Quantitative Comparisons of Antibody-Binding Sites of Platelet Glycoprotein IIb/IIIa in Aplastic Anemia and Idiopathic Thrombocytopenic Purpura

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Abstract. The numbers of antibody-binding sites of platelet glycoprotein (GP) IIb/IIIa on circulating platelets were analyzed using 4 kinds of antibodies in 34 aplastic anemia (AA) patients, 20 idiopathic thrombocytopenic purpura (ITP) patients, and 14 normal controls. The numbers of antibody-binding sites of CD41, CD41a, CD41b, and CD61 on platelets of the AA patients were less than in the normal controls (p <0.001). In the ITP patients, the numbers of sites for CD41 and CD41a were less than in normal controls (p <0.05). There were significant positive correlations between CD41 and CD41a, CD41b, and CD61 in the 3 groups. There were significant negative correlations between CD41 and CD41b and between CD41a and CD41b in the normal controls, but not in the AA or ITP patients. In summary, the numbers of the 4 antibody-binding sites of GPIIb/IIIa on platelets of AA and ITP patients are different from those in normal controls. Measurements of the antibody-binding sites of GPIIb/IIIa are not necessary for the differential diagnosis of AA and ITP. However, the differences in correlations between the numbers of epitopes in AA and ITP patients suggest that the epitopes of GPIIb/IIIa are altered in these diseases.

Keywords: platelet glycoprotein IIb/IIIa, aplastic anemia, idiopathic thrombocytopenic purpura

Introduction

Patients with aplastic anemia (AA) or idiopathic thrombocytopenic purpura (ITP) both exhibit thrombocytopenia in the peripheral blood. AA is usually idiopathic and refers to pancytopenia associated with severe reduction in the amount of hematopoietic tissue, including megakaryocytes, which results in deficient production of blood cells [1]. ITP is a syndrome of destructive thrombocytopenia due to the presence of autoantibodies with a normal or an increased number of bone marrow megakaryocytes [2,3]. Therefore, the life spans of circulating platelets in AA patients are normal, and those in ITP are reduced. The mean platelet volume (MPV) and platelet distribution width (PDW) have been used for the differential diagnosis of AA and ITP; both are increased in ITP but not in AA [3-9].

Expression of platelet glycoprotein (GP) IIb/IIIa has been reported to be altered in patients with coronary arterial disease and administration of platelet GPIIb/IIIa inhibitors is helpful for the prevention of thrombosis in these patients [10-12]. The autoantibodies in ITP patients are mainly against platelet-associated antigens, most commonly located on the platelet GPIIb/IIIa complex [13-16]. Furthermore, platelet GPIIb/IIIa inhibitors can cause ITP [17]. The GPIIb/IIIa complex on circulating platelets in ITP patients may be occupied by autoantibodies, and the available binding sites may be decreased in ITP patients but not in AA patients.

In this study, the numbers of antibody-binding sites of platelet GPIIb/IIIa on circulating platelets were analyzed using 4 kinds of antibodies to different epitopes in AA and ITP in order to
investigate differences between these 2 diseases that exhibit thrombocytopenia.

Materials and Methods

Patients. In this study, we enrolled 34 patients who were diagnosed with AA (14 males, 20 females) and 20 patients who were diagnosed with ITP (9 males, 11 females). Fourteen healthy volunteers who had not taken aspirin within 3 weeks were recruited as normal controls (5 males, 9 females). The ages of the patients ranged from 18 mo to 69 yr, with a median of 23.5 yr for the AA patients, 24.0 yr for the ITP patients, and 30.0 yr for the normal controls. We included 18 juvenile patients (13 AA and 5 ITP) who were less than 16 yr of age. The diagnoses of AA and ITP were based on routine hematological evaluation, bone marrow examination, and therapeutic responses. EDTA-anticoagulated peripheral blood samples were kept at room temperature and analyzed within 4 hr after collection.

Measurements of platelet count, MPV, and PDW. The platelet count, MPV, and PDW were measured using an automatic hematology analyzer (Sysmex XE-2100, Japan). The same EDTA-anticoagulated peripheral blood samples were analyzed as those used for flow cytometric measurements of surface GPIIb/IIIa.

