ORIGINAL ARTICLE

Plasma von Willebrand Factor Abnormalities in Patients with Essential Thrombocythemia

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Abstract

Plasma von Willebrand factor (vWF) in eleven patients with essential thrombocythemia was studied quantitatively and qualitatively to assess its role in the development of bleeding tendency. Five patients with prolonged bleeding time including three with clinical bleeding tendencies showed decreased ristocetin cofactor (VIIIIR:RCo) and factor VIII-related antigen (VIIIIR:Ag). On the other hand, six patients without prolongation of bleeding time had normal VIIIIR:RCo and VIIIIR:Ag. Analysis of vWF multimeric structures by SDS-agarose gel electrophoresis revealed lack of the larger multimers in the former group of patients. These abnormalities of plasma vWF were corrected when platelet counts became nearly normal by treatment with busulfan. Infusion of 1-diamino-8-Arginin-Vasopressin improved all the hemostatic abnormalities except for defective platelet aggregation seen in patients with essential thrombocythemia. These results indicate that qualitative and quantitative abnormality of vWF is the main causative factor in development of bleeding tendency in patients with essential thrombocythemia.

Key words: essential thrombocythemia, von Willebrand factor, SDS agarose gel electrophoresis, bleeding tendency
Introduction

It is well known that in spite of marked thrombocytosis, bleeding tendency is often one of the main clinical features in patients with essential thrombocythemia. However, the cause of bleeding tendency in this disorder has not been clarified yet. There have been a number of reports regarding to the qualitative platelet defects in patients with essential thrombocythemia. When platelet aggregation was measured \textit{in vitro} in the aggregometer, lack of response to epinephrine was most often observed and lesser degree to ADP and collagen.\textsuperscript{1} Arachidonic acid metabolism, which plays an important role in platelet activation, has been also extensively studied by several investigators. While oxygenation of arachidonate by cyclooxygenase was intact, lipoxygenase-catalysed oxygenation was found to be defective in platelets from patients with essential thrombocythemia.\textsuperscript{2,3} Alterations of platelet surface membranes were reported including decreased $\alpha$-adrenergic\textsuperscript{4} and prostaglandin D\textsubscript{2} receptors,\textsuperscript{5} and increased expression of Fc receptors.\textsuperscript{6}

However, none of these studies could demonstrate the clear causal relationship between the qualitative platelet abnormalities and bleeding tendencies.

Von Willebrand disease is a hereditary bleeding disorder characterized by a prolonged bleeding time and qualitative or quantitative abnormality of plasma von Willebrand factor (vWF), which is essential for platelets to adhere to subendothelium of vessel walls. This hereditary disorder is mostly inherited as autosomal dominant form.

Recently, similar hemostatic alterations have been reported to occur in patients with systemic lupus erythematosus,\textsuperscript{7} monoclonal gammopathy,\textsuperscript{8} lymphoproliferative disorders\textsuperscript{9} without apparent family history of bleeding tendencies. This clinical conditions are currently called acquired von Willebrand disease.

In this study, plasma vWF was analysed quantitatively and qualitatively in patients with essential thrombocythemia and it was demonstrated that the acquired von Willebrand disease was often associated with this disorder to be the main cause of bleeding tendencies.

Materials and Methods

Patients

Eleven patients aged from 29 to 72 were investigated. Among them five patients were male and six were female. The diagnosis of essential thrombocythemia was established based upon the following criteria of Murphy;\textsuperscript{10} platelet count of more than 1,000,000/μl, absence of Philadelphia chromosome, normal neutrophil alkali-phosphatase and no identifiable causes to induce thrombocytosis such as malignancy, infection, chronic inflammatory disease and previous history of splenectomy.
Preparation of platelet rich plasma and platelet suspension

Platelet rich plasma (PRP) was obtained by centrifugation at 50 g for 15 min from whole blood anticoagulated with one-tenth volume of 3.8% trisodium citrate. Platelet suspension was prepared as follows. PRP mixed with 15% volume of acid citrate dextrose (ACD) was centrifuged at 1,000 g for 10 min, and sedimented pellets were washed twice with modified Tyrode buffer (pH 7.4). Platelets were finally re-suspended in the same buffer at a concentration indicated.

