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Research Article

Asialo von Willebrand factor (AS-vWf) binds to and aggregates normal human platelets in the absence of ristocetin. Maximal specific binding of AS-vWf is 1-2 micrograms vWf protein/10(8) platelets. Despite the specificity of the binding, only 60% of the bound AS-vWf can be dissociated after equilibrium has been reached. We investigated the site of binding and the mechanism of aggregation of platelets by AS-vWf by (a) pre-incubating platelets with either of two monoclonal antibodies, one against glycoprotein Ib (GPIb) or a second against the glycoprotein IIb/IIIa complex (GPIIb/IIIa), and (b) varying the concentration of fibrinogen in the medium. The results of our studies indicate that AS-vWf binds initially to GPIb. This binding then results in the exposure of receptors for AS-vWf on GPIIb/IIIa. In the presence of plasma fibrinogen, both AS-vWf and fibrinogen bind to GPIIb/IIIa. In the presence of plasma fibrinogen, 50% more AS-vWf binds to the platelet, and this additional AS-vWf binds almost exclusively to GPIIb/IIIa. Despite this enhanced binding of AS-vWf in the absence of fibrinogen, platelet aggregation is much less than that which occurs in the presence of plasma fibrinogen. Comparative studies of AS-vWf binding to normal platelets and the platelets of patients with Glanzmann's thrombasthenia reveal decreased binding to the thrombasthenic platelets and a marked decrease in the extent of platelet aggregation. These studies indicate that […]

Asialo von Willebrand Factor Interactions with Platelets

Interdependence of Glycoproteins Ib and IIb/IIIa for Binding and Aggregation

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Abstract

Asialo von Willebrand factor (AS-vWf) binds to and aggregates normal human platelets in the absence of ristocetin. Maximal specific binding of AS-vWf is 1–2 μg vWf protein/10^8 platelets. Despite the specificity of the binding, only 60% of the bound AS-vWf can be dissociated after equilibrium has been reached. We investigated the site of binding and the mechanism of aggregation of platelets by AS-vWf by (*a*) pre-incubating platelets with either of two monoclonal antibodies, one against glycoprotein Ib (GPIb) or a second against the glycoprotein IIb/IIIa complex (GPIIb/IIIa), and (*b*) varying the concentration of fibrinogen in the medium. The results of our studies indicate that AS-vWf binds initially to GPIb. This binding then results in the exposure of receptors for AS-vWf on GPIIb/IIIa. In the presence of plasma fibrinogen, both AS-vWf and fibrinogen bind to GPIIb/IIIa. In the absence of plasma fibrinogen, 50% more AS-vWf binds to the platelet, and this additional AS-vWf binds almost exclusively to GPIIb/IIIa. Despite this enhanced binding of AS-vWf in the absence of fibrinogen, platelet aggregation is much less than that which occurs in the presence of plasma fibrinogen. Comparative studies of AS-vWf binding to normal platelets and the platelets of patients with Glanzmann's thrombasthenia reveal decreased binding to the thrombasthenic platelets and a marked decrease in the extent of platelet aggregation.

These studies indicate that AS-vWf binding to, and ensuing aggregation of, platelets is different from that observed with intact vWf protein when platelets are stimulated with either ristocetin or thrombin. The AS-vWf binds to GPIb which, in turn, makes additional AS-vWf receptors available on GPIIb/IIIa. If plasma fibrinogen is present, it competes with the AS-vWf for binding to GPIIb/IIIa and causes aggregation of platelets. In the absence of plasma fibrinogen, more of the AS-vWf binds to GPIIb/IIIa, but this AS-vWf is much less effective than fibrinogen in supporting platelet aggregation.

Introduction

The interaction between platelets and von Willebrand factor (vWf)[1] is important in both of the processes that contribute to normal primary hemostasis, that is, the adhesion of platelets to subendothelial surfaces and the subsequent aggregation of platelets (1, 2). Human vWf in the presence of ristocetin binds to the glycoprotein Ib (GPIb) of platelets that are either intact metabolically or have been fixed with formaldehyde. To date, no in vivo analogue of ristocetin has been found. DeMarco and Shapiro reported, however, that asialo vWf (AS-vWf) binds spontaneously and independently of ristocetin to the platelet surface and causes aggregation (3). They postulated that this represented a possible mechanism for the in vivo binding of vWf to human platelets and subsequent adhesion and aggregation.

We have undertaken an investigation to determine the site or sites of AS-vWf binding to human platelets, the effects of other plasma proteins on the binding, and the mechanism(s) of platelet aggregation induced by AS-vWf. Our studies employed (*a*) monoclonal antibodies specific for GPIb and the glycoprotein IIb/IIIa complex (GPIIb/IIIa), (*b*) plasmas containing variable concentrations of fibrinogen, and (*c*) the platelets from thrombasthenic patients.

