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. Granick

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 fibrinogen. 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 micromilliters 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 a fibrinogenemic 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.

PLASMA von Willebrand factor (vWF) is an essential cofactor for platelet adherence to vascular subendothelium at high wall shear rates.¹,² The molecule must first bind to the subendothelium in order to enable platelets to attach and spread along the subendothelial surface and subsequently form aggregates. The ability of vWF to support the agglutination of platelets in the presence of ristocetin forms the basis for the only widely used assay for the functional activity of plasma vWF. In general, the ristocetin cofactor (RCof) activity has been clinically useful for the evaluation of patients with von Willebrand’s disease.³,⁴ However, discrepancies between RCof activity and the bleeding time and severity of clinical bleeding have been noted.⁵,¹⁰ This suggests that the vWF-dependent mechanisms that physiologically mediate platelet-subendothelial interaction in primary hemostasis are not completely reflected in the RCof assay. Since structure-function investigations of vWF have largely relied on the RCof activity,¹¹-¹³ extension of the conclusions of these studies to the physiology of primary hemostasis may be somewhat problematic.

vWF is a multimeric glycoprotein whose carbohydrate content comprises 14% of its molecular weight.¹⁴ Approximately 4% of the glycoprotein is comprised of sialic acid residues.¹⁵ When purified vWF is treated with neuraminidase to remove its sialic acid, the resulting asialo-vWF (AS-vWF) demonstrates a 20% to 40% increase in RCof activity.¹⁵ Initial studies by De Marco and Shapiro¹⁶ and subsequent work from our laboratory and others¹⁵,¹⁷ have shown that AS-vWF binds to normal human platelets independently of ristocetin and induces platelet aggregation dependent on platelet glycoproteins Ib(GPⅠb) and IIb/IIIa (GPⅡb/Ⅲa) in the presence of plasma fibrinogen. It has been postulated that small amounts of AS-vWF that may be generated at sites of vascular disruption might enhance platelet adherence to subendothelium and subsequent platelet aggregation in primary hemostasis.¹⁴ To test this hypothesis, we have employed the Baumgartner technique, an ex vivo system that appears to simulate the in vivo events of primary hemostasis,¹⁸ to define whether AS-vWF was associated with enhanced platelet adherence to human arterial subendothelium. We have also treated AS-vWF with β-galactosidase to remove its terminal and penultimate galactose moieties. We compared asialo,agalacto-vWF (AS,AG-vWF) with AS-vWF and the native protein in attempt to address the role of carbohydrate moieties of vWF in its capacity to support platelet adherence.

MATERIALS AND METHODS

Perfusion procedure and morphologic evaluation. Human umbilical arteries were dissected free from umbilical cords obtained immediately after birth. Arterial segments, approximately 15 to 20 mm in length, were everted and de-endothelialized by brief exposure to air. As previously described,¹⁹ the vessels were treated with 0.1 mmol/L aspirin for one hour before being used in experiments, and were then washed four times in 0.2 mol/L Tris-HCl buffer (pH 7.35) to completely remove the aspirin before exposure of the vessels to perfused blood. The vessel segments were pretreated with aspirin to prevent prostaeycin production by vascular smooth muscle cells.²⁰ An annular perfusion chamber similar to those developed by Baumgartner²¹ was used. This device contains a rod on which everted umbilical artery segments are mounted such that the blood entering the chamber flows through the annular space between the subendothelial surface and the chamber wall, thereby exposing the subendothelium to platelets. The chamber has an effective annular width of 0.18 cm. This is the distance between the chamber wall and the central rod, minus the average thickness of the artery wall (~0.15 mm.) Steady, nonpulsatile flow across the chamber was produced by gravity using a plastic funnel connected to a peristaltic pump.
and Muggli. For each perfusion experiment, two cross sections of samples were warmed for five minutes at 37°C before perfusion. Differences between mean values, SEM, and Student's t-test were used to evaluate the significance 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 1; Fig 1). The majority of this surface coverage was represented by a monolayer of spread platelets (Table 1). 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 1; Fig 1). However, addition of 4 μg/mL AS-vWF caused a 69% reduction in platelet adherence (P < .001), (Table 1; 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 contain-
doses the postperfusion reduction of the platelet count of the
There was no significant difference in surface coverage with
samples contained no added plasma proteins. Mean values (±
with controls failed to reach statistical significance
ing purified vWF
concentration present in the AS-vWF perfusates, immedi-
doses of AS-vWF as low as 1 μg/mL (Fig 1). When doses of
groups. Platelet adherence was significantly inhibited by
AS-vWF preparations before their addition to whole
were not significantly different from those of the controls
the postperfusion platelet counts in the AS-vWF experiments
with control samples. By contrast, at doses up to 4 μg/mL,
platelet adherence was reduced by 48%, compared
before perfusion. The mean total platelet adherence (C + S)
platelet thrombus formation or postperfusion platelet counts
and would enhance platelet adherence to the subendothelial
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 per-
was 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 neu-
aminidase 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, immedi-
atly 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.