Platelet aggregation

Platelet aggregation was measured turbidimetrically using PRP according to the method of Born in an aggregometer (Scienco Co. USA). Platelet counts of PRP were adjusted to 300,000/μl by diluting the original PRP with the patient's own plasma. ADP (Sigma Co. USA), epinephrine (Daiichi Pharmaceutical Co. Japan) and collagen (Holm Chemi. West Germany) were used as aggregating agents.

Assay of von Willebrand factor

Ristocetin cofactor (VIIIIR:RCo) was assayed according to the method of Weiss with a slight modification. Platelet suspensions prepared from normal individuals were mixed with patient plasma at a ratio of 2:1 and the extent of ristocetin (1.2 mg/ml) induced platelet agglutination was determined. Ristocetin was a product of Lundbeck, Sweden.

Factor VIII-related antigen (VIIIIR:Ag) was determined immunologically by the method of Laurell.

Analysis of the multimeric structures of plasma and platelet vWF

The multimeric structures of plasma and platelet vWF were analyzed by thin layer sodium dodecyl sulphate (SDS) agarose gel electrophoresis according to the method of Ruggeri and Zimmerman. Plasma from patients or normal individuals was diluted by 20 times with sample buffer (0.01 M Tris-HCl, 0.001 M EDTA, 2% SDS, 8 M urea, pH 7.4) before it was applied to the agarose gel. Samples were also prepared for analysis of platelet vWF. PRP was obtained from whole blood mixed with one tenth volume of a solution containing 3.8% trisodium citrate, 10 mM leupeptin, 50 mM EDTA and 60 mM N-ethylmalaimide. Platelets were washed twice with modified Tyrode buffer, pH 7.4, containing 0.38% trisodium citrate, 0.1 mM leupeptin, 6 mM N-ethylmalaimide, and finally resuspended in the same buffer. These platelet suspensions were lysed with 10 times volume of sample buffer for electrophoresis. All samples were run at constant current of 20 mA until the samples reached the running gel and then at 10 mA until the tracking dye reached the end of the gel. vWF multimers were identified by exposing the dried gels to 125I-labelled affinity-purified antibodies to human vWF (a generous gift of Dr. T. S. Zimmerman) followed by autoradiography.
Analysis of platelet membrane glycoproteins

Platelets membrane glycoproteins were surface-labelled with neuraminidase/galactose oxidase/[3H]NaHB₄ labelling technique according to the method of Nurden et al.,¹⁶ and were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

vWF binding to platelets

A commercial Factor VIII concentrate, Haemate P (Hoechst Co. Japan), was precipitated with freshly prepared barium sulfate (0.5 M) to remove contaminating Factor II, VII, IX and X for 15 min at room temperature. After centrifugation at 1,000 g for 10 min, the supernatants were applied to Sepharose 4B column equilibrated with Owren's buffer. The void volume fractions containing proteins with ristocetin cofactor activity were pooled and concentrated. vWF thus purified was labelled with ¹²⁵I by lactoperoxidase oxidation technique.¹⁷

Platelet suspensions were incubated with ¹²⁵I-vWF (5 μg/ml) in the presence of ristocetin (1.2 mg/ml) for 3 min at room temperature. After incubation, platelets were separated by centrifugation at 7,000 g for 1 min through silicone oil, and the radioactivity of platelet pellets was measured by γ-counter. To determine non-specific binding, excess amount of non-labelled vWF was added prior to centrifugation. Specific binding was calculated by subtracting non-specific binding from total binding.

