A New Congenital Platelet Abnormality Characterized by Spontaneous Platelet Aggregation, Enhanced von Willebrand Factor Platelet Interaction, and the Presence of All von Willebrand Factor Multimers in Plasma

By Alessandra Casonato, Luigi De Marco, Mario Mazzucato, Vincenzo De Angelis, Dina De Roia, Fabrizio Fabris, Zaverio M. Ruggeri, and Antonio Girolami

A case is reported of a 49-year-old woman with a mild bleeding tendency. Her bleeding time, platelet count and size, plasma ristocetin cofactor activity, von Willebrand factor (vWF) antigen, and vWF multimeric pattern are all within normal limits. Spontaneous platelet aggregation is observed when citrated platelet-rich plasma (PRP) is stirred in an aggregometer cuvette. This aggregation is completely or only slightly diminished by an antilygycoprotein (GP) IIb/IIIa or by an anti-GPlb monoclonal antibody. The patient's PRP shows increased sensitivity to ristocetin. The distinct feature of this patient, also present in two family members studied, is that platelet aggregation is initiated by purified vWF in the absence of any other agonist. The vWF-induced platelet aggregation is abolished by anti-GPlb and anti-GP I b/IIla monoclonal antibodies and by EDTA (5 mmol/L). Apyrase inhibits the second wave of aggregation. Patient's platelets in PRP are four to six times more reactive to asialo vWF-induced platelet aggregation than normal platelets. The amount of radiolabeled vWF bound to platelets in the presence of either low concentration of ristocetin or asialo vWF was increased 30% compared with normal. The patient's platelet G P I b was analyzed by SDS page and immunoblotting and by binding studies with anti-GP I b monoclonal antibodies showed one band with slightly increased migration pattern and a normal number of G PIb molecules. Unlike the previously reported patients with pseudo or platelet-type von Willebrand disease, this patient has normal vWF parameters.

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EVERAL PATIENTS have been reported in the litera-
ture with a congenital bleeding disorder called platelet-
type von Willebrand's disease or pseudo von Willebrand's disease. This disease appears to be inherited as an autosomal dominant trait. Bleeding symptoms are mild and similar to classic von Willebrand's disease. Sometimes spontaneous platelet aggregation (SPA) and/or thrombocytopenia is observed. A common feature of all patients described so far is a heightened platelet sensitivity to aggregating following addition of ristocetin, which is accompanied by a decreased concentration of the higher molecular weight multimers of plasma von Willebrand factor (vWF). Platelet aggregation is induced by the addition of purified vWF or cryoprecipitate to patients' platelet-rich plasma (PRP) without the requirement for ristocetin. The defect of these disorders appears to be intrinsic to the platelet membrane that causes the absorption of vWF higher molecular weight multimers to GPlb.

We describe a patient with increased ristocetin-induced platelet aggregation, SPA, and platelet aggregation induced by high doses of purified vWF. Unlike patients with pseudo or platelet-type von Willebrand's disease, the full range of vWF multimers is present in her plasma and she also has normal vWF parameters. The defect appears to be at the level of the platelet membrane; therefore, the patient presents a previously undescribed platelet disorder.

CASE REPORT

The patient is a 49-year-old woman with a life-long mild bleeding tendency, eg, epistaxis and bleeding posttonsillectomy, postpartum, and posthysterectomy. Her father, who died of aortic rupture, also had a mild bleeding tendency. The patient's son and niece, investigated during the same study, also presented mild bleeding tendency.

MATERIALS AND METHODS

Bleeding times, platelet count, platelet size, ristocetin cofactor activity, and vWF antigen determinations were performed as previously described. All blood samples were obtained from patients and normal volunteers with their informed consent and in accordance with the Declaration of Helsinki. They had taken no medication for at least 2 weeks. PRP for aggregation and binding studies was prepared as previously described from blood collected into one tenth of 3.2% sodium citrate. Washed platelets for vWF binding studies were prepared from blood drawn into one sixth final volume of acid/citrate/dextrose, pH 4.5. Platelets were washed free of plasma constituents using the albumin density gradient technique previously described. Absorption of plasma vWF to normal platelets in the presence of ristocetin was performed as described by Ruggeri and Zimmerman.

