Unique Interactions of Asialo von Willebrand Factor With Platelets in Platelet-Type von Willebrand Disease

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The present studies demonstrate that platelets from patients with platelet-type von Willebrand disease show specific and saturable binding of asialo von Willebrand factor (AS-vWF) under conditions where such binding is not observed with normal platelets. Although specific binding of 125I-AS-vWF to formalin-fixed normal platelets could not be demonstrated, specific binding to fixed patient platelets was seen with an apparent Kd of 1.3 μg/mL and specific maximally bound ligand of 0.40 μg/10⁶ platelets. Preincubation of patient platelets with the anticyt glycoprotein lb (anti-GPIlb) monoclonal antibody AS-2 reduced total binding close to the level of computer-estimated nonspecific binding. In contrast, binding was not reduced by preincubation with anti-GPIIb/IIIa monoclonal antibody or with 5 mmol/L EDTA. Under stirring conditions, the binding of AS-vWF to fixed patient platelets was accompanied by a strong agglutination response. AS-vWF-induced agglutination was similarly observed in patient but not normal platelet-rich plasma (PRP) in the presence of 5 mmol/L EDTA. In the absence of EDTA, AS-vWF produced a full aggregation response in patient PRP at concentrations as low as 0.1 μg/mL in contrast to the 2 to 20 μg/mL required by normal PRP. Both thromboxane B2 formation and adenosine triphosphate secretion showed an AS-vWF concentration dependence paralleling the aggregation responses. These studies show that a major difference in the platelets from patients with platelet-type von Willebrand disease is the presence of an exposed, high-affinity binding site associated with GPIIb that recognizes AS-vWF.

von Willebrand factor (vWF) is a protein that plays a major role in platelet adhesive interactions. In an in vitro environment, platelets are agglutinated by vWF in the presence of the antibiotic ristocetin. This agglutination is dependent upon vWF binding to the platelets' at the platelet glycoprotein lb (GPIlb) receptor.1 The mechanism by which vWF may bind in vivo to GPIlb, in an environment lacking in ristocetin, is still unknown. After stimulation of platelets by thrombin,2 adenosine diphosphate3 and other agonists,4 vWF may additionally be demonstrated to bind at the GPIIb/IIIa receptor.6,7

In contrast to native vWF, desialylated or asialo vWF (AS-vWF) has been shown capable of inducing platelet aggregation in the absence of ristocetin.8 Aggregation was associated with the binding of AS-vWF to platelets and required the presence of free divalent cations. More recently, De Marco et al8 and Gralnick et al11 have used monoclonal antibodies to demonstrate that AS-vWF initially binds at the GPIlb locus in normal individuals. After this interaction, there is exposure of the GPIIb/IIIa fibrinogen receptor. AS-vWF additionally appears capable of binding to the GPIIb/IIIa receptor complex.

Patients with platelet-type von Willebrand disease exhibit an abnormality of vWF binding. Patient platelets bind vWF in the presence of significantly lower concentrations of ristocetin than are required by normal platelets.12 Additional, specific and saturable binding of native vWF by patient platelets has been demonstrated in the absence of ristocetin or any other platelet agonist, and native vWF induces aggregation of patient platelets.11 In view of the fact that desialylation changes the characteristics of vWF binding to platelets, we have undertaken the present studies to explore the interactions of AS-vWF with the abnormal platelets from patients with platelet-type von Willebrand disease.

METHODS

Purification and desialylation of vWF. Native human vWF was purified from human cryoprecipitate (provided by the Greater Syracuse Chapter of the American National Red Cross) as previously described.13 This vWF retained normal multimeric composition14 and ristocetin cofactor activity.15 The purified vWF in phosphate-buffered saline, pH 7.3, was then digested with neuraminidase from Vibrio cholerae (Calbiochem, La Jolla, CA) as described by De Marco et al at a ratio of 0.07 units enzyme/mg vWF. After a four-hour digestion, passage over a Sepharose 4B-CL column and subsequent dialysis against 0.02 mol/L Tris, 0.15 N NaCl, pH 7.3, the sialic acid content of the digested protein as well as of native vWF was assayed by the method of Warren.14 Desialylated vWF to be used in ligand binding studies was labeled with 125I by the method of Fraker and Speck.19

