CONCISE REPORT

Impaired Factor X and Prothrombin Activation Associated With Decreased Phospholipid Exposure in Platelets From a Patient With a Bleeding Disorder


Platelets from a platelet factor 3-deficient patient, which was first described by Weiss et al. (Am J Med 67:206, 1979), were found to be equally impaired in their ability to promote factor X and prothrombin activation. Compared to normal platelets, the patient’s platelets showed upon stimulation with thrombin plus collagen a much slower generation and a considerably lower level of platelet prothrombin- and factor X-converting activities. Treatment of stimulated platelets with phospholipases revealed a decreased exposure of negatively charged phospholipid at the outer surface of the patient’s platelets, relative to control’s. We suggest that the combined impairment of prothrombin- and factor X-converting activities in this patient is due to a defect in the mechanism by which phosphatidylserine becomes exposed at the outer surface of stimulated platelets.

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FORMATION of a hemostatic plug at sites of vascular injury requires concerted action of blood platelets and plasma coagulation factors. Platelets contribute to the arrest of bleeding by clumping together into aggregates and by promoting efficient coagulation, which consolidates the platelet plug with strands of fibrin. Apart from their participation in the contact system of coagulation, platelets promote conversion of factor X to Xa and of prothrombin to thrombin in the presence of other coagulation factors. Since both reactions are stimulated in vitro by negatively charged phospholipids, the procoagulant property of platelets has been partly attributed to membrane lipid or lipoprotein which becomes exposed upon platelet activation. Miletich et al. have shown that thrombin-activated platelets can bind factor Xa and that the binding site for factor Xa is associated with platelet-bound factor Va. Interestingly, they demonstrated that platelets from a patient with an isolated deficiency of platelet procoagulant activity, earlier described by Weiss et al., had a reduced number of factor Xa–factor Va binding sites. Impaired binding was shown to be reflected in a reduced ability of these platelets to enhance conversion of prothrombin to thrombin by factors Xa, Va, and calcium. Since the patient’s platelet factor Va activity released after thrombin treatment is normal, Miletich et al. concluded that an as yet unidentified membrane component essential for thrombin formation at the platelet surface is deficient in these platelets. Because the chemical nature of this component does not necessarily have to be phospholipid, it was of interest to see whether or not the platelets of this patient are able to enhance the other lipid-dependent coagulation reaction, ie, conversion of factor X to Xa by factors IXa, VIIIa, and calcium.

In this study, we report that these platelets are equally impaired in their ability to promote factor Xa and thrombin formation. In addition, we provide evidence that exposure of negatively charged phospholipids at the outer surface of activated platelets is substantially lower in this patient than observed with normal platelets.

MATERIALS AND METHODS

Platelets

Washed platelets were prepared as described previously. The patient M.S., with a moderately severe bleeding disorder, has been reported in detail by Weiss et al. She visited Maastricht for these studies and blood was taken from her on three occasions at the same time as control’s. Measurements of platelet activity in prothrombin and factor X activation, as well as platelet treatment with exogenous phospholipases, were conducted within a period of 18 hours after venipuncture. Platelet concentration was determined with a Coulter Counter (Hialeah, Fla). Patient’s platelets and control’s were adjusted to the same count.

Proteins

Coagulation factors VIIIC, IX, Xa, and prothrombin were purified from bovine blood according to established procedures as described elsewhere. Factors VIII, IXa, Xa, Va, and thrombin were prepared from their respective precursors. Molar concentrations of coagulation factors were determined by active site titration or by kinetic analysis. Phospholipase A2 (phosphatidyl-2-acylhydro-lase; EC 3.1.4.14) from Naja naja and bee venom and sphingomyelinase (sphingomyelin–cholinephosphohydrolase; EC 3.1.4.12) from Staphylococcus aureus were purified as described before. One international unit of phospholipase is defined as the amount of...
enzyme which hydrolyzes 1 μmol of phospholipid per minute under optimal conditions.

**Assays**

Prothrombin- and factor X-converting activities of platelets were calculated from the rates of thrombin and factor Xa formation determined with chromogenic substrates. In some experiments, platelet prothrombinase activity was measured in the absence of added factor Va. Incubation of (activated) platelets with phospholipases followed by lipid extraction and determination of surface-exposed phospholipids was performed in the same way as described elsewhere.4

**RESULTS**

**Platelet Prothrombinase Activity Without Addition of Factor Va**

Miletich et al7 showed that the patient’s platelets stimulated by thrombin had normal factor Va activity. Addition of factor Xa and prothrombin to these platelets resulted, however, in thrombin generation rates that were about three times lower than normal. Figure 1 shows that similar results were obtained in our laboratory using a chromogenic substrate assay to measure rates of thrombin formation. Platelets were activated by thrombin and the time course of generation of platelet prothrombinase activity was measured in the absence of added factor Va. At final concentrations of 0.2 nmol/L factor Xa and 4 μmol/L prothrombin, the patient’s platelets showed rates of thrombin formation which were about half of those observed with normal platelets. The factor Xa concentration employed was similar to that used in the above-mentioned study7 (5 ng Xa/mL) and approximated the reported dissociation constant of factor Xa for thrombin-activated platelets (~0.1 nmol/L).5

We have previously shown that stimulation by collagen plus thrombin makes platelets much more active in prothrombin activation (as well as in factor X activation) than stimulation by either collagen or thrombin separately.10 Figure 1 shows that this effect is also apparent in the absence of added factor Va. Despite the fact that the same factor Va activities were present in platelets stimulated by thrombin or by collagen plus thrombin, the latter activation procedure results in much higher prothrombin-converting activities. Nevertheless, collagen plus thrombin stimulated platelets from patient M.S. remain about 2½ times less active than stimulated control platelets.

