Characterization of the Unique Mechanism Mediating the Shear-dependent Binding of Soluble von Willebrand Factor to Platelets*

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Shinya Goto, Daniel R. Salomon, Yasuo Ikeda‡, and Zaverio M. Ruggeri§

From the Roon Research Center for Arteriosclerosis and Thrombosis, Division of Experimental Hemostasis and Thrombosis, Departments of Molecular and Experimental Medicine and of Vascular Biology, The Scripps Research Institute, La Jolla, California 92307

We have studied the mechanism of interaction between soluble von Willebrand factor (vWF), labeled with fluorescein isothiocyanate (FITC), and platelets exposed to shear in a cone-and-plate viscometer. A flow cytometer calibrated with fluorescent bead standards was used to calculate the number of molecules associated with each platelet in suspension. To validate the methods and reagents used, binding of the same labeled vWF was assessed in the presence of ristocetin or α-thrombin and found to be saturable, with a narrow and symmetric distribution on >90% of the platelets. As expected, essentially all bound ligand interacted exclusively with platelet membrane glycoprotein (GP) Ibα in the presence of ristocetin and with GP IbβIIa after stimulation with α-thrombin. In contrast, only a minor proportion (<20%) of the platelets exposed to shear were found to bind vWF, with no evidence for saturation and markedly decreased interaction when the platelet count was below 100,000 μL. Moreover, shear-induced vWF binding was blocked equally effectively by selected monoclonal antibodies against either GP Ibα or GP IbβIIa or against the respective binding sites in vWF. Thus, both receptors are involved in the process, possibly through initial transient interactions mediated by GP Ibα that lead to platelet activation and subsequent irreversible binding supported by GP IbβIIa. While the levels of shear stress theoretically applied to platelets in these experiments are above those thought to occur in the normal circulation, our findings demonstrate a unique vWF binding mechanism that is not mimicked by other known modulators and correlates with platelet aggregation. Similar processes may occur in response to lower shear stress when platelets are exposed to thrombogenic surfaces and agonists generated at sites of vascular injury during thrombus formation.

The role of von Willebrand factor (vWF)1 in platelet thrombus formation, particularly under conditions of high shear, is well established and supported by experimental evidence as well as clinical observations (1). It is known that platelets have two distinct binding sites for vWF, glycoprotein (GP) Ibα in the GP Ibα-IX-V complex and the integrin αIIbβ3 (GP IbβIIa complex) (2). Binding of soluble normal vWF to GP Ibα in the absence of flow requires the presence of exogenous modulators, like ristocetin (3) or botrocetin (4), while interaction with GP IbβIIa can only occur after platelet activation (5). In addition to vWF, the GP IbβIIa receptor can also bind fibrogen, fibronectin (6), and vitronectin (7), but the respective role of these proteins in thrombogenesis is still a topic for investigation.

Exposure of platelets to levels of shear higher than thought to occur in the normal circulation results in aggregation if soluble vWF is present (8); no other adhesive ligand can support this process (9). Shear-induced platelet aggregation may represent an important pathophysiological function of vWF, but the underlying mechanism is still poorly understood. It has been proposed that vWF binds to GP Ibα under the effect of shear, causing an increase in intracytoplasmic calcium ion levels and, consequently, GP IbβIIa activation; vWF interaction with activated GP IbβIIa would then mediate aggregation (10, 11). There is, however, no evidence that shear can promote the binding of vWF to GP Ibα; rather, there is only the indirect observation that anti-GP Ibα antibodies capable of inhibiting vWF binding mediated by other modulators can block shear-induced aggregation (9, 12). Thus, it is not known whether the characteristics of vWF binding to GP Ibα induced by shear are comparable to those defined in the presence of exogenous modulators like ristocetin or botrocetin.

We have performed studies to clarify the process of vWF interaction with platelets under shear. Our results demonstrate the existence of a unique mechanism that involves both GP Ibα and GP IbβIIa in supporting vWF binding and is not mimicked by the effect of exogenous modulators or platelet agonists inducing activation. The observed binding appears to correlate well with the occurrence of aggregation.