blood processing and platelet function studies. For studies in platelet-rich plasma (PRP), normal or patient blood was drawn into 1/10 vol of 3.8% sodium citrate and the PRP prepared by centrifugation at 900 g for 75 seconds at room temperature (22°C to 25°C) as previously described.15 Unless otherwise specified, for studies of formalin-fixed platelets, platelets were prepared from acid-citrate-dextrose–anticoagulated blood by the method of Macfarlane et al17 with the modification that the platelets were incubated for two hours at room temperature with a final concentration of 1.4% formalin in the absence of sodium azide. After formalin fixation, the platelets were resuspended in imidazole-buffered saline, pH 7.3, containing no divalent cations. Patient and normal platelets were alternatively processed and formalin fixed for 48 hours exactly as described by Takahashi and Shibata18 for a smaller number of experiments. For platelet function studies the platelet count was adjusted to 200,000/
nary infection (anaerobic lung abscess, day 244). He was then treated with restitution of corticosteroids and antibiotics (not shown). His PMN-IgG again decreased, his granulocyte count again rose, and his infections again resolved.

Patient G.D. (Fig. 5) is presented to illustrate a therapeutic response to alternate-day corticosteroids. He initially had increased PMN-IgG, severe granuloctyopenia, and profound malaise. He was begun on daily prednisone therapy (80 mg q.d.). Within 1 wk his malaise began to resolve. When studied on day 40, his PMN-IgG had decreased and his granulocyte count had become normal. Cessation of steroids (day 120) resulted in recurrent malaise, increased PMN-IgG, and granulocytopenia. He responded to reinstitution of steroids. On alternate-day prednisone (20 mg q.d.) he continues to feel well and has maintained a normal granulocyte count, despite a mild increase in PMN-IgG (day 310), now 15 mo after diagnosis.

DISCUSSION

We have adapted a radiolabeled antiglobulin test for the measurement of granulocyte-associated IgG in suspected immune granulocyte disorders. We observed increased levels of PMN-IgG in 12 of 16 patients with neutropenia of uncertain etiology and 8 of 8 patients with Felty’s syndrome (Fig. 2). All of these patients had cellular bone marrows with a relative increase in granulocyte precursors. In contrast, patients with neutropenia secondary to intrinsic bone marrow disease, chemotherapy-induced myelosuppression, or splenomegaly had normal levels of PMN-IgG. In two of our patients with increased PMN-IgG (A.S. and J.P.), an enlarged spleen or altered bone marrow granulocyte production may have contributed in part to the neutropenia. In all other patients, however, no other clinical explanation for the neutropenia was evident.

Ten of our patients with neutropenia of uncertain etiology had evidence for an underlying immunologic disorder, such as ITP or SLE. Five of these patients were thrombocytopenic (Table 1) with increased platelet-associated IgG, and one patient had IgG-induced thrombocytopenia and hemolytic anemia. This combination of neutropenia and ITP occurred more commonly than previously recognized. Three of our patients presented with neutropenia, increased PMN-IgG, weakness, and malaise and subsequently developed positive latex fixation titers. Two of these patients later developed arthralgias and one frank arthritis. Although presenting with neutropenia, these patients may ultimately develop classical rheumatoid arthritis and thus may represent a clinical variant of Felty’s syndrome.

All of the neutropenic patients with evidence of another immunologic disorder had increased levels of PMN-IgG. However, in several of these patients, neutropenia with increased PMN-IgG preceded other evidence of an underlying immunologic disorder. Our series of neutropenic patients, therefore, differs from the patients generally reported as having chronic idio
dopath neutropenia. Such patients usually do not have associated immunologic dysfunction and generally have decreased or ineffective granulocyte production. Only 2 of 6 such patients with neutropenia in the absence of associated immunologic disease had increased PMN-IgG in our series. The other four patients with isolated neutropenia and normal PMN-IgG were only distinguishable by a history of significant ingestion of major tranquilizers (Table 1).