**Comparison Between Platelet-Enhanced Factor X and Prothrombin Activation**

The abnormal prothrombinase activity of platelets from patient M.S. has been ascribed to a partial deficiency of specific factor Xa–factor Va binding sites on the platelets.67 The next experiments were designed to determine whether this deficiency is limited to prothrombinase activity or also affects the factor X-converting activity of these platelets. Both coagulant reactions were measured with added factors Xa, Va, and prothrombin, or factors VIIIa, IXa, and X, respectively. For prothrombinase measurements, saturating amounts of factor Va were added to make prothrombin activation independent of release and activation of platelet factor V. On stimulation of platelets from patient M.S. with collagen plus thrombin, both the development of prothrombinase activity and factor X converting activity were considerably less than observed with normal platelets (Fig 2). Ten minutes after platelet stimulation, both activities were about 25% to 30% of that of normal platelets (see also Table 1), while this difference was found to be much larger at shorter time periods of stimulation.

Prothrombin and factor X activation were also compared following ten minutes of stimulation of
platelets with thrombin or with Ca-ionophore A23187 (Table 1). In concordance with previous observations, platelet stimulation by thrombin produced a much smaller increase in prothrombin and factor X activation than stimulation by collagen plus thrombin. Nevertheless, the lower activity of the patient's platelets in both reactions was also apparent after thrombin stimulation. A remarkable effect was observed when the ionophore A23187 was used to activate platelets. Stimulation by A23187 evoked high prothrombin- and factor X-converting activities in normal platelets, whereas no significant enhancement of both coagulation reactions occurred with ionophore-stimulated patient's platelets. It should be mentioned that all stimulation procedures resulted in normal platelet release and aggregation, both in patient's and control platelets. Maximal stimulation of prothrombin and factor X activation was observed with platelets lysed by sonication, but no difference between patient and control was apparent.

**Phospholipid Exposure at Platelet Outer Surface**

Since the phospholipid composition of the patient's platelets was confirmed to be normal, platelets were tested for their ability to expose negatively charged phospholipids upon activation. Exposure of phospholipids at the outer surface of collagen plus thrombin-stimulated platelets was probed upon treatment of the cells with phospholipase A2 and sphingomyelinase, under conditions at which cell lysis remained below 10%. As shown in Table 2, patient's platelets differed markedly from normal platelets. Activated platelets from a control subject exposed amounts of phospholipid that fell within the range previously published, whereas platelets from patient M.S. had a diminished exposure of glycerophospholipids and a higher exposure of sphingomyelin. Since both factor X and prothrombin activation strongly depend on the presence of negatively charged phosphatidylserine, it is remarkable that the amount of this lipid which became exposed at the outer surface of activated platelets was about 2½ times less in the patient's platelets than in control's.

Treatment of ionophore-stimulated platelets with phospholipases for 20 minutes produced more than 50% breakdown of phosphatidylserine in normal platelets, whereas less than 20% of this lipid was degraded in the patient's platelets (data not shown). Unfortu-
Table 2. Phospholipid Degradation Under Nonlytic Conditions by Phospholipase A2 and Sphingomyelinase in Activated Platelets from Patient M.S. and Control

<table>
<thead>
<tr>
<th>Phospholipid Class</th>
<th>Percentage of Degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M.S.</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>17.9</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>22.4</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>23.9</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>58.0</td>
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Platelets (10^10 per milliliter) were stimulated for ten minutes with a combination of collagen (10 μg/mL) and thrombin (3.6 nmol/L), prior to a 20-minute incubation with phospholipase A2 (30 IU) and sphingomyel- nase (3.5 IU), as described before. Lipids were extracted and analyzed by two-dimensional thin-layer chromatography according to established procedures. Hydrolysis is expressed as the percentage of each phospholipid class, and data are the average of triplicate experiments from one blood sample of 200 mL.

The moderately severe bleeding disorder of patient M.S. has been ascribed by Weiss et al.6 to an isolated deficiency of platelet procoagulant activity, presumably due to an impaired ability of the platelets to accelerate prothrombin activation. Since thrombin-induced release and activation of platelet factor V was normal,6,7 a defect in the binding of coagulation factors to the platelet surface was suspected. Miletich et al.8 showed that factor Va promotes the binding of factor Xa to the platelet surface, while some unidentified platelet component ultimately limits the number of binding sites. It was subsequently demonstrated that binding of factor Xa-Va to platelets from patient M.S. was about 25% of the normal value, and that this was reflected in an impairment of the platelets to stimulate thrombin formation.7 The unidentified platelet component was suggested to be specific for factor Va binding, and most likely represents a membrane protein which is partly deficient in patient M.S.7,12 It should be mentioned, however, that binding studies with factor Va alone have not been reported with this patient's platelets.

The experiments presented in this report show that platelets from M.S. are equally defective in promoting prothrombin and factor X activation. This would exclude the possibility that the deficiency is limited to a binding site specific for factors Va-Xa. Although the patient's platelets can be moderately stimulated to enhance both factor X and prothrombin activation, the extent to which this occurs is always much less than that observed with stimulated control platelets. Especially, the time course of generation of both procoagulant activities following platelet stimulation is much slower than normal. Incubations with phospholipases suggest that the decreased stimulatory effect of the patient’s platelets is accompanied by a reduced exposure of anionic phosphatidylserine as compared to normal platelets. This would point to a defect in the mechanism by which anionic phospholipids become exposed at the outer surface during platelet activation. Since normal platelets show the highest procoagulant activity upon stimulation with Ca2+-ionophore,4 it is intriguing that this treatment is almost without effect on the patient’s platelets, despite normal release and aggregation. Therefore, the ultimate resolution of the defect might be revealed by an in-depth study with Ca2+-ionophore.

REFERENCES

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