**EXPERIMENTAL PROCEDURES**

Preparation of Washed Platelets—Blood was drawn from an antecubital vein of normal donors with a 19-gauge needle into plastic syringes and immediately transferred into polypropylene tubes containing trisodium citrate, pH 7.5, as anticoagulant (final concentration 0.13 M) and the ADP scavenger, apyrase (Sigma), to prevent platelet activation (final concentration 10 units/ml). All human subjects who participated

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‡ Present address: Dept. of Medicine, Keio University School of Medicine, Tokyo, Japan.

§ To whom correspondence should be addressed: The Scripps Research Institute, SBR-8, 10666 N. Torrey Pines Rd., La Jolla, CA 92037. Tel.: 619-554-8950; Fax: 619-554-6779.

1 The abbreviations used are: vWF, von Willebrand factor; GP, platelet membrane glycoprotein; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate.
in these studies were aware of the experimental nature of the research and gave their informed consent in accordance with the Declaration of Helsinki. Blood was divided into 1-ml aliquots in plastic microcentrifuge tubes and centrifuged at 12,000 revolutions/min for 15 s; the resulting supernatant plasma was removed and replaced with an equivalent volume of divalent cation-free HEPES-Tyrode buffer (10 mM HEPES, 140 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_{2}PO_{4}, 0.4 mM NaHCO_{3}, and 5 mM dextrose), pH 7.4 (2). The sedimented cells (including platelets and leukocytes on top of the erythrocyte cushion) were resuspended and centrifuged again at 12,000 revolutions/min for 15 s; the procedure of removing the supernatant fluid, containing progressively decreasing residual amounts of plasma proteins, and replacing it with fresh buffer was repeated a total of four times. After the last centrifugation, the cell pellet was resuspended in modified HEPES-Tyrode buffer, pH 7.4, containing 1.25 mM CaCl_{2} and 6.25 mg/ml of bovine serum albumin (BSA, Sigma), and a platelet-rich suspension was obtained by centrifuging the sample at 1,500 revolutions/min for 1 min. The platelet count in the final suspension was then adjusted to 240,000/μl using the same modified HEPES-Tyrode buffer with CaCl_{2} and BSA. These platelets were not significantly activated by the washing procedure since the expression of P-selectin, detected by flow cytometric analysis using the monoclonal antibody S-12 labeled with fluorescein isothiocyanate (FITC, Sigma), was not increased as compared to that measured in platelet-rich plasma (13) (the antibody S-12 was obtained as a generous gift from Dr. Rodger P. McEver of the Oklahoma Medical Research Foundation, Oklahoma City, OK). Moreover, functional reactivity of the washed platelets was well preserved since there was no response to the addition of either ristocetin (1.25 mg/ml, Sigma) or ADP (10 μM, Sigma) alone but prompt aggregation occurred when vWF or fibrinogen, respectively, were also added.