The finding of increased PMN-IgG in neutropenic patients with SLE and Felty’s syndrome is in agreement with previous reports using antiglobulin consumption techniques and suggests a role for increased PMN-IgG in the development of neutropenia. However, increased PMN-IgG may represent only one of several causes of neutropenia in patients with these disorders. In addition, we have observed that a few patients with seropositive rheumatoid arthritis or SLE with normal granulocyte counts had levels or PMN-IgG above our normal range (Fig. 2).
platelets of our patients with platelet-type von Willebrand disease appear to have lost the ability to be agglutinated by vWF in the absence of added ristocetin. They have, however, retained their increased responsiveness to quite low concentrations of ristocetin in comparison with fixed platelets obtained from normal donors.

In contrast to native vWF, the desialylated vWF produced a strong, concentration-dependent agglutination of the formalin-fixed patient platelets in the absence of added ristocetin. Normal platelets identically treated showed no agglutination response (Fig 5). Agglutination of the patient platelets was fully inhibited if the anti-GPIb antibodies AP-1 or AS-2 were added to the platelet suspension (25 to 40 μg/mL, final concentration) before the AS-vWF. Neither the anti-GPIIb/IIIa antibody AP-2 (40 μg/mL) nor the presence of EDTA (5 mol/L) inhibited this agglutination.

To distinguish whether the agglutination induced in patient platelets by AS-vWF was attributable to increased was capable of restoring responsiveness to vWF alone.14 In contrast, the addition of an equal volume of PPP to a suspension of fixed patient or normal platelets produced no agglutination in the absence of ristocetin. The subsequent addition of either cryoprecipitate or purified native vWF (1 to 4 U/mL ristocetin cofactor activity) also failed to produce an agglutination response. Identical results were obtained when platelets were formalin fixed by the method used by Takahashi and Shibata.20 Thus, after formalin fixation, the

Fig 3. Platelet aggregation, ATP secretion, and thromboxane B2 production in citrated PRP as a function of AS-vWF concentration in the absence (open symbols) or presence (solid symbols) of 5 mmol/L EDTA added before the AS-vWF. After completion of the lumiagregation run, the cuvette was removed and thromboxane production subsequently determined.

Fig 4. Agglutination of formalin-fixed patient platelets in the presence of native vWF (1.5 U/mL ristocetin cofactor, added as cryoprecipitate before the beginning of trace) and varying concentrations of ristocetin. Ristocetin additions are marked by an upward deflection of traces. The final concentration of ristocetin (mg/mL) is indicated at end of each trace. (A, C) Platelets from a patient with platelet-type von Willebrand disease. (B, D) Platelets from a normal control.

Fig 5. Agglutination of formalin-fixed platelets as a function of AS-vWF concentration.
AS-vWF binding or, instead, to a platelet abnormality subsequent to the initial binding step, we studied the binding of $^{125}$I-AS-vWF to fixed platelets. Binding was performed at room temperature under nonstirring conditions. Analysis of the binding of $^{125}$I-AS-vWF to patient platelets in the absence of any inhibitor (Fig 6, top panel, buffer condition) by the LIGAND$^{23}$ nonlinear curve fitting and Scatchard analysis program (Fig 7) produced estimates of an apparent dissociation constant of 1.3 $\mu$g/mL and specific maximally bound ligand of 0.40 $\mu$g/10$^8$ platelets. There was a relatively low residual variance (mean square) of 60 for this fit. The 0.8% nonspecific binding (0.26 $\mu$g/10$^8$ platelets at a 25-$\mu$g/mL ligand concentration) estimated by the program for the buffer condition was quite close to the actual binding observed when the platelets were preincubated with the anti-GPIb monoclonal antibody AS-2 (Fig 6, top panel). In contrast, neither the anti-GPIIb/IIIa monoclonal antibody AP-2 (Fig 6, top panel) nor the presence of 5 mmol/L EDTA (data not shown) produced any inhibition of binding. When fixed platelets from normal individuals were used in place of patient platelets in these studies (Fig 6, bottom panel, buffer condition), a specific binding component could not be identified by computer analysis. Moreover, no significant difference in binding to normal platelets was seen in the presence of AS-2 or AP-2, thus suggesting that the observed binding was in fact nonspecific in nature.