Ristocetin-induced platelet agglutination (RIPA)

RIPA was measured in an aggregometer using PRP under the following experimental conditions. The extent of maximum agglutination was determined in the presence of 1.2 mg/ml of ristocetin. (1) PRP isolated from healthy individuals or patients was diluted with their own plasma to adjust the platelet count to 300,000/μl. (2) PRP was mixed with 15% volume of ACD followed by centrifugation at 1,000 g for 10 min. After decanting the supernatants, normal or patient's platelets were resuspended in patient's or normal plasma, respectively, with the platelet count of 300,000/μl. (3) Fifty μl of buffer, normal or patient's plasma was incubated with 25 μl of monoclonal antibody against platelet membrane glycoprotein Ib (GPIb) (60 μg/ml) for 5 min at 37°C. Twenty five μl of this mixture was added to 200 μl of normal PRP before measuring RIPA. Monoclonal antibody was kindly provided by Dr. M. Handa, Scripps Research Institute, USA.

Assay of glycocalcin in plasma

Glycocalcin in plasma was measured by the competitive inhibition assay according to the modified method of Coller.¹⁸ Fifty μl of buffer, normal or patient's plasma was incubated with 10 μl of anti-GPIb monoclonal antibody (3 μg/ml) for 30 min at 37°C.
To the microtiter wells coated with purified GPIb, fifty μl of the above mixture was then added. Monoclonal antibody was quantified by enzyme-linked immunosorbent assay (ELISA) using goat peroxidase-conjugated anti-mouse IgG antibody. A hundred μl of O-phenylenediamine in citrate phosphate buffer containing 0.03% hydrogen peroxide, pH 5, was used as a substrate. After incubation for 30 min, optical density was measured at 395 nm.

The results were expressed by the following formula:

\[
\text{% Change} = \frac{\text{OD in buffer wells} - \text{OD in sample wells}}{\text{OD in buffer wells}} \times 100
\]

Factor VIII coagulant activity (VIII:C) was determined by the extent of correction of a kaolin activated partial thromboplastin time of severe hemophilia A by adding patient's plasma.

Bleeding time was measured by the template method of Mielke.

1-Diamino-8-D-Arginin-Vasopressin (DDAVP) (Kyowa Hakko, Japan) was administered intravenously at a dose of 0.4 μg/kg body weight diluted with 100 ml of normal saline over a 30 min period.

Results

The clinical data of eleven patients with essential thrombocythemia were summarized in Table 1. Among eleven patients investigated, three patients had the apparent clinical bleeding tendency. Patient □□□□ and □□□□ showed excessive and prolonged bleeding after dental extraction, and patient K. F. frequently showed epistaxis. On the other hand, four patients had a history of thrombotic events. There were no patients who had both bleeding and thrombotic complications. Bleeding time was prolonged in five patients including three patients mentioned above, who had clinical bleeding tendency. On the other hand, the other six patients including four patients with a history of thrombosis had normal bleeding time.

The assay of vWF and VIII:C was performed in all patients and the results were shown in (Fig. 1).

Both plasma VIIIIR:RCo and VIIIIR:Ag were decreased in five patients who had prolonged bleeding time (bleeders). On the other hand, they were normal in six patients who had normal bleeding time (non-bleeders). In bleeders the mean values of plasma VIIIIR:RCo and VIIIIR:Ag were 42% and 55%, respectively, while 90% and 125%, respectively, in non-bleeders. The mean value of VIII:C was within normal range both in bleeders and non-bleeders, although it was lower in bleeders than in non-bleeders.

In nine patients (three bleeders and six non-bleeders), plasma vWF multimeric structures were analysed by SDS-agarose gel electrophoresis. All of three bleeders...
Table 1  Clinical Data of Eleven Patients with Essential Thrombocythemia

