Role and Initiation Mechanism of the Interaction of Glycoprotein Ib With Surface-Immobilized von Willebrand Factor in a Solid-Phase Platelet Cohesion Process

By Shizuko Tsuji, Mitsuhiko Sugimoto, Mitsuhiro Kuwahara, Kenji Nishio, Yukihiro Takahashi, Yoshiihiro Fujimura, Yasuo Ikeda, and Akira Yoshioka

To know the role and initiation mechanism of the interaction of glycoprotein (GP) Ib with surface-immobilized von Willebrand factor (vWF), we examined the effect of shear stress on platelet binding to vWF-coated plates using a cone-and-plate type viscometer capable of loading various levels of shear stress. The extent of platelet binding to immobilized vWF reached a plateau at the shortest period tested (20 seconds) under high shear stress (90 dyne/cm²), whereas 9 to 12 minutes was necessary for saturable platelet binding under static conditions. This shear effect, which was found to be dependent on the vWF-GP Ib interaction, was observed even under the lowest shear stress (1.5 dyne/cm²) examined. In contrast with the high shear effect previously reported to initiate the interaction of GP Ib with soluble vWF, these results indicate that relatively low levels of shear stress can promote the interaction of GP Ib with surface-immobilized vWF. This effect of shear stress was observed regardless of the manner in which vWF was immobilized, suggesting that immobilization itself and not, as previously hypothesized, a conformational change in vWF induced by direct adsorption to the surface is responsible for the enhanced GP Ib binding. Thus, the present findings suggest that the vWF-GP Ib interaction contributes optimally to rapid platelet cohesion on a thrombogenic surface when vWF is in a static state and when platelets are moved by an appropriate rheological force such as low shear stress.

In the complex process of platelet plug formation essential for in vivo primary hemostasis, resting platelets first adhere to subendothelial components at sites of vascular damage and thereby become activated and cohered on the surface. Of various extracellular ligands, von Willebrand factor (vWF) immobilized and exposed to the subendothelium at the injured vessel wall appears to be physiologically critical as a thrombogenic surface on which platelet aggregates form, because individuals with a congenital functional defect of vWF, von Willebrand disease, are known to exhibit a bleeding tendency.

Our current understanding of the mechanism of platelet cohesion on the surface-immobilized vWF is that the initial platelet contact with the surface is mediated by the interaction of vWF with the specific platelet receptor glycoprotein (GP) Ib/IX complex. Firm platelet binding to the surface is then induced by the irreversible binding of vWF with another platelet receptor, GP Ib/IIa complex, via an intraplatelet signal transduction pathway. Indeed, the above scenario is supported by experiments under flow conditions as well as by static experiments. Because either platelet binding or the vWF-GP Ib interaction can occur on the vWF surface even under static conditions, the exact role or significance of shear stress in those events remains unclear, despite the previous observation that surface-bound vWF is the major ligand to support platelet adhesion to the surface under high shear stress condition. In this regard, Ikeda et al reported that extremely high experimental shear stress (>80 dyne/cm²) can induce the interaction of soluble vWF with GP Ib, suggesting a role for shear stress in the in vivo pathogenesis of arterial thrombotic lesions. However, this finding, obtained in a liquid phase, does not explain all events in normal hemostasis, because in vivo hemostasis definitely requires solid-phase platelet aggregation, yet high levels of shear stress are found in only limited locations in vivo.

Although numerous studies using nonphysiologic agonists, such as ristocetin or botrocetin, have contributed to an understanding of the vWF-GP Ib interaction in the liquid phase, little is known about the interaction of surface-immobilized vWF with GP Ib in the solid-phase platelet aggregation process that seems essential for in vivo primary hemostasis. In the present work, we analyzed this crucial interaction in the solid phase under various shear stress conditions. We show that, as compared with static conditions, platelet binding to the vWF-coated surface is rapidly saturated as a function of vWF-GP Ib interaction even under low shear stress conditions. This effect of low shear force in promoting the solid-phase vWF interaction with GP Ib was also observed even when vWF is only indirectly immobilized by surface-coated anti-vWF antibodies. Thus, under the described conditions, both static vWF and the movement of platelets induced by an appropriate rheological force such as low shear stress are essential for maximal contribution of the vWF-GP Ib interaction to rapid platelet cohesion on the vWF-coated surface.

