Asialo-von Willebrand Factor Inhibits Platelet Adherence to Human Arterial Subendothelium: Discrepancy Between Ristocetin Cofactor Activity and Primary Hemostatic Function

By Jeffry B. Lawrence and Harvey R. Gralnick

Platelet adherence at high wall shear rates requires plasma von Willebrand factor (vWF). Clinically, the ristocetin cofactor (RCof) activity is the only widely available assay for vWF function. When purified vWF is treated with neuraminidase to yield asialo-vWF (AS-vWF), its RCof activity is increased by 20% to 40%. AS-vWF binds to normal human platelets independently of ristocetin and induces platelet aggregation in the presence of fibrogenin. To determine whether AS-vWF also shows an enhanced capacity to support platelet adherence to subendothelium, we used the Baumgartner technique. Intact vWF, AS-vWF, or AS-vWF treated with β-galactosidase (asialo, agalacto-vWF; AS,AG-vWF) was added to normal citrated whole blood before perfusion over human umbilical artery segments (wall shear rate, 2,600 sec⁻¹). Four micrograms per milliliter AS-vWF caused a 69% reduction in total platelet adherence compared with citrated whole blood (P < .001), and 4 μg/mL AS,AG-vWF led to a 48% reduction (P < .006). With 4 μg/mL intact vWF, the platelet adherence values were not significantly different from the controls. No significant differences in subendothelial platelet thrombi or postperfusion platelet counts were evident among any of the groups. In reconstituted afibrinogenemic perfusates, 4 μg/mL AS-vWF caused a 42% reduction in platelet adherence (P < .05). Thus, AS-vWF is a potent inhibitor of platelet adherence, despite its enhanced RCof specific activity. Abnormalities in vWF carbohydrate may play a role in impaired primary hemostasis in some patients with von Willebrand’s disease.

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(Varistatic A Series; Manostat Corp, New York) as previously described.6,21 For all experiments citrated whole blood (final whole blood concentration 10.88 mmol/L sodium citrate), or reconstituted citrated blood composed of washed platelets, platelet-poor plasma (PPP) and washed red cells, was warmed for five minutes at 37°C and then perfused across the chamber at 37°C and recirculated at a rate of 300 mL/min for five minutes. The corresponding wall shear rate, 2,600 sec⁻¹, closely approximates conditions physiologically present in the microvasculature.22

After a perfusion experiment, the arterial segment was washed by perfusing 0.2 mol/L Tris-HCl buffer (pH 7.35) through the chamber for five minutes. The segment was fixed in 4% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) and cross sections were made from the central 5 to 8 mm portion of the segment. As previously described,14 the sections were dehydrated in graded ethanol solutions and propylene oxide, embedded in an oriented manner in epoxy resin (LX-112; Ladd Research Industries, Burlington, VT), and stained with toluidine blue and basic fuchsin. 0.8 μm sections were evaluated morphometrically by light microscopy according to Baumgartner and Muggli.18 For each perfusion experiment, two cross sections of the arterial segment were evaluated over their entire surfaces at 10 μm intervals, and the platelet-subendothelial interactions were categorized as either C, contact platelets attached but not spread on the surface, or S, platelets spread on the surface. The latter category may include spread platelets with superimposed platelet aggregates (thrombi) that extend 5 μm or more above the subendothelial surface. Platelet adherence is defined as C + S, and each parameter is expressed as a percentage of the total number of points counted (~1,500) per vessel segment. All values are expressed as the mean ± SEM, and Student’s t test was used to evaluate the significance of differences between mean values.

Perfusate preparation. Donors for all of the perfusion studies were normal hospital personnel who had no bleeding history and were not receiving medications that interfered with platelet function. Pre- and postperfusion complete blood counts were performed on all blood samples by an electronic counting device (Model S Plus; Coulter Electronics, Hialeah, FL). The mean preperfusion values (and ranges) were as follows: hematocrit, 43.6% (39.0% to 46.0%); and platelet count, 329,000/μL (252,000 to 405,000/μL). A portion of these donors led to platelet adherence values not significantly different from the controls (Table I; Fig 1).