Plasma vWF multimeric composition. Plasma was electrophoresed in a 1.4% agarose gel containing sodium dodecylsulfate, and vWF multimers were detected using a radiolabeled antibody and autoradiography as previously described. Autoradiographs were scanned with a laser densitometer (LKB, Uppsala, Sweden).

Purification of normal vWF (NvWF) and preparation of Asialo vWF (A vWF). Purified NvWF was obtained from cryoprecipitates following a previously described procedure. Ristocetin cofactor activity and multimeric composition were determined for each vWF preparation as described. Purified NvWF was treated with protease-free neuraminidase from Vibrio cholerae (Calbiochem, Behring Corp, La Jolla, CA) to prepare AS vWF as previously described.
described. Sialic acid content was measured according to Warner. Monoclonal antibodies. Monoclonal antibodies to GPIb (LJ-Ib1 and LJ-Ib10) and GPIIb/IIIa complex (LJ-CP8) were prepared and characterized as described. LJ-Ib1 is specific for GPIb and completely inhibits ristocetin-induced vWF binding to platelets and AS vWF binding. LJ-Ib1 epitope is located between residues His-1 and Ala-291 of the α-chain of GPIb. LJ-Ib10 epitope is located between residues Ala-238 and Ala-291 of the α-chain. LJ-CP8 is specific for the GPIIb/IIIa complex of stimulated or nonstimulated platelets and completely inhibits the binding of vWF, fibrinogen, and fibronectin to platelets. IgG were purified using protein A-Sepharose. Monovalent Fab' fragments from LJ-Ib1 IgG were prepared by the pepsin digestion method followed by reduction and alkylation as previously described.

Radioiodination of proteins. Radioiodination was performed with I125 (Amersham Corp, Arlington Heights, IL) using Iodogen (Pierce Chemical, Rockford, IL) according to the method of Fraker and Speck.

Binding studies. The methods used to measure the binding of radiolabeled monoclonal IgG, vWF (ristocetin-dependent binding), and AS vWF to platelets have been previously described in detail. Binding of I125-vWF was measured in washed platelets in the presence of three different concentrations of ristocetin (0.4, 1.2, and 1.6 mg/mL final concentration). Binding of radiolabeled monoclonal antibodies and I125-AS vWF was measured in citrated PRP with increasing concentrations of radiolabeled monoclonal antibodies (LJ-Ib1, LJ-Ib10, LJ-CP8, and increasing concentrations of I125-AS vWF. Binding parameters were calculated using the computer-assisted program LIGAND assuming molecular masses of 170 Kd for monoclonal antibodies and of 275 Kd for NvWF and AS vWF. Nonspecific binding was calculated as a fitted parameter.

Immunoblotting. Washed platelets were lysed in TRIS 0.02 mol/L, NaCl 0.15 mol/L, pH 7.35 containing sodium dodecylsulfate (SDS) 200 mg/mL, EDTA 10 mmol/L, NEM 5 mmol/L, and PMSF 1 mmol/L, and the lysates were treated with dithiothreitol. Immunoblotting of the platelet lysates was performed as previously described.

Platelet aggregation. Platelet aggregation was measured in a Chrono-Log Lumiaggregometer (Chrono-Log Corp, Havertown, PA). The siliconized cuvette was maintained at 37°C and the suspension was stirred at 1,200 rpm. ADP and adrenalin were from Sigma Chemical Co. Ristocetin was from Maxisc-Brunelli (Mi, Italy) and collagen from Horm-Chemie (Munich, Federal Republic of Germany).

RESULTS

Basic laboratory studies. Several sequential platelet count and bleeding times were normal in the patient, her son, and her niece. Platelet size was within the control range. The patient’s platelet aggregation by collagen, epinephrine, and adenosine diphosphate was normal (Table 1), but doses of ristocetin were as low as 0.75 mg/mL final concentration, a concentration that fails to induce aggregation in normal individuals in our laboratory, induced platelet aggregation that was greatly reduced by the prior addition of Fab’ fragment of an anti-GPIb monoclonal antibody (LJ-Ib1). The aggregation with 0.3 mg/mL of ristocetin may be accounted for by the spontaneous platelet aggregation that occurs in patients citrated PRP upon stirring in an aggregometer. vWF Ag and vWF RCo determinations were within normal limits (Table 1). The absorption of patient’s plasma vWF by normal washed platelets in the presence of various concentrations of ristocetin (ranging from 0.2 to 2.0 mg/mL) showed a curve similar to that obtained with normal plasma vWF (not shown).