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Hb (g/dl)</th>
<th>WBC (×10^3/μl)</th>
<th>PLT</th>
<th>NAP</th>
<th>Splenomegaly</th>
<th>Ph 1</th>
<th>Bleeding Time IVY</th>
<th>VIII: Ag</th>
<th>VIII: RCo</th>
<th>RIPA</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>1.</td>
<td>47</td>
<td>F</td>
<td>10.8</td>
<td>16900</td>
<td>222</td>
<td>290</td>
<td>(−)</td>
<td>(−)</td>
<td>13.5 min.</td>
<td>66%</td>
<td>54%</td>
<td>62%</td>
<td>16%</td>
</tr>
<tr>
<td>2.</td>
<td>66</td>
<td>M</td>
<td>14.0</td>
<td>8400</td>
<td>126</td>
<td>192</td>
<td>(−)</td>
<td>(−)</td>
<td>4.5</td>
<td>130</td>
<td>85</td>
<td>114</td>
<td>0</td>
</tr>
<tr>
<td>3.</td>
<td>32</td>
<td>F</td>
<td>13.0</td>
<td>9100</td>
<td>160</td>
<td>N.D.</td>
<td>(−)</td>
<td>(−)</td>
<td>10.5</td>
<td>64</td>
<td>44</td>
<td>54</td>
<td>0</td>
</tr>
<tr>
<td>4.</td>
<td>48</td>
<td>F</td>
<td>12.7</td>
<td>11200</td>
<td>149</td>
<td>N.D.</td>
<td>(−)</td>
<td>(−)</td>
<td>12.0</td>
<td>110</td>
<td>49</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>5.</td>
<td>54</td>
<td>M</td>
<td>13.0</td>
<td>7800</td>
<td>114</td>
<td>N.D.</td>
<td>(−)</td>
<td>(−)</td>
<td>3.5</td>
<td>164</td>
<td>125</td>
<td>82</td>
<td>0</td>
</tr>
<tr>
<td>6.</td>
<td>74</td>
<td>F</td>
<td>11.1</td>
<td>6300</td>
<td>108</td>
<td>156</td>
<td>(−)</td>
<td>(−)</td>
<td>6.5</td>
<td>150</td>
<td>190</td>
<td>132</td>
<td>0</td>
</tr>
<tr>
<td>7.</td>
<td>51</td>
<td>M</td>
<td>16.2</td>
<td>9900</td>
<td>170</td>
<td>306</td>
<td>(−)</td>
<td>(−)</td>
<td>4.0</td>
<td>125</td>
<td>115</td>
<td>130</td>
<td>0</td>
</tr>
<tr>
<td>8.</td>
<td>55</td>
<td>F</td>
<td>16.9</td>
<td>13900</td>
<td>172</td>
<td>258</td>
<td>(−)</td>
<td>(−)</td>
<td>2.0</td>
<td>176</td>
<td>115</td>
<td>120</td>
<td>0</td>
</tr>
<tr>
<td>9.</td>
<td>29</td>
<td>F</td>
<td>14.1</td>
<td>6900</td>
<td>195</td>
<td>243</td>
<td>(−)</td>
<td>(−)</td>
<td>19.5</td>
<td>135</td>
<td>75</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>10.</td>
<td>57</td>
<td>F</td>
<td>10.2</td>
<td>16200</td>
<td>479</td>
<td>148</td>
<td>(−)</td>
<td>(−)</td>
<td>over 15.0</td>
<td>68</td>
<td>55</td>
<td>34</td>
<td>6</td>
</tr>
<tr>
<td>11.</td>
<td>33</td>
<td>M</td>
<td>13.0</td>
<td>7400</td>
<td>105</td>
<td>264</td>
<td>(−)</td>
<td>(−)</td>
<td>3.5</td>
<td>106</td>
<td>86</td>
<td>102</td>
<td>8</td>
</tr>
</tbody>
</table>

WVF in Essential Thrombocythemia
showed abnormal electrophoretic patterns of vWF multimers. vWF larger multimers were absent in the patient (case ?) who had prominent bleeding tendencies. In case A.I. and ?, vWF larger multimers were found to be present, but much decreased in quantities. All of non-bleeders revealed normal vWF multimeric structures (Fig. 2).

In two patients (case and ?), severity of bleeding tendencies and hemostatic examinations before and after busulfan therapy were evaluated to see whether these hemostatic abnormalities were acquired in nature or not (Table 2).

