RAPID COMMUNICATION

Defective Adhesion of Blood Platelets to Vascular Microfibrils in the Bernard-Soulier Syndrome

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Bernard-Soulier syndrome (BSS) platelets, which lack the membrane glycoprotein complex Ib-IX, do not adhere to subendothelium. The adhesion of platelets from two patients with BSS to subendothelial microfibrils (MFs) and type I collagen was compared in an in vitro assay adapted to patients with low platelet count. With both patients, platelet adhesion to MFs was strongly defective, whereas the adhesion to collagen was normal. The involvement of GPIb in the MFs-induced platelet adhesion was confirmed by the inhibitory effect of a MoAb (AN561) to the von Willebrand (vWF) factor binding domain of GPIb. The adhesion of platelets to MFs thus requires GPIb-IX and an axis MFs-vWF-GPIb-IX seems therefore to be prevalent in the reactivity of platelets with subendothelium.

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THE BERNARD-SOULIER syndrome (BSS) is a rare platelet disorder in which a prolonged bleeding time associates a thrombocytopenia with enlarged platelets: their aggregation by adenosine diphosphate (ADP), collagen, and epinephrine was shown to be normal, but they failed to agglutinate with the antibiotic ristocetin in the presence of normal plasma. A major defect of BSS was shown by various investigators to be a defective platelet adhesion to subendothelium. As reviewed by Nurden et al., the characteristic feature of BSS platelets is the quantitative defect in glycoprotein Ib (GPIb), a major sialoglycoprotein of the platelet membrane that is involved in platelet adhesion to subendothelium. GPIb is composed of a large α subunit that is linked to a small β subunit: the α subunit has been shown to bear receptor sites for von Willebrand factor (vWF), a high molecular weight plasma protein that is also present in subendothelium. The thrombogenicity of subendothelium is caused by the reactivity of several components with platelets. Two main vascular macromolecules have been shown to react with platelets and induce their adhesion and subsequent aggregation. These components are collagen (for review, see Barnes et al.) and microfibrils (MFs). Previous studies from our laboratory have shown that GPIb was directly involved in the adhesion of platelets by MFs. Moreover, vWF binds to MFs, and this binding seems to be a prerequisite for the interaction between platelets and MFs. We have recently described an adhesion assay in which we showed that in the MFs-platelet interaction, vWF is indispensable to the early step of adhesion. This assay has been applied to two patients with BSS to estimate the adhesion of their platelets to MFs, as compared with type I collagen. This report describes a defect in the adhesion of BSS platelets to MFs that contrasts with their normal adhesion to collagen.

MATERIALS AND METHODS

Case report. B and V. are two unrelated patients with BSS. Their case reports were described previously. Diagnosis was made on the basis of thrombocytopenia (patient B., 18,000 platelets/μL; patient V., 60,000 platelets/μL) with large platelets, prolonged bleeding time, and absence of platelet agglutination with ristocetin despite normal vWF-related properties. Platelet aggregation to collagen, ADP, and epinephrine were found to be normal. A lack in GPIb was described for both patients.

Indium-labeled platelets. All studies were performed after informed consent. Blood was withdrawn from a normal volunteer (control) or from the patient by venepuncture into a one-sixth volume of acid-citrate-dextrose (NIH formula A). The blood sample was allowed to settle for 2 hours at room temperature in 5-mL plastic tubes. The platelet-rich plasma (PRP) was removed by aspiration and PGE, (Sigma, St Louis, MO) was added at a 10−7 mol/L final concentration. Platelet poor plasma (PPP) was obtained by centrifugation of the resting blood at 1,000 g for 20 minutes at 20°C. Platelets were labeled by indium tropolonate (CIS Biointernational, GIF/Yvette, France) as previously described. Platelets were then washed three times in Patscheke washing buffer, pH 6.5, containing PGE1 10−7 mol/L final concentration. Control and patient platelets were finally resuspended at a concentration of 40 × 105 per μL.

Collagen. Acid soluble calf skin collagen was purchased from Diagnostica Stago (Asnières, France), resuspended, and polymerized according to the instructions of the manufacturer.

MFS. MFs were extracted from human umbilical arteries by 6 mol/L guanidinium chloride following a procedure previously described. The absence of type VI collagen was verified by enzymelinked immunosorbent assay (ELISA) and Western blot analysis using a polyclonal anti-type VI antibody donated by Dr S. Ayad (University of Manchester Medical School, Manchester, England). In the adhesion assays, the MFs were sonicated after addition of 40 μL of PPP for 400 μL of platelet suspension. This is the amount of plasma necessary for an optimal platelet response to MFs.