MATERIALS AND METHODS

Reagents. Prostaglandin E1 (PGE1), apyrase, cytochalasin D, RDGS, and RFDS tetrapeptides were all purchased from Sigma Chemical Co (St Louis, MO). NHS-LC biotin was from Pierce (Rockford, IL). Proxidase-conjugated streptavidin was from Zymed.
Human vWF was purified from cryo-precipitates as described. Multimer analysis by sodium dodecyl sulfate (SDS)-1.5% agarose gel electrophoresis was performed as described to confirm that the purified vWF possessed the highest molecular weight multimer. The purification and characterization of a snake venom protein, jaraaca GP Ib-BP, has been described. Jaraaca GP Ib-BP binds to the overlapping regions of the vWF binding site on GP Ib of platelets and thus totally blocks the vWF-GP Ib interaction at concentrations up to 5 μg/mL without affecting any platelet GP IIb/IIIa functions.

Monoclonal antibodies (MoAbs). The anti-vWF MoAbs used in the present study have been characterized in detail. NMC-4 reacts with the GP Ib-binding domain comprising vWF residues 449-728 and completely abolishes the in vitro vWF-GP Ib interaction at a concentration of 10 μg/mL. Antibody 2.2.9 (provided by Dr. Z.M. Ruggeri, The Scripps Research Institute, La Jolla, CA) recognizes the carboxy-terminal region of the vWF subunit and does not affect any adhesive functions of vWF. All of the antiplatelet MoAbs used in this study have also been characterized. LJ-P4 and LJ-CP8 (kind gift from Dr. Z.M. Ruggeri) are both anti-GP Ib/IIa antibodies; the former exhibits minimal effects on platelet function and the latter completely abolishes the binding of soluble adhesive proteins, including vWF and fibrinogen, to GP Ib/IIa. Both antibodies react with GP Ib/IIa in an activation-independent manner. AP-1 (donated by Dr. T.J. Kunicki, The Scripps Research Institute) is an anti-GP Ib antibody that totally blocks the vWF-GP Ib interaction in vitro at a concentration of 10 μg/mL. Mouse IgG were purified by chromatography on protein A-Sepharose (Pharmacia & Upjohn Co., Piscataway, NJ) and 2.2.9, were obtained by established protocols involving pepsin digestion of intact IgGs at low pH, followed by the collection of flow-through fractions from protein A-Sepharose columns. All intact IgG and F(ab')2 fractions of selected IgGs, NMC-4 and 2.2.9, were obtained by established protocols involving pepsin digestion of intact IgGs at low pH, followed by the collection of flow-through fractions from protein A-Sepharose columns. All intact IgG and F(ab')2 fractions were concentrated to 1 to 3 mg/mL, dialyzed with 20 mMol/L phosphate-buffered saline (PBS; pH 7.35), and stored at -70°C until used.

Biotinylation of LJ-P4 IgG. Purified IgG of LJ-P4 (3 mg) was dialyzed for 18 hours at 4°C against 0.1 mol/L NaHCO3 containing 0.1 mol/L NaCl. NHS-LC biotin (0.6 mg dissolved in dimethyl sulfoxide) was added to the dialized IgG solution and the mixture was incubated for 2 hours in the dark at room temperature (RT). The resulting biotinylated LJ-P4 IgG was dialyzed against PBS for 18 hours at 4°C and stored at -70°C until used.

Preparation of washed platelets. Human blood from a healthy volunteer was drawn by venipuncture into 1/10 vol of 3.8% (w/v) trisodium citrate. Platelet-rich plasma (PRP) was obtained by centrifuging the citrated blood at 200g for 20 minutes. Washed platelets were prepared from PRP by the albumin-density method of Walsh et al. Finally, washed platelets were resuspended and adjusted to 4 × 10^5/μL with HEPES-Tyrode buffer (0.01 mol/L HEPES, 0.14 mol/L NaCl, 2.7 mmol/L KCl, 0.4 mmol/L NaH2PO4/2H2O, 12 mmol/L NaHCO3, 5 mmol/L dextrose, pH 7.35) and kept under gentle shaking at 37°C until used. Before the assay, 1 mmol/L CaCl2 was added to the washed platelet preparation.

