Deficiency of Intact Thrombospondin and Membrane Glycoprotein Ia in Platelets With Defective Collagen-Induced Aggregation and Spontaneous Loss of Disorder

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Platelets from a patient with a severe lifelong bleeding tendency, which later spontaneously disappeared, lacked intact thrombospondin and glycoprotein (GP) Ia. Before disappearance of the bleeding disorder, results of coagulation studies and platelet aggregation in response to adenosine diphosphate (ADP), arachidonic acid, thrombin, A23187, epinephrine, and ristocetin were normal. In contrast, aggregation only occurred in the presence of collagen or wheat germ agglutinin at unusually high doses of these agonists. The platelets adhered normally to purified bovine and human type I collagen, and they did not spread in the presence of methylated type I collagen. No collagen-induced clot retraction was observed. Two-dimensional gel electrophoretic analyses of platelet proteins and immunologic studies showed that intact thrombospondin and GP Ia were absent. Aggregation in response to collagen could be restored by adding thrombospondin. Disappearance of the bleeding tendency occurred at the onset of menopause; subsequent analyses revealed that thrombospondin and GP Ia were present in platelets and that collagen-induced platelet aggregation was normal. These results suggest that both thrombospondin and GP Ia are essential in collagen-induced platelet aggregation. The spontaneous disappearance of the bleeding tendency may have been related to hormonal influences.

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NORMAL PLATELET function requires the presence of specific receptors on the platelet surface that interact with molecules from the vessel wall or plasma. Platelet interaction with collagen is a crucial event in hemostasis and in the pathogenesis of thrombosis and probably atherosclerosis. A putative role as the platelet receptor for collagen has been proposed for proteins such as fibronectin, von Willebrand factor (vWF), and thrombospondin. These substances are released from the α-granule and have been shown to bind collagen and membrane constituents of activated platelets; however, because collagen also activates washed platelets, a collagen receptor probably exists on the membrane surface of unstimulated platelets. So far, two platelet membrane proteins have been identified that bind to the soluble α1(1) chain of chick skin collagen or to insoluble human collagen. On the other hand, the zymogen form of platelet factor XIII has been proposed as the receptor in collagen-induced platelet aggregation. Nieuwenhuis et al have recently shown that platelets from a patient with a mild bleeding disorder did not respond to collagen and failed to express surface glycoprotein (GP) Ia. Here, we describe an isolated defect in collagen-induced platelet aggregation and its association with the lack of intact thrombospondin and platelet GP Ia.

PATIENT, MATERIALS, AND METHODS

Platelets from a 52-year-old female patient (Sigrid S.) with a severe bleeding tendency were studied. The patient and her parents indicated that the bleeding tendency had existed since early childhood. The symptoms were intensive menstrual and frequent spontaneous petechial bleeding. Some menstrual bleeding episodes had necessitated blood transfusions, and two severe postoperative hemorrhages had occurred. The bleeding tendency surprisingly disappeared in the autumn of 1986, and a fracture of the tibia in February 1987 was not accompanied by abnormal bleeding. The onset of menopause was observed at this time. Six examinations of the patient were performed before and three after disappearance of the bleeding tendency. Informed consent was obtained from the patient before each examination, and blood was drawn without cufing by using a 19-gauge butterfly hypodermic needle. The blood was anticoagulated by the methods that follow. Bleeding times were determined after insult with Simplate II blades (Gödecke, Freiburg, FRG). Clot retraction was measured according to Benthaus. Blood for control purposes was obtained from healthy female and male adult volunteers.

All studies on coagulation and platelets were performed at least twice on different dates.

Components of platelet granules. The serotonin content in platelets was measured according to Drummond and Gordon. Platelet factor 4 (PF4) and β-thromboglobulin (β-tg) in plasma were measured by means of radioimmunoassays (RIAs: Abbott Laboratories, Darmstadt, FRG, and Amersham Buchler, Braunschweig, FRG, respectively).

Platelet adhesion and spreading. The adhesion of platelets to purified human and bovine fibrillar type I collagen in a static system was measured according to Legrand et al by using gel filtration on Sepharose 2B (Pharmacia, Inc, Freiburg, FRG). Platelet spreading on Zapon varnish (Schminke, Krefeld, FRG) in the presence of collagen was assayed as described by Balleisen.

Aggregation studies. Platelet aggregation was performed according to Born and Gross. Platelet numbers were adjusted to 2 × 10^5/μL by dilution with autologous platelet-poor plasma (PPP). Aggregation was measured in response to adenosine diphosphate (ADP), epinephrine, arachidonic acid, the calcium ionophore A23187, wheat germ agglutinin (all from Sigma Chemical Co, Taukirchen, FRG), ristocetin (Paezol, Frankfurt, FRG), thrombin (Behringwerke AG, Marburg, FRG), and different collagen. Purified, acid-soluble, type I and type III collagen from calf skin and human type I collagen were kindly provided by Dr J. Rauterberg, Münster, FRG. Bovine type I collagen was methylated according to Rauterberg and Kühn. For studies on the influence of thrombospondin on aggregation, platelet-rich plasma (PRP) from the patient was incubated with 10 μg/mL of highly purified thrombospondin.
Fig 1. LABP-stained Western blots (A, B) and silver-stained (C, D) two-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gels of platelet proteins from control subjects (A, C) and from the patient during the bleeding disorder (B, D). Thrombospondin (TSP) is missing in B and D, and additional smaller proteins are visible in B (arrows).

