Subunit Composition of Plasma von Willebrand Factor in Patients With the Myeloproliferative Syndrome


In order to evaluate the role of proteolysis in acquired von Willebrand's disease (vWD) associated with the myeloproliferative syndrome, we have determined the relative quantity of von Willebrand factor (vWF) fragments as compared with the intact 225 kDa subunit in four patients. The plasma vWF of each individual lacked large multimers; each had a prolonged bleeding time; and both platelet and leukocyte counts were elevated. Plasma was obtained from blood drawn into 1 mmol/L leupeptin, 6 mmol/L N-ethylmaleimide, and 5 mmol/L EDTA to prevent in vitro proteolysis. vWF was isolated from plasma by immunoadsorbent chromatography, reduced, subjected to SDS-5% polyacrylamide gel electrophoresis, and immunoblotted with a mixture of 55 anti-vWF monoclonal antibodies. In three patients with essential thrombocytosis (ET) the 176 and 140 kDa fragments were increased in proportion to the intact 225 kDa subunit indicating increased proteolysis. Treatment of one ET patient with CCNU (Lomustine) decreased the platelet count and, to a lesser extent, the white blood cell count. This was associated with a correction of the bleeding time, a partial correction of the multimeric abnormality, and a lessening of vWF cleavage. In a patient with polycythemia rubra vera (PRV) the proportion of the 176 kDa fragment was increased to the upper limit of normal but there was no definite evidence of increased proteolysis. These studies provide evidence that proteolysis plays a role in the acquired von Willebrand's disease associated with the myeloproliferative syndrome. However, other mechanisms must also be considered.

ACQUIRED von Willebrand's disease (vWD) has been described recently in patients with the myeloproliferative syndrome and high leukocyte and platelet counts.1 2 The multimeric pattern of these patients' von Willebrand factor (vWF) resembled that of patients with inherited Type II vWD in that the largest and sometimes the intermediate multimers were missing.3 As in normal plasma, each multimer could be resolved into three or more bands. The multimeric patterns of most patients showed a relative increase of the fastest moving band in each multimer, similar to IIA and IIB vWD. Normalization of the platelet and leukocyte count usually led to a progressive normalization of the multimeric pattern. In several patients, however, some abnormalities were still detectable when platelet counts were normal.

We have recently studied the proportion of intact vWF subunit, as compared to smaller proteolytic fragments in inherited Type II vWD variants.4 Evidence for increased in vivo proteolysis, as compared to that of normal plasma vWF, was found in Types IIA and IIB vWD. On the other hand, little or no proteolysis could be detected in Types IIC, IID, and IIE even though large multimers were also missing from plasma in these variants. In order to determine if proteolysis might contribute to the acquired absence of large vWF multimers seen in the myeloproliferative syndrome, we have examined the subunit composition of vWF from four patients in whom the large multimers were absent. Increased proteolysis was demonstrated in three individuals with essential thrombocytosis (ET), whereas no definite evidence of increased proteolysis could be detected in a fourth patient with polycythemia rubra vera (PRV). These results suggest not only that proteolysis participates in the pathogenesis of acquired vWD in association with the myeloproliferative disorder, but also that additional mechanisms should be considered.

MATERIALS AND METHODS

Reagents. Leupeptin was acquired from Chemicon (El Segundo, Calif); N-ethylmaleimide (NEM) from Sigma Chemical Co. (St. Louis, Mo); electrophoresis pure reagents from Bio-Rad (Richard- mond, Calif); rabbit antiserum IgG was from Zymed (San Francisco, Calif). All other reagents were of the highest grade available.

Patients. Patients studied included three women (Patients 1–3) with essential thrombocytosis (ET), aged 26, 45, and 69 years, and one woman (Patient 4) with polycythemia rubra vera (PRV), aged 69 years. Clinical laboratory data is presented in Table I. Factor VIII, vWF:Ag, ristocetin cofactor activity (vWF:RCO), and bleeding times were determined as described.5

Preparation of plasma samples. Blood was drawn from a large antecubital vein. The blood was collected in polypropylene syringes with 1/10th final volume of 3.8% sodium citrate containing sufficient leupeptin, N-ethylmaleimide (NEM), and EDTA to give final concentrations in the blood of 1.0 mmol/L, 6 mmol/L, and 5 mmol/L, respectively. Platelet-poor plasma was obtained by immediate centrifugation at 3,200 rpm (approximately 2,700 g) for 20 minutes at 23 °C. The plasma was rapidly separated from the cells and stored at −70 °C. Platelet-rich plasma was prepared by immediate centrifugation at 2,200 rpm (approximately 1,100 g) for 75 seconds at 23 °C × 3, with aspiration and pooling of the platelet-rich plasma after each spin.