Evaluation of platelets bound to surface-immobilized vWF under static or shear stress conditions. A modified cone-and-plate type viscometer capable of continuous measurement of shear-induced platelet aggregation (SIPA) was used to load platelets with various shear stress forces. The apparatus and basic principles of its operation have been described in detail. Two different approaches to immobilization of vWF were used in this study. First, in the assay involving the direct coating of vWF, a polyethylenehexacyclate plate (2.2-cm diameter) was coated with 3 mL of purified vWF (20 μg/mL) or BSA for 18 hours at 4°C. This concentration of vWF in a coating solution gave maximum platelet binding to the plate, as determined by preliminary experiments. After blocking the nonsaturable site on the surface of the plate with 7 mL of 1% BSA for 1 hour at RT, the plate was washed three times with 5 mL of PBS. At this point in the process, to examine the possibility that the temperature of the vWF surface may affect the results, the plate was filled with 5 mL of PBS and was kept at 37°C for 1 hour before use in some indicated experiments. Then, after PBS was aspirated from the plate, 0.5 mL of washed platelets (3 × 10^7/μL) was placed into the plate, and various shear forces were immediately applied by the SIPA apparatus for different durations at RT. In static experiments, platelets were placed into the plate without loading shear stress for the corresponding reaction time. The plate was then immediately washed twice with PBS containing 4 μmol/L PGE, (PBS-PGE) to remove nonadherent platelets, and 4 mL of 1% formaldehyde in PBS-PGE was then added for 15 minutes to fix the platelets bound to the plate. After washing twice with PBS-PGE, some of the platelets were subjected to May-Grünewald-Giemsa staining for microscopic observations, and other plates were treated with 2 mL of biotinylated LJ-P4 IgG (0.5 μg/mL) for 30 minutes at RT. After washing twice with PBS-PGE, platelets were incubated with 2 mL of peroxidase-conjugated streptavidin (0.25 μg/mL) for 15 minutes and washed twice with PBS-PGE, and 2 mL of o-phenylenediamine substrate (1 mg/mL) was added for 5 minutes. The reaction was terminated with 2 mol/L H2SO4, and the extent of platelet binding to the plate was evaluated based on the intensity of the color developed, measured at optical density (OD) of 492 nm, which represents the amount of biotinylated vWF bound to the GP Ib/IIa complex of platelets bound to the plate. In some inhibition experiments, washed platelets were preincubated with PGE1, apyrase, ethylenediamine-N,N,N’,N’-tetraacetic acid disodium salt (EDTA), cytochalasin D, jaraaca GP Ib-BP, tetrapetides (RGDS or RFDS), or antiplatelet MoAbs (AP-1 or LJ-CP8) for 30 minutes at 37°C. In other inhibition studies, the vWF-coated plate was pretreated with 4 mL of anti-vWF antibody NMC-4 F(ab')2 (10 μg/mL) for 1 hour at RT.

In the second approach to vWF immobilization, a plate was coated with 5 mL of anti-vWF antibody NMC-4 F(ab')2 (10 μg/mL) or 2.2.9 F(ab')2 (10 μg/mL) for 18 hours at 4°C. After saturating the plate with BSA and washing three times as described above, 3 mL of purified vWF (20 μg/mL) or PBS as a control was added for 1 hour at RT. The plates, containing vWF captured by anti-vWF MoAbs coated on the surface, were washed three times with PBS and used for additional experiments. Subsequent experimental procedures were identical to those used in the direct vWF-coating system.

