Glycoprotein Ib, von Willebrand Factor, and Glycoprotein IIb:IIIa Are All Involved in Platelet Adhesion to Fibrin in Flowing Whole Blood

By Roy R. Hantgan, Georg Hindriks, Richard G. Taylor, Jan J. Sixma, and Philip G. de Groot

We have investigated the molecular basis of thrombus formation by measuring the extent of platelet deposition from flowing whole blood onto fibrin-coated glass coverslips under well-defined shear conditions in a rectangular perfusion chamber. Platelets readily and specifically adhered to fibrin-coated coverslips in 5 minute perfusion experiments done at either low (300 s⁻¹) or high (1,300 s⁻¹) wall shear rates. Scanning electron microscopic examination of fibrin-coated coverslips after perfusions showed surface coverage by a monolayer of adherent, partly spread platelets. Platelet adhesion to fibrin was effectively inhibited by a monoclonal antibody (MoAb) specific for glycoprotein (GP) Ib:IIa. The dose-response curve for inhibition of adhesion by anti-GP Ib:IIa at both shear rates paralleled that for inhibition of platelet aggregation. Platelet aggregation and adhesion to fibrin were also blocked by low concentrations of prostacyclin. In contrast, anti-GP IIb reduced adhesion by 40% at 300 s⁻¹ and by 70% at 1,300 s⁻¹. A similar pattern of shear rate-dependent, incomplete inhibition resulted with a MoAb specific for the GP IIb:IIIa recognition region of von Willebrand factor (vWF). Platelets from an individual with severe von Willebrand's disease, whose plasma and platelets contained essentially no vWF, exhibited defective adhesion to fibrin, especially at the higher shear rate. Addition of purified vWF restored adhesion to normal values. These results are consistent with a two-site model for platelet adhesion to fibrin, in which the GP IIb:IIIa complex is the primary receptor, with GP IIb:IIIa providing a secondary adhesion pathway that is especially important at high wall shear rates.

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A DHESION OF PLATELETS to the fibrin network is a critical event in thrombus formation, especially in narrow regions of the vasculature characterized by rapid blood flow and high wall shear rates. Defining the cell surface receptors and intracellular adhesive molecules involved in this process is important to an understanding of normal hemostasis, and to the design of therapeutic strategies to prevent the development of thrombosis.

The importance of blood flow to platelet adhesion and fibrin deposition on the subendothelium has been demonstrated in ex vivo experiments carried out with everted vessel wall segments in an annular perfusion chamber. With nonanticoagulated whole blood from normal donors, increased wall shear rates resulted in increased platelet adhesion but decreased fibrin deposition on both the subendothelial surface and the platelets themselves. Interestingly, fibrin deposition on platelets was normal in studies with Glanzmann's thrombasthenic patients, but was markedly decreased in Bernard-Soulier patients. However, with citrated blood, the number of adherent platelets was decreased in Glanzmann's thrombasthenia, Bernard-Soulier syndrome, and severe von Willebrand's disease in a shear rate-dependent manner.

The observation of normal platelet adhesion and thrombus formation in perfusion experiments carried out with blood from a patient with severe congenital fibrinogen deficiency emphasizes the importance of other adhesive proteins, especially von Willebrand factor (vWF), in these processes. Moreover, the origin and multimeric composition of vWF strongly influences its ability to support platelet-platelet and platelet-vessel wall interactions, especially under high shear conditions. For example, Frangos et al. have shown that unusually large multimers of vWF, present in plasma and secreted from endothelial cells subjected to shear stress, play a major role in shear-induced platelet aggregation. Stel et al. and de Groot and Sixma have demonstrated that at high wall shear rates, both vWF in the subendothelium and plasma vWF are required for optimal platelet adhesion to the subendothelium and the extracellular matrix of cultured endothelial cells.