For experiments using reconstituted blood, washed platelets and red cells were obtained from a normal donor, and PPP was obtained from a patient with congenital afibrinogenemia. This PPP contained less than 5 μg/mL fibrinogen, as measured by the tanned red cell hemagglutination assay, and had a thrombin time >300 seconds. The washed platelets and sedimented red cells were prepared from whole blood collected in 12% citrate/phosphate/dextrose/adenine anticoagulant (final whole blood concentrations: 1.9 mmol/L citric acid, 11.0 mmol/L sodium citrate, 2.0 mmol/L dextrose, 0.2 mmol/L adenine, 2.0 mmol/L monobasic sodium phosphate). Platelet-rich plasma (PRP) was obtained by centrifugation at 25°C for ten minutes at 200 × g. The PRP was diluted 1:1 in Ringer’s citrate dextrose buffer (RCD), pH 6.5, containing 43 ng/mL prostaglandin E1 (PGE1; Sigma Chemical Co, St Louis). RCD was comprised of 71.9 mmol/L NaCl, 0.7 mmol/L KCl, 0.6 mmol/L CaCl2, 0.8 mmol/L NaHCO3, 20.0 mmol/L trisodium citrate, 27.8 mmol/L dextrose. The PRP diluted in RCD with PGE1 was centrifuged at 25°C for 20 minutes at 1,100 × g. The platelet pellet was washed with RCD, pH 6.5, containing 43 ng/mL PGE1, centrifuged at 25°C for 20 minutes at 1,100 × g, and then washed with RCD, pH 6.5, without PGE1. After centrifugation at 25°C for 20 minutes at 1,100 × g, the final platelet pellet was resuspended in RCD, pH 7.2, without PGE1. The sedimented red cells were washed twice in saline dextrose buffer (154 mmol/L NaCl, 111 mmol/L dextrose). The platelets, red cells, and afibrinogenemic PPP were combined to yield a hematocrit of 40% and a platelet count of 246,000 per microliter. Four micrograms per milliliter of purified vWF was added to a portion of this reconstituted blood, and 4 μg/mL of AS-vWF was added to the remainder before perfusion.

Protein purification and carbohydrate modification. The vWF protein was purified from human cryoprecipitate by chromatography on Sepharose 4B as previously described.23 Protein concentrations were estimated by the method of Lowry et al.24 The basic acid was removed from intact vWF by the addition of Clostridium perfringens neuraminidase (Sigma Chemical Co) as previously described.25 Briefly, 0.02 to 0.04 U neuraminidase per milligram protein (1 U of enzyme activity defined as the amount that released 1 μmol of sialic acid per minute at 37°C from α1 glycoprotein) was incubated at 37°C with purified vWF for two hours. The enzyme contained no protease activity when reacted with [¹⁴C] globin.13 Sialic acid was measured by the method of Warren,25 using N-acetyl neuraminic acid as the standard. The sialic acid released by neuraminidase was quantified by the same method, except that the acid hydrolysis step was omitted.23 The mean sialic acid content of the vWF protein was 144 ± 15 nmol/mg (n = 3). Neuraminidase treatment for two hours released >97% of the sialic acid. The neuraminidase was not removed from the AS-vWF preparations before their addition to the perfusates. Therefore, control experiments were performed in which neuraminidase was added to whole blood at a concentration of .004 U/mL, a final concentration 2.5 to 10 times higher than the concentration of neuraminidase present in the AS-vWF perfusates. AS-vWF was treated with β-galactosidase, prepared from Streptococcus pneumoniae (gift of Dr G. Ashwell, Bethesda, MD), to remove its ultimate and penultimate galactose residues as previously described.25 Briefly, 0.005 U β-galactosidase per milligram protein was added to AS-vWF and incubated at 37°C for eight hours. This enzyme was also protease free.26 The released free galactose was measured in an assay using galactose dehydrogenase as described,26 and the total galactose content of vWF was determined by hydrolysis in 2 mol/L hydrochloric acid at 100°C for 90 minutes, followed by assay for free galactose.26 The mean total galactose measured by acid hydrolysis was 139 ± 24 nmol/mg (n = 3). Treatment with β-galactosidase removed approximately 72% of this galactose (range 63% to 79%).