RESULTS

Dose-dependence and time course of platelet binding to surface-immobilized vWF. Under static conditions with a fixed reaction time of 30 minutes, platelet binding to the vWF-coated plate increased and appeared to be saturable with increasing numbers of platelets added, whereas no significant platelet binding was observed on the BSA-coated plate (Fig 1). Consistent with those findings, microscopic analysis showed that platelets finally bound to the surface were adherent (Fig 1). In a time-course experiment using a fixed concentration of platelets (3 × 10^7/μL), platelet binding detected on the vWF-surface increased as a function of time, and binding was saturated in 9 to 12 minutes under static conditions (Fig 2). By contrast, under high shear stress conditions (90 dyne/cm²), platelets bound to the surface more...
Effects of functional inhibitors of platelets on platelet binding to the vWF surface under static or high shear stress conditions. The effects of various platelet inhibitors on platelet binding to immobilized vWF were examined using a fixed platelet count (3 × 10^6/μL) and reaction time (6 minutes) under both static and high shear stress (90 dyne/cm²) conditions. For experimental high shear (30 dyne/cm²), the relevant value of the in vivo artery was also examined. In all conditions, binding was almost completely inhibited by EDTA and PGE₁, modestly inhibited by cytochalasin D, and only minimally affected by the adenosine diphosphate (ADP) scavenger, apyrase. Both the GP IIb/IIIa blockers, RGDS tetrapeptide and anti-GP IIb/IIIa MoAb LJ-CP 8, sig-

rapidly and binding was saturated in 20 to 40 seconds, whereas the platelet binding capacity at the saturation point did not differ significantly between these two conditions (Fig 2). Preheating of the plate at 37°C did not significantly affect the results in terms of the time course and the capacity of platelet binding to the vWF surface in the present experimental approach (Fig 2). We therefore performed the following experiments using the plate kept at RT. Microscopic findings in bound platelets under high shear stress were basically indistinguishable from those in static conditions (results not shown). Although a shear stress force of 90 dyne/cm² induces liquid-phase platelet aggregation in the presence of soluble vWF, no appreciable platelet aggregation in solution was observed in the present experiments (results not shown), possibly due to the lack of soluble vWF in the washed platelet preparation.

Fig 1. Dose-dependent platelet binding to the vWF-coated plate in static conditions. (Top panel) Washed platelets at various concentrations were added to the vWF-coated plate. After a 30-minute reaction time in static conditions, the plate was immediately washed and fixed with formaldehyde and the extent of platelet binding to the plate was evaluated based on color intensity at OD 492 nm as described in the Materials and Methods. Data are given as the mean and range of three separate experiments. (Bottom panel) Light microscopy images of platelets bound to (A) the vWF- and (B) BSA-coated plate at a platelet count of 3 × 10²/μL. Original magnification of micrographs × 400.

Fig 2. Time-dependent increase in platelet binding to the vWF-coated plate under static and high shear stress (90 dyne/cm²) conditions. (Top panel) Washed platelets at a fixed count of 3 × 10²/μL were added to the vWF-coated plate. The platelet binding reaction in static conditions was terminated by formaldehyde fixation at 20 seconds, 40 seconds, 1 minute, 3 minutes, 6 minutes, 9 minutes, 12 minutes, and 18 minutes, respectively. The amount of platelets bound to the plate at each reaction time is expressed as the color intensity. Solid and open symbols represent results using the vWF- and BSA-coated plate, respectively. Circles represent results obtained from the regular experiment using the plate kept at RT, and triangles are from the experiments using the plate preincubated at 37°C. (Bottom panel) Same as above, except that a shear stress of 90 dyne/cm² was applied for each reaction time. Note that platelet binding to the vWF plate was more rapidly saturated under 90 dyne/cm² shear stress.
Table 1. Effect of Various Inhibitors of Platelet Function on Platelet Binding to Surface-Immobilized vWF Under Static and High Shear Stress (30 or 90 dyne/cm²) Conditions