Methods

Blood was drawn from normal subjects and patients by use of a two-syringe technique. A 19-gauge needle was used and the initial 5–10 ml of blood was used for other laboratory tests. The blood from the second syringe was placed into polypropylene tubes containing 0.1 ml sodium citrate anticoagulant, final concentration 10.9 mM. Platelet-rich plasma (PRP) was prepared from whole blood by removal of the plasma-platelet layer after centrifugation at 750 relative centrifugal force (RCF) for 3 min at 25°C. In some experiments, the platelets were washed free of erythrocytes and leukocytes by the preparation of PRP and one wash of the erythrocytes with Hepes buffer, pH 7.35 (4). The PRP and wash were then placed on a discontinuous arabinogalactan gradient 20% (3 ml) and 10% (5 ml) and platelets were separated from plasma proteins by centrifugation at 2,000 RCF for 30 min. The platelets (>85% recovery) were then resuspended in autologous or homologous normal plasma or plasma from two patients with congenital afibrinogenemia. Plasma was obtained from two patients with congenital afibrinogenemia who had thrombin time > 300 s, and no detectable fibrinogen by the Clauss technique or tanned erythrocyte hemagglutination inhibition, i.e., a level < 0.5 μg/ml. Mixing studies were performed to ensure that neither afibrinogenemic plasma contained antifibrinogen antibodies. Each afibrinogenemic plasma was incubated with normal plasma samples (1:1 ratio) at 1 and 60 min and were analyzed for fibrinogen content by the method of Clauss and for fibrinogen antigen content by radial immunodiffusion or tanned erythrocyte hemagglutination inhibition. Normal plasma diluted with normal serum or Tris-NaCl (0.05 M Tris, 0.1 M NaCl, pH 7.4) served as a control. Mixing studies demonstrated that both afibrinogenemic plasmas were free of antifibrinogen antibodies. Blood was also collected from two patients with thrombasthenia who have been previously well-characterized, and the PRP was prepared in the same manner as the PRP from normal blood (5, 6).

Purified proteins. The Factor VIII (FVIII)/vWf protein was purified

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1. *Abbreviations used in this paper:* AS-vWf, asialo von Willebrand factor; DTE, dithierythritol; FVIII, Factor VIII; GPIb, glycoprotein Ib; GPIIb/GPIIIa, glycoprotein IIb/IIIa complex; PRP, platelet-rich plasma; RCF, relative centrifugal force; vWf, von Willebrand factor.

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from human cryoprecipitate by chromatography on Sepharose 4B. The ascending limb of the protein peak of the void volume was collected, concentrated by ammonium sulfate precipitation, dialyzed, and used as previously described (7). Labeling of the protein was performed either by use of the technique of limited oxidation of terminal galactose residues by galactose oxidation and reduction of these residues by tritiated [^{3}H]potassium borohydride as previously described (7), or by iodination with ^{125}I by means of immobilized lactoperoxidase (8, 9).

Fibrinogen was purified from normal plasma by ammonium sulfate precipitation and further purified on DEAE cellulose chromatography as previously described (10). Albumin used was purchased from Miles Laboratories, Inc., Research Products Div. (Pentex bovine albumin Fraction V; Elkhart, IN). Purified cold insoluble globulin was a generous gift of Dr. Claudine Mauzier, Centre Regionale de Transfusion, Lille, France.

Platelet aggregation. Normal or thrombasthenic platelets (PRP or platelets separated by arabinogalactan and resuspended in plasma, both at 200,000/μl) were studied in a Chrono-Log Lumi aggregometer (Chrono-Log Corp., Havertown, PA) or a Payton aggregometer (Payton Associates Inc., Buffalo, NY) before and after the addition of various concentrations of AS-vWf (range, 0.22 to 88 μg/ml final concentration). Quantification was done by measurement of the initial slope of aggregation. In some experiments, monoclonal antibodies against GPIb or GPIIb/IIIa were added to the platelets 1 min before the AS-vWf. Time course of binding of AS-vWf to, and aggregation of, platelets was performed by addition of 50 μl AS-vWf (2.0–6.0 μg/ml final concentration) to 0.4 ml of PRP for 30 min. At specific intervals, the platelets were separated from the plasma and the radioactivity in the platelet pellet was counted.

Binding studies. Binding studies of intact and AS-vWf were performed in an aggregometer with constant stirring at 900 rpm at 37°C. In each case, to 0.4 ml of platelets (200,000/μl) suspended in either normal plasma or afibrinogenemic plasma, 50 μl AS-vWf was added (final concentration from 0.22 to 88.0 μg/ml) and incubated for 15 min. Platelet-bound radioactive vWf was separated from free vWf by centrifugation of platelets through a 30-mm cushion of 10% arabinogalactan (2,000 RCF for 30 min). The amount of vWf bound to 10^8 platelets was constant in samples centrifuged from 2 to 30 min. An aliquot of the supernatant was removed and counted. The platelet pellet was recovered and dissolved in 1 ml newborn calf serum tissue solubilizer (Amersham Corp., Arlington Heights, IL) overnight at room temperature. Then 50 μl acetic acid was added and the dissolved platelet pellet was placed into 10 ml of Econoflor-2 (New England Nuclear, Boston, MA) and counted. More than 95% of the platelets was recovered in the pellet and when plasma was substituted for platelets in the experiments, <1% of the radiolabeled AS-vWf was present in the bottom of the tube. Nonspecific binding was determined by the addition of a 50-fold excess of unlabeled AS-vWf in the presence of the labeled material.