**Fig 6.** Binding of $^{125}$I-AS-vWF to formalin-fixed platelets. Platelet suspensions were brought to a final concentration of 30 $\mu$g/mL antibody by adding to 100 $\mu$L of a 10$^9$ platelets/mL suspension of formalin-fixed (A) patient or (B) normal platelets 30 $\mu$L AS-2 (anti-GPIb), 30 $\mu$L AP-2 (anti-GPIIb/IIIa), or 30 $\mu$L buffer. After ten minutes at room temperature, 25 $\mu$L of varying concentrations of $^{125}$I-AS-vWF was added to bring AS-vWF to the final concentrations indicated. After a further 30-minute incubation at room temperature under nonstirring conditions, the platelets were then centrifuged through a layer of 20% sucrose containing 2% BSA and the platelet-associated radioactivity counted.

**Fig 7.** Scatchard plot of the same experiment for the buffer condition of patient platelets shown in Fig 6. Bound/free v bound ($\mu$g/10$^8$ platelets) represents the specific binding and is based on an estimate of 0.8% nonspecific binding by the LIGAND$^{23}$ computer program. Computer estimates of specific maximally bound ligand and of the dissociation constant for binding were 0.40 $\mu$g/10$^8$ platelets and 1.3 $\mu$g/mL, respectively, with a residual variance (mean square) of 60 for the fit.
When the binding of $^{125}$I-AS-vWF to patient platelets was measured in the presence of $>30$-fold (wt/wt) excess of native vWF, the total amount of $^{125}$I-AS-vWF bound was decreased by 20% to 25% (Table 1). This inhibition represented only approximately one third that achievable by the presence of anti-GPIb monoclonal antibody. Ristocetin greatly enhanced $^{125}$I-AS-vWF binding to the patient platelets (Table 1). This enhancement was prevented if native vWF was present in the incubation mixture at the time of ristocetin addition.

**DISCUSSION**

The present studies have established that platelets from patients with platelet-type von Willebrand disease show specific and saturable binding of AS-vWF under conditions where such binding is not observed with normal platelets. Thus, binding of AS-vWF to platelets in the absence of ristocetin is demonstrable after formalin fixation of patient platelets, but not to formalin-fixed platelets from normal subjects. This binding is blocked by monoclonal antibody directed against platelet GPIb but not by antibody against GPIIb/IIIa or by the divalent cation chelator EDTA. This interaction now defines a third major ligand-binding abnormality of these platelets, following the prior demonstration of (a) enhanced ristocetin sensitivity for interaction of native vWF and (b) the binding of native vWF to metabolically active platelet-type von Willebrand disease platelets in the absence of any stimulating agent. The present findings provide further evidence for an abnormality of the GPIb receptor complex in platelet-type von Willebrand disease.

The binding of AS-vWF to formalin-fixed patient platelets resulted in the agglutination of these platelets. Agglutination could also be demonstrated when AS-vWF was added to fresh patient PRP in the presence of 5 mmol/L EDTA. In contrast, as previously reported by other workers, no agglutination of normal PRP could be demonstrated in the presence of EDTA. Under conditions where the platelets were metabolically active (PRP without added EDTA), thromboxane formation and dense granule secretion by patient platelets was observed at AS-vWF concentrations at least an order of magnitude lower than that required for normal platelets.

De Marco et al. and Gralnick et al. have previously shown that in normal citrated PRP AS-vWF binds initially to GPIb and subsequently to the exposed GPIIb/IIIa receptor. The present studies indicate that in platelet-type von Willebrand disease platelets AS-vWF can bind to GPIb receptor sites even in the absence of free divalent cations. It is presumably the increased reactivity of these binding sites for AS-vWF in the patient platelets that leads in the presence of calcium ions to increased platelet activation, with subsequent thromboxane formation, dense granule secretion, and true aggregation. As in the case of normal platelets, the precise mechanism(s) by which binding at the GPIb site leads to platelet activation and to exposure of the GPIIb/IIIa receptor remains unknown.