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Stasis % Inhibition</th>
<th>30 dyne/cm²</th>
<th>90 dyne/cm²</th>
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<tbody>
<tr>
<td>EDTA (5 mmol/L)</td>
<td>97 ± 3</td>
<td>97 ± 4</td>
<td>98 ± 4</td>
</tr>
<tr>
<td>PGE₁ (4 μmol/L)</td>
<td>96 ± 5</td>
<td>90 ± 7</td>
<td>89 ± 8</td>
</tr>
<tr>
<td>Cytochalasin D (20 μmol/L)</td>
<td>72 ± 9</td>
<td>52 ± 10</td>
<td>58 ± 12</td>
</tr>
<tr>
<td>Apyrase (5 U/mL)</td>
<td>12 ± 6</td>
<td>5 ± 5</td>
<td>9 ± 4</td>
</tr>
<tr>
<td>GPⅠb/Ⅲa blockers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lj-CP8 (100 μg/mL)</td>
<td>94 ± 11</td>
<td>88 ± 9</td>
<td>90 ± 7</td>
</tr>
<tr>
<td>RGDS (200 μg/mL)</td>
<td>77 ± 8</td>
<td>61 ± 10</td>
<td>65 ± 9</td>
</tr>
<tr>
<td>RFDS (200 μg/mL)</td>
<td>9 ± 3</td>
<td>8 ± 4</td>
<td>5 ± 4</td>
</tr>
<tr>
<td>vWF-GPⅠb blockers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP-1 (10 μg/mL)</td>
<td>19 ± 8</td>
<td>92 ± 6</td>
<td>90 ± 7</td>
</tr>
<tr>
<td>NMC-4 (10 μg/mL)*</td>
<td>23 ± 10</td>
<td>94 ± 7</td>
<td>92 ± 6</td>
</tr>
<tr>
<td>Jararaca GPⅠb-BP (5 μg/mL)</td>
<td>21 ± 9</td>
<td>93 ± 8</td>
<td>94 ± 8</td>
</tr>
</tbody>
</table>

Washed platelets (3 × 10⁵/μL) were incubated with each platelet inhibitor for 30 minutes at 37°C and added to the vWF-coated plate for 6 minutes under static or high shear stress (30 or 90 dyne/cm²) conditions. Platelet binding to the vWF plate was evaluated based on the color intensity of OD 492 nm. Results are expressed as the mean ± SD percentage of inhibition, assuming 100% inhibition on the BSA-coated plate and 0% inhibition when platelets were not incubated with inhibitors.

* This experiment was performed using the vWF-coated plate that was pretreated with NMC-4.

Effect of various inhibitors on platelet binding. To test the effect of various inhibitors on platelet binding, washed platelets (3 × 10⁵/μL) were incubated with each platelet inhibitor for 30 minutes at 37°C and added to the vWF-coated plate for 6 minutes under static or high shear stress (30 or 90 dyne/cm²) conditions. Platelet binding to the vWF plate was evaluated based on the color intensity of OD 492 nm. Results are expressed as the mean ± SD percentage of inhibition, assuming 100% inhibition on the BSA-coated plate and 0% inhibition when platelets were not incubated with inhibitors.

Inhibition of platelet binding. The effect of various inhibitors on platelet binding was evaluated under static and high shear stress conditions. EDTA, PGE₁, and Cytochalasin D were found to significantly inhibit platelet binding (Table 1). These results confirmed that platelets did adhere to the vWF surface by binding of surface-immobilized vWF with platelet GPⅠb/Ⅲa activated via intraplatelet mechanisms. In contrast with the results described above, in which no significant difference was observed between static and high shear stress conditions, blockers of the vWF-GPⅠb interaction (anti-GPⅠb antibody AP-1 and GPⅠb-binding snake protein jararaca GPⅠb-BP) induced almost complete inhibition of platelet binding under high shear but only partial inhibition in static conditions (Table 1).

Evaluation of GPⅠb-dependency and the rapidity in platelet cohesion to the vWF surface under shear stress conditions. In static conditions, overall platelet cohesion to vWF-coated plates pretreated with anti-vWF antibody NMC-4 showed slightly delayed time course, although the final platelet binding capacity was almost unchanged. By contrast, platelet cohesion on the NMC-4-pretreated vWF surface under high shear stress was totally blocked at each reaction time point, indicating that platelet cohesion under high shear was strictly dependent on the vWF-GPⅠb interaction (Fig 3). To determine whether this effect is specific for such high shear stress, we examined the inhibitory effects of the GPⅠb-blocker, jararaca GPⅠb-BP, under various shear stress levels at a fixed reaction time of 6 minutes. Interestingly, the percentage of GPⅠb-dependent platelets of the total amount bound to the vWF surface, which was calculated based on the inhibitory effect of jararaca GPⅠb-BP, increased as a function of increasing shear stress loaded less than 6 dyne/cm², and GPⅠb-dependency became saturable at more than 6 dyne/cm² (Table 2). To confirm the relationship between GPⅠb-dependency and the accelerated rate of platelet cohesion, a time-course study under conditions of low shear stress (<6 dyne/cm²) was performed using platelets in the presence or absence of jararaca GPⅠb-BP. Under those experimental conditions, saturation of platelet binding occurred more rapidly when the shear stress was increased stepwise, and the inhibition study using jararaca GPⅠb-BP indicated that the total amount of slowly progressing platelet cohesion independent of GPⅠb decreased inversely as a function of increasing shear stress force. The patterns of platelet cohesion at 6 dyne/cm² with or without jararaca GPⅠb-BP were almost identical as those observed under high shear stress of 90 dyne/cm² (Fig 4).