Labeling of Ligands with Fluorescein Isothiocyanate—Plasma vWF was purified from cryoprecipitate and characterized as described previously (2). The purified protein supported both ristocetin-induced and shear-induced aggregation of washed platelets in a manner similar to that seen with platelet-rich plasma (13). Purified vWF was labeled with FITC according to the method described for labeling IgG, with slight modifications (14, 15). Briefly, after adjusting the pH of the purified vWF to 9.5 with 5% sodium carbonate, a 10 mg/ml solution of FITC in dimethyl sulfoxide (Me_{2}SO, Sigma) was added to achieve a final fluorescein/protein (F/P) weight ratio of 1.60 and the mixture was incubated for 10 min at room temperature (22–25°C). The FITC-labeled platelet protein was separated from free FITC by gel permeation chromatography on a Sephadex G-25 PD-10 column (Pharmacia Biotech Inc.) equilibrated with HEPES buffer (10 mM HEPES and 140 mM NaCl), pH 7.4. The concentration of FITC-labeled vWF was calculated by spectrophotometric analysis (14), according to the formula: vWF (mg/ml) = \([A_{280} - (0.35 \times A_{280})]/0.7\), where 0.7 is the extinction coefficient for purified vWF. The concentration of fluorescein was calculated from the A_{496} with the extinction coefficient of 200/474. The molar F/P ratio was then calculated on the basis of the known molecular mass of vWF and weight ratio was 1:10. A final molar F/P ratio between 1 and 3 was kept at 8.5 to avoid denaturation of the molecule and the F/P ratio was determined on the basis of the known molecular mass of vWF and weight ratio was 1:10. A final molar F/P ratio between 1 and 3 was kept at 8.5 to avoid denaturation of the molecule and the F/P ratio was determined on the basis of the known molecular mass of vWF and weight ratio was 1:10. A final molar F/P ratio between 1 and 3 was kept at 8.5 to avoid denaturation of the molecule and the F/P ratio was determined on the basis of the known molecular mass of vWF and weight ratio was 1:10. A final molar F/P ratio between 1 and 3 was kept at 8.5 to avoid denaturation of the molecule and the F/P ratio was determined on the basis of the known molecular mass of vWF and weight ratio was 1:10. A final molar F/P ratio between 1 and 3 was kept at 8.5 to avoid denaturation of the molecule and the F/P ratio was determined on the basis of the known molecular mass of vWF and weight ratio was 1:10. A final molar F/P ratio between 1 and 3 was kept at 8.5 to avoid denaturation of the molecule and the F/P ratio was determined on the basis of the known molecular mass of vWF and weight ratio was 1:10. A final molar F/P ratio between 1 and 3 was kept at 8.5 to avoid denaturation of the molecule and the F/P ratio was determined on the basis of the known molecular mass of vWF and weight ratio was 1:10. A final molar F/P ratio between 1 and 3 was kept at 8.5 to avoid denaturation of the molecule and the F/P ratio was determined on the basis of the known molecular mass of vWF and weight ratio was 1:10. A final molar F/P ratio between 1 and 3 was kept at 8.5 to avoid denaturation of the molecule and the F/P ratio was determined on the basis of the known molecular mass of vWF and weight ratio was 1:10. A final molar F/P ratio between 1 and 3 was kept at 8.5 to avoid denaturation of the molecule and the F/P ratio was determined on the basis of the known molecular mass of vWF and weight ratio was 1:10. A final molar F/P ratio between 1 and 3 was kept at 8.5 to avoid denaturation of the molecule and the F/P ratio was determined on the basis of the known molecular mass of vWF and weight ratio was 1:10. A final molar F/P ratio between 1 and 3 was kept at 8.5 to avoid denaturation of the molecule and the F/P ratio was determined on the basis of the known molecular mass of vWF and weight ratio was 1:10. A final molar F/P ratio between 1 and 3 was kept at 8.5 to avoid denaturation of the molecule and the F/P ratio was determined on the basis of the known molecular mass of vWF and weight ratio was 1:10. A final molar F/P ratio between 1 and 3 was kept at 8.5 to avoid denaturation of the molecule and the F/P ratio was determined on the basis of the known molecular mass of vWF and weight ratio was 1:10.
Shear-dependent vWF Binding to Platelets