Studies to examine the specificity of binding were performed both with normal platelets in PRP and normal platelets resuspended in Hepes buffer. Intact vWf, purified fibronectin, purified human albumin, and human fibrinogen were tested for their effectiveness in displacing binding of ^{3}H-AS-vWf. The competing proteins were added at concentrations of at least 100-fold (weight/weight) excess concentration of the AS-vWf. The ^{3}H- or ^{125}I-vWf and the competing proteins were added simultaneously to 0.8×10^8 platelets in either PRP or Hepes buffer. The samples were incubated for 15 min, and bound ligand was then separated from free ligand as described above. Percentage inhibition of AS-vWf binding was calculated by means of AS-vWf binding without any competing protein at 100%. The amount of labeled vWf in the assay varied between 1 and 2 μg/ml and the maximum amounts of the competing proteins in the assay were: intact vWf, 0.250 mg/ml; fibronectin, 0.375 mg/ml; albumin, 0.5 mg/ml; and fibrinogen, 0.4 mg/ml.

Carbohydrate modification of vWf. The intact vWf was desialated by the addition of *Clostridium perfringens* neuraminidase (0.02–0.04 U/mg protein with 1 U of enzyme defined as the amount that released 1 μmol of sialic acid/min at 37°C from α_1-glycoprotein). The enzyme was purified as previously described (11) and stored in acetate buffer (pH 5.5) containing 3% bovine serum albumin. The enzyme contained no protease activity by assay against [^{14}C]globin (11). Sialic acid was measured by the Warren technique (12) with *N*-acetyl neuraminic acid as the standard. The sialic acid released from the FVIII/vWf protein by neuraminidase was assayed by the same method, except that the acid hydrolysis step was omitted (12).

Glyoxyl agarose electrophoresis. Multimeric structure of the intact and AS-vWf protein before and after radiolabeling was analyzed by glyoxyl agarose electrophoresis and radioautography (13). The antibody used was a rabbit anti-human FVIII/vWf antibody which had been immunoaffinity purified. In some studies ^{125}I-AS-vWf was used in the binding studies for analysis of the multimeric structure of platelet-bound ligand. The platelets were lysed with 30 μl of Tris-EDTA-sodium dodecyl sulfate (SDS) buffer, pH 8.0 (10 mM Tris HCl, 1 mM EDTA containing 5% SDS). The samples were incubated for 20 min at 60°C and the extracted ^{125}I-AS-vWf protein was analyzed by glyoxyl agarose electrophoresis and radioautography.

Monoclonal antibodies. Purified IgG or the F(ab)$_2$ fragment of three monoclonal antibodies was used in the study. The first monoclonal antibody, 6D1, had previously been characterized as a monoclonal antibody against the GPIb complex that totally blocks ristocetin-induced platelet aggregation (14). The second monoclonal antibody, 10E5, has been characterized as being directed against GPIIb/IIIa and totally blocks fibrinogen binding to platelets (15). The third monoclonal antibody used in the study is directed against T lymphocyte antigen and did not react with platelets (Bethesda Research Laboratories, Gaithersburg, MD). This monoclonal antibody was used as a control.

All three monoclonal antibodies were tested at doses ranging from 0.01 to 72.3 μg/ml and were added to normal or thrombasthenic PRP 1 min before the AS-vWf. The respective F(ab)$_2$ fragments were tested from 0.01 to 3.0 μg/ml (6D1) and from 0.01 to 15.0 μg/ml (10E5) final concentration. In other experiments, arabinogalactan-separated platelets were resuspended in normal or afibrinogenemic plasma and the monoclonal antibodies were added individually before the addition of the AS-vWf. In all studies, the concomitant controls included the monoclonal antibody not directed against platelets and identical incubation mixtures in which buffer replaced the monoclonal antibodies. The amount of inhibition of the vWf binding was calculated in comparison with the appropriate controls.

Results

vWf protein. The intact vWf protein purified from normal cryoprecipitate, which also contained FVIII procoagulant activity, had a multimeric structure on glyoxyl agarose electrophoresis similar to that seen in normal plasma; in particular, the largest multimeric structures were present (Fig. 1). Sialic acid content of the FVIII/vWf protein was 141±12 nmol/mg. Neuraminidase treatment for 2 h released >91% of the sialic acid (range, 92–97%). After removal of the sialic acid, the multimeric pattern of the AS-vWf showed a slight anodal shift of all the multimers consistent with the expected reduction in molecular weight of ~5%. On SDS-polyacrylamide gel electrophoresis, neither intact vWf nor AS-vWf entered a 5% gel (Fig. 2 *b*). After reduction, however, a single subunit of ~230,000 mol wt was seen with both preparations (Fig. 2, *a* and *b*). The subunit of the AS-vWf always migrated faster than that of the intact vWf; however, the molecular weight determinations were always within 5,000 (Fig. 2). The ^{3}H- and ^{125}I-labeled AS or intact vWf were indistinguishable from their respective unlabeled proteins.

