Platelet Adhesion to Fibronectin in Flow: Dependence on Surface Concentration and Shear Rate, Role of Platelet Membrane Glycoproteins GP IIb/IIIa and VLA-5, and Inhibition by Heparin

By Sara Beumer, Martin J.W. IJsseldijk, Philip G. de Groot, and Jan J. Sixma

Platelet adhesion to purified surface-immobilized fibronectin under flow conditions was investigated. Fibronectin was found to support attachment and spreading of platelets. The extent of platelet spreading depended on the amount of immobilized fibronectin. An antiglycoprotein (anti-GP IIb/IIIa) antibody and an Arg-Gly-Asp (RGD)-containing peptide inhibited adhesion almost completely, whereas antibodies directed against platelet GP Ia/IIa (very late antigen 5) inhibited by 50%. Similar results with the antibodies and the peptide were found in a static system. A comparison of different anticoagulants showed no difference in adhesion using citrate or hirudin. However, unfractionated heparin (UFH) or low-molecular-weight heparin (LMWH) as the only anticoagulant or in combination with citrate maximally blocked adhesion. These results indicate that platelet adhesion to fibronectin in flow involves several receptors, is highly RGD-mediated, does not require physiologic levels of divalent cations, and can be inhibited by direct binding of heparin to the fibronectin surface.

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Menzel, Braunschweig, Germany) were cleaned in 80% alcohol, rinsed in distilled water, and dried thoroughly. For adhesion, coverslips were incubated for 1 hour with a 300 μg/mL fibronectin solution in phosphate-buffered saline (PBS; 10 mmol/L phosphate buffer, pH 7.4, and 0.15 mol/L NaCl). Spraying, the other technique used to apply protein to the surface, was performed with a retouching airbrush (Badger model 100, Badger Brush CO, Franklin Park, IL) in such a way that each layer of protein had been dried before the next layer was sprayed. In that case, 100 μL per square coverslip or 33 μL per circular coverslip of a fibronectin solution in 0.05 mol/L ammonium acetate pH 7.4 was sprayed to reach the indicated surface concentration. After coating or spraying, the glass coverslips were incubated with a 1% human albumin solution for 1 hour to block a specific adhesion to glass. No adhesion was found on glass coverslips coated with albumin alone.

ECM. Human umbilical vein endothelial cells were isolated and grown to confluence as described. Cells of the second passage were seeded on glass coverslips coated with gelatin. Cells were removed with 0.1 mol/L NH₄OH for 15 minutes at room temperature, and subsequently, the matrices were washed three times with PBS.

Polyclonal Antibodies

Polyclonal antihuman fibronectin antibodies [F(ab')₂ fragments] were purchased from Cappel Organon Teknika Corporation (West Chester, PA) in lyophilized form and dissolved in PBS.

Monoclonal Antibodies (MoAbs)

MoAb C17, directed against the IIIa chain (β₃) of the GP IIb/IIIa complex, was a gift from Dr A.E.G.Kr. von dem Borne (CLB, Amsterdam, The Netherlands) and was used at a concentration of 8 μg/mL, which was 10 times the dose necessary to completely inhibit adenosine diphosphate (ADP)-induced platelet aggregation. The MoAbs anti-α₁ (B12/G2) and anti-β₁ (AIIB2) integrin (both by Biomedical culture media) were provided by Dr C. Damsky (University of California, San Francisco) and used at a 1:20 dilution (a lower dilution did not result in more inhibition of adhesion). The D-arginyl-glycyl-L-aspartyl-L-tryptophan (dRGDW) peptide was generously provided by Dr J. Bouchaudon (Rhône-Poulenc-Rorer, Chemistry Department, Centre de Recherche de Vitry, Vitry sur Seine, France).

Antibodies to be tested were added to the perfusate (flow) or platelet suspension (static) 45 minutes before performing the experiment. The preincubation time of the dRGDW peptide was 15 minutes.