Monoclonal Antibodies—All the murine monoclonal antibodies used in these experiments were obtained and characterized as described previously. They were purified using protein A (Sigma) chromatography according to published procedures (18). Lj-1b1 (IgG1) reacts with the amino-terminal 45-kDa domain of GP Ibα containing the vWF-binding site (19–21) and inhibits completely the vWF-GP Ib interaction under all experimental conditions tested (22); in particular, this antibody blocks shear-induced platelet aggregation (9). Of note, Lj-1b1 has no effect on α-thrombin binding to platelets (23). Lj-1b10 (IgG2a) reacts with the 45-kDa amino-terminal domain of GP Ibα between residues Ala238 and Arg293 (19) and inhibits α-thrombin but not vWF binding to GP Ibα (23–25). Lj-Cp8 (IgG1) reacts with the GP IIb-IIIa complex and blocks the activation-dependent binding of soluble ligands to this receptor (16, 26) as well as platelet aggregation and thrombus formation under all experimental conditions tested (26), including shear-induced aggregation (9). Lj-P5 (IgG1) is directed against the GP Ibα-IIa complex and, as a monoclonal Fab fragment, inhibits selectively the binding of soluble vWF but not fibrinogen to activated platelets (27); it also inhibits platelet thrombus formation on denuded subendothelium under high flow conditions (26). Lj-P9 (IgG2b) is also directed against the GP Ibα-IIa complex and has high affinity comparable to that of Lj-P5; it inhibits both vWF and fibrinogen binding to activated platelets (27). Lj-152b6 (IgG1) reacts with the vWF site that includes the Arg-Gly-Asp sequence recognized by GP Ibα-IIa, but has no cross-reactivity with other ligands of this receptor containing the same sequence (28). This antibody selectively inhibits vWF binding to GP Ibα-IIa as well as platelet aggregation and thrombus formation under high shear conditions (9, 26). NMC-4 (IgG1) reacts with the A1 domain of vWF including the disulfide bond between residues Cys509 and Cys695 (29, 30) and blocks the interaction between vWF and GP Ibα under all the experimental conditions tested; it also inhibits shear-induced platelet aggregation (11, 31). Lj-229 (IgG1) reacts with a COOH-terminal domain of vWF present in the dimeric fragment IIb (residues 1366–2050 of the mature vWF subunit) generated by Staphylococcus aureus V8 protease (32, 33); it has no known inhibitory activity on vWF function. Lj-C3 (IgG1) reacts with the NH2-terminal domain of vWF corresponding to residues 1–272 of the mature subunit; it inhibits the interaction of vWF with coagulation factor VIII (34) but has no demonstrable effects on vWF-platelet interactions. Monovalent Fab fragments of all these antibodies were prepared according to methods previously described (35). Briefly, IgG was incubated for 4.5 h at 37 °C with 4–8% (w/w) mercaptoethanol (Sigma) preactivated by cysteine; the optimal concentration of enzyme was determined for each antibody in preliminary trials by monitoring IgG digestion with pepsin, a protease with a specificity (i.e., the two control antibodies had no inhibitory effect). In contrast, IgG and Fab fragments of anti-GP Ibα and anti-GP Ibα-IIa antibodies gave consistently similar results.

RESULTS

Flow Cytometric Measurement of the Binding of FITC-labeled vWF to Platelets in the Presence of Ristocetin or after Stimulation with α-Thrombin—These experiments were performed to validate the methodology and reagents used for the study of shear-induced vWF binding to platelets. In agreement with data reported in the literature and obtained with 125I-labeled vWF (2), saturable binding of FITC-labeled vWF was observed both in the presence of ristocetin or after stimulation with α-thrombin (Fig. 2). Scatchard-type analysis of the corresponding isotherms yielded values for apparent Bmax (ristocetin, 42125 ± 6511 vWF subunit molecules/platelet; α-thrombin, 39425 ± 11002) and Kd (ristocetin, 5.3 ± 2.3 μg/ml; α-thrombin, 10.8 ± 2.2 μg/ml) consistent with those published previously (2). Moreover, the effect of specific monoclonal antibodies on

![Flow Cytometric Measurement of the Binding of FITC-labeled vWF to Platelets in the Presence of Ristocetin or after Stimulation with α-Thrombin](image)
thrombin-induced vWF binding to platelets. These assays were performed as described in the legend to Fig. 2, except that a fixed concentration (15 μg/ml) of FITC-labeled vWF was used, and 10 μl of the appropriate monoclonal antibody solution (purified IgG or Fab) was added to platelets, replacing an equivalent volume of buffer, before adding the ligand (in the case of experiments with thrombin, antibodies were added during the first activation step). Anti GP Ibα IgG were used at a final concentration of 150 μg/ml; anti-GP Ibα-IgG at 50 μg/ml; anti vWF Fab at 500 μg/ml. The number of vWF molecules bound/platelet in the presence of antibodies is expressed as percent of the value measured in control mixtures without antibody. Data are the mean ± S.E. of four to eight experiments. Upper panel, ristocetin-mediated binding; note that antibody NMC-4 inhibits completely. Lower panel, binding induced by activation with α-thrombin.