Analyses of antibody-binding sites of platelet GPIIb/IIIa. Platelet-rich plasma (PRP) was collected from peripheral blood by centrifugation. The platelet count of the PRP was measured using a hematology analyzer (Sysmex XE-2100) and the PRP was diluted to a platelet count of 10 × 10^9/L using phosphate-buffered saline (PBS; pH 7.4). Flow cytometric measurements of the antibody-binding sites of platelet GPIIb/IIIa on circulating platelets were performed as previously described [18], using 4 monoclonal antibodies to CD41, CD41a, CD41b, and CD61 and a negative control. Briefly, 5 µl of fluorescein isothiocyanate (FITC)-labeled anti-CD41 antibody (Serotec, Oxford, UK) was added to 50 µl of diluted PRP and mixed. Similarly, 10 µl of FITC-conjugated anti-CD41a (Becton Dickinson, San Jose, CA, USA), anti-CD 41b (Pharmingen, San Diego, CA, USA), phycoerythrin (PE)-conjugatedanti-CD61 (Pharmingen), and a negative control (γ1/γ2a, Becton-Dickinson, Rutherford, NJ, USA) were added to 50 µl of the diluted PRP and mixed. Each mixture was incubated in the dark at room temperature for 15 min. After washing with 3 ml of PBS, the platelets were resuspended in 0.5 ml of PBS and analyzed using a flow cytometer (FACSCalibur, Becton-Dickinson) and CellQuest program (Becton-Dickinson). The quantum FITC and PE mean equivalent soluble fluorochrome (MESF) kits (Bangs Laboratories, Fishers, IN, USA) were run along with samples for comparative fluorescence analysis. Platelets and control beads were gated using the forward scattering (FSC) and side scattering (SSC) characteristics on a dot plot (Fig. 1A). The results were expressed as mean geometric fluorescence intensities, and the MESF values were calculated using the QuickCal program (version 2.3, Bangs Laboratories).

Statistics. The numbers of antibody-binding sites of platelet GPIIb/IIIa on the circulating platelets of the AA and ITP groups were compared to those of normal controls by the t test. Pearson’s correlation coefficient (r) was used to assess the correlations between the numbers of the 4 antibody-binding sites in the 3 groups of subjects.

Results

Measurements of platelet count, MPV, and PDW. The platelet counts of peripheral blood samples from the AA and ITP patients were much lower than the normal controls (290 × 10^9/L). There was no significant difference in the platelet counts between the AA (47.0 × 10^9/L) and ITP patients (41.8 × 10^9/L) (p = 0.654). The MPV of the AA patients was 9.75 fl and that of the ITP patients was 10.8 fl (p = 0.07). The PDW of the AA patients was 11.5%, which was significantly lower than that of the ITP patients (13.7%; p = 0.008).

Antibody-binding sites of platelet GPIIb/IIIa (Table 1, Fig. 1). The fluorescence levels of platelets stained with negative control antibody were <101. The number of antibody-binding sites of CD41 on
Fig. 1. Dot scattergram of platelets using forward scatter (FSC) and side scatter (SSC) parameters illustrates well-aggregated platelets (A). The histograms of platelets after staining with anti-glycoprotein antibodies show different fluorescence intensities. The platelets of patients with idiopathic thrombocytopenic purpura (C, 43,300) and aplastic anemia (D, 24,800) show less fluorescence intensities (MESF) than those of the normal control (B, 92,200) after staining with FITC-conjugated anti-CD41.

Table 1. Numbers (MESF, mean ± SD) of variable antibody-binding sites of platelet glycoprotein IIb/IIIa on circulating platelets in patients with aplastic anemia (AA) and idiopathic thrombocytopenic purpura (ITP) and in normal control subjects.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>CD41</th>
<th>CD41a</th>
<th>CD41</th>
<th>CD61</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA patients (n = 34)</td>
<td>33,200±24,600*</td>
<td>24,300±11,900</td>
<td>5,360±1,470*</td>
<td>91,500±22,000*</td>
</tr>
<tr>
<td>ITP patients (n = 20)</td>
<td>46,600±29,100*</td>
<td>30,200±16,500*</td>
<td>7,350±4,310</td>
<td>102,000±27,900</td>
</tr>
<tr>
<td>Normal controls (n = 14)</td>
<td>71,400±31,300</td>
<td>49,300±21,000</td>
<td>8,630±5,450</td>
<td>122,000±25,700</td>
</tr>
</tbody>
</table>