Preincubation of Glass Coverslips with Heparin

To test the effect of different heparins, fibronectin sprayed coverslips were incubated for 1 hour at room temperature with the indicated heparin solution diluted in PBS. As a control, coverslips were incubated with PBS alone. The heparins tested were unfractionated heparin (UFH; Rovi Laboratories, Madrid, Spain), a low-molecular-weight heparin (LMWH; Fragmin) obtained from Kabi Pharmacia AB (Stockholm, Sweden) and a series of heparins, purified from unfractionated heparin, with increasing average MW: El 17 (8 kD), El20 (18 kD), and El21 (21 kD), which were generously provided by Dr G. van Dedem (Akzo, Diosynth bv, Oss, The Netherlands).

Comparison of Different Anticoagulants

The anticoagulant always constituted one tenth of the total volume. The following anticoagulants were tested (in parentheses the final concentration is mentioned): trisodium citrate (11 mmol/L), UFH (5 U/mL), LMWH (20 U/mL), or hirudin (80 U/mL; Pentapharm, Basel, Switzerland) supplemented with soybean trypsin inhibitor (SBTI; 50 μg/mL). The effect of a combination of heparin with citrate was studied using citrate anticoagulated blood to which heparin was added.

Perfusion Studies

Perfusion studies were performed in a parallel-plate perfusion chamber with well-defined rheologic characteristics designed to accommodate duplicate glass coverslips. Whole blood obtained by venipuncture from healthy volunteer donors was anticoagulated with one-tenth volume 110 mmol/L trisodium citrate, unless mentioned otherwise. Whole blood (15 mL) was prewarmed at 37°C for 5 minutes and then recirculated for 5 minutes through the perfusion chamber which contained two protein-covered glass coverslips at the indicated wall shear rate. After perfusion, the coverslips were removed and rinsed with 10 mmol/L HEPES buffer containing 150 mmol/L NaCl, pH 7.35. They were then fixed 0.5% glutaraldehyde, dehydrated in methanol and stained with May-Grünwald-Giemsa as described previously. Platelet adhesion was evaluated with a light microscope at 1,000× magnification and the coverage was measured with an Image Analyzer (AMS 40-10, Saffron Walden, UK). Platelet coverage, expressed as the percentage of the surface covered with platelets, is the average of 60 fields/coverslip.

Quantification of platelet spreading was performed using the IBAS image-analysis system (Zeiss/Kontron, Eching, Germany). The surface of 100 randomly chosen single platelets was measured and is presented in square micrometers.

Static Adhesion Assay

Static adhesion experiments were performed on fibronectin-sprayed circular glass coverslips placed in a 24-well plate (Costar, Cambridge, MA). For these experiments, platelets were washed as previously described and resuspended in fibroenin-depleted plasma at a platelet count of 250,000/μL. The adhesion assay was performed for 1 hour at 37°C. Platelets were stained and platelet coverage was determined as mentioned above.

Patient Studies

Two patients with Glanzmann’s thrombasthenia were studied. One patient, used in the first experiment, has been characterized and described before (patient C.V.d.P.W.). Flow-cytometric analysis showed the absence of GP IIb/IIIa with a normal GP Ib level for both patients. Both patients had normal platelet counts.

Statistical Analysis

Significance of difference between means of total surface coverage of two samples was calculated with a paired Student’s t-test. Comparison of two series of coverslips obtained in one experiment, as is the case with the patient studies, was performed with an unpaired Student’s t-test. Comparison of means of single platelet coverage was performed using a model I one-way analysis of variance (ANOVA) followed by a Student Newman-Keuls (SNK) multiple comparison test (two-tailed test). The validity of the underlying assumptions of the ANOVA was tested by the Fmax test, to test for heterogeneity, and by examining the correlation between the means and variances. All statistical analyses were performed according to Sokal and Rohlf. P values of less than .05 were assumed to indicate significance.