Studies with model systems composed of platelets isolated free of plasma proteins have clearly shown that the Ca²⁺-dependent, noncovalent glycoprotein (GP) IIb:IIIa complex is the principal platelet receptor responsible for binding fibrinogen, soluble fibrin polymers, and the fully assembled fibrin clot under static conditions. However, other lines of evidence suggest that GP Ib, in conjunction with vWF, may provide an alternate pathway for adhesion of fibrin to the platelet surface. This concept is supported by reports of the ability of polymerizing fibrin to attach to stirred, formaldehyde-fixed normal platelets in a process that depends on the availability of plasma vWF and platelet GP Ib. In addition, a complex interplay between fibrin monomer and GP Ib has been described that results in increased binding of platelet-released vWF to thrombin-stimulated platelets.

Development of the rectangular perfusion chamber has provided a valuable experimental tool for study of interactions of platelets with surface-bound purified proteins under well-defined shear conditions. Studies done with mono-

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meric and fibrillar collagen, which had been deposited on glass coverslips by a spray coating technique, have shown the importance of both fibronectin and vWF in platelet adhesion, especially under conditions of high shear rate.26-28 In this report, we describe the application of these coating and perfusion techniques to the study of platelet adhesion to fibrin-coated coverslips and provide evidence for the involvement of both glycoproteins Ib and IIb:IIa, as well as vWF in platelet-fibrin interactions in flowing whole blood.

**EXPERIMENTAL PROCEDURES**

**Preparation of Protein-Coated Coverslips**

**Fibrinogen.** Human fibrinogen (IMCO, Stockholm, Sweden) or fluorescein-labeled fibrinogen29-30 at 3 mg/mL in 0.3 mol/L NaCl was dialyzed versus 0.05 mol/L ammonium acetate, pH 7.4, containing 0.01 mg/mL soybean trypsin inhibitor (Sigma, St Louis, MO) overnight at 4°C and clarified by centrifugation, and the fibrinogen concentration was determined by absorbance measurements as previously described.21,30

Eighteen-millimeter square glass coverslips were cleaned in 80% ethanol, rinsed in distilled water and incubated with poly-lysine (1 mg/mL in phosphate-buffered saline). The coverslips were then coated with fluorescein-fibrinogen using a Badger Model 100 airbrush (Franklin Park, IL) to spray a fine mist of protein solution at 1.0 mg/mL in the volatile ammonium acetate buffer to obtain a protein density of 100 μg/coverslip, following procedures developed for spray-coating with collagen.30 Control 5-minute perfusion experiments showed that platelets exhibited minimal adhesion to poly-lysine coated coverslips: the percent surface covered was found to be 1.7% ± 1.2% at 300 s⁻¹ and 0.3% ± 0.3% at 1,300 s⁻¹. Therefore, blocking steps were not used subsequent to coating coverslips with fibrinogen or fibrin.

**Fibrin.** Right angle light scattering intensity measurements31 were used to monitor the rate/extent of fibrin formation using 1.0 mg/mL fibrinogen in 0.05 mol/L ammonium acetate, pH 7.4. Clotting was initiated by addition of human α-thrombin (provided by Dr J. Fenton; specific activity, 2,265 NIH U/mg) to a final concentration of 0.3 NIH U/mL. At approximately 30 seconds, a sharp increase in scattering intensity was observed, and by 3 minutes, long fibers composed of 18 ± 1 fibrin molecules per cross section had formed. At 30 minutes, the average fiber thickness was 38 ± 5 fibrin molecules/cross section (mean ± SD of three experiments). Although thicker fibers (86 ± 2 fibrin molecules/cross section) result at physiologic ionic strength,31 the light scattering data confirm that coarse fibrin fibers were formed in 0.05 mol/L ammonium acetate buffer, pH 7.4.

These solution conditions were subsequently used to coat coverslips with a matrix of clotted fibrin. Thrombin (provided by Dr J. Fenton or purchased from Sigma; specific activity, 3,080 NIH U/mg; final concentration 0.3 to 0.4 NIH U/mL) was added to fibrinogen (1.0 mg/mL) in ammonium acetate buffer. Before the gel point (approximately 1 minute), the solution was sprayed onto glass coverslips (which had been cleaned with ethanol, thoroughly rinsed with distilled water, and dried in air or, in selected cases, treated with polylysine and then dried) to achieve a coating density of 100 μg per coverslip. The fibrin-coated coverslips were allowed to air dry for 1 to 2 hours at room temperature before use in perfusion experiments. Epifluorescence examination of randomly selected coverslips coated with fluorescein-fibrin revealed that the surface was coated with a dense network of brightly fluorescent fibrin strands, and that the fibrin coat remained intact after perfusions.