Shear-dependent vWF Binding to Platelets

Fig. 3. Effect of monoclonal antibodies on ristocetin and thrombin-induced vWF binding to platelets. These assays were performed as described in the legend to Fig. 2, except that a fixed concentration (15 μg/ml) of FITC-labeled vWF was used, and 10 μl of the appropriate monoclonal antibody solution (purified IgG or Fab) was added to platelets, replacing an equivalent volume of buffer, before adding the ligand (in the case of experiments with thrombin, antibodies were added during the first activation step). Anti GP Ibα IgG were used at a final concentration of 150 μg/ml; anti-GP Ibα-IgG at 50 μg/ml; anti vWF Fab at 500 μg/ml. The number of vWF molecules bound/platelet in the presence of antibodies is expressed as percent of the value measured in control mixtures without antibody. Data are the mean ± S.E. of four to eight experiments. Upper panel, ristocetin-mediated binding; note that antibody NMC-4 inhibits completely. Lower panel, binding induced by activation with α-thrombin.

Fig. 4. Flow cytometric analysis of shear-induced vWF binding to platelets. The two upper panels show the distribution of forward and side light scattering (representing essentially particle size and granularity) measured in a mixture of platelets (400 μl of the suspension described in the legend to Fig. 3; final count 1.92 × 10^7/μl) and FITC-vWF (100 μl of a solution in HEPES buffer; final concentration 15 μg/ml) before and after exposure to shear (10,800 dynes/cm² for 6 min at 24 °C). After rotation in the cone-and-plate viscometer (or incubation without agitation at the same temperature for the samples not exposed to shear), 100 μl of the suspension was mixed with 400 μl of HEPES-Tyrode buffer, pH 7.4 (with no BSA or divalent cation), and kept on ice protected from light until analyzed in the flow cytometer. Particles both larger (R1) and smaller (R3) than single platelets (R2) appeared after exposure to shear, representing aggregated platelets and platelet-derived microparticles, respectively. The two lower panels show the fluorescence distribution in the same experimental mixtures. Analysis of the whole population of particles in suspension without gating for size (lower left) demonstrated a small number of highly fluorescent elements appearing in the sample exposed to shear as compared to the distribution seen before exposure to shear. After gating for size (lower right), it became apparent that the fluorescent particles seen after exposure to shear correspond to aggregated platelets (R1).

Shear-induced Platelet Aggregation and vWF Binding—The effect of applying shear to washed platelets in the presence of FITC-labeled vWF (15 μg/ml) was evaluated by flow cytometric analysis (Fig. 4). After shearing, there was a clear change in the size and granularity of particles in suspension, as judged by side and forward light scattering. The occurrence of platelet aggregation (appearance of larger particles, indicated as R1) and activation (appearance of smaller particles, indicated as R3, corresponding to microparticles released from stimulated platelets). Only particles with the size of aggregated platelets showed a change in the distribution of fluorescence intensity and increased fluorescence, whereas particles with the size of single platelets (indicated as R2) showed no such increase (Fig. 4). Because of the assumption that the primary vWF-binding site on platelets is GP Ibα, we reasoned that we could use antibodies directed against GP Ibα to prevent shear-induced platelet aggregation, thus avoiding nonspecific vWF trapping in aggregates and allowing demonstration of specific binding to single platelets. Indeed, in the presence of the anti-GP Ibα antibody LJ-CP 8 aggregation was reduced >80%, activation-dependent release of microparticles was unchanged, and a fraction (~20%) of the single platelet population became fluorescent with bound FITC-vWF (Fig. 5). These results suggest that, in the absence of inhibitory antibodies against GP Ibα, all the platelets that bind vWF become part of aggregates. In fact, examination by epifluorescence microscopy confirmed that single platelets but no aggregates were positive for bound FITC-labeled vWF in the presence of the anti-GP Ibα antibody, whereas only aggregates were positive in the absence of the antibody (data not shown here). The antibody LJ-Ib1, which is known to inhibit vWF binding to GP Ib, inhibited the shear-induced vWF interaction with single platelets as well as the release of microparticles (Fig. 5). In agreement with previously published results (9), this antibody completely inhibited shear-induced aggregation even in the absence of GP Ibα blockade (not shown). The specificity of shear-dependent vWF binding to single platelets was demonstrated by an experiment in which FITC-labeled fibrinogen (Fig. 6) or FITC-labeled BSA were added to platelets exposed to shear in the presence of nonlabeled vWF (the latter was necessary to support shear-induced aggregation). Both fluorescent ligands were incorporated into the platelet aggregates (although less than vWF), consistent with nonspecific trapping; however, minimal binding to single platelets was
observed when aggregation was blocked by addition of the antibody LJ-CP8.