RESULTS

Characteristics of Platelet Adhesion to Fibronectin Sprayed Coverslips

Concentration dependence. Fibronectin adsorbed to glass coverslips resulted in platelet adhesion and spreading...
Fig 1. Characterization of platelet adhesion to immobilized fibronectin. (A) shows concentration dependence; coverslips were sprayed with various amounts of fibronectin and perfused for 5 minutes at a shear rate of 300 s\(^{-1}\). (B) shows shear-rate dependence; coverslips sprayed with 5 \(\mu\)g fibronectin/cm\(^2\) were perfused for 5 minutes at different shear rates. (C) shows time dependence; coverslips sprayed with 5 \(\mu\)g fibronectin/cm\(^2\) were perfused for different times at shear rate 300 s\(^{-1}\). (D) Dose-dependent inhibition of platelet adhesion to fibronectin by polyclonal antifibronectin Fab\(^{\prime}\) fragments. Fibronectin-sprayed coverslips were incubated for 1 hour with an increasing concentration of polyclonal antifibronectin antibodies after which they were perfused for 5 minutes at a shear rate of 300 s\(^{-1}\). All perfusions were performed with whole citrate anticoagulated blood. Values are mean \pm\ SEM obtained in two or three independent experiments.

Shear-rate dependence. Fibronectin-sprayed coverslips were perfused for 5 minutes at different shear rates. Platelet coverage reached a maximum at a shear rate of 300 s\(^{-1}\), after which it decreased to a minimum of 4% surface coverage at a shear rate of 1,300 s\(^{-1}\) (Fig 1B). Platelet spreading occurred over the whole range of shear rates. All further experiments were performed at a shear rate of 300 s\(^{-1}\).

Time dependence. Fibronectin-sprayed coverslips were perfused for 1, 3, 5, and 10 minutes at a shear rate of 300 s\(^{-1}\). Platelet coverage showed a linear increase in time up to 10 minutes (Fig 1C). We chose 5 minutes as perfusion time in the subsequent inhibition experiments.

Specificity of platelet adhesion to fibronectin. To ascertain that platelet adhesion to the fibronectin-sprayed coverslips was specific, the surface was preincubated with an increasing concentration of polyclonal anti fibronectin Fab\(^{\prime}\)\(_2\) fragments. Platelet adhesion was completely blocked at a...
Fig 2. Morphology (full face) of platelets adhering to fibronectin. Coverslips sprayed with different concentrations of fibronectin were perfused for 5 minutes at a shear rate of 300 s⁻¹. The surface coverage of 100 single platelets was measured per concentration. Values are mean ± SEM of three independent experiments. SNK test

P < .05: 1.25 µg/cm² = 2.5 µg/cm² = 5 µg/cm² > 10 µg/cm² > 20 µg/cm².

concentration of 500 µg/mL with an IC₅₀ between 100 and 200 µg/mL (Fig 1D). Control F(ab')₂ fragments had no effect at a concentration of 500 µg/mL (result not shown)

Identification of Platelet Receptors in Adhesion to Fibronectin.

Antibody studies. To examine the role of different platelet membrane receptors in platelet adhesion to fibronectin, various MoAbs directed to these receptors were tested in both the perfusion and the static system (Table 1). Perfusions were performed with whole blood while the static experiments were performed with washed platelets reconstituted

Table 1. Platelet Adhesion to Fibronectin in the Presence of Specific Antibodies or Prostacyclin

