Increased Platelet–Fibrinogen Affinity in Patients With Myeloproliferative Disorders

By Raffaele Landolfi, Raimondo De Cristofaro, Massimo Castagnola, Erica De Candia, Giuseppe D’Onofrio, Giuseppe Leone, and Bruno Bizzi

Patients with myeloproliferative disorders (MPD) are known to have some abnormalities of platelet glycoproteins (Gp). Quantitative changes of the Gp Ib, IIb-IIIa, and/or their glucidic content have been reported. Since the Gp IIb-IIIa complex plays a major role in fibrinogen binding by activated platelets, we measured the platelet fibrinogen affinity in nine patients with polycythemia vera (PV) and one subject with chronic myeloid leukemia (CML) by the aggregometric method of Marguerie. In all patients the Kd of the platelet fibrinogen reaction was significantly decreased as compared to controls, with evidence in two cases with PV of a heterogeneity of platelet–fibrinogen receptor sites. The measurement of 125I-labeled fibrinogen–platelet binding, performed in seven patients (five PV and two CML), showed receptor populations with increased (Kd1 = 0.58 ± 0.3 x 10^7 mol/L) and normal affinity (Kd2 = 5.12 + 3.1 x 10^7 mol/L). These results demonstrate a heterogeneity of platelet–fibrinogen receptors in these patients and may explain the thrombotic diathesis of MPD subjects.

PATIENTS with myeloproliferative disorders (MPD) have an increased incidence of both thrombotic and hemorrhagic episodes that have been attributed to quantitative and qualitative abnormalities. Among qualitative changes major abnormalities of MPD platelet membrane and granular glycoproteins (Gp) have been recognized. In a recent study a reduced sialylation and a defect in glucose-mannose glycosylation of Gp Ib, IIb, IIIa, and IIb have been reported. Because the Gp IIb-IIIa complex plays an important role in fibrinogen-specific binding by activated platelets, we investigated the platelet-fibrinogen interaction in patients with polycythemia vera (PV) and chronic myeloid leukemia (CML). The affinity for fibrinogen of platelets from normal controls and from MPD subjects was measured by the aggregometric technique of Marguerie et al. and by measuring the platelet binding of 125I-labeled fibrinogen. In MPD patients both methods showed a heterogeneity of platelet receptors for fibrinogen with evidence of binding sites with increased affinity.

MATERIALS AND METHODS

Clinical Material

This study was conducted according to the principles in the Declaration of Helsinki. The patients were from the Department of Hematology of the Catholic University of Rome. Eleven patients with MPD were studied. Nine of these patients had PV, and two had CML. The platelet count of our patients varied from 1.4 to 13.9 x 10^9/mL. One of the patients had been splenectomized. Two patients had histories of a major thrombotic episode, and one showed clinical evidence of hemorrhagic tendency. Drugs were discontinued for patients receiving antiaggregating therapy ten days before the study.

Preparation of Platelets

Venous blood from patients and controls was collected in plastic tubes containing the anticoagulant sodium citrate (final concentration 0.38%). Platelet rich plasma (PRP) was obtained by centrifugation at 120 g for 20 minutes. Platelets were then gel filtered twice on a 25 x 1.5-cm Sepharose 4B column equilibrated with HBMT without albumin and glucose. Because the Gp IIb-IIIa complex plays an important role in fibrinogen-specific binding by activated platelets, we investigated the platelet-fibrinogen interaction in patients with polycythemia vera (PV) and chronic myeloid leukemia (CML). The affinity for fibrinogen of platelets from normal controls and from MPD subjects was measured by the aggregometric technique of Marguerie et al. and by measuring the platelet binding of 125I-labeled fibrinogen. In MPD patients both methods showed a heterogeneity of platelet receptors for fibrinogen with evidence of binding sites with increased affinity.

Preparation of Fibrinogen

Human adult fibrinogen (grade L), purchased from Kabi (Stockholm), was purified by gel filtration on a 2 x 100-cm Sephadex G 200 column equilibrated with HEPES-buffered modified Tyrode's solution (HBMT), pH 7.2, containing 0.2% bovine serum albumin (BSA), 0.13 mol/L NaCl, 2.6 mmol/L KCl, 0.39 mmol/L NaH2PO4, 12 mmol/L NaHCO3, 10 mmol/L HEPES, and 5.5 mmol/L glucose. Platelet concentration was then adjusted to 1.5 to 2.0 x 10^8/mL.

Aggregometric Studies

Evaluation of platelet-fibrinogen interaction was performed by testing the aggregatory response of washed platelets to excess adenosine diphosphate (ADP) in the presence of various concentrations of fibrinogen. In a typical experiment 0.25 mL of platelet suspension in a plastic cuvette was stirred at 1,000 rpm at 37°C in an Elvi 840 dual-channel aggregometer connected to a double-pen recorder. At 30-sec intervals 10 µL of 0.28 mol/L CaCl2, 10 µL of fibrinogen solution (final concentrations 0.010 to 0.21 mg/mL) and 10 µL of 280 µmol/L ADP were consecutively added. The initial slope of each aggregometric curve was used to evaluate the effect of the various fibrinogen concentrations on platelet aggregatory response. In all cases aggregometric studies were performed within two hours of blood collection.