An additional series of experiments compared platelet adhesion to coverslips that were spray-coated with fluorescein-fibrin alone with those that were subsequently coated with 10 μmol/L phenyl-α-lanuglycyl-chloromethylketone, an irreversible thrombin inhibitor. This 2,000-fold molar access (inhibitor: thrombin) was used to insure complete inhibition of thrombin activity; previous results obtained with a sensitive light scattering kinetic assay of fibrin formation indicated thrombin to be fully inhibited in less than 5 minutes under these conditions.32

Collagen, vWF, albumin. Poly-lysine-coated coverslips were coated with collagen type III31 or vWF30 and then blocked with bovine serum albumin, as previously described.33 Platelets adhered minimally to glass or albumin-coated coverslips; less than 1% of either surface covered at either 300 s⁻¹ or 1,300 s⁻¹ in 5 minute perfusion experiments.

**Isolation and Characterization of Monoclonal Antibodies**

Ascites fluids were provided by the following individuals: anti-GPIIb (monoclonal antibody [MoAb] 6D-1, Dr B. Colter);34 anti-GPllb:IIa (MoAb HIP-1-D, Dr W. Nichols);35 anti-vWF588-728 (MoAb RAG35, Dr J.A. van Mourik4). One to two milliliter aliquots of each ascites fluid were diluted 1:1 with binding buffer consisting of 1.5 mol/L glycine, 3 mol/L NaCl, pH 8.9, and were applied to a 1 × 4 cm column of Protein A Sepharose (Pharmacia, Piscataway, NJ). After exhaustive washing with binding buffer, adsorbed IgG was eluted with 0.1 mol/L sodium citrate, pH 4.0 followed by dialysis against phosphate-buffered saline (PBS). IgG concentrations were determined by absorbance measurements at 280 nm, using an extinction coefficient of 1.5 mg/mL/cm.35

The ability of the anti-GPllb:IIa IgG to inhibit platelet aggregation was determined with platelet-rich plasma (blood anticoagulated with 1/10 vol 110 mmol/L trisodium citrate) which was stimulated with 25 μmol/L adenosine diphosphate (ADP) at 37°C in a Payton multichannel aggregometer (Payton, Scarborough, Canada) with stirring at 900 rpm.36,37 Similarly, the concentrations of the anti-GP1b and anti-vWF588-728 IgG preparations required to inhibit platelet aggregation were determined by aggreometry with platelet-rich plasma in the presence of 1 mg/mL Ristocetin.38,39

**Inhibition of Aggregation by Prostacyclin**

The ability of prostacyclin (Upjohn, Kalamazoo, MI) to inhibit platelet aggregation was determined by aggreometry of platelet-rich plasma (stimulated by 25 μmol/L ADP, as described above), after the addition of small aliquots of PG1, in 0.1 mol/L NaOH to achieve plasma concentrations ranging from 0 to 235 nmol/mL. Control experiments involved addition of the equivalent volume of 0.1 mol/L NaOH.