MEFS = Mean equivalent soluble fluorochrome.
*p value <0.05 vs normal controls.
the circulating platelets in the AA patients was 33,200 ± 24,600, which was less than in the normal controls (71,400 ± 31,300; p <0.001). The numbers of antibody-binding sites of CD41a (24,300 ± 11,900) and CD41b (5,360 ± 1,470) in the AA patients were less than in the normal controls (49,300 ± 21,000, p <0.001 and 8,630 ± 5,450, p = 0.005). Further, the number of antibody binding sites of CD61 in the AA patients was 91,500 ± 22,000, which was less than in the normal controls (122,000 ± 25,700, p <0.001). The numbers of antibody-binding sites of CD41 (46,600 ± 29,100) and CD41a (30,200 ± 16,500) in the ITP patients were less than in the normal controls (p = 0.024 and p <0.005). However, the numbers of antibody-binding sites of CD41b (7,350 ± 4,310) and CD61 (102,000 ± 27,900) in patients with ITP were not significantly different from those in normal controls (p = 0.507 and p = 0.051). Although statistically insignificant (p >0.05), the numbers of the 4 different antibody-binding sites of platelet GPIIb/IIIa in patients with AA were less than those in patients with ITP.

The correlations between the numbers (MESF) of the 4 antibody-binding sites of GPIIb/IIIa on the circulating platelets in the normal controls and in patients with AA and ITP are as follows: CD41 correlated well with CD41a in all 3 groups (r >0.8, p <0.0001), but showed negative correlation with CD41b only in the normal controls (r = -0.7058, p = 0.0033). Further, there was no significant correlation between the AA and ITP patient groups (p >0.05). CD41 did not correlate with CD61 in any of the 3 groups (p >0.05). CD41a was negatively correlated with CD41b in the controls (r = -0.7058, p = 0.0033), but there was no significant correlation between the AA and ITP patient groups (p >0.05). CD41a did not correlate with CD61 in any of the 3 groups (p >0.05); in contrast, CD41b correlated well with CD61 in all 3 groups (r >0.7, p <0.01).

Discussion

AA is diagnosed when the peripheral blood shows pancytopenia or bicytopenia, and the bone marrow is hypocellular without other abnormalities [1]. ITP is diagnosed when patients have thrombocytopenia, show a normal peripheral blood film except for thrombocytopenia, and have adequate numbers of megakaryocytes in the bone marrow [1]. It is important to determine if thrombocytopenia is a result of decreased platelet production or increased platelet destruction. Although MPV and other platelet indices are helpful in the differential diagnosis of AA and ITP, the bone marrow examination provides valuable information about platelet production, including the number of megakaryocytes and the degree of platelet production, and it is necessary for the differential diagnosis of AA and ITP [3-9]. MPV and PDW have been reported to be higher in ITP than in AA [3-9]. In this study, the platelet counts in the AA and ITP patients were much lower than in the normal controls, and the PDW value was increased in the ITP patients (p = 0.008). Although the mean value of MPV was higher in ITP than in AA, the difference was statistically insignificant, possibly due to the relatively small sample sizes in this study.

Platelet-associated anti-GPIIb/IIIa antibodies are frequently detected in ITP patients [8]. A minority of AA cases are associated with immunological diseases, and platelet-associated anti-GPIIb/IIIa antibodies are found in a minority of the normal population [1,8]. The autoantibodies in ITP are mainly against platelet-associated antigens, and most commonly located on the platelet GPIIb/IIIa complex [13-16]. Platelet GPIIb/IIIa inhibitors can cause ITP [17]. Therefore, the GPIIb/IIIa complex on the circulating platelets in ITP may be occupied by autoantibodies and the available binding sites can be decreased in ITP and not in AA. However, because the circulating platelets in ITP are larger in size and younger than those found in AA [8], they can influence the number of antibody-binding sites of platelet GPIIb/IIIa. Further, the maximal numbers of binding sites of these antibodies depend on the conformational intactness of GPIIb/IIIa [19,20]. Decreases in the binding of antibodies to the platelet GPIIb/IIIa complex due to membrane abnormalities have been reported in several myeloproliferative disorders [21,22]. However, whether or not the platelets in AA and ITP patients have any membrane abnormalities remains unknown. The numbers of binding sites available to exogenous antibodies of different epitopes on platelets of AA and ITP patients have
not been studied previously. An ITP patient may have platelet-associated antibodies and plasma antibodies that differ in their specificities [19,23].