Before treatment, both patients had bleeding tendencies with prolonged bleeding time, prominent thrombocytosis, decreased VIIIR:RCo and VIIIR:Ag and defective epinephrine- and collagen-induced platelet aggregation. When platelet counts decreased after treatment with busulfan, VIIIR:RCo and VIIIR:Ag returned to normal accompanied by shortening of bleeding time and disappearance of bleeding tendencies. Although ADP and collagen-induced platelet aggregation became normal after treatment in case ?, platelet aggregation induced by epinephrine remained impaired even after treatment. In case ?, platelet aggregation induced by three different agonists continued to be defective during treatment.
61 vWF in Essential Thrombocythemia

Fig. 2 Plasma vWF multimeric structures of nine patients with essential thrombocythemia. Plasma vWF was separated by SDS-agarose gel electrophoresis using 1.4% low gelling temperature agarose followed by autoradiography as described in the method. Fluorography of plasma vWF multimers was shown. C indicates control individuals and * indicates patients with prolonged bleeding time.

Table 2 Hemostatic Examinations before and after Treatment with Busulfan in Two Patients with Essential Thrombocythemia

<table>
<thead>
<tr>
<th>Case</th>
<th>Before</th>
<th>After</th>
<th>Case</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleeding Time (min.)</td>
<td>15&lt;</td>
<td>5.3</td>
<td>Bleeding Time (min.)</td>
<td>13.5</td>
<td>7.0</td>
</tr>
<tr>
<td>Plt. count (10⁴/µl)</td>
<td>245</td>
<td>72</td>
<td>Plt. count (10⁴/µl)</td>
<td>222</td>
<td>28</td>
</tr>
<tr>
<td>VIII: C (%)</td>
<td>135</td>
<td>160</td>
<td>VIII: C (%)</td>
<td>66</td>
<td>200</td>
</tr>
<tr>
<td>VIII R: Ag (%)</td>
<td>75</td>
<td>135</td>
<td>VIII R: Ag (%)</td>
<td>54</td>
<td>140</td>
</tr>
<tr>
<td>VIII R: RCo (%)</td>
<td>34</td>
<td>95</td>
<td>VIII R: RCo (%)</td>
<td>62</td>
<td>146</td>
</tr>
<tr>
<td>Plt. Aggregation (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epinephrine (1 µg/ml)</td>
<td>0</td>
<td>0</td>
<td>Epinephrine (1 µg/ml)</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>ADP (4 µM)</td>
<td>26</td>
<td>90</td>
<td>ADP (4 µM)</td>
<td>37</td>
<td>50</td>
</tr>
<tr>
<td>Collagen (2 µg/ml)</td>
<td>6</td>
<td>80</td>
<td>Collagen (2 µg/ml)</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

Busulfan was discontinued one week prior to the hemostatic examinations. After treatment with busulfan, bleeding tendency disappeared clinically.
Fig. 3 Change of multimeric structures of plasma vWF during the course of illness (case ). Analysis of vWF multimers was performed with 1.4% low gelling temperature agarose gel as described in the method. Samples were obtained before and after treatment with busulfan. Platelet count was $222 \times 10^4$ and $50 \times 10^4/\mu l$, respectively.

In case , multimeric structures of plasma vWF were also analyzed before and after treatment. Lack of vWF larger multimers was observed before treatment, while vWF multimeric structures were normal after treatment (Fig. 3).

To see the correlations between platelet counts and VIIIIR:RCo, these two parameters were measured simultaneously during the course of illness in all patients and were plotted in (Fig. 4a and Fig. 4b). Figure 4a represented the patients who had prolonged bleeding time, while the data from patients with normal bleeding time were shown in (Fig. 4b). In patients with prolonged bleeding time, significant inverse correlations were observed between platelet counts and VIIIIR:RCo. On the other hand, no significant correlations were seen in non-bleeders.