Adhesion assay. A procedure derived from the method described by Santoro and Cunningham was adapted as follows. After addition of different concentrations (between 20 and 100 μg) of collagen and MFs were added to 400 μL of indium-labeled platelets and the suspensions were stirred for 5 minutes at room temperature. The suspen-
sions were then deposited onto Isopore membranes (Millipore, Bedford, MA) (5 μm pore diameter): free nonadhering platelets filtered through, whereas adhering platelets remained on the filter associated to collagen or MFs. Controls were performed by filtering a platelet suspension incubated without inducer. The filters were washed three times with saline, and platelet adhesion was determined by measuring the radioactivity associated to the membrane in a gamma counter. The results were expressed as the number of platelets present on the filter according to the specific activity of the platelet suspension. The values of the control (platelet suspension in the absence of inducer) were removed from the results. The assays were performed in duplicate and the results were the mean values of these duplicates.

MoAbs: AN51 was purchased from Dako laboratories (Dako-patts, Glostrup, DK). FMC 25 and WM 23 were gifts from Dr M. Berndt (University of Sidney, Australia). Platelets suspensions were incubated for 30 minutes at room temperature with 2, 5, or 10 μg/mL (final concentration) of the antibodies. As control, the platelets were incubated with mouse nonimmune serum. The results were the mean (±SD) of three different experiments in duplicate.

RESULTS

Platelet adhesion in BSS. The adhesion of platelets to collagen and MFs was measured in the assay using Isopore membrane. Because of differences in their number of platelets, patients B. and V. have been separately studied. The very low platelet amount present in the PRP from patient B. (18,000 platelets/μL) did not allow concentration of the suspension to 1 × 10^5 platelets/μL (standard platelet concentration in the assay), and the maximal platelet concentration reached with this patient was 0.3 × 10^5/μL: one single dose of MFs (50 μg) was therefore applied. In such a condition, the adhesion of his platelets to MFs was nil, as compared with 0.45 × 10^5 adhering platelets retained on the filter for the control: this corresponds to the mean value for platelet adhesion to MFs (12 determinations), i.e., 0.47 ± 0.12 × 10^5 platelets in the same condition of platelet concentration, for which no adhesion to collagen was observed. In this regard, we previously showed that the adhesion depends on the platelet concentration: in this assay 30,000 platelets corresponds to the threshold in the adhesion to MFs that this threshold is higher for collagen, as seen from the lack of reactivity of the control platelets at the same platelet concentration. The fact that MFs are more reactive for platelets than collagen has already been mentioned and the smaller diameter of the MFs can offer to platelets a greater reactive surface area than the large type I collagen fibers.

Figure 1 shows the results obtained with patient V. A weak adhesion of her platelets to MFs was observed, with a reduction of 33%, 69%, and 90% in the adhesion, as referred to control platelets, after incubation with 20, 50, and 100 μg of MFs, respectively. In contrast, the adhesion of the same platelets to collagen was normal or slightly increased (2.9 × 10^5 adhering platelets for the patient v 1.27 × 10^5 adhering platelets for the control). The size of the platelets did not interfere in this assay because in the two controls (platelets deposited in the absence of inducer), the number of platelet retained on the filter was equal to 1.04 × 10^5 for the patient and 1.08 × 10^5 for the control. These blank values have been subtracted from the amount of adhering platelets retained on the filter. The average number (±SD) of normal platelets (10 donors) adhering to 20 and 50 μg MFs was, respectively, 2.1 × 10^5 ± 0.2 for 20 μg and 4 × 10^5 ± 0.5 for 50 μg of MFs, and the average number of platelet adhering to 50 μg of collagen was 1.16 × 10^5 ± 0.2. The values of platelet adhesion for the donor were thus in accordance with this average.

Platelet adhesion in the presence of anti GPIb MoAbs. Three MoAbs directed against different domains of the GPIb-IX complex were used: the antibody FMC 25, which recognizes GPIX, the antibody An 51, which reacts with the 45-Kd N-terminal part of GPIba that bears the vWF binding domain, and the antibody WM 23, which recognizes the central 84-Kd macroglycopeptide of GPIba. As shown in Fig 2, among these antibodies, AN 51 was the only one that significantly affected the adhesion of platelets to MFs. Inhibitions of 60%, 67%, and 70% were found with, respectively, 2, 5, and 10 μg/mL AN 51 final concentrations. The MoAbs WM 23 and FMC 25 were without effect. None of these antibodies affected the adhesion of platelets to collagen (not shown).