Multimeric composition of plasma vWF. vWF multimeric pattern performed in the patient’s and her niece’s plasma showed that all multimers were present as in a normal control (Fig 1). Scanning analysis of the gels also showed that all vWF multimers (including the largest) were present in a proportion similar to that in normal plasma.

Spontaneous platelet aggregation. To investigate spontaneous platelet aggregation, the patient’s citrated PRP was stirred in the aggregometer cuvette at 37°C without addition of aggregating agent; 10 to 25% of aggregation was seen on

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Table 1. Main Laboratory Data of the Patient

<table>
<thead>
<tr>
<th></th>
<th>Patient</th>
<th>Normal Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet count (10⁶/mL)</td>
<td>2.76</td>
<td>1.5 - 3.5</td>
</tr>
<tr>
<td>Bleeding time (sec) (min)</td>
<td>7</td>
<td>6 ± 1.4</td>
</tr>
<tr>
<td>vWF:Ag</td>
<td>1.04</td>
<td>0.5 - 1.5</td>
</tr>
<tr>
<td>vWF:RCo</td>
<td>1.27</td>
<td>0.5 - 1.5</td>
</tr>
<tr>
<td>Platelet aggregation (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP μmol/L (final concentration)</td>
<td>73.8</td>
<td>56 ± 14</td>
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<tr>
<td>Adrenaline 10 μmol/L</td>
<td>71.6</td>
<td>60 ± 11</td>
</tr>
<tr>
<td>Collagen 1 μg/mL</td>
<td>95</td>
<td>70 ± 18</td>
</tr>
<tr>
<td>Ristocetin 1.5 mg/mL</td>
<td>89.2</td>
<td>72 ± 12</td>
</tr>
<tr>
<td>Ristocetin 0.75 mg/mL</td>
<td>70.3</td>
<td>0</td>
</tr>
<tr>
<td>Ristocetin 0.3 mg/mL</td>
<td>10-20</td>
<td>0</td>
</tr>
</tbody>
</table>

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Fig 1. Multimeric composition of plasma vWF. A normal multimeric composition was found in a normal (N), the patient (P), and her niece (G). Electrophoresis was run in an agarose gel containing sodium dodecyl sulfate; vWF multimers were detected by autoradiography after reaction with a radiolabeled antibody. The arrow at the top indicates the interface between stacking and running gels; the arrow at the bottom indicates the dye front. The cathode is at the top of the figure.
different occasions. Figure 2 shows that the spontaneous platelet aggregation was completely blocked by the addition of an anti-GPIIb/IIIa monoclonal antibody (LJ-CP8) and only marginally affected by an anti-GP Ib monoclonal antibody (Fab' fragment of LJ-Ibl). EDTA (5 mmol/L final concentration) blocked SPA completely (not shown).

Platelet aggregation induced by purified NvWF. The addition of purified NvWF (80 and 140 μg/mL final concentration) to the patient's stirring citrated PRP induced a significant platelet aggregation in a dose-dependent fashion (Fig 3). Platelet aggregation induced by the highest dose of purified NvWF (140 μg/mL) was considered as 100%, and this aggregation was completely inhibited by EDTA (5 mmol/L) and by the anti-GP Ib/IIIa monoclonal antibody (LJ-CP8) (Fig 3), whereas the Fab' fragment of the anti-GP Ib monoclonal antibody (LJ-Ibl) reduced it by 85% to 90% but did not block it completely (Fig 3). Apyrase (5 U/mL) completely blocks the second wave of aggregation induced by purified NvWF (Fig 3). Purified NvWF induced platelet aggregation also in the niece's and the son's PRP only when used at higher (>180 μg/mL) concentrations. PRP from controls did not show aggregation using concentrations of NvWF as high as 350 μg/mL.