Characteristics of Shear-induced vWF Binding to Platelets—In view of the data presented above, all experiments aimed at characterizing the interaction between vWF and single platelets under shear were performed in the presence of the anti-GP IIb-IIIa antibody LJ-CP8 to prevent aggregation. It should be carefully noted, however, that although binding was measured in the presence of this antibody, GP IIb-IIIa was eventually shown to be required to support the interaction (see below). In contrast to treatment with ristocetin or stimulation with a-thrombin, conditions that result in the binding of >90% of the platelets in suspension of >10,000 molecules of vWF subunit interacting with GP Ib or GP IIb-IIIa, respectively (Fig. 2), <10% of the platelets exposed to shear bound >10,000 molecules of vWF subunit even after addition of 60 μg/ml of vWF in solution (Fig. 7). In order to obtain the distribution of fluorescence on the positive single platelet population, experi-
ments were performed where increasing amounts of FITC-labeled vWF were added to platelets either in the presence or absence of the function blocking anti-GP Ib antibody, LJ-Ib1 (as mentioned above, the anti-GP IIb-IIIa antibody LJ-CP8 was always present in these experiments). After shearing, the values of fluorescence measured in the presence of the antibody were subtracted from those measured in absence; thus, the observed distribution corresponds to bound vWF that can be inhibited by the anti-GP Ibα antibody. Each curve represents the mean from four different experiments.

Shear-dependent vWF Binding to Platelets

FIG. 7. Flow cytometric analysis of the dose-dependent binding of FITC-labeled vWF to platelets exposed to shear. All experiments presented here were performed as described in the legend to Fig. 5, in the presence of antibody LJ-CP8 (50 μg/ml) to block aggregation, with or without addition of antibody LJ-Ib1 (150 μg/ml) to block GP Ibα function (as indicated by two different lines) and in the presence of the indicated concentrations of FITC-labeled vWF. The figure shows the distribution of particles in the R2 population (single platelets as shown in Fig. 5) as a function of the calculated number of vWF molecules bound (see "Experimental Procedures" for the method used for calculation). The insets show the distribution obtained after subtracting the values of bound vWF measured in the presence of the anti-GP Ibα antibody, LJ-Ib1, from those measured in its absence; thus, the observed distribution corresponds to bound vWF that can be inhibited by the anti-GP Ibα antibody. Each curve represents the mean from four different experiments.

FIG. 8. Dose-response analysis of shear-induced vWF binding to platelets and correlation with aggregation. The upper panel shows the dose-response curve of specific (inhibited by anti-GP Ibα antibody) shear-induced vWF binding calculated from the median of the distribution observed for each experimental point (see Fig. 7). Each point is the mean ± S.E. of two to six different experiments. The lower panel shows the relationship between shear-induced vWF binding and platelet aggregation (based on single platelet count, i.e. the values shown represent the percent decrease in the number of single platelets in suspension, or 100% residual single platelets, after shear versus before shear). One to four experiments were performed for each point, and the mean is shown when more than one experiment was performed. Regression analysis demonstrated the positive correlation between the two parameters. Aggregation was measured in mixtures prepared as described for measuring binding, but without the monoclonal antibodies.