**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 sialytransferases 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
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 GPIIb/IIIa, 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/IIIa. 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/IIIa 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/IIIa 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 GPIIb/IIIa 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-acetylglucosamine 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

### Table 2. Postperfusion Percentage Reduction in the Platelet Counts of Perfusates Containing Added vWF or Carbohydrate-Modified vWF

<table>
<thead>
<tr>
<th>Added Protein</th>
<th>Percent Postperfusion Platelet Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n = 10)</td>
<td>36 ± 3</td>
</tr>
<tr>
<td>4 µg/mL vWF (n = 3)</td>
<td>34 ± 3</td>
</tr>
<tr>
<td>1 µg/mL AS-vWF (n = 2)</td>
<td>38</td>
</tr>
<tr>
<td>2 µg/mL AS-vWF (n = 3)</td>
<td>46 ± 9</td>
</tr>
<tr>
<td>4 µg/mL AS-vWF (n = 7)</td>
<td>43 ± 5</td>
</tr>
<tr>
<td>4 µg/mL AS,AG-vWF (n = 7)</td>
<td>43 ± 3</td>
</tr>
</tbody>
</table>

De-endothelialized human umbilical arteries were perfused with citrated blood at 37°C for five minutes at a wall shear rate of 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.

### Table 3. Effect of AS-vWF on Platelet Adherence With Reconstituted Afibrinogenemic Blood

<table>
<thead>
<tr>
<th>Protein Added to Blood</th>
<th>Percentage of Contact (C)</th>
<th>Percentage of Spread (S)</th>
<th>Total Platelet Adherence (C + S)</th>
<th>Percentage Postperfusion Platelet Count Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 µg/mL vWF (n = 2)</td>
<td>5.69</td>
<td>40.56</td>
<td>46.25</td>
<td>14.8</td>
</tr>
<tr>
<td>4 µg/mL AS-vWF (n = 2)</td>
<td>4.25</td>
<td>23.35*</td>
<td>27.60*</td>
<td>19.6</td>
</tr>
</tbody>
</table>

Reconstituted perfusates were prepared from washed normal red cells, washed normal platelets; and citrated PPP from a patient with congenital afibrinogenemia (hematocrit, 40%; platelet count, 246,000/µL). The specified amounts of native plasma vWF or AS-vWF were added to the samples before perfusion at 37°C for five minutes over de-endothelialized human umbilical artery segments at a wall shear rate of 2,600 sec⁻¹. Mean values are shown and the indicated values are significantly different between the two groups.

*P < .02.
†P < .05.
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. 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. 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. 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.

ACKNOWLEDGMENT

We thank Wendy S. Kramer for excellent technical assistance with morphometry and Sherette Ferrell for typing the manuscript.

REFERENCES


From www.bloodjournal.org by guest on October 3, 2017. For personal use only.


Asialo-von Willebrand factor inhibits platelet adherence to human arterial subendothelium: discrepancy between ristocetin cofactor activity and primary hemostatic function

JB Lawrence and HR Gralnick