Platelet aggregation induced by AS vWF. Complete platelet aggregation in the patient's PRP was seen using AS vWF concentration as low as 0.42 μg/mL with faster kinetics at increasing AS vWF concentrations (Fig 4). A pattern similar to the patient's was shown in her niece's PRP. In contrast, no aggregation was seen in PRP from a normal individual with 1.7 μg/mL of AS vWF (Fig 4). Concentra-

![Increased Light Transmittance](image1)

**Fig 3.** Platelet aggregation induced by N vWF: the effect of monoclonal antibodies, EDTA, and apyrase. Citrated platelet-rich plasma (final count 3.0 x 10^5 platelets/mL) from the patient was stirred in the aggregometer with the addition of (at arrow) different concentrations of NvWF, as described: Trace 1: NvWF (140 μg/mL final concentration) induced platelet aggregation in the presence of EDTA (5 mmol/L). Trace 2: NvWF (140 μg/mL) induced platelet aggregation in the presence of LJ-CP8 IgG (70 μg/mL final concentration). Trace 3: NvWF (140 μg/mL) induced platelet aggregation in the presence of LJ-Ibl Fab' fragment (135 μg/mL). Trace 4: NvWF (140 μg/mL) induced platelet aggregation in the presence of apyrase (5 ATP'-ase units/mL final concentration). Traces 5 and 6: NvWF (80 μg/mL and 140 μg/mL, respectively) induced platelet aggregation in the presence of TRIS-saline buffer, pH 7.4.

![Increased Light Transmittance](image2)

**Fig 4.** Asialo von Willebrand factor (ASvWF) induced platelet aggregation on normal and patient's platelet-rich plasma. Citrated platelet-rich plasma from a control donor and the patient (final platelet count 2.5 x 10^5 platelets/mL) were stirred in the aggregometer with the addition (at arrow) of different concentrations of AS vWF, as described: Trace 1: AS vWF (1.7 μg/mL final concentration) induced platelet aggregation of normal PRP. Trace 2: AS vWF (1.7 μg/mL final concentration) induced platelet aggregation of patient's PRP in the presence of LJ-Ibl Fab' fragment (137 μg/mL). Traces 3, 4, and 5: AS vWF (0.22, 0.42, and 1.7 μg/mL, respectively) induced platelet aggregation of patient's PRP.
A NEW CONGENITAL PLATELET ABNORMALITY

Table 2. Binding of Monoclonal Antibodies to Normal and Patient’s Platelets

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Normal (kd/mol/L)</th>
<th>Normal (mol/PLT)</th>
<th>Patient (kd/mol/L)</th>
<th>Patient (mol/PLT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LJ-IB1</td>
<td>1.15 x 10^{-7}</td>
<td>0.37 x 10^{-7}</td>
<td>1.18 x 10^{-7}</td>
<td>0.53 x 10^{-7}</td>
</tr>
<tr>
<td>LJ-IB10</td>
<td>0.16 x 10^{-7}</td>
<td>0.16 x 10^{-7}</td>
<td>0.29 x 10^{-7}</td>
<td>0.29 x 10^{-7}</td>
</tr>
<tr>
<td>LJ-CP8</td>
<td>35,100 ± 3,600</td>
<td>34,200 ± 3,800</td>
<td>31,700 ± 0.02</td>
<td>31,800 ± 0.02</td>
</tr>
</tbody>
</table>

Table 3. Ristocetin-Induced vWF Binding to Normal and Patient’s Platelets

<table>
<thead>
<tr>
<th>Ristocetin (mg/mL, final concentrations)</th>
<th>0.4</th>
<th>1.2</th>
<th>1.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>0.40 x 10^{-7} ± 0.02</td>
<td>0.35 x 10^{-7} ± 0.03</td>
<td>0.21 x 10^{-7} ± 0.02</td>
</tr>
<tr>
<td>Normal</td>
<td>31,800 ± 2,400</td>
<td>35,100 ± 4,300</td>
<td>31,700 ± 3,900</td>
</tr>
</tbody>
</table>

DISCUSSION

The patient reported in this study shows platelet aggregation induced by concentrations of ristocetin lower than those necessary to induce aggregation in normal controls. Moreover, platelet aggregation is initiated by the addition of purified NvWF. Unlike patients with pseudo or platelet-type von Willebrand’s disease, whose plasma vWF lacks the higher molecular weight multimers and who have a low ristocetin cofactor, our patient has a normal plasma vWF multimeric structure and a normal ristocetin cofactor. Therefore, the patient may represent a variant of the platelet-type or pseudo von Willebrand’s disease group.