125I-Labeled Fibrinogen Binding Studies

Labeling procedures. Purified human fibrinogen was labeled with carrier-free Na 125I using the iodogen technique. The specific activity of 125I-labeled fibrinogen was 200 µCi/mg of protein.
Table 1. Kd Values of the Platelets Fibrinogen Reaction in Normal Subjects and in Ten MPD Patients Calculated by the Aggregometric Method

<table>
<thead>
<tr>
<th></th>
<th>n = 10</th>
<th>Kd (μmol/L)</th>
<th>r</th>
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<tbody>
<tr>
<td>Normal subjects†</td>
<td>0.26 ± 0.06</td>
<td>0.98 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>MPD patients†</td>
<td>0.26 ± 0.06</td>
<td>0.98 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>CF (PV)</td>
<td>0.05</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>DP (PV)</td>
<td>0.05</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>SA (PV)</td>
<td>0.10</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>GF (PV)</td>
<td>0.05</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>NG (PV)</td>
<td>0.06</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>MM (PV)</td>
<td>0.08</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>LG (PV)</td>
<td>0.05</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>CM (PV)</td>
<td>0.09</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>BE (PV)</td>
<td>0.06</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>MS (CML)</td>
<td>0.06</td>
<td>0.96</td>
<td></td>
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†p < .001.

Clotting ability of fibrinogen was not significantly affected by the labeling procedure.

Measurement of platelet 125I-fibrinogen binding. Two hundred and fifty microliters of gel-filtered platelet suspensions was incubated for 20 minutes at 37°C with 1 mmol/L CaCl₂, 10 μmol/L ADP, and 125I-labeled fibrinogen at final concentrations ranging between 15 and 1,200 nmol/L. To measure the nonspecific binding at the various fibrinogen concentrations, ADP and CaCl₂ were omitted. At the end of incubation 100 μL of the platelet suspensions were stratified on 400 μL of 20% sucrose in plastic cuvettes. After centrifugation for two minutes at 12,000 rpm, the supernatants were aspirated, and the tips of the tubes containing the platelet pellets were cut and counted in a Hewlett Packard gamma counter. The equilibrium dissociation constant and the number of receptor sites were calculated by plotting the data according to Klotz. The platelet-fibrinogen reaction was analyzed by both double-reciprocal plot and Hill plot (equation 2). The Kd values obtained from the two types of analyses were not significantly different. Only Kd values calculated by double-reciprocal plot have been reported (Table 1). In ten normal subjects the Kd of the platelet-fibrinogen reaction ranged from 1.6 to 3.5, with an average value of 2.6 ± 0.6 x 10⁻⁵ mol/L. In MPD patients the Kd values ranged from 0.2 to 0.9 x 10⁻⁵ mol/L (mean 0.65 ± 0.18). There was no correlation between blood platelet counts and Kd values in these patients. Except in two patients with PV, correlation coefficients of straight lines of double-reciprocal plots were always >0.94. Hill coefficients in the same group were not significantly different from 1. In two PV patients in whom lower correlation coefficients in double-reciprocal plot were found, Hill plot analysis suggested the presence of two different groups of receptors. As

RESULTS

Aggregometric Studies

Washed platelets from each subject were first tested for ADP-induced aggregation in absence of fibrinogen. Under these conditions no aggregation could be induced. Platelet-fibrinogen affinity was evaluated according to the classical receptor occupancy theory. Accordingly dose–response curves meet each of the following equations:

\[
\frac{1}{S} = \frac{1}{F} \times \frac{Kd}{S_{max}} + \frac{1}{S_{max}}
\]

and

\[
\log \frac{S}{(S_{max} - S)} = n \log F - \log Kd
\]

where F, Kd, S, and n are fibrinogen concentration, dissociation constant of the reaction, the initial slope of aggregation, and Hill coefficient, respectively. Smax, obtained from the aggregation experiments, was also controlled by plotting the data according to Klotz. The platelet-fibrinogen reaction was analyzed by both double-reciprocal plot (equation 1) and Hill plot (equation 2). The Kd values obtained from the two types of analyses were not significantly different. Only Kd values calculated by double-reciprocal plot have been reported (Table 1). In ten normal subjects the Kd of the platelet-fibrinogen reaction ranged from 1.6 to 3.5, with an average value of 2.6 ± 0.6 x 10⁻⁵ mol/L. In MPD patients the Kd values ranged from 0.2 to 0.9 x 10⁻⁵ mol/L (mean 0.65 ± 0.18). There was no correlation between blood platelet counts and Kd values in these patients. Except in two patients with PV, correlation coefficients of straight lines of double-reciprocal plots were always >0.94. Hill coefficients in the same group were not significantly different from 1. In two PV patients in whom lower correlation coefficients in double-reciprocal plot were found, Hill plot analysis suggested the presence of two different groups of receptors. As
shown in Fig 1, two different Kd in platelets from the same donor could be calculated. Of these two values one is close to the mean Kd of normal subjects, while the other is similar to the value generally observed in MPD patients. In both cases the Hill coefficients in the half-saturation region were 0.36 and 0.38, respectively. In MPD patients Smax and slope values at the various fibrinogen concentrations were consistently higher than in normal subjects (Fig 2).