<table>
<thead>
<tr>
<th>Addition</th>
<th>Platelet Coverage (% of control)</th>
<th>Flow</th>
<th>Static</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-α₂ integrin</td>
<td></td>
<td>53.3 ± 7.0* (4)</td>
<td>51.6 ± 4.8† (4)</td>
</tr>
<tr>
<td>Anti-β₁ integrin</td>
<td></td>
<td>50.3 ± 5.9* (4)</td>
<td>54.3 ± 5.6* (4)</td>
</tr>
<tr>
<td>Anti-GP IIb-IIIa</td>
<td></td>
<td>14.6 ± 2.3† (4)</td>
<td>14.1 ± 4.0† (7)</td>
</tr>
<tr>
<td>Prostacyclin</td>
<td></td>
<td>4.0 ± 0.7* (4)</td>
<td>15.5 ± 2.9† (5)</td>
</tr>
</tbody>
</table>

Fibronectin-sprayed coverslips were perfused for 5 minutes with whole blood. The static adhesion assay was performed for 1 hour at 37°C with washed platelets reconstituted in fibronectin-depleted plasma at 250,000 platelets/µL. MoAbs were added 45 minutes before the perfusion or static adhesion assay. The amount of MoAb anti-GP IIb/IIIa added was 10 times the concentration that is required for maximal inhibition of its functional activity in the appropriate other test system (see Experimental Procedures). Prostacyclin was preincubated for 10 minutes in a concentration of 30 nmol/L. Platelet adhesion is expressed as percentage of the surface coverage found when nothing was added. The values represent mean ± SEM with the number of determinations in parentheses.

* P < .01.
† P < .05.
‡ P < .001.
in fibronectin-depleted plasma to rule out possible influence by plasma fibronectin. In both systems, antibodies directed against both chains of the VLA-5 receptor (anti-α5 and anti-β1 integrin) were able to inhibit platelet adhesion significantly by about 50%. No reduction in platelet spreading could be observed. An antibody against the GP IIb/IIIa receptor nearly completely abolished platelet adhesion to fibronectin in flow as well as in the static system. With this antibody, there was no platelet spreading. A similar reduction in adhesion and change in morphology was found when platelets were preincubated with prostacyclin.

When fibronectin was added back to the fibronectin-depleted plasma in static experiments, or when platelet-rich plasma was used, the extent of inhibition by all the antibodies remained the same (results not shown), although in the latter case, the absolute amounts of surface coverage were lower (probably because of less platelet activation during the preparation of the platelet suspension).

**Patient study.** To further examine the contribution of GP IIb/IIIa, perfusions were performed with whole blood from two different Glanzmann’s thrombasthenia patients, who lack GP IIb/IIIa on the platelet membrane. The absence of GP IIb/IIIa caused almost total inhibition of platelet adhesion (Table 2). The remaining adherent platelets were contact platelets. In both experiments, we found the control values to be in the range of what is normally found (19.3 ± 8.3; mean ± SD; n = 45). This indicates that in both experiments, the fibronectin surface was of good quality and that the large decrease in adhesion with Glanzmann’s platelets was related to their defect.

**Inhibition by the dRGDW peptide.** To further investigate the importance of GP IIb/IIIa and VLA-5, which are both RGD-containing fibronectin receptors, the effect of the RGD-containing peptide dRGDW was examined (Fig 4). Both under flow and static conditions, this peptide completely abolished platelet adhesion to fibronectin, although the IC50 values were different (flow, 17 μmol/L; static condition, 4 μmol/L). In both systems, a gradual loss of platelet spreading was observed.

**Effect of Heparin**

**Addition of heparin to the perfusate.** Divalent cations have been described to influence ligand binding to GP IIb/IIIa as well as VLA-5, and because these two receptors are important for adhesion to fibronectin, as described above, the presence of divalent cations at physiologic levels may very well change adhesion. Therefore, we compared platelet adhesion on coverslips perfused with either heparin or citrate anticoagulated blood. Because heparin, apart from leaving the cation concentrations at physiologic levels, may influence platelet function in general or may affect platelet adhesion to fibronectin by binding to the heparin-binding domain of this molecule (see below) we also included the thrombin inhibitor hirudin as anticoagulant in the perfusion assay (Table 3, left column). UFH, in a concentration of 5 U/mL, almost completely abolished platelet adhesion, leav-

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**Table 2. Adhesion of Glanzmann’s Thrombasthenia Platelets to Fibronectin**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Platelet Coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.7 ± 3.6</td>
</tr>
<tr>
<td>GT</td>
<td>1.2 ± 0.2*</td>
</tr>
</tbody>
</table>

Whole blood from two different GT patients or healthy controls was perfused for 5 minutes over fibronectin-sprayed coverslips. Values are means ± SD obtained from four independent coverslips.