Several findings distinguish a recently described subgroup of von Willebrand’s disease from the patient reported in the present paper. Ristocetin cofactor activity and vWF absorbance to normal platelets were both normal in our patient, whereas a low ristocetin cofactor activity and increased adsorbance of plasma vWF to normal platelets was seen in the aforementioned patients. Furthermore, platelet aggregation occurred in our patient’s PRP upon addition of large doses of purified vWF and in the absence of ristocetin, which suggests a platelet membrane rather than a plasmatic abnormality.

The inhibitory effect of monoclonal antibodies against GPIb and the GPIIb/IIIa complex on platelet aggregation induced by purified vWF demonstrates that both receptors are involved in this phenomenon. Furthermore, the inhibitory effect of EDTA on platelet aggregation demonstrates that the aggregation response depends on divalent cations. These results are similar to those reported in the literature for patients with platelet-type von Willebrand’s disease. In our patient apyrase abolished the second wave of aggregation, suggesting that endogenous ADP is an absolute requirement for platelet aggregation when the inducer acts as a weak
agonist, as it was postulated for IIB vWF induced platelet aggregation, in contrast to the previously reported data by Miller et al. Our findings suggest that the mechanism of vWF-induced aggregation in our patient may follow the pattern reported for AS vWF and IIB vWF-induced platelet aggregation. vWF bound to the GPIb of our patient’s platelets and this binding was followed by the release of ADP and subsequent exposure of the GPIb/IIIa complex to which vWF and/or fibrinogen bound, triggering the aggregation.

Spontaneous platelet aggregation has been previously reported only in one family with platelet-type vWD, in few patients with type IIB vWD, and in a congenital bleeding disorder called Montreal syndrome. Spontaneous platelet aggregation in our patient was blocked only by the anti-GPIb monoclonal antibody. A possible explanation for these results is that some platelet GPIb molecules have already vWF bound that is not displaceable by the anti-GPIb monoclonal antibody known to block binding of vWF to GPIb. Occupancy of few GPIb molecules with vWF may be sufficient in our experimental conditions and upon stirring to induce the exposure of GPIb/IIIa that triggers spontaneous platelet aggregation.

The results obtained with the asialo vWF-induced platelet aggregation also showed an enhanced interaction of vWF with the patient’s platelets. These results are similar to those recently reported by Miller et al.

Unlike other investigators, we were unable to detect an increased binding or an altered affinity of monoclonal antibodies against GPIb. We reproducibly observed a slightly faster than normal migrating GPIb band in the patient’s platelets, suggesting a molecular or carbohydrate abnormality or defect, which appears to be different from that reported in two families with platelet-type von Willebrand’s disease.

The data on the binding studies showed a 30% increased vWF bound to platelets, which correlates with the enhanced aggregation observed with low concentrations of ristocetin and with asialo vWF.

Our studies suggest that a congenital structural change in or near the platelet binding site for vWF might be responsible for the platelet abnormalities seen in our patient. As already postulated, it is possible that occupancy of the vWF receptor on GPIb in circulating platelets results in impaired adhesive properties as a consequence of the inability of the occupied receptor to interact with subendothelial vWF at sites of injury. The lack of thrombotic episodes in this patient, as well as in IIB vWD patients, even after DDAVP (1-Desamino-8-D-arginine-vasopressin) infusion (which means appearance of large vWF multimers in blood), signifies that formation of stable occluding thrombi requires a greater degree of activation than that achieved following occupancy of GPIb-vWF receptors in circulating platelets.

REFERENCES

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20. Fraker DJ, Speck JC: Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a, 6a-diphenylglycoluril. Biochem Biophys Res Commun 80:849, 1978


A new congenital platelet abnormality characterized by spontaneous platelet aggregation, enhanced von Willebrand factor platelet interaction, and the presence of all von Willebrand factor multimers in plasma

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