Effect of various modes of vWF immobilization on platelet cohesion under static and shear stress conditions. To test the previously suggested hypothesis that a conformational...
Table 2. Effect of Jararaca GP Ib-BP on Platelet Binding to the Surface-Im mobilized vWF Under Static or Shear Stress Conditions

<table>
<thead>
<tr>
<th>Shear Level (dyne/cm²)</th>
<th>Control (100%)</th>
<th>BSA-Coated (0%)</th>
<th>+Jararaca GP Ib-BP</th>
<th>GP Ib-Dependency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stasis 0.515</td>
<td>0.087</td>
<td>0.421</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>0.397</td>
<td>0.076</td>
<td>0.208</td>
<td>59</td>
</tr>
<tr>
<td>3.0</td>
<td>0.422</td>
<td>0.094</td>
<td>0.173</td>
<td>76</td>
</tr>
<tr>
<td>6.0</td>
<td>0.419</td>
<td>0.102</td>
<td>0.124</td>
<td>93</td>
</tr>
<tr>
<td>30.0</td>
<td>0.465</td>
<td>0.088</td>
<td>0.118</td>
<td>92</td>
</tr>
<tr>
<td>60.0</td>
<td>0.436</td>
<td>0.104</td>
<td>0.117</td>
<td>96</td>
</tr>
<tr>
<td>90.0</td>
<td>0.477</td>
<td>0.081</td>
<td>0.114</td>
<td>94</td>
</tr>
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</table>

Washed platelets (3 x 10⁵/µL) in the presence or absence of jararaca GP Ib-BP (5 µg/mL) were added to the vWF-coated (or BSA-coated) plate for 5 minutes under static or shear stress conditions. Values are based on the color intensity of OD 492 nm, reflecting the amount of platelets finally bound to the plate. GP Ib-dependency values were calculated from the following formula: d = 100 - [(c - b)/(a - b)] (%).

change in the GP Ib-binding domain of vWF generated by adsorption to the surface underlies the enhanced susceptibility of immobilized vWF for GP Ib.4-6 vWF immobilized indirectly by antibodies coated on the surface was tested in the present system. Antibody 2.2.9, which recognizes the carboxy-terminal region of vWF and does not affect any adhesive functions of vWF, was used to capture vWF on the plate surface. The amount and GP Ib-dependency of platelet cohesion in the 2.2.9 capturing system, as judged by the color intensity, was basically indistinguishable from that seen in the assay with directly immobilized vWF under both static and high shear stress (30 or 90 dyne/cm²) conditions (Fig 5). Furthermore, even when vWF was captured on the plate by antibody NMC-4, which recognizes the crucial region of the GP Ib-binding site and completely blocks the vWF-GP Ib interaction, the amount and GP Ib-dependency of platelet cohesion under both conditions were comparable to those found using the direct vWF coating method as well as in the 2.2.9 capturing system (Fig 5).