Abbreviation: GT, Glanzmann’s thrombasthenia.

* P < .01.

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**Table 3. Effect of Different Anticoagulants on Platelet Adhesion to Fibronectin**

<table>
<thead>
<tr>
<th>Anticoagulant</th>
<th>Citrate Absent (%)</th>
<th>Citrate Present (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hirudin + SBTI</td>
<td>115.1 ± 17.1</td>
<td>ND</td>
</tr>
<tr>
<td>UFH</td>
<td>18.9 ± 7.0*</td>
<td>19.4 ± 4.11</td>
</tr>
<tr>
<td>LMWH</td>
<td>43.4 ± 8.5*</td>
<td>46.1 ± 5.24</td>
</tr>
</tbody>
</table>

Fibronectin-sprayed coverslips were perfused for 5 minutes with whole blood anticoagulated with a 1/10 volume of anticoagulant to reach a final concentration of either 11 mmol/L trisodium citrate, 80 U/mL hirudin + 50 μg/mL SBTI, 5 U/mL UFH, or 20 U/mL LMWH (left column) or 11 mmol/L trisodium citrate to which 5 U/mL UFH or 20 U/mL LMWH was added (right column). Platelet adhesion is expressed as percentage of the surface coverage found with citrated blood. Values represent mean ± SEM with the number of determinations in parentheses.

Abbreviation: ND, not determined.

* P < .05.
† P < .1.
‡ P < .001.
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ing only some contact platelets, while 20 U/mL LMWH caused a reduction of 55% without changing platelet morphology compared with perfusing with citrated blood. By contrast, anticoagulation with hirudin, in combination with the factor Xa inhibitor SBTI to absolutely prevent thrombin formation (no fibrinopeptide A was detected in the perfusate after perfusion), did not significantly alter adhesion. To confirm that the effect of heparin did not correlate with free divalent cation concentrations, perfusions were executed with citrated blood to which heparin was added (Table 3, right column). Also then, both heparins inhibited adhesion and the degree and nature of inhibition was the same as in the absence of citrate. For both heparins, the extent of inhibition differed highly between individuals: in the absence of citrate, the inhibition by UFH and LMWH varied between 59% and 95% and 28% and 84%, respectively. In the presence of citrate these values were 72% and 93% and 35% and 62%. Titration of heparin to citrated blood showed a dose-dependent inhibition by both UFH and LMWH (Fig 5). For UFH, half-maximal inhibition was reached at 2 µg/mL (0.25 U/mL), whereas a concentration of 8 µg/mL (1 U/mL) was sufficient to reach 80% inhibition. For LMWH, the IC₅₀ was 8 µg/mL (1.2 U/mL) reaching maximal, though not complete inhibition at 25 µg/mL (3.7 U/mL).