The removal of >91% of the sialic acid did not affect the FVIII:C or the FVIIIR:Ag levels as compared with the intact material. However, the specific activity of the vWf (measured in the ristocetin cofactor assay) increased ~20% after desialation (intact vWf, 112 U/mg, and AS-vWf protein, 139 U/mg, with

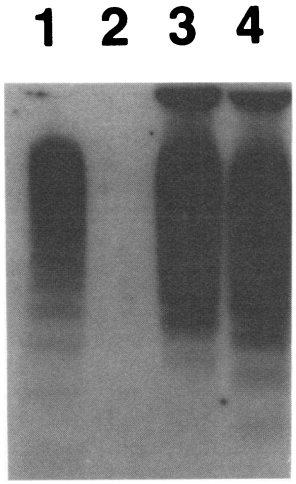

Figure 1. Electrophoretic analysis of purified vWf protein. The samples were electrophoresed in 1.25% glyoxyl agarose in the presence of SDS. The sample in lane *1* is pooled normal plasma; in lane *2* is the plasma from a patient with severe von Willebrand's disease (<3% FVIII:C, FVIIIR:Ag, and vWf activity); in lane *3* is the intact purified FVIII/vWf protein; and in lane *4* is vWf after neuraminidase treatment and removal of 92% of the sialic acid. Lanes *3* and *4* contained ~0.6 ng of vWf protein. The gels were developed with a ^{125}I-rabbit anti-vWf antibody as previously described (13). The anode is at the bottom. Note the mild shift of the migration of the multimers of the AS-vWf protein (lane *4*) as compared with intact vWf (lane *3*). This shift is a result of the change in molecular weight of the multimers by removal of the sialic acid.

1 U defined as the activity of 1 ml of plasma). Radiolabeling did not affect the specific biologic activity.

Aggregation of platelets by AS-vWf. The addition of AS-vWf to normal PRP induced platelet aggregation. At lower doses of AS-vWf, the aggregation had a biphasic pattern, but at higher doses, there was only a single major wave of aggregation. In four time-course experiments, when 2–6 μg/ml of AS-vWf was added to normal PRP, 63% (range, 56–74%) of the AS-vWf bound to the platelets within the first minute. The onset of aggregation was directly related to the amount of AS-vWf bound to the platelets. In four experiments, aggregation began when a mean of 0.174 μg/ml AS-vWf bound to 10^8 platelets (n = 4; range 0.156 to 0.183) (Fig. 3). More than 83% of the total AS-vWf present in the reaction mixture was bound at the onset of aggregation (n = 4; range 80–87%). Platelets separated from plasma proteins by the arabinogalactan gradient and resuspended in homologous or autologous plasma behaved identically to PRP, whereas the arabinogalactan-

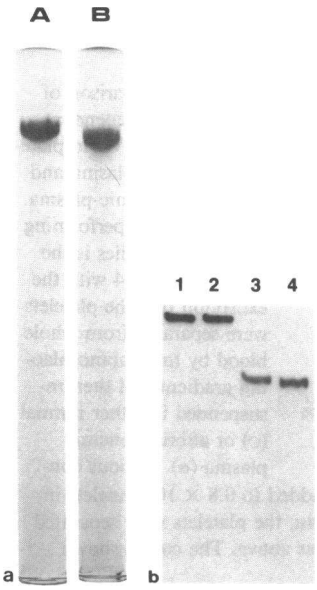

Figure 2. Electrophoretic analysis of purified vWf. (*a*) Purified vWf was analyzed before and after desialation with α-D-neuraminidase. The samples were reduced with 50 mM dithierythritol (DTE) and heated at 100°C for 3 min. 5 μg of intact (lane *A*) and asialo (lane *B*) vWf were applied to a 5% acrylamide 0.25% bisacrylamide gel. The gel was stained with Coomassie Blue. The cathode is at the bottom. (*b*) The intact (lanes *1* and *3*) and asialo (lanes *2* and *4*) ^{125}I-vWf were analyzed by radioautography after electrophoresis on a 5% polyacrylamide gel. In lanes *1* and *2* are the intact (lane *1*) and asialo (lane *2*) proteins before reduction with DTE and in lanes *3* and *4* are the same proteins after reduction with DTE.