RESULTS

When citrated whole blood from normal donors was perfused over umbilical artery segments for five minutes at a wall shear rate of 2,600 sec⁻¹, morphometric analysis showed approximately 50% of the subendothelial surface to be covered with adherent platelets (Table I; Fig 1). The majority of this surface coverage was represented by a monolayer of spread platelets (Table I). Approximately 1% of the surface was occupied by platelet thrombi extending 5 μm or more above the subendothelium.

Addition of 4 μg/mL purified vWF to whole blood from these donors led to platelet adherence values not significantly different from the controls (Table I; Fig 1). However, addition of 4 μg/mL AS-vWF caused a 69% reduction in platelet adherence (P < .001), (Table I; Fig 1). With 4 μg/mL AS-vWF, platelet spreading (S) was significantly reduced relative to both the controls (P < .001) and the perfusates containing 4 μg/mL purified vWF (P < .005). By contrast, the AS-vWF significantly inhibited surface cover-
age with contact platelets (C) relative to perfusates containing purified vWF (P < .02), whereas the inhibition compared with controls failed to reach statistical significance (P > .20). There was no significant difference in surface coverage with platelet thrombi between any of the experimental or control groups. Platelet adherence was significantly inhibited by doses of AS-vWF as low as 1 μg/mL (Fig 1). When doses of AS-vWF > 4 μg/mL were added to whole blood, further reductions in platelet adherence were produced, but at these doses the postperfusion reduction of the platelet count of the perfusate became significantly greater than that observed with control samples. By contrast, at doses up to 4 μg/mL, the postperfusion platelet counts in the AS-vWF experiments were not significantly different from those of the controls (Table 2). Since the neuraminidase was not removed from the AS-vWF preparations before their addition to whole blood, control experiments were performed in which neuraminidase was added to whole blood at a dose of .004 U/mL, a dose 2.5 to 10 times higher than the final whole blood concentration present in the AS-vWF perfusates, immediately before warming the samples for five minutes at 37°C before perfusion. The mean total platelet adherence (C + S) for these experiments (n = 2), 44.58%, was not significantly different from that of the controls (Table 1).

When 4 μg/mL AS,AG-vWF was added to normal whole blood, platelet adherence was reduced by 48%, compared with controls (P < .005; Table 1; Fig 1). As with AS-vWF, S was significantly reduced by AS,AG-vWF, whereas the inhibition of C failed to achieve statistical significance (Table 1). AS,AG-vWF caused no significant differences in platelet thrombus formation or postperfusion platelet counts (Table 2) compared with controls.

Since platelet aggregation caused by AS-vWF requires plasma fibrinogen, a reconstituted afibrinogenemic perfusate was prepared to define whether the inhibition of platelet adherence observed with AS-vWF resulted from platelet microaggregate formation in the circulating blood. Four micrograms per milliliter of purified vWF was added to a portion of this perfusate and 4 μg/mL of AS-vWF was added to the remainder before perfusion. Even in the absence of plasma fibrinogen, AS-vWF caused a 42% reduction in platelet adherence compared with native vWF (P < .05; Table 3). Postperfusion platelet counts in the two groups were not significantly different (Table 3).

**DISCUSSION**

The observation that low concentrations of AS-vWF are capable of directly binding to and aggregating platelets in the absence of ristocetin led earlier investigators to propose the hypothesis that sialidases or sialyltransferases derived from platelets or other cells may physiologically generate AS-vWF from plasma vWF at sites of vascular injury. The AS-vWF would be deposited at sites of endothelial cell injury and would enhance platelet adherence to the subendothelial...
Control samples contained no added plasma proteins. Mean values of citrated blood at 37°C for five minutes at a wall shear rate 2,600 sec⁻¹ are shown and the indicated values are significantly different between the two groups.

De-endothelialized human umbilical arteries were perfused with citrated blood at 37°C for five minutes at a wall shear rate 2,600 sec⁻¹. Control samples contained no added plasma proteins. Mean values (± SEM) are shown. None of the experimental values showed a statistically significant difference from the controls.