Preincubation of coverslips. Fibronectin-sprayed coverslips were incubated before perfusion with UFH or LMWH to investigate whether heparin was able to inhibit platelet adhesion by direct binding to immobilized fibronectin (Fig 6). UFH dose-dependently reduced adhesion showing half-maximal inhibition at a concentration of 3 µg/mL (0.36 U/mL) and maximal inhibition at a concentration of 50 µg/mL (6.25 U/mL). The reduction in platelet adhesion was accompanied by a gradual loss of platelet spreading, with the highest heparin concentration showing only contact platelets (Fig 7B). When we pretreated the coverslips with LMWH, no effect could be observed up to a concentration of 1 mg/mL (148 U/mL). We further analyzed the effect of heparins of different size, varying in MW between 8 and 21 kD, which were purified from UFH (Fig 8). At a concentration of 100 µg/mL, where UFH showed 95% inhibition, the three heparins with the lowest molecular weights (E117, ~ 8 kD; E118, ~ 12 kD; E119, ~ 15 kD) displayed an inhibition of platelet adhesion of 40%. Heparin E120 (~ 18 kD) reduced adhesion further by 70%. Treatment with heparins E117 to E120 did not lead to reduced platelet spreading (Fig 7C). Only the heparin with the highest molecular weight (E121, ~ 21 kD) blocked adhesion almost completely because of a decrease in the number of platelets attached and complete loss of platelet spreading (Fig 7D).

Platelet Adhesion to the ECM

The contribution of fibronectin-dependent adhesion to the ECM was investigated (Table 4). Preincubating the surface with polyclonal antifibronectin inhibited adhesion significantly by 35%. To obtain information about the role of VLA-5 in a more complex system, as the ECM is, the effect of the antibody directed against the α₅ integrin was investigated. No inhibition could be observed using a concentration that we had found to be maximally inhibitory for platelet adhesion to isolated fibronectin. The same was found with the antibody directed to GP IIb/IIIa. Preincubation of the ECM with UFH also had no effect on adhesion, even when we used a concentration of 1 mg/mL (not shown).

DISCUSSION

In this study, we investigated platelet adhesion to purified fibronectin, one of the major components of the vessel wall. Until now, most studies focused on the role of fibronectin in the adhesion process to other surfaces, such as the subendothelium of the vessel wall, the ECM of cultured endothelial cells, or collagens. To get more insight in the direct interaction between the platelet and fibronectin, and to find out how this interaction can be influenced, we chose fibronectin itself as adhesive surface.
Platelet adhesion to sprayed fibronectin showed a high and homogeneous surface coverage (19.4% ± 8.3%; mean ± SD; n = 45). We found that the surface concentration was of critical importance to platelet spreading. At 5 μg/cm², platelet spreading was still optimal, whereas raising the concentration up to 20 μg/cm² resulted in a gradual loss of platelet spreading. It is difficult to explain this phenomenon. Electronmicroscopic examination in cryosection showed that a concentration of 5 μg/cm² already resulted in a continuous layer of protein on the glass coverslip (result not shown). A higher concentration would only result in more layers of protein. Apparently this influences the exposure or accessibility of adhesive sites in the fibronectin molecule, especially sites that are involved in platelet spreading. It is conceivable that these sites become covered.

The adhesion to fibronectin was optimal at a shear rate of 300 s⁻¹, with some adhesion occurring at a shear rate of 1,300 s⁻¹. Adhesion to isolated fibronectin differs in this respect from fibronectin-dependent adhesion to collagen type I and III, or to more complex systems as the ECM of cultured endothelial cells or the subendothelium of the vessel wall, which also occurs at shear rates higher than 300 s⁻¹. An explanation for this may be that the interaction between a platelet and fibronectin alone is not strong enough to resist high shear forces and that at higher shear rates, fibronectin needs the help of other adhesive proteins to support platelet adhesion.

Platelet adhesion to fibronectin under static conditions has been reported to depend both on VLA-5 and GP IIb/IIIa. We found that both receptors are equally important under flow and static conditions. Under both conditions, antibodies against the two different chains of VLA-5 reduced adhesion to 50% of the control value without effecting the extent of platelet spreading, which suggests a role for VLA-5 in initial adhesion to fibronectin.