**Perfusion Procedures**

Perfusates were carried out in a parallel plate perfusion chamber with well-defined rheologic characteristics and that was designed to accommodate glass microscope coverslips.40 Whole blood obtained by venipuncture from healthy, volunteer donors was anticoagulated with ½ vol 110 mmol/L trisodium citrate. Perfusates were prewarmed to 37°C for 5 minutes, then recirculated through the perfusion chamber, which contained two protein-coated coverslips, for 5 minutes at wall shear rates of either 300 s⁻¹ or 1,300 s⁻¹. The coverslips were removed, rinsed with 10 mmol/L HEPES, 150 mmol/L NaCl, pH 7.4, then fixed in 1% glutaraldehyde, dehydrated in methanol, and stained with May-Grünwald-Giemsa.40-42 The ability of anti-GPIb, anti-GPllb:IIa, and anti-vWF588-728 to inhibit platelet adhesion to fibrin was determined in a series of whole blood perfusion experiments. Control ascites fluid, as well as ascites containing these antibodies (each diluted 200-fold), purified monoclonal antibodies, or PBS were added to whole blood samples at least 30 minutes before perfusion experiments. The ability of PG1, to
inhibit platelet adhesion to fibrin was determined in a similar manner. Small aliquots of PGI$_2$ or 0.1 mol/L NaOH were added to whole blood samples at the time of obtaining blood, and immediately before the start of the perfusions. The inhibitor concentration in the plasma was calculated based on a 40% hematocrit.

**Evaluation of Platelet Adhesion**

The extent of surface coverage by adherent platelets was evaluated by light microscopy of stained coverslips at 1,000 x magnification, aided by an image analyzer (Quantimet 720; Imago, Royston, UK) that was interfaced to the microscope. Thirty to forty randomly selected areas of each coverslip were evaluated, and the results from duplicate coverslips were averaged. Statistical significance of the difference between means in the presence/absence of potential inhibitors was calculated with the Student's $t$ test.

Selected glutaraldehyde-fixed coverslips were dehydrated through a graded ethanol series, then treated with hexamethyldisilazane (Polysciences, Washington, PA), air-dried, and sent to the MICROMED facility at Bowman Gray School of Medicine, where they were sputter-coated with gold-palladium and viewed at 15 kV using a Philips 501 Scanning Electron Microscope (Eindhoven, The Netherlands).

**Studies With a Patient With Severe von Willebrand’s Disease**

Perfusion experiments were done with citrated blood drawn from a patient whose plasma and platelets contained essentially no vWF (less than 0.05 U/mL ristocetin cofactor activity and less than 0.01 U/mL vWF:Ag). The effect of exogenous vWF on adhesion of platelet patients was examined by the addition of purified vWF to an activity of 1 U/mL to samples of the patient’s blood before perfusions.

**RESULTS**

**Characteristics of Platelet Adhesion to Fibrin-Coated Coverslips**

Results obtained in a series of whole blood perfusion experiments carried out in a rectangular perfusion chamber showed that platelets readily and specifically adhered to the fibrin network that was obtained by spray coating glass coverslips with a fine mist of fibrinogen plus thrombin. As shown in Fig 1, platelets exhibited minimal adhesion to glass coverslips or those coated with albumin, whereas 13% ± 3% of the fibrin-coated surface was covered with platelets during 5 minute perfusion experiments carried out at low shear rate (300 s$^{-1}$) and 15% ± 7% coverage at high shear rate (1,300 s$^{-1}$). Evaluation of this data by the $t$ test confirmed that the extent of platelet adhesion to fibrin was the same at both shear rates ($p = .4$). Our results are consistent with those of Chiu et al, who found that at shear rates ranging from 45 s$^{-1}$ to 180 s$^{-1}$, the extent of fibrin surface coverage by adherent platelets exceeded the low levels obtained with either albumin coating or with a clean glass surface. We also found that platelet adhesion to fibrin was independent of the platelet count, over the range 1.2 to 2.8 x 10$^9$ mL$^{-1}$ (Fig 1, inset).

Additional experiments (data not shown) showed that the extent of platelet adhesion was similar with surfaces coated with either fluorescein isothiocyanate (FITC)-labelled fibrinogen, fibrin, or fibrin plus phenyl-alanyl-prolyl-arginyl-chloromethyl-ketone (10$^{-3}$ mol/L), an irreversible thrombin inhibitor. These observations show that residual thrombin, which may remain bound to the fibrin network, does not play a significant role in platelet adhesion to fibrin.