DDAVP has been used for treatment of bleeding tendency in vWF, since this agent is able to release vWF from endothelial cells when injected intravenously. To elucidate the mechanism of deficient vWF larger multimers in patients with essential thrombocythemia, DDAVP was administered to the patient (case ) who showed the prolonged bleeding time and lack of vWF larger multimers. Before and after infusion, platelet count, bleeding time, plasma VIIIIR:Ag, VIIIIR:RCo and platelet aggregation were measured simultaneously (Table 3).

Platelet counts were not significantly changed, but bleeding time became shortened.
vWF in Essential Thrombocytopenia

Fig. 4(a) Correlations between VIIIR: RCo and platelet counts in patients with prolonged bleeding time. 
- indicates the patients with prolonged bleeding time only.
○ indicates the patients with clinical bleeding tendencies.

Fig. 4(b) Correlations between VIIIR: RCo and platelet counts in patients with normal bleeding time.
Table 3 Hemostatic Examinations before and after Administration of DDAVP

<table>
<thead>
<tr>
<th></th>
<th>Before</th>
<th>30'</th>
<th>60'</th>
<th>120'</th>
<th>240'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plt. count (10^4/μl)</td>
<td>168</td>
<td>183</td>
<td>174</td>
<td>192</td>
<td>159</td>
</tr>
<tr>
<td>Bleeding time (Ivy)</td>
<td>10'30''</td>
<td>8'30''</td>
<td>8'00''</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIII: C (%)</td>
<td>100</td>
<td>264</td>
<td>220</td>
<td>230</td>
<td>220</td>
</tr>
<tr>
<td>VIII R: Ag (%)</td>
<td>49</td>
<td>210</td>
<td>210</td>
<td>230</td>
<td>210</td>
</tr>
<tr>
<td>VIII R: RCo (%)</td>
<td>36</td>
<td>196</td>
<td>190</td>
<td>176</td>
<td>148</td>
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<tr>
<td>Plt. Aggregation (%)</td>
<td>90</td>
<td>90</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP (4 μM)</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen (2 μg/ml)</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epinephrine (1 μg/ml)</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DDAVP (0.4 μg/kg body weight) diluted with 100 ml of normal saline was injected to Case intravenously over 30 min. period. After infusion was completed, blood samples were drawn for the hemostatic examinations at the time indicated. Bleeding time was also measured 60, 120 min. after infusion.

after DDAVP was infused. VIIIIR:Ag and VIIIIR:RCo were both markedly increased in 30 min after administration of DDAVP, and remained elevated for subsequent four hours.

Defective collagen- and epinephrine-induced platelet aggregation, which is the common finding in patients with essential thrombocythemia, remained unchanged one hour after DDAVP infusion.

Multimeric structures of vWF were also analysed after infusion of DDAVP. The appearance of the vWF larger multimers were clearly seen in the plasma obtained at 30 min after the infusion, indicating that DDAVP caused the normalization of plasma vWF not only quantitatively but also qualitatively. vWF larger multimers remained to be present in samples obtained at four hours after infusion. On the other hand, no significant change was observed in platelet vWF multimeric structures (Fig. 5). These results indicate that shortening of bleeding time after DDAVP is closely associated with increase of plasma vWF larger multimers.

Mechanism of development of vWF abnormalities were then investigated. Since GPIb is known to function as a receptor of vWF, platelet membrane glycoproteins were analyzed by SDS polyacrylamide gel electrophoresis in seven patients with essential thrombocythemia. Labelling pattern and electrophoretic mobility of GPIb in seven patients including two bleeders were found to be normal as illustrated in (Fig. 6).