### Table 4. Effect of Anti-VLA-5, Heparin, and Antifibronectin on Platelet Adhesion to ECM of Cultured Endothelial Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Platelet Coverage (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-α5 integrin</td>
<td>102.7 ± 4.5 (3)</td>
</tr>
<tr>
<td>Anti-GP IIb/IIIa</td>
<td>93.5 ± 13.5 (2)</td>
</tr>
<tr>
<td>UFH</td>
<td>109.0 ± 14.0 (2)</td>
</tr>
<tr>
<td>Polyclonal antifibronectin</td>
<td>63.5 ± 2.4* (6)</td>
</tr>
</tbody>
</table>

The ECM was perfused with whole blood for 5 minutes at a shear rate of 300 s⁻¹. The antibody directed to the α5 integrin and GP IIb/IIIa were added to the perfusate 45 minutes before perfusion. Preincubation of the ECM with 100 μg/mL UFH in PBS or polyclonal antifibronectin was for 1 hour. Platelet adhesion is expressed as percentage of the amount of surface coverage found in the control (no treatment). Values represent mean ± SEM with the number of determinations in parentheses.

* P < .001.
adhesion. This finding is in agreement with the 50% reduction of adhesion by different anti-VLA-5 antibodies that has been found before for platelet adhesion of nonactivated platelets to solid-phase fibronectin in a static system.\textsuperscript{8,17} When we used a combination of the two antibodies in flow, the adhesion was not further decreased (result not shown), suggesting that no \( \beta_1 \) integrin other than VLA-5 contributes to the adhesion.

Inhibition of GP IIb/IIIa by an antibody or absence of this receptor, as with Glanzmann’s thrombasthenia patients, resulted in almost complete inhibition of adhesion. The only platelets left were contact platelets, suggesting that both initial adhesion and subsequent spreading were affected. Although the extent of inhibition, 50%, was less than that found by us, Piotrowicz et al\textsuperscript{8} have shown that adhesion of nonactivated platelets to adsorbed fibronectin in a static system can be inhibited by an antibody against GP IIb/IIIa. An explanation for this discrepancy might be the degree of activation of the platelets, which determines the activity of GP IIb/IIIa. Piotrowicz et al performed their experiments with platelets treated with prostaglandin E\(_2\) (PGE\(_2\)), thereby keeping the platelets in a nonactivated state. We performed our experiments in the absence of PGE\(_1\), allowing platelets to become slightly activated during the washing procedures and possibly resulting in a higher contribution of GP IIb/IIIa. However, we found that different degrees of platelet manipulation (washed platelets compared with platelet-rich plasma), which may result in a different extent of platelet activation, did not change the extent of inhibition by the anti-GP IIb/IIIa antibody. From this we conclude that possible platelet activation caused by the washing procedure was not a determining factor in our experiments.

When we used prostacyclin in the static system we were, in contrast with Piotrowicz et al,\textsuperscript{8} not able to detect any adhesion at all. The reason for this difference is not clear, but might reflect a difference in sensitivity between the research systems used. In flow, prostacyclin was also found to be almost completely inhibitory. Earlier studies have shown that prostacyclin can inhibit VLA-6-mediated adhesion to laminin\textsuperscript{4} and platelet spreading on the subendothelium of the vessel wall\textsuperscript{40} that highly depends on GP IIb/IIIa.\textsuperscript{41} Thus, prostacyclin inhibits adhesion irrespective of the receptors involved. The mechanism by which prostacyclin inhibits adhesion is not yet clear. By increasing the intraplatelet cyclic adenosine monophosphate (cAMP) concentration, prostacyclin may have a direct effect on platelet receptors as has been described for GP Ib\(_\alpha\).\textsuperscript{42} Another option is that prostacyclin exerts its effect in an indirect way by affecting components of the cytoskeleton.\textsuperscript{43} Both the \( \beta_1 \) integrin\textsuperscript{44} and GP IIb/IIIa\textsuperscript{45} have been described to be associated with cytoskeletal components. It is conceivable that changes in the cytoskeleton may change the signal from the receptor into the cell or change receptor function itself.