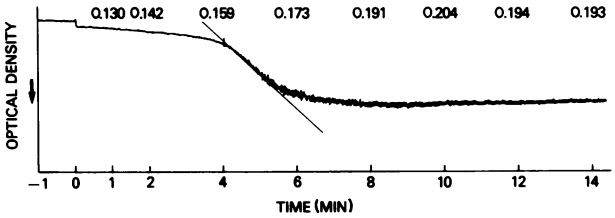

Figure 3. Time course of binding of AS-vWf protein to platelets. Purified AS-vWf protein was used at a final concentration of 5 μg/ml (range, 2–6 μg/ml). At time 0, the AS-vWf was added to 0.4 ml of platelets in PRP (0.8 × 10^8 platelets) in the aggregometer (900 rpm) at 37°C. The aggregation began at 4 min when 0.159 μg AS-vWf had bound to 10^8 platelets (values in the upper portion of the figure). The line through the aggregation tracing depicts the calculation of the initial slope, 55 mm/min. At the onset of aggregation >80% of the AS-vWf is already bound to the platelets.

separated platelets resuspended in afibrinogenemic plasma did not aggregate until 0.31 μg of AS-vWf bound to 10^8 platelets (n = 5; range, 0.24–0.46 μg). Although more AS-vWf was bound to these platelets, they showed a marked reduction in the initial slope of aggregation (mean, 10.2 vs. 66.8 mm/min; n = 4), i.e., ~12% of normal. Thrombasthenic PRP also had a markedly reduced aggregation response to AS-vWf. The initial slope of aggregation was only 11% of that found with normal PRP.

Binding studies. The data of the time course of binding are presented above. In concentration-dependent binding studies we have found good agreement between the amount of binding and the onset of aggregation. In five other experiments, the onset of aggregation occurred when at least 0.12 and not more than 0.20 μg were bound to 10^8 platelets. The mean value in these experiments, which were not time-course experiments, was 0.152 μg of AS-vWf bound/10^8 platelets. Similar studies with platelets in afibrinogenemic plasma revealed that aggregation did not take place until a mean of 0.415 μg of AS-vWf bound to the platelets in the presence of the afibrinogenemic plasma (range, 0.26–0.62 μg bound/10^8 platelets). On two occasions with thrombasthenic PRP, aggregation was not seen until 0.30 and 0.55 μg bound 10^8 platelets, respectively.

Intact vWf, fibrinogen, fibronectin, and albumin were unable to displace AS-vWf binding to the platelet surface in PRP. Intact vWf, fibronectin, and albumin resulted in no displacement, and fibrinogen displaced only 5%. When these platelets were suspended in Hepes buffer, the AS-vWf binding was only 15–20% of that observed in PRP and aggregation did not occur. None of the competing proteins displaced >18% of AS-vWf binding. Maximum concentration of fibrinogen displaced 10% AS-vWf binding, intact vWf displaced 5% AS-vWf, and fibronectin displaced 18% AS-vWf. Albumin had no effect. The percentage inhibition was calculated relative to the total binding of ^{3}H-vWf to the platelet in the presence of buffer rather than of competing ligands. Binding of AS-vWf to platelets was saturable, and the labeled and unlabeled AS-vWf competed on a equal molar basis (Figs. 4 and 5). At saturation, binding to normal platelets ranged between 0.94 and 2.02 μg/10^8 platelets (mean, 1.41 μg/10^8 platelets; n = 5) (Table I). In contrast, the binding to platelets in afibrinogenemic plasma was 51% greater, with a mean value of 2.04 μg/10^8 platelets (range, 1.29–2.63 μg/10^8 platelets; n = 5; Fig. 6 and Table I). At saturation, binding to thrombasthenic platelets was reduced as compared with the normal PRP performed

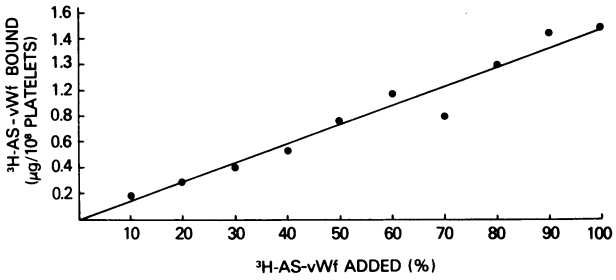

Figure 4. Binding of ^{3}H-AS-vWf protein and unlabeled AS-vWf protein to platelets in stirred PRP. The relative proportion of unlabeled and ^{3}H-AS-vWf was varied in this experiment. The total FVIII/vWf concentration mixture was kept constant at 54 μg/ml. 50 μl of a mixture of labeled and unlabeled protein was added to 0.4 ml of PRP (0.8 × 10^8 platelets), which was then placed in the aggregometer (900 rpm) for 15 min at 37°C.

simultaneously (0.96 μg/10^8 thrombasthenic platelets vs. 1.41 μg/10^8 normal platelets in one experiment and 0.60 μg/10^8 platelets vs. 0.98 μg/10^8 platelets in another; a reduction of 32 and 39%, respectively) (Table II).

Dissociation experiments were carried out by addition of a 40-fold excess of unlabeled AS-vWf to 0.4 ml of normal PRP 15 min after the addition of the 50 μl of radiolabeled AS-vWf. At the same time, parallel incubation mixtures were diluted with the same amount of Hepes buffer. Samples were taken from each mixture at various times, and the ^{3}H-AS-vWf remaining bound was determined; 48% of the AS-vWf was dissociated at 60 min, 68% at 90 min.

