffer containing the indicated ion concentration. Before dialysis, 20 U/mL LMWH and 20 U/mL sulphatohirudin was added to the plasmas. Reconstituted perfusates were prepared with 40% red blood cells and 190,000 platelets/mL. Perfusions were performed for 5 minutes at a shear rate of 300/s. (A) Concentration dependence of adhesion on Mg2+. All the perfusates contained 2 mmol/L Ca2+. (B) Concentration dependence of adhesion on Ca2+. All the perfusates contained 2 mmol/L Mg2+.
anticoagulated blood in the presence or absence of antibodies against VLA α6-chain (Fig 4). Without antibodies, platelet adhesion to ECM increased with increasing shear rate, reaching a maximum at 800/s, and with a gradual decrease at higher shear rates. Addition of anti-α6-chain antibody to the perfusate partly inhibited platelet adhesion at shear rates between 100 and 300/s, but there was no significant effect on platelet adhesion at shear rates of 800/s and higher.

**Effect of antilaminin.** The effect of an antilaminin antibody on the adhesion of platelets to ECM is shown in Fig 5. The antilaminin antibody completely inhibited the adhesion of platelets to purified laminin (Table 2). There was, however, no inhibition of platelet adhesion at all shear rates tested when ECM was preincubated with antilaminin.

### DISCUSSION

The results presented here show that platelets in flowing blood adhere rapidly and specifically to purified laminin.

The specificity of the interaction of laminin with platelets is supported by several observations. Firstly, the binding was dependent on the concentration of the laminin coated on the coverslip and reached a level 100 times higher than the background adhesion to albumin-coated glass coverslips. Secondly, platelet adhesion to laminin was not supported by adhesive proteins present in plasma. Thirdly, platelet adhesion was mediated completely by the laminin receptor α6β1 (VLA-6) on platelets. Furthermore, in contrast to adhesion to adhesive proteins present in plasma, platelet adhesion to laminin completely depended on the presence of millimolar concentrations of divalent cations in the perfusate (Fig 3).

The characteristics of the adhesion to laminin are fundamentally different from the adhesion to purified plasma proteins such as fibronectin, fibrinogen, and vWF that can be present in the vessel wall. Adhesion to laminin strongly depends on the presence of cations. A significant difference, both in total extent and shear rate dependency, was found when laminin adhesion was compared in the presence or absence of Mg²⁺ and Ca²⁺. Experiments with reconstituted perfusates, in which the Mg²⁺ and Ca²⁺ concentrations were varied, showed an optimal adhesion to laminin at a concentration of 2.0 mmol/L Mg²⁺ and 0.5 mmol/L Ca²⁺. It has been shown before under static conditions that the interaction of laminin with VLA-6 was dependent on the presence of Mg²⁺ ions or Mn²⁺ ions, probably via upregulating the functional expression of the integrin on the platelet membrane. In contrast to the static

### Table 3. Effect of Plasma Proteins on Platelet Adhesion to Laminin

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Addition</th>
<th>300/s</th>
<th>1,300/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control plasma</td>
<td></td>
<td>31.3 ± 1.2</td>
<td>3.3 ± 0.6</td>
</tr>
<tr>
<td>vWF-free plasma</td>
<td>vWF</td>
<td>32.3 ± 1.2</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>vWF-free plasma</td>
<td></td>
<td>31.2 ± 0.6</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td>Fibronectin-free plasma</td>
<td>vWF</td>
<td>27.1 ± 2.3</td>
<td>3.8 ± 2.0</td>
</tr>
<tr>
<td>Fibronectin-free plasma</td>
<td>Fibronectin</td>
<td>25.7 ± 2.5</td>
<td>ND</td>
</tr>
</tbody>
</table>

Citrated plasma deficient on vWF or fibronectin was dialyzed after the addition of 20 U/mL LMWH and 20 U/mL hirudin against HEPES buffer pH 7.4 containing 1 mmol/L Mg²⁺ and 2 mmol/L Ca²⁺.