DISCUSSION

To investigate platelet binding to surface-immobilized vWF under various shear stress conditions, we used a modified cone-and-plate type viscometer capable of continuous loading of various shear stress, instead of a flow chamber widely used for platelet adhesion studies.8-10,24-26 Although our experimental approach might be physiologically less relevant than the flow chamber system, which is assumed to reproduce in vivo rheological circumstances, it does enable quantitative evaluation of the bound platelets in a defined time course. In addition, to solely analyze the effect of shear levels on the interaction of surface-immobilized vWF, but not the soluble vWF, with GP Ib in the initial phase of platelet adhesion process, we used washed platelets containing no soluble vWF. Unlike an experiment using whole blood or PRP, the multilayer platelet thrombus growth, which could be observed in an experiment using whole blood or PRP, does not occur in the present assay, due to the lack of plasma adhesive proteins, such as fibrinogen or vWF, which may be involved in the secondary platelet aggregates accumulation. In fact, unlike platelet aggregation in a liquid phase induced by exogenously added agonists as well as by high shear stress,11,12 the observed inability of apyrase to block platelet binding suggested that a local increase in ADP concentrations, which seems essential for platelet thrombus growth, was not required for the monolayer or oligolayer platelet binding to the vWF surface observed in the present approach. Another important feature of the present assay is that it detects only the platelets that are firmly bound to the surface via platelet activation process (platelet cohesion), due to multiple washing steps in the assay, and does not reflect platelets temporarily attached to the surface (primary platelet adhesion). Indeed, regardless of whether the vWF-GP Ib interaction plays a role, the irreversible binding of vWF to activated GP IIb/IIIa was absolutely required for the final platelet cohesion to the surface, as shown by the inhibitory effects of the GP IIb/IIIa blockers, PGE, and cytochalasin D.
The present study supports previous observations that, in the absence of exogenous agonists, platelets can bind to the surface vWF independently of rheological circumstances. However, platelet cohesion to the surface progressed more rapidly under shear stress conditions, even at the lowest shear stress loaded, as compared with a static condition. The promoting effect on platelet binding to the vWF surface observed in relatively low levels of shear stress was found to be derived from the vWF-GP Ib interaction, and, consistent with previous observations, the vWF-GP Ib interaction was also observed under static conditions. However, note that GP Ib-independent platelet cohesion was predominant in this situation. The mechanism or physiological relevance of predominant GP Ib-independent platelet cohesion observed under static or low shear stress (<6 dyne/cm²) conditions is presently unknown, but may reflect spontaneous platelet activation during the washing steps in the assay process. Interestingly, GP Ib-independent platelet cohesion was completely abolished under conditions with shear stress greater than 6 dyne/cm², raising the possibility that shear stress forces peel off platelets that are loosely bound to the surface via the GP Ib-independent mechanism.

Because the levels of experimental shear stress sufficient to induce such effects neither initiate the interaction of soluble vWF with GP Ib nor induce platelet aggregation in a liquid phase under the described conditions, it is clear that surface-immobilized vWF, unlike soluble vWF, is susceptible to GP Ib. To address the question of how surface immobilization determines this enhanced susceptibility of vWF for GP Ib, we compared the effects of direct and indirect vWF immobilization, and found nearly identical patterns of platelet cohesion in both systems. Our results argue against a major role for a postulated conformational change within the GP Ib-binding domain of vWF generated by its adsorption to the plastic surface, which is highly negatively charged, and several lines of evidence suggesting that charge properties of both the ligand and receptor are critical for this interaction. Moreover, we found that platelet cohesion occurred comparably in the antibody capturing system using an antibody that blocks the vWF-GP Ib interaction. Because vWF is a multimeric protein, we speculate that the vWF subunits not directly captured can interact with GP Ib even when a blocking antibody is used for capture. Thus, the attachment of vWF to the surface, regardless of the manner of immobilization, may be critical for the initiation of the vWF-GP Ib interaction in a solid phase. This interpretation may also explain the functional role of subendothelial components, such as collagens or heparin-like glycosaminoglycans that bind to vWF, in the solid-phase platelet cohesion process. Although there is as yet no definitive evidence that these molecules upregulate the vWF-GP Ib interaction by binding to vWF in liquid-phase platelet aggregation studies, the observation that even an inhibitory anti-vWF antibody can effectively support the solid-phase platelet cohesion strongly suggests that all of these vWF-binding subendothelial components can contribute to platelet aggregate formation in vivo on the surface via the vWF-GP Ib interaction.

Together, our results indicate the requirement for both static vWF and platelets moving by an appropriate rheological force such as low shear stress for the full contribution of the vWF-GP Ib interaction to rapid platelet cohesion on the surface. In this regard, the contact frequency of platelets with vWF might be critical, because, as compared with static platelets and immobilized vWF or platelets plus soluble vWF
together in a moving phase, contact frequency is presumably much increased when platelets are moving above the immobile vWF surface.

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