The mechanism of the inhibition of platelet adherence caused by AS-vWF is not understood at present. Sakariassen et al demonstrated that plasma vWF must first bind to the subendothelium in order to support platelet adherence at high wall shear rates. It is possible that AS-vWF may not bind to the subendothelial surface as readily as native plasma vWF. If this is the case, the binding of the AS-vWF to the platelet GPIb and GPIb/IIa, as demonstrated by us and others, could block these platelet receptors from interacting with the subendothelial-bound native plasma vWF. This would inhibit platelet adherence because vWF binding to GPIb is required for platelets to attach to the subendothelium normally. Furthermore, we and others have also shown that normal platelet spreading along the subendothelial surface requires intact GPIb/IIa. This mechanism is consistent with our observation that AS,AG-vWF is less potent as an inhibitor of platelet adherence than is AS-vWF (Table 1; Fig 1), for it has been shown that AS,AG-vWF binds with a lower affinity to platelet GPIb than does AS-vWF. Thus, AS,AG-vWF would be expected to compete less readily for binding sites on GPIb, and probably the GPIb/IIa complex, with subendothelial-bound native plasma vWF, leading to decreased inhibition of normal platelet adherence compared with AS-vWF.

An alternate mechanism to explain the decreased platelet adherence would involve the binding of AS-vWF and AS,AG-vWF to the subendothelium with similar affinities to that of native plasma vWF, yet they may be less efficient in supporting platelet attachment and spreading than the native molecule. Recent studies by Girma et al suggest that the amino-terminal portion of vWF contains distinct sites responsible for binding to GPIb and collagen, while the GPIb/IIa binding site appears to be near the carboxy-terminal end of vWF. Thus it is possible that the collagen-binding site on vWF may not be affected by removal of carbohydrate, whereas the GPIb and/or GPIb/IIa attachment sites may require a full complement of sialic acid and galactose to maintain the capacity of vWF to support platelet adherence. The observed inhibition of platelet adherence does not appear to be due merely to platelet aggregation causing an effective thrombocytopenia in the perfused blood, since the postperfusion platelet counts in the control and experimental groups were not significantly different (Table 2).

Furthermore, in the experiments using afibrinogenemic reconstituted blood, platelet adherence was significantly inhibited by AS-vWF (Table 3), despite the fact that AS-vWF-induced platelet aggregation is dependent on the presence of plasma fibrinogen.

Previous studies from our laboratory have demonstrated deficiencies in sialic acid, galactose, and N-acetylgalactosamine in vWF purified from patients with type IIb von Willebrand's disease (vWD). This suggests that the inhibited platelet adherence we have observed with AS-vWF and AS,AG-vWF may have clinical relevance. Sakariassen et al showed that infusion of 1-deamino (8-D-arginine) vasopressin (DDAVP) into type IIb vWD patients caused reduced platelet adherence to subendothelium, presumably due to the release of increased amounts of their qualitatively abnormal vWF into the plasma. Although the carbohydrate composition of the patients' vWF was not described, the data of Sakariassen et al support our findings that qualitatively abnormal vWF can impair platelet adherence to subendothelium. Based on our current observations and our previous findings, it is likely that AS,AG-vWF binds with a lower affinity to platelet GPIb than does AS-vWF.
studies of vWD, it is possible that some vWD patients show impaired primary hemostasis as a consequence of inhibited platelet adherence due to carbohydrate deficiency or other structural defects of their plasma vWF.\textsuperscript{30,31}

Our findings suggest that the ability of plasma vWF to support platelet adherence to subendothelium requires preservation of the sialic acid content of the glycoprotein. By contrast, the RCoF activity of vWF is enhanced by 20% to 40% by the removal of the negatively charged sialic acid residues.\textsuperscript{15} This discrepancy between vWF function in primary hemostasis and the RCoF assay is reflected in the clinical observation that some patients with vWD show RCoF levels that fail to correlate with their bleeding times and hemorrhagic diatheses.\textsuperscript{5-10} It further suggests that concepts regarding structure-function relationships in vWF must be tested in other systems in addition to the RCoF assay to ensure their physiologic relevance.

REFERENCES


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