FIG. 9. Flow cytometric analysis of the dose-dependent binding of FITC-labeled vWF to platelets exposed to shear. The figure shows the distribution of particles in the R2 population (single platelets as shown in Fig. 5) as a function of the calculated number of vWF molecules bound (see "Experimental Procedures" for the method used for calculation). The insets show the distribution obtained after subtracting the values of bound vWF measured in the presence of the anti-GP Ibα antibody, LJ-Ib1, from those measured in its absence; thus, the observed distribution corresponds to bound vWF that can be inhibited by the anti-GP Ibα antibody. Each curve represents the mean from four different experiments.

FIG. 10. Flow cytometric analysis of the dose-dependent binding of FITC-labeled vWF to platelets exposed to shear. The figure shows the distribution of particles in the R2 population (single platelets as shown in Fig. 5) as a function of the calculated number of vWF molecules bound (see "Experimental Procedures" for the method used for calculation). The insets show the distribution obtained after subtracting the values of bound vWF measured in the presence of the anti-GP Ibα antibody, LJ-Ib1, from those measured in its absence; thus, the observed distribution corresponds to bound vWF that can be inhibited by the anti-GP Ibα antibody. Each curve represents the mean from four different experiments.
ligand (NMC-4), also inhibited shear-dependent binding. Control antibodies defined as noninhibitory in the assay modulated by ristocetin were also without effect on shear-dependent binding. The unexpected result was that two antibodies known to block the interaction of vWF with GP IIb-IIIa, one against the receptor (LJ-P5) and the other against the domain of vWF containing the Arg-Gly-Asp integrin recognition sequence (LJ-152B/6), consistently inhibited vWF binding to platelets exposed to shear (Table I). Thus, unlike ristocetin- or thrombin-induced binding, the stable interaction of vWF with platelets under shear appears to require ligand binding to both GP Ib and GP IIb-IIIa, either in a sequential or concurrent fashion. Of note, all antibodies known to inhibit the ristocetin-mediated vWF-GP Ib interaction also prevented shear-induced binding and aggregation; however, not all those interfering with vWF binding to GP IIb-IIIa on platelets stimulated by α-thrombin had the same effect on platelets exposed to shear, although they all inhibited aggregation. Like the latter group of antibodies, the synthetic peptide GRGDSP, containing the Arg-Gly-Asp integrin recognition sequence, was effective in blocking shear-induced aggregation but not vWF binding (not shown).

**DISCUSSION**

These studies provide experimental evidence that shear can modulate the binding of vWF to platelets eliciting a unique mechanism of interaction that requires two platelet receptors, the GP IbIX-V complex and the integrin αIIbβ3 (GP IIb-IIIa). While there is no other example of a dual receptor requirement for soluble vWF binding to platelets, our findings are reminiscent of conditions previously established for the adhesion of nonstimulated platelets to surface-bound vWF, a process that depends on both GP Ib and GP IIb-IIIa to become irreversible (36). There are obvious limitations in the methodology we have used to perform these studies, essentially dictated by the nature of the phenomena being evaluated. Since aggregation always occurs when platelets are exposed to high levels of shear in the presence of vWF, it was necessary to use a blocking anti-GP IIb-IIIa antibody in order to study vWF binding to
Shear-dependent vWF Binding to Platelets

The monoclonal antibodies against GP Ib and GP IIb-IIIa (all intact IgG) were tested at the final concentration of 150 and 50 μg/ml, respectively; the Fab fragments against vWF were at 500 μg/ml. The inhibition of shear-induced vWF binding was indicated as positive when the results obtained with a given antibody were essentially the same as seen in the presence of LJ-Ib1; negative, when the results were as seen in the absence of any antibody.

| Antibodies tested | Inhibition of vWF binding |
|-------------------|---------------------------|
| Anti-GP Ib<sub>x</sub> | LJ-Ib1 | + |
| | LJ-Ib10 | - |
| Anti-GP IIb-IIIa<sub>1</sub> | LJ-P5 | + |
| | LJ-P9 | - |
| Anti-vWF | NMC-4 | + |
| | LJ-229 | - |
| | LJ-C3 | - |
| | LJ-1528/6 | + |