Immunosolization of plasma vWF. vWF was immunosolized by incubation of 1 to 5 mL of plasma with 1 to 2.5 mL of agarose beads covalently linked with anti-vWF monoclonal antibody, as described.6 After extensive washing, the vWF was eluted with 2 mL of 2% SDS, 0.1 mol/L Tris (pH 8.0) at 60 °C, and the eluate was concentrated using Amicon Centricron 30 microconcentrators to a final volume of approximately 100 μL.

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Table 1. Clinical Data of the Four Patients With Myeloproliferative Disease Included in the Study

<table>
<thead>
<tr>
<th></th>
<th>1 (ET)</th>
<th>2 (ET)</th>
<th>30’ Post DDAP</th>
<th>4 (ET)</th>
<th>4 (PRV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4/10/85*</td>
<td>4/17/85</td>
<td>5/8/85</td>
<td>Pre DDAP</td>
<td>2,760</td>
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<tr>
<td>Platelet count (x 10^3/mL)</td>
<td>2,240</td>
<td>1,960</td>
<td>348</td>
<td>2,745</td>
<td>388</td>
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<tr>
<td>WBC (x 10^9/mL)</td>
<td>19.2</td>
<td>21.5</td>
<td>16.9</td>
<td>13.2</td>
<td>38.8</td>
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<tr>
<td>RBC (x 10^12/mL)</td>
<td>5.64</td>
<td>5.53</td>
<td>5.49</td>
<td>4.10</td>
<td>5.02</td>
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<tr>
<td>Hct</td>
<td>37.7</td>
<td>36.7</td>
<td>36.4</td>
<td>38.9</td>
<td>38.8</td>
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<tr>
<td>Bleeding time (min)</td>
<td>&gt;15</td>
<td>&gt;15</td>
<td>8</td>
<td>10</td>
<td>5.5</td>
</tr>
<tr>
<td>Factor VIII (U/dL)</td>
<td>92</td>
<td>110</td>
<td>176</td>
<td>74</td>
<td>225</td>
</tr>
<tr>
<td>vWF:Ag (U/dL)</td>
<td>112</td>
<td>84</td>
<td>171</td>
<td>52</td>
<td>215</td>
</tr>
<tr>
<td>vWF:RCo (U/dL)</td>
<td>64</td>
<td>42</td>
<td>166</td>
<td>54</td>
<td>138</td>
</tr>
</tbody>
</table>

Abbrev: essential thrombocytosis, ET; polycythemia rubra vera, PRV.

CCNU (Lomustine) 160 mg given PO.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. SDS-5% polyacrylamide gels were prepared according to Laemmli. The vWF was reduced by dithiothreitol (final concentration 65 mmol/L) for 15 minutes at 60°C in pH 7.0 polyacrylamide gel sample buffer. Following SDS-5% polyacrylamide electrophoresis the samples were transferred to nitrocellulose and reacted with a pool of 55 anti-vWF monoclonal antibodies, all of which reacted with the reduced 225 kDa subunit. The nitrocellulose was then reacted with 125I-rabbit antimouse IgG antibody. The relative concentrations of the intact vWF subunit and its proteolytic fragments were estimated by identifying each band by autoradiography and then excising each band and counting them in a Packard Auto-Gamma Scintillation Spectrometer, United Technologies Instrument Co, Downer’s Grove, IL. The plasma from each patient was processed on four separate occasions and the means and ranges of counts were determined from these combined analyses.

RESULTS

Prior to therapy in all four patients, large vWF multimers were absent from plasma (Figs 1 and 2). In the three ET patients (patients 1, 2, and 3), the fastest moving band in each multimer was relatively increased. In the PRV patient (patient 4), the relative concentration of each band within a given multimer was similar to that of normal vWF.

In order to show that the presence or absence of cells in plasma did not affect the relative concentration of the vWF fragments, the following experiments were performed. vWF was immunopurified from plasma prepared by centrifugation of blood at 2,700 g for 15 minutes prior to freezing and from plasma that had been recentrifuged at 100,000 g prior to freezing. Comparison of the relative concentration of fragments showed no differences. Similarly, vWF was immunopurified from platelet-rich plasma that had been incubated for 18 hours at 37°C and compared with vWF from plasma that had been doubly centrifuged as above and then similarly incubated. Again no differences were seen in the relative concentration of fragments.