**Evaluation by Scanning Electron Microscopy**

Fibrin-coated coverslips were also examined using scanning electron microscopy (SEM) to determine the percentage of surface covered with platelets and the extent of platelet spreading. Platelets adhered to fibrin-coated coverslips as a monolayer or small clusters; raised aggregates of platelets exceeded the low levels obtained with either albumin-coated glass or with a clean glass surface. Treatment of platelets with 88 nmol/L PGI$_2$ before adhesion reduced the extent of spreading (Fig 2B) and adhesion at low and high shear rates (3.4% ± 2.4% and 4.1% ± 5.2% surface coverage, respectively). The extent of platelet spreading was quantified using SEM and graded as having few pseudopods versus several pseudopods and some spreading. Using this criteria, there was little difference between low and high shear rates (73% and 86% spread, respectively), but PGI$_2$ markedly inhibited spreading (22% spread at low shear and 14% spread at high shear rate).

**Identification of the Receptors Involved in Platelet Adhesion to Fibrin**

The effects of anti-GPIIb/IIIa and anti-GPIb MoAbs on platelet adhesion to fibrin at both 300 s$^{-1}$ and 1,300 s$^{-1}$ are...
summarized in Fig 3, in which the average extent of surface coverage by adherent platelets is shown for a series of experiments carried out in the presence/absence of these MoAbs (at concentrations in excess of 8 μg/mL). Clearly, the greatest inhibition resulted with anti-GPIIb:IIIa; adhesion was reduced ninefold at 300 s⁻¹ (i.e., from a control value of 13.5% surface covered to a value of 1.5% in the presence of antibody) and sevenfold at 1,300 s⁻¹. A monoclonal antibody specific for GPIb caused a 1.5-fold reduction in the extent of platelet adhesion to fibrin at the lower shear rate and a threefold reduction at the higher shear rate (both significant at P < .01).

Complementary results were obtained with anti-vWF, which recognizes an epitope on vWF contained within the N-terminal GPIb binding region. While the 20% reduction in adhesion determined at 300 s⁻¹ was not statistically significant (P = .3), the threefold inhibition at 1,300 s⁻¹ was significant (P < .01) and comparable with the results obtained with anti-GPIb.

Comparable results were obtained with ascites fluids containing each of the MoAbs. In contrast, control ascites showed no significant effects on platelet adhesion to fibrin at shear rates of 100, 500, or 1,300 s⁻¹. The ratio of the percent surface covered by adherent platelets in the presence of control ascites (200-fold diluted) to that in its absence was 1.09 ± 0.12 over the range 100 to 1,300 s⁻¹. Further studies of the concentration- and shear rate-dependence of the inhibition of platelet adhesion to fibrin by each of these purified monoclonal antibodies were done to investigate their mechanisms of action.

Inhibition of Aggregation and Adhesion by an Anti-GPIIb:IIIa MoAb

As can be seen in Fig 4, low concentrations of anti-GPIIb:IIIa effectively inhibited platelet adhesion to fibrin at both 300 s⁻¹ and 1,300 s⁻¹, and also inhibited ADP-stimulated platelet aggregation. Each data set was analyzed in terms of the following competitive inhibition model (Equation 1): F(I) = IC₅₀/[IC₅₀ + [MoAb]], in which F(I) is a measure of the relative extent of adhesion or aggregation in the presence of a particular antibody concentration, MoAb, compared with the control value in the absence of antibody, and the IC₅₀ is the MoAb concentration at which half-maximum inhibition results. These parameters were determined with a nonlinear regression routine (ENZFITTER, Elsevier-BIOSOFT, Cambridge, UK) and are summarized in Table 1.