Abbreviation: ND, not determined.

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**Fig 5.** Effect of antilaminin fragment E8 on platelet adhesion to endothelial extracellular matrix. Whole blood anticoagulated with 20 U/mL LMWH was perfused for 5 minutes over the extracellular matrix of cultured endothelial cells at different shear rates. Before perfusion, the matrix was incubated for 1 hour with 1:40 diluted antilaminin fragment E8 (○). Control matrices were preincubated with an unrelated ascites (●). As a control, purified laminin also was incubated with the E8 antibody for the same period and at the same concentration and perfused in the same way. Platelet adhesion was expressed as a percentage of the matrix covered with platelets. Error bars denote the SD of data obtained with four coverslips perfused with blood of the same donor. Experiments with blood of two other donors gave similar results, although the absolute amount of platelet coverage varied due to the variations in platelet number, hematocrit, and vWF level between the donors.

**Fig 4.** Effect of anti-VLA α6-chain antibody on platelet adhesion to endothelial extracellular matrix. Whole blood anticoagulated with 20 U/mL LMWH was perfused for 5 minutes over the extracellular matrix of cultured endothelial cells at different shear rates. (○) No addition; (●) 10 μg/mL anti-VLA α6-chain. Platelet adhesion was expressed as percentage of the matrix covered with platelets. Error bars denote the SD of data obtained from four coverslips perfused with blood of the same donor. Experiments with two other donors gave similar results, although the absolute amount of platelet adhesion to the matrix differed due to the variation in platelet number, hematocrit, and vWF levels in the plasmas of the donors.
incubations in which Ca\(^{2+}\) seems to inhibit binding of platelets to laminin,\(^9\) platelet adhesion to laminin under flow conditions depends also on the presence of Ca\(^{2+}\) ions. At a shear rate of 300/s platelet adhesion increases four times in the presence of physiologic concentrations of Ca\(^{2+}\). It has been described that laminin has the ability to form large aggregated structures probably necessary to maintain laminin in a functional conformation.\(^{35,48}\) This activity is Ca\(^{2+}\) dependent. In the presence of chelating agents, laminin is easily extractable from basement membranes.\(^49\) A possible explanation for the Ca\(^{2+}\) dependence of platelet adhesion to laminin is that a certain conformation is necessary for laminin to increase the concentration of adhesive epitopes, making a multiple interaction between a platelet and the laminin surface possible. This multiple interaction increases the affinity of the platelet for the surface, enabling the platelet to withstand shear forces. Under static conditions without shear forces, this increased affinity is not necessary.

Laminin is known to contain two major cell adhesion domains, fragment E8 on the terminal half of the long arm and fragment P1 or E1, which is present in the central part of the molecule.\(^{13,14,42}\) Within the P1 fragment an RGD (Arg-Gly-Asp) sequence is present (position 1123-1125 of the A-chain).\(^50\) The P1 cell binding site is probably a cryptic adhesion site because it requires proteolytic cleavages of the molecule before it is expressed.\(^47\) This potential cell binding site is not involved in the adhesion of platelets to laminin, because no inhibition was found with the RGD-sequence containing peptides. In contrast, a complete inhibition of platelet adhesion was found with polyclonal antibodies against the fragment E8, indicating that this domain present at the end of the long arm of laminin participates in the adhesion of platelets. This finding is consistent with the idea of \(\alpha_6\beta_1\) as an E8 receptor.\(^44,45\)