Binding of vWF to patient's platelets was also determined in seven patients to see
whether GPIb in this patient's function properly as vWF receptor. There was no significant difference among normal controls, bleeders, and non-bleeders in vWF binding to platelets (Table 4). It is well known that addition of ristocetin to a stirred platelet suspension in the presence of vWF causes platelets to agglutinate. This ristocetin-induced platelet agglutination (RIPA) was found to be nearly absent in all patients with essential thrombocythemia, irrespective of the value of plasma vWF. This finding was unexpected since RIPA is now considered to represent in vitro approximation of in vivo process such as platelet adhesion to subendothelium. The following experiments were, therefore, carried out to clarify the reason for the defective RIPA. RIPA was measured under the various experimental conditions as described in the method. When normal platelets were resuspended in patient's plasma, RIPA was absent. On the other hand, RIPA was normally demonstrated when patient's platelets were resuspended in normal plasma as shown in (Fig. 7). This result indicated that the patient's plasma contained some factors to inhibit RIPA, while the patient's platelets were not responsible for the defective RIPA in essential thrombocythemia. To explore the causative factors in plasma for defective RIPA, the following experiments were performed. It is well
Fig. 6  Autoradiography of $^3$H-labelled platelet membrane glycoproteins in patients with essential thrombocythemia. Normal or patient's platelet membrane glycoproteins labelled with neuraminidase/galactoseoxidase/$^3$HNaHB$_4$ was separated by SDS polyacrylamide 5–15% gradient gel electrophoresis followed by autoradiography. N.C. indicates normal control and * indicates the patients with prolonged bleeding time.

Table 4  Binding of vWF to Platelets from Patients with Essential Thrombocythemia

<table>
<thead>
<tr>
<th>bound/total (%)</th>
<th>bound/total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>20.0 ± 1.3 (n = 5)</td>
</tr>
</tbody>
</table>

125I-labelled vWF (50 µg/ml) was incubated with normal or patient's platelet suspensions (final platelet concentration $30 \times 10^4/\mu l$ for 3 min at room temperature in the presence of 1.2 mg/ml of ristocetin. Platelet pellets were separated by centrifugation at 7,000 g for 1 min through silicon oil and binding from total binding as described in the method. The extent of binding was expressed by bound/total $\times 100\%$.

* indicates the patients with prolonged bleeding time.
known that RIPA is completely inhibited by anti-GPIb monoclonal antibody. When this monoclonal antibody was preincubated with patient's plasma at room temperature for 5 min, its inhibitory effect on RIPA was abolished. Plasma from normal individuals had minimal effect on this monoclonal antibody to inhibit RIPA (Fig. 8). These results indicated the presence of some substances in patient's plasma which could react with anti-GPIb monoclonal antibody. To further define the exact nature of these substances, competitive inhibition assay of glycocalicin, soluble proteolytic fragment of GPIb in plasma was performed. As shown in Table 5, plasma glycocalicin was found to be significantly increased in five patients with essential thrombocythemia examined.

Discussion

Plasma vWF from patients with essential thrombocythemia was studied. Among eleven patients investigated, five patients including three patients with clinical bleeding tendency showed decreased plasma vWF and prolonged bleeding time. Analysis of vWF multimeric structures showed vWF was also qualitatively abnormal in these patients. On the other hand, the other six patients including four patients with a history
Fig. 8 Effect of plasma on the ability of anti-GPIb monoclonal antibody to inhibit RIPA. Fifty μl of buffer, normal or patient’s plasma was incubated with 10 μl of anti-GPIb monoclonal antibody (6 μg/ml) for 5 min at room temperature. To normal PRP, 20 μl of the above mixture was added and RIPA was recorded as described in the method.