Identification of platelet proteins. Platelet proteins were separated by two-dimensional gel electrophoresis and either stained with silver or analyzed by the lectin-avidin-biotin-peroxidase (LABP) technique as described by Kehrel et al. Thrombospondin was identified in Western blots of platelet proteins by using a monoclonal antibody prepared according to Vischer et al. and a second peroxidase-coupled antibody (Bio-Rad, Munich).

RESULTS AND DISCUSSION
Studies on coagulation excluded any coagulation disorder including von Willebrand’s disease. Platelet morphology was...
normal by light and electron microscopy. The bleeding time was prolonged (15 to three to six minutes in normal individuals). The platelet serotonin content (1 µg/10^9 TZ) and plasma levels of β-tg (43.7 ng/mL) and PF4 (5.9 ng/mL) were normal.

**Platelet proteins.** Analysis of platelet proteins by two-dimensional gel electrophoresis followed by silver staining or by the LABP technique (Fig 1) showed that both GP Ia and intact thrombospondin were absent. No residual spots attributable to these two glycoproteins could be detected when twice the normal amount of protein was used in the analysis. Intact thrombospondin could not be detected in immunoblots using a polyclonal antithrombospondin antibody (Fig 2). Small spots of glycoproteins related immunologically to thrombospondin and reacting with the same lectins were detected by LABP and immunoblot techniques. These proteins were found in the lower–molecular weight range of 70,000 to 80,000 daltons. It is possible that they are fragments of thrombospondin produced by in vivo proteolysis. Alternatively, their presence may indicate that synthesis of incomplete or altered thrombospondin molecules had occurred. GP Ib and IIb/IIIa were present as usual.

After the bleeding tendency had disappeared, protein analyses revealed that both intact thrombospondin and GP Ia were present in the patient’s platelets.

**Adhesion to fibrillar collagen.** The interaction of the patient’s platelets with human and bovine fibrillar type I collagen was normal as estimated in a static system according to Legrand et al. Adhesion values were 81% with human and 83% with bovine collagen (controls, 83% ± 10% and 83% ± 7%, respectively; n = 10). These observations contrast with those of Nieuwenhuis et al who also studied platelets deficient in GP Ia. Using the same method, these authors found only 4.4% ± 0.7% adhesion to fibrillar human type I collagen. In our studies, however, it seems unlikely that platelet adhesion to collagen is mediated directly by GP Ia because the adhesion levels were completely normal despite the absence of GP Ia.

**Spreading of platelets.** Unlike adhesion, spreading of the patient’s platelets was abnormal. In the presence of 0.1 mg/mL methylated bovine type I collagen, only 10% of the platelets spread on Zapon varnish (controls, 63% ± 6%). When 0.2 mg/mL methylated type I collagen was used, the amount of spreading was similar (9.9%; control, 82% ± 4%).

Using a Baumgartner perfusion chamber, Nieuwenhuis et al also observed abnormally low levels of spreading of GP Ia–deficient platelets.

**Clot retraction.** In PRP, no clot retraction was observed in response to a collagen stimulus. Because fibrinogen clotting and platelet activation are required for clot retraction, this experiment indicates that collagen was unable to activate the platelets.
Platelet aggregation. Platelet aggregation in response to ADP (3 μmol/L), ionophore A23187 (1.5 μmol/L), epinephrine (4 μmol/L), thrombin (0.15 U/mL), arachidonic acid (1.0 mmol/L), and ristocetin (1.0 mg/mL) was normal. In contrast, aggregation in response to wheat germ agglutinin or collagen occurred only at high concentrations and was not as complete as with control platelets (Fig 3). Because the aggregation defect is not absolute, it is possible that the putative collagen receptor GP Ia is not totally absent or that there are other collagen receptors that function at higher collagen concentrations. Our results support the suggestion by Nieuwenhuis et al20 that GP Ia is the receptor for wheat germ agglutinin–induced platelet activation.

When PRP from normal donors was mixed with PPP from the patient, normal aggregation occurred in response to collagen, whereas impaired aggregation of the patient’s platelets was still observed in the presence of normal PPP. This demonstrates that the reduced ability to aggregate was not due to a circulating inhibitory factor. Preincubation of the patient’s PRP with highly purified thrombospondin as shown in Fig 4, however, normalized collagen-induced aggregation (Fig 5). After the bleeding tendency had disappeared, collagen-induced aggregation of the platelets was found to be normal. This finding correlates with the disappearance of the bleeding tendency and with the appearance of thrombospondin and GP Ia. It is significant that the addition of purified human thrombospondin normalized collagen-induced platelet aggregation.

The results support the hypothesis that thrombospondin is involved in platelet aggregation.21 This hypothesis is also strengthened by observations that secreted thrombospondin binds to the surface of stimulated and even of resting platelets.22 Furthermore, antithrombospondin antibodies inhibit thrombin-, collagen-, and A23187-induced platelet aggregation and the secretion-dependent secondary phase of ADP-induced aggregation.21 The specific mechanism by which thrombospondin participates in platelet-collagen interactions remains to be elucidated. The unexpected disappearance of the tendency of the patient to petechial skin bleeding and spontaneous hematomas in 1986 occurred a few weeks after menstrual bleeding had stopped, ie, ostensibly at the onset of menopause. We speculate, therefore, that disappearance of the bleeding disorder may be associated with hormonal changes.

REFERENCES

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