Cell adhesion to laminin is mediated by specific cell surface receptors. Four different receptors have been shown to function as laminin receptor: VLA-1, VLA-2, VLA-3, and VLA-6.\(^6,28\) Three \(\beta_1\) integrins have been identified on human platelets, \(\alpha_2\beta_1\) (VLA-2), \(\alpha_5\beta_1\) (VLA-5), and \(\alpha_6\beta_1\) (VLA-6).\(^6\) The specificity of an integrin as a laminin receptor appears to be determined by the cell type expressing the receptor. Both under static and under flow conditions, antibodies directed against \(\alpha_6\) and \(\beta_1\) completely inhibited platelet adhesion. No inhibition was found with antibodies against \(\alpha_2\) and \(\alpha_5\). Platelets do not express VLA-1, VLA-3, and VLA-4. These results show that VLA-6 is the only VLA-protein present on the platelet membrane involved in the adhesion of platelets to laminin under flow conditions. After completion of this work, it was reported that the interaction of washed human platelets with laminin under static conditions was mediated by a laminin-binding 67-Kd protein.\(^47\) At the moment we do not know the role of the 67-Kd protein in platelet adhesion under flow conditions in the presence of all other blood cells. Pilot experiments in which the synthetic peptide Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg (1 \(\mu\) mol/L) that blocks laminin mediated adhesion to the 67-Kd laminin binding protein was added to the perfusate had no significant influence on platelet adhesion at a shear rate of 300/s (not shown). In static studies we have shown that the same synthetic peptide interfering with the 67-Kd laminin receptor did not inhibit platelet adhesion.\(^9\) The results presented here clearly show the absolute necessity of the expression of VLA-6 on the platelet membrane for normal platelet adhesion to laminin in flowing blood.

Platelet adhesion to laminin is completely inhibited by preincubating platelets with prostacyclin. The binding of platelets to laminin does not require activation of platelets\(^8\) or expression of GPIIb:IIIa. Prostacyclin is able to inhibit the spreading of platelets on surfaces.\(^4\) Scanning electron microscopy pictures showed platelets spread over the laminin surface (Fig 2). Inhibition of platelet spreading by prostacyclin probably decreases the affinity of the platelets for the surface and platelets may then be washed away by the flowing blood. In their studies with washed platelets resuspended in Dulbecco's modified Eagle's medium, Ill et al\(^6\) found that the platelets did not spread on laminin. The Mg\(^{2+}\) concentration in Dulbecco's medium is less than 0.5 mmol/L and probably too low to allow proper spreading of the platelets. In experiments with citrated blood under flow, contact platelets are easily washed away from the surface and therefore not seen in our experiments.

When perfusions were performed over endothelial cell matrices in the presence of antibodies against VLA-6, a partial inhibition of about 60% was found at shear rates below 800/s. This finding indicates that at these lower shear rates platelet adhesion to more complex substrata, containing a number of different adhesive molecules, also depends on the presence of VLA-6. It has been shown before that platelet adhesion to endothelial cell matrices at these lower shear rates depends on the presence of GPIIb but not on the presence of GPIIb:IIIa. Studies with a patient lacking platelet membrane glycoprotein VLA-2 have shown that also VLA-2 is involved in the adhesion of platelets to subendothelial structures. VLA-2 is the receptor for collagen. At the moment, three different platelet membrane receptors, GPIb, VLA-2, and VLA-6, have been found involved in the adhesion of platelets at lower shear rate. How these receptors cooperate and what their sequence of action is remains unknown. Whether VLA-6 interacts with laminin in the endothelial cell matrix is not certain because antibodies against laminin that are able to inhibit completely the adhesion to purified laminin have no influence on the adhesion to the endothelial cell matrix. A possible explanation might be that the antibody used is directed against mouse laminin and does not recognize human laminin. Another explanation is that an as yet unknown protein exists in the extracellular matrix that interacts with VLA-6. To date, however, no other adhesive proteins have been described as interacting with VLA-6.

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REFERENCES

44. Paulsson M, Aumailley M, Deutzmann R, Timpl R, Beck K,


44. Sonnenberg A, Linders KJT, Modderman PW, Damsky CH, Aumailley M, Timpl R: Integrin recognition of different cell-binding fragments of laminin (P1, E3, E8) and evidence that α6β1 but not α6β4 functions as a major receptor for fragment E8. J Cell Biol 110:2145, 1990


Platelet adhesion to laminin: role of Ca2+ and Mg2+ ions, shear rate, and platelet membrane glycoproteins

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