The ability of formalin-fixed patient platelets to bind AS-vWF has permitted the study of AS-vWF binding restricted to the platelet GPIb receptor without the complexity of subsequent GPIIb/IIIa exposure that is seen in fresh platelets. Indeed, preincubation of the fixed patient platelets with the anti-GPIIb/IIIa antibody AP-1 (Fig 6) showed no diminution of the binding by AS-vWF. Even at concentrations as low as 0.4 to 1.0 g/mL AS-vWF, significant binding to patient platelets was detected. This concentration range is quite similar to that at which a significant degree of AS-vWF-induced agglutination of patient platelets is detected. Thus, with AS-vWF concentrations for which approximately 0.1 to 0.5 g/mL of ligand are bound per $10^8$ platelets, a strong agglutination response results. This is also very similar to the amount of AS-vWF that has been found necessary to bind to fresh normal platelets for an aggregation response to be observed. It seems likely, therefore, that the major difference in the platelets from platelet-type von Willebrand disease patients is the increased affinity or more accessible binding of AS-vWF to the GPIb site. Once AS-vWF has bound to the required number of receptors per platelet, aggregation may ensue. Because fixed normal platelets are unable to bind comparable numbers of AS-vWF molecules, such agglutination would not be observed.

In the present studies native vWF appeared unable to produce an agglutination reaction in patient platelets in the absence of ristocetin. This finding is in contrast to the report by Takahashi and Shibata where such an agglutination response was reported. It may well be that, although the patients with platelet-type von Willebrand disease in the present studies and those reported in the Japanese studies show major phenotypic similarities, the precise abnormalities at the vWF receptor region are not identical. Thus, although an abnormal band pattern of GPIb has been reported in the Japanese patients, no GPIb structural abnormalities have yet been found in studies of this disorder by ourselves or others. The qualitatively abnormal and high-affinity association of AS-vWF with the platelets from our patients with platelet-type von Willebrand disease may prove a useful tool for studies of this disorder.

**Table 1. AS-vWF Binding to Platelets From Patients With Platelet-Type von Willebrand Disease**

<table>
<thead>
<tr>
<th>Condition</th>
<th>$^{125}$I-AS-vWF Bound (Percentage of Control)</th>
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<tbody>
<tr>
<td>Buffer control</td>
<td>100</td>
</tr>
<tr>
<td>Anti-GPIb antibody AP-1 (40 g/mL)</td>
<td>35.0 ± 6.1 (6)</td>
</tr>
<tr>
<td>Native vWF (44 µg/mL)</td>
<td>76.7 ± 4.1 (6)</td>
</tr>
<tr>
<td>Ristocetin (1 mg/mL)</td>
<td>585.2 ± 113.9 (6)</td>
</tr>
<tr>
<td>Native vWF (44 µg/mL) + ristocetin</td>
<td>93.7 ± 12.9 (6)</td>
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A 25-µL aliquot of $^{125}$I-labeled AS-vWF was added to 100 µL of a $10^8$ platelets/mL suspension of formalin-fixed patient platelets in imidazole-buffered saline to bring the final concentration of AS-vWF to 1.2 µg/mL. After 30 minutes' incubation at room temperature under nonstirring conditions, the platelets were centrifuged through a layer of 20% sucrose containing 2% BSA and the platelet-associated radioactivity counted.

Before addition of the $^{125}$I-labeled AS-vWF, native vWF, ristocetin, the combination of both, anti-GPIb monoclonal antibody AP-1, or an equal volume of buffer was incubated with the platelet suspension for ten minutes at room temperature under nonstirring conditions. The total $^{125}$I-AS-vWF bound under each experimental condition is expressed as a percentage of the mean total binding for the buffer control. Values shown are means ± SEM for (n) experiments.
tool for the identification of the underlying structural abnormality in these platelets.

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