In this study, the antibody-binding sites of GPIIb/IIIa for 4 kinds of antibodies on circulating platelets were all decreased in the AA patients (p <0.05). Since the immunological process, including the presence of autoantibodies to these molecules, is a minor aspect of the pathogenesis of AA, this result could be due to the intrinsic nature of the platelets. The clinical significance of the finding that the 4 different antibody-binding sites of the GPIIb/IIIa complex are all decreased in AA is not clear. However, platelet GPIIb/IIIa complex is the membrane protein that mediates platelet aggregation [24-26]. GPIIb/IIIa in activated platelets is known to bind 4 soluble adhesive proteins: fibrinogen, von Willebrand factor (VWF), fibronectin, and vitronectin [27]; in particular, the binding of fibrinogen and VWF to GPIIb/IIIa causes platelet aggregation [27]. GPIIb/IIIa inhibitors can increase bleeding risks [28]. Therefore, it appears plausible that the decrease in the platelet count itself and the decrease in the antibody-binding sites of the platelet GPIIb/IIIa complex may jointly contribute to the bleeding tendency in AA patients.

The numbers of antibody-binding sites of CD41 and CD41a in the ITP patients were less than in the normal controls (p <0.05), but in the cases of CD41b and CD61, the numbers were not significantly different from the normal controls (p >0.05). We suggest that the antibody-binding sites of CD41 and CD41a may be occupied by autoantibodies and that the available binding sites may be decreased in ITP. It is also possible that the autoantibodies in ITP may bind to the antibody-binding sites of CD41 and CD41a with a greater affinity than to those of CD41b and CD61. Usually, ITP patients do not exhibit a bleeding tendency even though their platelet counts are extremely low. The reason for this has not been established in previous studies. From the results of our study, it appears that this can be partly due to the relatively normal numbers of CD41b and CD61 antibody-binding sites or a possible difference in the binding sites between soluble proteins and antibodies. It is interesting to note that, although statistically insignificant (p >0.05), the numbers of 4 different antibody-binding sites of the platelet GPIIb/IIIa complex in the AA patients were less than those in the ITP patients.

GPIIb (integrin α2b, CD41) is a calcium-dependent noncovalently associated heterodimer and contains a heavy chain (GPIIbα) and a light chain (GPIIbβ) linked by a single disulfide bond. Integrin α2b chain interacts with the integrin β3 subunits (CD61) to form the platelet glycoprotein complex GPIIb/IIIa [25]. The results of this study indicate that CD41 is closely connected to CD41a and neither of them is connected with CD61. Further, the results show that CD41b correlates with CD61 in all 3 groups of subjects. Both CD41 and CD41a showed negative correlation to CD41b in the control group. Whether there is relevance between the numbers of the 3 epitopes—CD41, CD41a, and CD41b—on the integrin α2b chain has not been studied. However, there could be a substitutional relationship between these 3 epitopes or the glycoprotein subunits could undergo transformations after binding with antibodies or soluble proteins. Experiments have not been performed in this regard. Hence, studies are warranted regarding the three-dimensional conformation of the GPIIb/IIIa complex and the changes that occur after its binding with various antibodies.

In conclusion, the numbers of 4 different antibody-binding sites of GPIIb/IIIa in platelets of AA and ITP patients are different from those in normal controls. Measurements of the antibody-binding sites of GPIIb/IIIa are not necessary for the differential diagnosis of AA and ITP. However, differences in the correlations between the numbers of epitopes in AA and ITP patients have been detected. This suggests that there are alterations in the epitopes of GPIIb/IIIa in these diseases.

References

Platelet GPIIb/IIIa in aplastic anemia and idiopathic thrombocytopenic purpura