Analysis of the data obtained in perfusion experiments at both 300 s⁻¹ and 1,300 s⁻¹ by Equation 1 yielded IC₅₀ values in the range of 3 to 5 μg/mL. As can be seen in Fig 4, platelet adhesion to fibrin was reduced approximately 8-fold at anti-GPIIb:IIIa concentrations greater than 10 μg/mL. The solid line in Fig 4, which was calculated with Equation 1 and the IC₅₀ value of 1.7 μg/mL determined for inhibition of
Platelet adhesion to fibrin is mediated through the GPIb–vWF complex, which is activated by prostacyclin (PGI2). Inhibition of platelet aggregation and adhesion to fibrin is observed at higher concentrations of PGI2, demonstrating the role of this molecule in platelet function. The relationship between platelet activation and adhesion to fibrin is complex, as shown in Fig. 4, which illustrates the inhibition of platelet aggregation and adhesion to fibrin-coated coverslips as a function of the concentration of anti-GPIb:IIa. Open circles represent the results of ADP-stimulated aggregation, while solid symbols represent the results of 5-minute whole blood perfusion experiments at a wall shear rate of 300 s⁻¹. The data is expressed as the percent surface covered by adherent platelets, normalized by control values in the absence of antibody. The solid line was calculated from Equation 1, and the inhibition parameters are determined by fitting this data to Equation 1. As evidenced by the data in Fig. 4, the effects of PGI2 on platelet aggregation and adhesion to fibrin are concentration-dependent, with IC₅₀ values observed in the presence of saturating concentrations of MoAb.

Inhibition of Agglutination and Adhesion by an Anti-GPIb MoAb

The anti-GPIb preparation used here was an effective inhibitor of platelet agglutination, as shown by the data in Fig. 5 (○), where the inhibition of platelet aggregation and adhesion to fibrin-coated coverslips is expressed as the percent surface covered by adherent platelets normalized by control values in the absence of antibody. The inhibition of platelet adhesion at both low and high shear rates is notable, as is the steep, concentration-dependent inhibition with an IC₅₀ of 1.8 pg/mL. The high shear inhibition data are also reasonably well described by Equation 2, as evidenced by the agreement between the long-dashed line and the solid squares in Fig. 5. At 300 s⁻¹, concentrations of anti-GPIb ≥8 µg/mL reduce adhesion threefold at MoAb concentrations in excess of 8 µg/mL, corresponding to a Bmin of 0.32 and an IC₅₀ of 1.8 µg/mL.

Role of GPIb and vWF in Platelet Adhesion to Fibrin

An additional series of experiments was done to test the hypothesis that GPIb, in conjunction with vWF, is involved in binding platelets to fibrin. A modified competitive inhibition model (Equation 2) mathematically allows for the possibility that blocking a single epitope on a receptor may result in only partial inhibition of platelet function. The anti-GPIb was found to be a more effective and reproducible inhibitor at the higher shear rate (1,300 s⁻¹), with adhesion to fibrin reduced threefold at MoAb concentrations in excess of 8 µg/mL, corresponding to a Bmin of 0.32 and an IC₅₀ of 1.8 µg/mL. The high shear inhibition data are reasonably well described by Equation 2, as evidenced by the agreement between the long-dashed line and the solid squares in Fig. 5.
Platelet-Fibrin Interactions Studied With Blood From a Patient With Severe von Willebrand's Disease

A critical test of the role of vWF in platelet-fibrin interactions was provided by a perfusion experiment that used whole blood from a patient whose plasma and platelets contain essentially no vWF. As seen in Fig 6, at 300 s⁻¹ platelets from this individual exhibited a small but significant reduction (P = .03) in adhesion, with approximately 10% of the fibrin surface covered by adherent platelets compared with 13% surface coverage obtained with blood from a normal donor in parallel perfusions carried out on the same day. Addition of purified vWF (final concentration 1 U/mL) did not significantly (P = .5) alter the adhesion seen under these low shear conditions with the patient's blood.Defective adhesion was most evident at 1,300 s⁻¹, where only 6% of the fibrin-coated surface was covered with adherent platelets, compared with 17% for the control (significant at P = .01). Addition of purified vWF caused a threefold increase (significant at P < .01) in patient platelet adhesion at 1,300 s⁻¹, to a value comparable with the control donor (summarized in Table 2).

The vWF-deficient blood also provided an additional means to test the specificity of inhibition observed with normal platelets and antibodies directed against GPIIb:IIIa, GPIb, and vWF. As can be seen in Table 2, anti-GPIIb:IIIa caused substantial inhibition of platelet deposition onto fibrin with both control and patient blood at both low and high shear rates. This result is consistent with the major role of the GPIIb:IIIa complex in promoting platelet adhesion to fibrin, as documented earlier in this report.