Another important consideration is that, at present, there is no clear understanding of what responses are evoked when platelets in suspension are exposed to a shear field. According to fluid dynamics theory, the surface of a body moving freely in...
a streaming fluid is not subject to tangential forces imposed by the flow (37); thus, it is unlikely that platelets are subjected to a significant shear stress when moving freely with the liquid as single particles. We hypothesize that if more than one platelet is transiently interacting with a vWF multimer (Fig. 11), greater shear stress may be exerted on the larger, more irregular “particle.” Increased shear stress on the membrane may combine with the consequences of proximity, established during the transient binding of several platelets to a vWF multimer, and lead to activation (38–41). Such a mechanism could favor the local availability of greater concentrations of released ADP, a synergistic agonist that has been known to be required for shear-induced aggregation (42), and could also account for the greater efficiency of larger vWF multimers in mediating shear-induced activation and aggregation. Limiting factors in a process like this would be a low platelet count, in agreement with our experimental results, and the relatively low concentration of soluble large vWF multimers present, thus explaining why only a small proportion of platelets in suspension appear to bind vWF. These hypotheses will have to be tested by future experiments.

Our results indicate that the interaction of vWF with GP IIb-IIIa is absolutely required to achieve irreversible shear-induced binding to the platelet surface, but this cannot be prevented by blocking the pool of GP IIb-IIIa molecules accessible to inhibitory antibodies and peptides on the membrane of resting platelets. As a possible explanation of this observation, we suggest that vWF interacts with GP IIb-IIIa molecules, initially not accessible to antibodies, that become exposed on the platelet surface only after GP Ib-mediated activation (Fig. 11). The surface expression and functional integrity of a previously internal pool of GP IIb-IIIa molecules has been well documented after platelet stimulation (16). These newly exposed molecules may be more easily occupied by vWF already interacting with GP Ibx on the platelet surface than by competing antibodies or peptides in solution. If this concept is correct, it is not immediately apparent why an anti-GP IIb-IIIa antibody like LJ-P5 can inhibit shear-induced vWF binding when other antibodies with well established inhibitory activity on GP IIb-IIIa function (like LJ-CP8) cannot. The unique effect of LJ-P5 may be due to its epitope specificity, since this is the only antibody available to us that has been well documented to inhibit selectively soluble vWF binding to activated GP IIb-IIIa (27). This property, coupled with the high affinity of the antibody (and presumably fast on rate) may allow more effective blockade of newly exposed GP IIb-IIIa in competition with vWF. It is consistent with the proposed model that blocking the Arg-Gly-Asp sequence in vWF (as with the antibody LJ-CP8) cannot.

Our results also indicate that vWF binding to GP Ib becomes stable after GP IIb-IIIa is also engaged in the interaction (concurrent binding) or whether only GP IIb-IIIa supports vWF binding to platelets after activation mediated by the transient interactions with GP Ib (sequential binding).

In conclusion, our studies provide evidence for a unique mechanism of interaction between soluble vWF and platelets that is not mimicked by other known modulators of vWF binding to GP Ib. These findings are likely to be relevant for normal hemostasis as well as pathological thrombosis even though the levels of shear stress theoretically applied to platelets in these experiments are above those thought to occur in the normal circulation of man and other mammals, for which estimates of maximal shear rate vary from 800 to 2000 s−1 (43). In fact, it is conceivable that similar processes occur even in response to lower shear when platelets are exposed to thrombogenic surfaces and agonists generated at sites of vascular injury during thrombus formation. For example, some key aspects of the mechanism described here, notably the sequential requirement for two distinct receptors, appear to be similar if not identical to those involved with platelet adhesion to surface-bound vWF even in the absence of flow (36). Further progress in this important area of platelet physiopathology should come from a better understanding of the nature of the vWF-GP Ib bond and the subsequent mechanisms of platelet activation.

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