The multimeric structure of the AS-vWf bound to and eluted from the platelets was similar to that of the AS-vWf added to the platelets (Fig. 7).

Studies with monoclonal antibodies. The AS-vWf concentrations were 3.5 and 7.0 μg/ml in studies of the inhibition of binding by the monoclonal antibody. The 6D1 antibody, directed against GPIb, totally inhibited the binding of AS-vWf to normal platelets and blocked platelet aggregation at 10 μg/ml final concentration (Fig. 8). In contrast, the 10E5 antibody, directed against GPIIb/IIIa blocked only 44% of the AS-vWf binding to normal platelets, even when the antibody concentration was as high as 73 μg/ml. Although the inhibition of AS-vWf was only partial, inhibition of platelet aggregation was complete at this concentration of 10E5. At lower concentrations of 10E5 (5.8 μg/ml) AS-vWf binding remained inhibited to nearly the same extent (42%), but platelet aggregation did occur. At even lower concentrations, there was less inhibition of the binding of the AS-vWf and the initial slope of aggregation increased. In contrast, with 6D1 at 0.22 μg/ml, AS-vWf binding was inhibited 58% but aggregation still occurred (Fig. 7). Compared on a molar basis, the inhibition of binding of AS-vWf was similar when purified IgG and F(ab)$_2$ fragments were used.

Table I. Effect(s) of Plasma Fibrinogen and Monoclonal Antibodies on AS-vWf Binding to Platelets

	AS-vWf bound (μg/10^8 platelets)		
	Control	With antibody 6D1*	With antibody 10E5‡
A. Normal plasma§	1.41 (0.94–2.02)‖	0	0.90 (0.58–1.04)
B. Afibrinogenemic plasma	2.04 (1.29–2.63)	0¶	1.01 (0.62–1.07)
Percent increase B/A	45	—	11

n = 5.
* 6D1 is against GPIb.
‡ 10E5 is against GPIIb/IIIa.
§ Arabinogalactan-separated platelets were resuspended in normal or afibrinogenemic plasma, 200,000/μl.
‖ Observed range of values.
¶ In three experiments, binding was totally inhibited and in one experiment 0.05 μg AS-vWf bound 10^8 platelets.

When AS-vWf concentrations were used to achieve saturation, the 6D1 antibody still inhibited 100% of the binding of the AS-vWf protein to normal platelets, whereas 10E5 inhibited 36% (0.51 μg/10^8) of the AS-vWf binding (Table I). With thrombasthenic platelets, 6D1 again inhibited 100% of the binding of AS-vWf, but 10E5 had only a minimal effect,

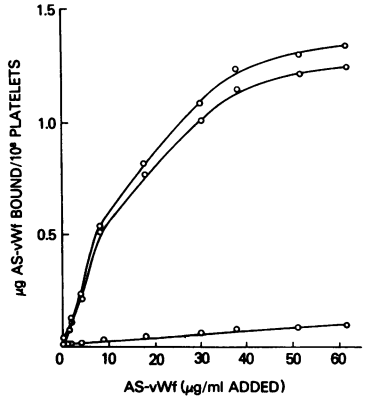

Figure 5. Concentration-dependent binding of ^{3}H-AS-vWf to platelets. ^{3}H-AS-vWf protein in increasing concentrations was added to PRP (0.8 × 10^8 platelets) in an aggregometer (900 rpm) at 37°C. The amount of ^{3}H-AS-vWf protein bound to the platelet pellet was measured. Nonspecific binding was measured in parallel mixtures by the addition of a 50-fold excess of unlabeled AS-vWf protein to the mixtures with the ^{3}H-AS-vWf protein. The top curve represents total binding, the middle curve represents specific binding, and the bottom line represents nonspecific binding. The nonspecific binding averaged 4.5% (range, 2.0–8.8%).

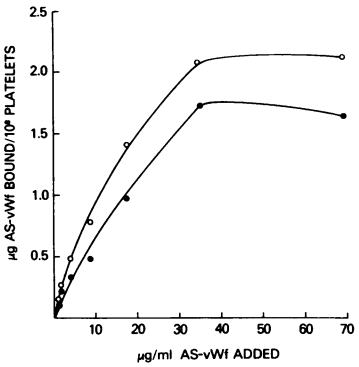

Figure 6. Comparison of concentration-dependent binding of AS-vWf to platelets in normal plasma and in afibrinogenemic plasma. The method of performing the binding studies is the same as in Fig. 4 with the exception that the platelets were separated from whole blood by the arabinogalactan gradient and then resuspended in either normal (○) or afibrinogenemic plasma (●). Various concentrations of AS-vWf protein were added to 0.8 × 10^8 platelets in an aggregometer at 37°C. After 15 min, the platelets were separated and specific binding was determined as above. The curves shown represent specific binding.