At 300 s⁻¹, anti-GPIb caused only a 20% to 25% reduction in adhesion with both normal and patient platelets. The effects of anti-GPIb were more pronounced at 1,300 s⁻¹, where patient platelet adhesion was reduced twofold and control platelet adhesion threefold by this MoAb (Table 2). The inhibition observed with the vWF-deficient blood raises the possibility that fibronectin, which can also bind to fibrin, may influence the role of vWF in platelet-fibrin interactions.
PLATELET ADHESION TO FIBRIN

Table 2. Platelet Adhesion to Fibrin for Severe von Willebrand Disease Patient and Normal Blood

<table>
<thead>
<tr>
<th>Addition to Blood</th>
<th>% Surface Covered With Platelets</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Patient</td>
</tr>
<tr>
<td>300 s⁻¹</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>9.8 ± 1.1</td>
</tr>
<tr>
<td>vWF/PBS</td>
<td>9.0 ± 1.4</td>
</tr>
<tr>
<td>Anti-GPllb:llla</td>
<td>1.3 ± 0.3*</td>
</tr>
<tr>
<td>Anti-GP1b</td>
<td>7.8 ± 2.7</td>
</tr>
<tr>
<td>Anti-vWF 449-728</td>
<td>8.3 ± 0.8</td>
</tr>
<tr>
<td>1,300 s⁻¹</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>6.2 ± 1.5</td>
</tr>
<tr>
<td>vWF/PBS</td>
<td>20.1 ± 1.2*</td>
</tr>
<tr>
<td>Anti-GPllb:llla</td>
<td>2.2 ± 0.1*</td>
</tr>
<tr>
<td>Anti-GP1b</td>
<td>3.3 ± 0.1*</td>
</tr>
<tr>
<td>Anti-vWF 449-728</td>
<td>4.5 ± 1.2</td>
</tr>
</tbody>
</table>

Platelet adhesion to fibrin-coated glass coverslips during 5-minute perfusion experiments with whole blood from a patient with severe von Willebrand's disease and a normal donor, expressed as percent surface covered with platelets ± SD. Additions to blood: vWF, 1 U/mL (patient) or equivalent volume of PBS (normal); anti-GPllb:llla, 11 μg/mL HP1-1D; anti-GP1b, 8 μg/mL ID-1; anti-vWF 449-728, 10 μg/mL RAg35. *Values that differ significantly (P < .03) upon addition of the reagents.

and to a platelet receptor distinct from GPllb:llla,²⁸ may be important for platelet-fibrin adhesive interactions. Significant inhibition by anti-vWF 449-728 was only observed with control platelets at the higher shear rate. The modest reduction in adhesion observed with anti-vWF 449-728 added to vWF-deficient blood was not statistically significant at either shear rate (Table 2).

DISCUSSION

The results presented here demonstrate that platelets in flowing whole blood adhere rapidly and specifically to fibrin, and that this adhesion occurs through two distinct pathways: a direct mechanism involving fibrin binding to the glycoprotein IIb:IIIa complex and an indirect mechanism in which von Willebrand factor provides an additional link between platelet glycoprotein Ib and fibrin. Our results suggest that the alternative adhesion system provided by GPIb:vWF is especially important in anchoring platelets tightly to a fibrin clot in rapidly flowing blood.

The observation that the GPIIb:IIIa complex is the primary receptor responsible for platelet adhesion to the fully assembled fibrin network under flow conditions in whole blood adds a new dimension to our understanding of the molecular basis of platelet-fibrin interactions. Previous results from steady state radiolabeled ligand binding studies with soluble fibrin polymers,¹⁹,²⁶ direct observation of platelet-bound fibrin protofibrils and fibrin fibers by electron microscopy,⁴⁵,⁴⁶ and quantitation of platelet-fibrin network binding by microfluorimetry²¹,⁴³ have established a major role for the GPIIb:IIIa complex in platelet-fibrin association in model systems composed of gel filtered platelets and purified proteins. The close relationship between prostacyclin inhibition of platelet activation/aggregation and platelet adhesion to fibrin supports the postulate that platelet activation is a prerequisite for assembly of the functional GPIIb:IIIa receptor complex required for platelet-fibrin interactions.²¹,⁴²,⁴³