<table>
<thead>
<tr>
<th>Normal control</th>
<th>28.6±0.4 (%) (n=4)</th>
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<tbody>
<tr>
<td>Patient</td>
<td></td>
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<tr>
<td></td>
<td>44.7</td>
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<tr>
<td></td>
<td>42.2</td>
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<td>41.5</td>
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<tr>
<td></td>
<td>44.1</td>
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<td></td>
<td>32.2</td>
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Fifty μl of buffer, normal or patient’s plasma was incubated with 10 μl of anti-GPIb monoclonal antibody 3 μg/ml in microtiter wells for 30 min at room temperature, and 50 μl of the above mixture was added to another microtiter wells coated with purified GPIb. Antibody bound was detected by ELISA using goat-peroxidase conjugated anti-mouse IgG antibody. The results were expressed as % change as described in the method. * indicates the patients with prolonged bleeding time.
of thrombosis had normal plasma vWF and bleeding time was not prolonged. We believe that the decrease of plasma vWF plays an important role in bleeding diathesis in patients with essential thrombocythemia because of the following reasons. (1) Rapid appearance of vWF larger multimers without any significant improvement of defective epinephrine- and collagen-induced platelet aggregation was coincided with shortening of bleeding time after infusion of DDAVP. (2) Disappearance of clinical bleeding diathesis and normalization of bleeding time were accompanied by increase of plasma vWF after successful busulfan treatment. (3) There was a significant inverse correlations between the extent of thrombocytosis and the value of plasma vWF in patients with prolonged bleeding time.

The mechanism of the development of acquired vWF in patients with essential thrombocythemia is not clear at present time. Acquired vWD associated with systemic lupus erythematosus and lymphoproliferative disorders have been reported. In case of systemic lupus erythematosus, autoantibody against vWF was demonstrated (7). In case of lymphoproliferative disorders, it was speculated that malignant cells adsorbed plasma vWF, resulting in reducting of plasma vWF level. In cases of essential thrombocythemia, no antibody against vWF was demonstrated. It is possible that autonomously proliferating platelets adsorb plasma vWF leading to acquired vWD.

Analysis of vWF multimeric structure by SDS agarose gel electrophoresis has made it possible to identify the qualitative defect of vWF. Several subtypes of congenital von Willebrand disease have been identified on the basis of vWF multimeric structures. Von Willebrand disease associated with essential thrombocythemia is similar to type IIb or platelet type von Willebrand disease in view of absence of larger multimers of vWF with normal intermediate and smaller multimers and rapid appearance of larger multimers after infusion of DDAVP. However, there was a distinct difference in RIPA between congenital von Willebrand disease and acquired one associated with essential thrombocythemia. In type IIb or platelet type von Willebrand disease, RIPA was heightened because of increased interaction of plasma vWF with its binding site on platelet membrane, glycoprotein Ib, while RIPA was defective in essential thrombocythemia. It is note to mention that defective RIPA was observed in all patients examined irrespective of the value of plasma vWF.

It is well known that purified GPIb and glyocalcin, inhibit RIPA. Recently it was also shown that glyocalcin, which is the cleaved fragment of GPIb by Ca++ dependent protease circulates in normal plasma. We, therefore, measured glyocalcin in plasma from patients with essential thrombocythemia and found it was significantly increased. This may explain the defective RIPA in essential thrombocythemia. The fact that RIPA returned to normal after platelet counts were decreased (data not shown) suggests that the increase of glyocalcin in plasma was due to increase of platelet count and/or turnover rate. It is probable that marked thrombocytosis may
increase the chance of contact with each other followed by release of their constituents, such as ADP, serotonin and Ca\(^{++}\) dependent protease by platelet activation.

Analysis of platelet membrane glycoproteins and assay of vWF binding to platelets failed to identify the quantitative and qualitative abnormality of GPIb in essential thrombocythemia.

Recently it was shown that vWF larger multimers could bind platelets in the presence of ADP and thrombin.\(^1\) Binding of vWF larger multimers to platelets may occur when platelets are activated, so that plasma vWF is decreased. Since the total means of platelets are greatly increased, the total quantity of vWF adsorbed to platelet surface may be increased to result in acquired vWD. The question however remains why plasma vWF was normal in patients without prolonged bleeding time in spite of similarly increased platelet counts.

One possibility is that plasma vWF binding to platelet is less in such patients, because platelets are less sensitive to ADP and thrombin, thus not producing reduction in plasma vWF. Second is the different role of endothelial cells in production and release of vWF. Further studies will be definitely needed to solve this important problem.

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References

vWF in Essential Thrombocythemia