Both VLA-5 and GP IIb/IIIa have been described as receptors whose ligand binding can be regulated by divalent cations.\textsuperscript{18,37,38} However, we did not find a difference in platelet adhesion or platelet spreading when we compared citrate and the thrombin inhibitor hirudin as anticoagulants, indicating that physiologic divalent cation concentrations are not necessary for optimal adhesion. In contrast, heparin showed inhibition of adhesion both in the absence and presence of citrate. On a molar basis, UFH was \( \sim 12\) times more active than LMWH. A probable explanation for the inhibitory effect of these two heparins may be a direct effect on the platelets because heparin has been shown to bind to platelets,\textsuperscript{36,61} thereby influencing platelet functions.\textsuperscript{39} The inhibition by heparin was highly donor specific. The reason for this variation is not yet understood. One can think of a donor-dependent clearance of heparin by plasma proteins, containing a heparin-binding domain, or by platelet proteins, such as platelet factor 4.\textsuperscript{46} Another source of variation may be the number of heparin-binding sites on the platelets.

Another possibility is that the heparins tested exerted their effect by binding to the heparin-binding domain of fibronectin, thereby possibly blocking adhesive sites. For this reason, we used the heparins to preincubate the fibronectin surface before perfusion. UFH showed almost complete inhibition, whereas LMWH had no effect, even at a high concentration of 1 mg/mL. There are two possible explanations for this difference; (1) LMWH does not bind to fibronectin, and (2) LMWH does bind to fibronectin, but cannot, because of its size, block adhesive sites. The latter option is supported by different observations. First, Ingham et al\textsuperscript{49} have shown that heparin with an MW of 5 kD, which is the size of LMWH, does not have a lower affinity for solid-phase fibronectin than heparins of higher molecular weight. Second, when we examined the inhibitory effect of heparins of different size between 8 and 21 kD, we observed a sharp decrease in adhesion between 18 and 21 kD. This suggests that heparin does not block an adhesive site at the heparin-binding site itself. This is in agreement with the finding that in a static system the heparin-binding domain does not support platelet adhesion at all.\textsuperscript{50} In flow, we have also found that the heparin-binding domain obtained by proteolytic cleavage of fibronectin with cathepsin D does not support adhesion (result not shown). When we consider that (1) UFH is a mixture of heparins with sizes up to 30 kD, (2) both UFH and E121 inhibit adhesion in a similar fashion as the anti GP IIb/IIIa antibody or by preincubation of the surface with UFH at concentrations that were completely inhibitory for adhesion to isolated fibronectin. The noninhibitory effect of UFH may be explained by assuming that the heparin-binding sites of fibronectin are already occupied by heparin or heparan sul-
fate present in the matrix. The other data indicate that, apparently, adhesion to fibronectin in the ECM at low shear rate can only be blocked at the site of the ligand and not by blocking the receptors VLA-5 and GP IIb/IIIa. This suggests the presence of a third receptor for fibronectin on the platelet membrane that is not RGD-mediated. Further research will be required to show the character of this receptor and to determine the domain of fibronectin that is responsible for the interaction.

In conclusion, platelet adhesion to isolated fibronectin in flow depends on at least two different receptors, GP IIb/IIIa and VLA-5, and is consequently a highly RGD-mediated process. In contrast, in one specific situation, namely platelet adhesion to fibronectin in the ECM at a low shear rate, these receptors do not seem to be crucial. These results indicate that caution is needed translating data from a simplified to a more complex system. However, other situations are conceivable in which the interactions between fibronectin and GP IIb/IIIa and/or VLA-5 may very well be of importance. We refer to platelet adhesion at high shear rates (>1,000 s⁻¹) to the subendothelium in which both fibronectin and GP IIb/IIIa are involved. Furthermore, we know that both fibronectin and GP IIb/IIIa are implicated in platelet aggregation. The results presented in our paper may contribute to a better understanding of the role of fibronectin in these processes.

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