Table II. Effect(s) of Monoclonal Antibodies on AS-vWf Binding to Platelets

	AS-vWf bound ($\mu g/10^8$ *platelets*)					
	Control		With antibody 6D1*		With antibody 10E5‡	
	1§	2	1	2	1	2
A. Normal PRP‖	1.41	0.98	0	0	0.84	0.66
B. Thrombasthenic PRP	0.96	0.60	0	0	0.89	0.56
Percent decrease B/A	32	39	—	—	+6	15

* As in Table I, 6D1 is against GPIb.
‡ As in Table I, 10E5 is against GPIIb/IIIa.
§ Two experiments performed.
‖ Normal and thrombasthenic PRP were used at 200,000 μl.

inhibiting just 7–8% of the binding (Table II). In afibrinogenemic plasma, 6D1 inhibited 100% of the AS-vWf binding and completely blocked aggregation. 10E5 was 100% more effective in inhibiting AS-vWf binding in afibrinogenemic plasma than in normal plasma (1.03 vs. 0.51 μg inhibited/10^8 platelets), but it did not affect the magnitude of AS-vWf platelet aggregation (Table I).

Discussion

Vermylen et al. (16–18) first observed that neuraminidase-treated human cryoprecipitate aggregated platelets in PRP but did not aggregate washed platelets unless plasma fibrinogen was added. The aggregation was partially inhibited by EDTA. DeMarco and Shapiro demonstrated that purified AS-vWf bound to the surface of the platelets and caused human platelets to aggregate even in the absence of ristocetin (3). They further demonstrated that the binding was saturable and that the aggregation was inhibited by EDTA. They suggested that AS-vWf bound to GPIb since the PRP of two patients

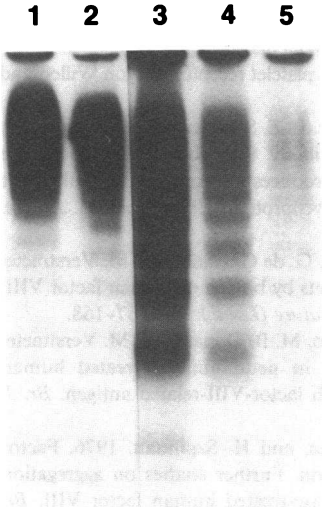

Figure 7. Glyoxyl agarose electrophoresis of ^{125}I-intact and AS-vWf protein. Lane *1*, intact ^{125}I FVIII/vWf protein; lane *2*, ^{125}I AS-vWf protein; lanes *3–5*, ^{125}I-AS-vWf protein bound to platelets in PRP at 37°C for 15 min in an aggregometer. The ^{125}I vWf was extracted from the platelet pellet after separation from unbound ligand. In lane *3*, 0.290 μg was bound to 10^8 platelets; in lane *4*, 0.083 μg were bound per 10^8 platelets; and in lane *5*, 0.033 μg were bound per 10^8 platelets. The pattern of multimers in lanes *3–5* is similar to that in lane *2*, the ^{125}I-AS-vWf used in the binding studies.

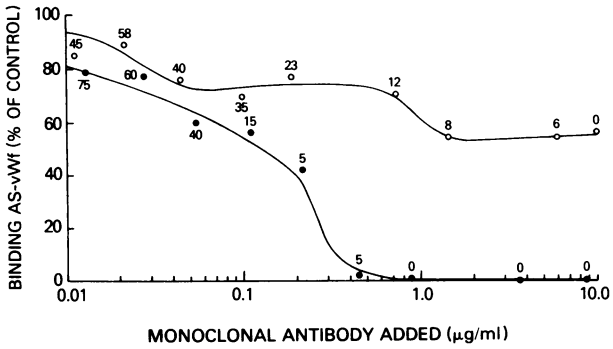

Figure 8. The effect of monoclonal antibodies on AS-vWf binding to, and aggregation of, platelets. Two monoclonal antibodies, one against GPIb (closed circles) and a second against GPIIb/IIIa complex (open circles) were added to 0.4 ml of PRP (0.8×10^8 platelets) for 1 min before the addition of ^{3}H-AS-vWf protein. The mixture was placed into the aggregometer at 900 rpm at 37°C for 15 min; the platelets were separated and the bound vWf protein was assayed. Controls included a monoclonal antibody directed against a lymphocyte marker not present on platelets and an equal volume of buffer added in place of monoclonal antibody. The AS-vWf concentration was 3.25 μg/ml. Neither the nonplatelet monoclonal antibody nor the buffer controls changed AS-vWf binding to platelets. The antibody against GPIb totally inhibited binding at a 1 μg/ml final concentration. At lower concentrations, binding increased, as did the amount of platelet aggregation. The antibody against GPIIb/IIIa inhibited only 44% of the FVIII/vWf binding even when added at concentrations as high as 72 μg/ml (data not shown). At doses <10 μg/ml of this antibody, platelet aggregation occurred. The initial slope of aggregation was 7 mm/min when 56% of the AS-vWf bound with the antibody against GPIIb/IIIa, but when 56% of the ligand bound in the presence of the antibody against GPIb, the aggregation was between 15 and 40 mm/min.

with Bernard-Soulier syndrome did not aggregate after the addition of AS-vWf protein.