DISCUSSION

In BSS, the adhesion of platelets to MFs is absent or strongly diminished: it was shown that the deficient expression of GPIba on the platelet membrane is responsible for the poor ability of platelets to adhere to subendothelium. This defect must undoubtedly be ascribed to the nonreactivity of subendothelial MFs with BSS platelets, as evidenced by the fact that their adhesion to purified collagen was not diminished. Assessing that the lack of adhesion of BSS platelets to subendothelium is caused by defective adhesion to subendothelial MFs, and not to collagen, which as shown in this report, is normal for patient V.'s platelets, leads to the confirmation that the MFs have a preeminent importance in

![Figure 1](https://www.bloodjournal.org) Adhesion of BSS (patient V.) and normal platelets to collagen and MFs. Adhesion of control (light bars) and BSS (dark bars) 111 indium-labeled platelets to 20, 50, and 100 μg of MFs or 50 μg of type I collagen. Platelets incubated with the adhesion inducer are then filtered through 5-μm pore Isopore filter. Adhering platelets are calculated from the radioactivity retained on the filter.
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the thrombogenicity of subendothelium. It must be strongly underlined that the 10 to 15-nm thrombospondin containing MFs (TSP-MFs) share no identity with any fibrillar collagen. In particular, they are not related to type VI collagen that, in its native state, also forms microfibrillar structures that differ in their diameter (5 nm), organization, and antigenicity from TSP-MFs, as shown by immunohistochemical studies.

The present study clearly confirms that the platelet surface glycoprotein involved in the MFs-platelets interaction is GPIb. This had already been suggested from other observations from our laboratory that applied the Baumgartner’s perfusion chamber system to show an inhibition of normal platelet adhesion to rabbit aortic subendothelium by the IgG AN 51, a MoAb directed against the N-terminal vWF binding region of GPIb. This inhibition, observed with untreated arteries, was amplified on aortic segments that had been incubated with collagenase to digest fibrillar collagen without altering the MFs. In this regard, it should be remembered that an IgG antibody against GPIb, developed in a multitransfused BSS patient, inhibited the interaction of normal platelets as well as of thrombasthenic platelets (with normal GPIb expression but lack of expression of the GPIb-IIIa complex on the membrane) to subendothelium.

A prevalent role of the N-terminal part of GPIbα in the interaction of platelets with MFs has been confirmed by the inhibitory effect of AN 51 on the MF-induced platelet adhesion and aggregation. In contrast, GPIIX or the GPIbα macroglycopeptide were shown to be not involved in the platelet-MFs interaction, as shown by the inability of their corresponding MoAbs FMC 25 and WM 23 to inhibit the initial step of adhesion. That the AN 51 antibody does not completely inhibit the microfibril-induced platelet adhesion can be explained by the heterogenous composition of MFs in which at least two different constituents were shown to be involved in their reactivity with platelets: a 97-Kd protein that binds vWF, and a thrombospondin (TSP)-derived constituent. This led us to propose that the MFs/platelet interaction would result from a complex phenomenon in which at least two platelet membrane receptors would act: GPIb for the recognition of vWF bound to the 97-Kd protein and the not yet identified receptor(s) for the recognition of the TSP constituent of the MFs. AN 51 consequently only affects the vWF-dependent phase of this complex phenomenon.

We have previously demonstrated that the adhesion of platelets to MFs and their subsequent aggregation imperatively required the presence of vWF. Recent data using immunogold labeling showed the preferential association of vWF to microfibrils in subendothelium. As mentioned, the assays were performed in the presence of 40 μL of plasma that, as previously demonstrated, is necessary for the occurrence of platelet aggregation and adhesion to MFs. This requirement for plasma is justified by the necessity to bring to the medium a minimal amount of vWF that binds to MFs and, as already proposed, becomes reactive with GPIb on the platelet surface. Consequently, this explains why the absence or deficiency in the expression of GPIb on the surface of BSS platelets and the occupancy of the GPIb-vWF binding domain by AN 51 result in their defective adhesion to MFs. Our present observations thus strongly support a model that explains the involvement of vWF and GPIb in the subendothelium-platelet interaction: MFs, which are abundant just beneath the endothelium, in the vicinity of the basement membrane are vascular constituents to which vWF preferentially binds, and the binding of vWF to MFs, which allows its recognition by GPIb, was presented as a triggering step in the platelet-subendothelium interaction.

In conclusion, in BSS, the hemorrhagic diathesis, which has been ascribed to a poor reactivity of platelets with the vessel wall, is obviously caused by a defective adhesion of platelets to the noncollagenous microfibrils that are abundant in the subendothelium.

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