However, the results from perfusion experiments presented here have also demonstrated the importance of an additional receptor:ligand system in binding platelets tightly to fibrin under high shear conditions. A monoclonal antibody specific for GPIIb²² was found to exhibit shear rate-dependent inhibition of platelet adhesion to fibrin; a maximum threefold reduction in the extent of adhesion was found at 1,300 s⁻¹. Interestingly, only 40% inhibition was found in perfusion experiments at 300 s⁻¹, while this same anti-GPIIb antibody had no effect on clotted fibrin binding to thrombin-stimulated platelets under static conditions.⁴³

Complementary results were obtained with a MoAb that recognizes an epitope contained within the N-terminal GPIb recognition region of the multidommainal vWF molecule.³⁴ The pattern of shear rate-dependent inhibition obtained with this reagent was quite similar to that determined with the anti-GP1b MoAb. Moreover, this same anti-vWF 449-728 antibody was also incapable of blocking the binding of clotted fibrin to thrombin-stimulated platelets in a quiescent system.⁴³

Consistent with the postulate that the GPIb:vWF pathway represents an alternative adhesion mechanism in flowing whole blood, the dose-response curves for inhibition of platelet adhesion to fibrin by both the anti-GP1b and anti-vWF 449-728 antibodies were well-described by an inhibition model that quantitatively allows for the possibility that an antibody can block binding of a ligand to one type of receptor while not effecting binding to another site.⁴⁴

Perhaps the most convincing evidence for a role for vWF in mediating platelet adhesion to fibrin comes from our results with blood from a patient with severe von Willebrand's disease, whose plasma and platelets contained essentially no vWF. Perfusion experiments showed near-normal platelet adhesion to fibrin at 300 s⁻¹, whereas a threefold reduced adhesion was observed at 1,300 s⁻¹. Addition of purified vWF to normal plasma levels fully corrected the defective adhesion at the higher shear rate, but was without effect at the lower shear rate. These results raise the possibility that normal levels of vWF in both plasma and platelets are required for optimal platelet adhesion to fibrin over a wide range of shear rates. In this context, it is interesting to note that platelet-released vWF, as well as the unusually large vWF multimers normally present in plasma, contribute to shear-induced platelet aggregation.¹²,¹³

While the rectangular perfusion chamber has proven to be an excellent tool with which to study the interactions of platelets with the extracellular matrix and purified plasma proteins,¹⁵,¹⁸ extrapolation of the results we have obtained to in vivo hemostasis and thrombosis must be made with caution. For example, all results reported here have been obtained with citrated blood. Other investigators have reported differences between native and citrated blood in platelet adhesion experiments done with everted vessel wall segments in an annular perfusion chamber.⁵³ Fortunately, data obtained in ongoing studies of platelet adhesion to fibrin, done with blood anticoagulated with low molecular weight heparin, have shown effects of anti-GP1b, anti-GPIIb: IIIa, and anti-vWF 449-728 that are quite similar to those
reported here (de Groot, personal communication, February, 1990).

Some questions concerning the role of vWF in mediating platelet adhesion to fibrin require further study. For example, the proposed alternative GPIb:VWF:fibrin pathway clearly requires a tight association between the fibrin and von Willebrand factor molecules in order to promote platelet adhesion to fibrin under high shear conditions, where dissociation events are favored.\(^2\) Evidence for noncovalent binding of vWF multimers to immobilized fibrin has been presented by Loscalzo and Handin, who have reported an apparent dissociation constant of 15 \(\mu\)g/mL for this process.\(^3\) Reports that high molecular weight multimers of vWF can bind to the platelet GPIb:IIa complex even in the presence of high concentrations of fibrinogen\(^4\)–\(^6\) raise the possibility that this mechanism could also be important for platelet adhesion to fibrin in flowing whole blood. Additional studies are underway to investigate this possibility.

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