To define better the interactions between AS-vWf and platelets, we have studied the effect(s) of plasma fibrinogen and monoclonal antibodies directed against GPIb and GPIb/IIIa on the amount and sites of AS-vWf binding to, and aggregation of, both normal and thrombasthenic platelets. The results of our studies indicate that there are two sites of binding of AS-vWf on the surface of platelets: GPIb and GPIIb/IIIa. In normal PRP, approximately two-thirds of the AS-vWf binds to GPIb and one-third to GPIIb/IIIa. The binding to the GPIb does not change appreciably in afibrinogenemic plasma. However, the binding to the GPIIb/IIIa complex increases by ~100%. This suggests that the AS-vWf binding to GPIIb/IIIa is, in part, inhibited by plasma fibrinogen. This is further supported by studies showing that the antibody against GPIIb/IIIa inhibits much more of the binding of AS-vWf to platelets in afibrinogenemic plasma (1.03 μg/10^8 platelets) than in normal plasma (0.51 μg/10^8 platelets).

Further evidence of the importance of the two platelet glycoproteins in binding comes from our studies with thrombasthenic platelets. The thrombasthenic platelets bound ~37% less AS-vWf than did normal platelets. This reduction of binding is almost identical to that seen with the antibody against GPIIb/IIIa in normal PRP (36%). As expected, this antibody produced only minimal inhibition of the binding of AS-vWf to thrombasthenic platelets.

We believe that the binding of AS-vWf to GPIIb/IIIa occurs after binding to GPIb. This hypothesis is supported by the observation that the GPIb antibody could completely block the binding of AS-vWf to normal and thrombasthenic platelets. Thus, the AS-vWf protein binding to GPIb must be pivotal in the making available of GPIIb/IIIa for the binding of additional AS-vWf. However, our data also indicate that the binding of AS-vWf to GPIb is not directly responsible for more than a small fraction of the observed aggregation process. Rather, our data suggest that it is more important for fibrinogen to bind to GPIIb/IIIa than AS-vWf for the platelet/platelet interaction. The evidence to support this is: (*a*) Thrombasthenic platelets had normal amounts of GPIb and normal binding of AS-vWf to GPIb and yet only had 10% of the aggregation response of normal platelets in normal plasma; (*b*) The antibody to GPIIb/IIIa, when added to platelets in normal plasma, inhibited the binding of AS-vWf by only 35–40% but inhibited the aggregation by ~90%. (*c*) When the AS-vWf binding was performed in the absence of fibrinogen, despite a 51% enhancement of binding of AS-vWf, the aggregation response was only 10–12% of that in normal plasma containing fibrinogen.

It was paradoxical that inhibition of platelet aggregation became incomplete when the concentration of 10E5 antibody was decreased from 11.6 to 5.8 μg/ml even though the inhibition of the binding of AS-vWf was only minimally altered. The probable explanation is that reducing the 10E5 concentration permitted the exposure of a small fraction of the GPIIb/IIIa sites to which fibrinogen, but not vWf, bound. This is consistent with studies we and others have reported showing that fibrinogen competes effectively with vWf for these sites (19–21).

Our current data extend the previously reported findings of the site of vWf binding to platelets. Ristocetin-induced vWf binding is to the GPIb and can be blocked only by the antibody against GPIb (14, 20, 22, 23). No effect was seen with the GPIIb/IIIa antibody (19, 20). Intact vWf binds to platelets independently of ristocetin when the platelets are stimulated with thrombin, ADP, or the combination of ADP and epinephrine (19, 20, 24–26). This binding appears to be uniquely to GPIIb/IIIa, since the antibody to GPIb does not inhibit binding or aggregation and both the antibody to GPIIb/IIIa and plasma fibrinogen can effectively and totally inhibit the binding (19–21). The binding of the AS-vWf protein appears to be unique in that it initially binds to GPIb, which then modifies the platelet to expose GPIIb/IIIa. Both AS-vWf and fibrinogen can bind to this site, and significant platelet aggregation seems to require that this site be occupied in part by fibrinogen. Studies with the monoclonal antibodies, thrombasthenic platelets, and afibrinogenemic plasma support this concept. We cannot state from our results whether AS-vWf and fibrinogen have structural homologies that are recognized on a common site of the GPIIb/IIIa (27), but even if they did have such a site one would have to postulate another site on AS-vWf that interacts with the GPIb.

The physiologic role, if any, for AS-vWf remains speculative. Since only relatively small amounts of AS-vWf were required to initiate aggregation (~0.17 $\mu g/10^8$ platelets representing <2% of the amount of vWf present in 1 ml of normal plasma), it is conceivable that desialation by enzymes on the surface of, or released by, platelets or other cells could generate a physiologically significant amount of AS-vWf. Direct confirmation of an event like this will be required, however, before any such mechanism can be accepted.

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