Evaluation of reduced vWF immunopurified from patient plasmas showed that the relative proportions of the 176 and 140 kDa polypeptides were significantly increased in patients 1, 2, and 3, and that of the intact 225 kDa subunit decreased (Figs 3 and 4). In patient 4, the proportions of the subunit and fragments were within the normal range, although the 176 kDa fragment was at the upper limits of normal.
Patient 1 was treated with CCNU (Lomustine), following which the platelet count and bleeding time decreased to normal. The leukocyte count was also reduced but remained above normal (Table 1). These changes were accompanied by a correction of the bleeding time and partial normalization of the plasma vWF multimeric structure (Figs 1 and 2). Intermediate-sized multimers returned, although not in their usual quantity. The relative proportion of bands within each multimer also became more similar to that of normal vWF. These changes were associated with a partial return toward normal of the proportion of fragments in relation to the intact subunit (Figs 3 and 4).

1-desamino-8-D-arginine vasopressin (DDAVP) was administered to patient 2. This was followed by a shortening of the bleeding time from ten minutes to 5.5 minutes, a more than twofold increase in vWF:RCo, a threefold increase in Factor VIII, and a fourfold increase in vWF:Ag (Table 1). The largest multimers returned transiently. However, there was no correction of the relative proportions of the bands within each multimer nor was there a reduction in the proportion of vWF fragments in comparison with the intact subunit (Figs 3 and 4).

DISCUSSION

The participation of in vivo proteolysis in the pathogenesis of some forms of inherited vWF has been suggested by two recent studies. Gralnick and coworkers provided evidence that Type IIA vWF was more susceptible to proteolysis than normal vWF. They, as well as Batlle and colleagues, showed that in vitro loss of intermediate-sized multimers could be prevented in some patients by including NEM, leupeptin,
and EDTA in the anticoagulant into which blood was collected. In unpublished studies, we have shown that in vitro generation of vWF fragments can also be suppressed by these inhibitors. Evidence for increased in vivo proteolysis of IIA and IIB vWF derived from studies of Zimmerman et al, who showed increased proportions of the 176 and 140 kDa fragments relative to the intact 225 kDa subunit in these two disorders. On the other hand, little or no proteolysis of the 225 kDa subunit could be demonstrated in Types IIC, IID, and IIE vWD.

Types IIA and IIB vWD have in common with the three essential thrombocytosis patients a similar alteration in the relative proportion of bands that comprise each multimer. In both the inherited vWD subtypes, as well as in the acquired form exhibited by these three patients, there was a relative increase in the fastest moving band in each multimer. This was associated in each case with evidence of increased proteolytic cleavage of vWF, as evidenced by increased proportions of the 176 and 140 kDa species. In contrast to Types IIA and IIB, there was no decrease in the relative quantity of the 189 kDa polypeptide.

Patient 4 differed from the others in that she had polycythemia rubra vera and did not show evidence of increased proteolysis of the vWF subunit, even though large multimers were absent from plasma. She also did not show the alteration in relative concentration of bands comprising each multimer. It is possible that the technique used for demonstrating increased proteolysis was not sufficiently sensitive to detect an abnormality in this case. Alternatively, the vWF abnormality in this patient may have a different basis from that of patients 1, 2, and 3.

The source and nature of the enzyme(s) responsible for vWF proteolysis in patients 1, 2, and 3 is unknown at present. Kunicki and coworkers have shown that the platelet calcium-activated protease can cleave vWF with resultant loss of large multimers. Although each of the patients studied above had markedly elevated platelet counts initially, correction of the platelet count in patient 1 after treatment with CCNU was associated with only a partial diminution of the proteolytic and multimeric abnormalities. In addition, incubation of the vWF present in platelet-rich plasma produced no increase in proteolysis compared to acellular plasma. Moreover, the white blood count was also elevated in each of these patients and leukocytes are known to be the source of a number of enzymes with the potential of cleaving vWF.

In summary, absence of large vWF multimers and alteration in the relative concentration of the bands comprising the multimers can be an acquired abnormality that may accompany the myeloproliferative syndrome. Proteolysis appears to contribute to this structural abnormality, although other causes may be operative.

REFERENCES

disease, but minimal in variants with aberrant structure of individual oligomers (Types IIC, IID and IIE). J Clin Invest 77:947, 1986
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U Budde, JA Dent, SD Berkowitz, ZM Ruggeri and TS Zimmerman