Binding Studies

125I-labeled fibrinogen binding studies were performed in seven patients, six of them (five PV and one CML) previously studied with the aggregometric method. Results obtained in normal controls and MPD patients are summarized in Table 2.

In six normal controls the mean Kd value was \(3.5 \pm 1.5 \times 10^{-7}\) mol/L, with 18,000 \(\pm 2,500\) receptor sites/platelet. In all cases Klotz's and Scatchard's analysis of binding data provided evidence of a single class of receptors (Figs 3 and 4).

Binding studies of patients with MPD showed two different platelet-fibrinogen binding sites with mean Kd values of 0.58 \(\pm 0.3\) and \(5.12 \pm 3.1 \times 10^{-7}\) mol/L and with 1,830 \(\pm 750\) and 8,100 \(\pm 1,800\) receptor sites/platelet, respectively (Figs 3 and 5). The results obtained in patients with PV and CML were comparable. The data reported in Table 2 derived from Scatchard's analysis. Klotz's plot was used to evaluate the saturation degree of receptors and gave values of Kd and number of sites comparable to Scatchard's plot.

**DISCUSSION**

Platelet-fibrinogen interaction is central to the physiologic process of platelet aggregation. Platelet affinity for fibrinogen has been studied by both radioisotopic and aggregometric techniques. In normal subjects Kd values ranging from 0.03 to 5.6 \(\mu\)mol/L have been reported. The different estimation of the normal Kd values by the various authors can probably be attributed to different methods of platelet preparation. When the aggregometric and radioisotopic methods were compared in the same study, similar Kd values were obtained. Also in this study the two techniques gave similar Kd values in normal subjects. In addition, a homogeneous receptor-site population was found. Both the Kd values and the homogeneity of receptor population are in agreement with the findings of Marguerie et al.

In patients with MPD, aggregometric studies and 125I-fibrinogen-binding experiments gave apparently discordant

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**Table 2. Binding Studies With 125I-Labeled Fibrinogen in Normal Subjects and in MPD Patients**

<table>
<thead>
<tr>
<th></th>
<th>Normal subjects</th>
<th>MPD patients</th>
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<tr>
<td>Kd ((\mu)mol/L)</td>
<td>(0.35 \pm 0.15)</td>
<td>(0.03 \pm 0.02)</td>
</tr>
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</table>

*The number of the fibrinogen receptor sites/platelet for each receptor class is reported in brackets.
results. In fact, while the radioisotopic method showed in all cases a double class of platelet-fibrinogen receptors, the aggregometric technique resulted in the same finding in only two MPD subjects. Since the high-affinity receptor sites found by the radioisotopic method had a Kd value comparable to the Kd value derived from the aggregometric method, it is likely that the binding of fibrinogen to these sites could induce the full biological effect (ie, aggregation), which masks the normal-affinity-receptor-population occupancy.

The existence of a double class of platelet-fibrinogen receptors in MPD could probably be attributed to a heterogeneity of platelet population rather than to the presence of different receptor classes in the same platelet. This hypothesis requires further study. The reduced number of fibrinogen receptor sites on MPD platelets did not cause a reduction of maximal MPD platelet aggregatory response; on the contrary, maximal MPD platelet aggregatory response increased (Fig 2).
Although a modified sensitivity to ADP cannot be excluded, this finding suggests that within a reasonable range of receptor sites the aggregatory response depends largely upon the thermodynamic factors that regulate the fibrinogen-receptor interaction. The molecular modifications that cause the increase of MPD platelet affinity for fibrinogen must be demonstrated by further studies. The reduced glycosylation of Gp IIb and IIa in MPD platelets reported recently by Clezardin et al\textsuperscript{5} may account for the modified affinity for fibrinogen. In fact the function of Gp sugars in modulating the receptor-ligand affinity is well known.\textsuperscript{21} The enhanced platelet-fibrinogen affinity may play a role in the thrombotic diathesis of MPD patients. Indeed, modifications of other platelet-membrane Gp could cause an abnormal MPD platelet interaction with other adhesive proteins and with subendothelium,\textsuperscript{22} thus contributing to the thrombo-hemorrhagic tendency of these patients.

REFERENCES

Increased platelet-fibrinogen affinity in patients with myeloproliferative disorders [see comments]

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