Uremic Platelets Have a Functional Defect Affecting the Interaction of von Willebrand Factor With Glycoprotein IIb-IIIa

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Uremic patients have an impaired platelet function that has been related to membrane glycoprotein (GP) abnormalities. Using a perfusion system, we have studied the interaction of normal and uremic platelets with vessel subendothelium (SE) under flow conditions. Reconstituted blood containing washed platelets, purified von Willebrand factor (vWF) (1 U/mL), and normal washed red blood cells was exposed to de-endothelialized rabbit segments for 10 minutes at two different shear rates (800 and 1,600 seconds⁻¹). In some experiments a monoclonal antibody to the GPIIb-IIIa complex (EDU3) was added to the perfusates. With normal platelets, the percentage of the vessel covered by platelets (%CS) was 23.1% ± 3.7% at 800 seconds⁻¹ and 30% ± 4.3% at 1,600 seconds⁻¹. Platelets were observed in contact or forming monolayers on vessel SE. EDU3 inhibited the spreading of normal platelets. The %CS (11.1% ± 3.3%) was statistically decreased (P < .01) and most of the platelets were observed in contact with the vessel surface. These data indicate that, under flow conditions, the interaction of vWF with GPIIb-IIIa can support the spreading of normal platelets in the absence of exogenous fibrinogen. Under the same experimental conditions, the interaction of uremic platelets with SE was markedly impaired at both shear rates studied (P < .01 v normal platelets). The presence of EDU3 did not modify the interaction of uremic platelets. These results confirm the impairment of the platelet adhesion observed in uremic patients. Furthermore, they indicate the presence of a functional defect in the interaction of vWF with GPIIb-IIIa. The fact that perfusions with normal and uremic platelets in the presence of an antibody to the GPIIb-IIIa complex did not show any differences gives indirect evidence on a functionally normal interaction vWF/GPIIb in uremic patients.

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BINDING OF fibrinogen to glycoprotein IIb-IIIa (GPIIb-IIIa) is known to play a prevalent role in mediating the aggregation of platelets. In recent years it has been suggested that GPIIb-IIIa would play an important role not only in mediating platelet-platelet interaction but in participating in the regulation of platelet-subendothelium interaction. Under flow conditions, Glanzmann's thrombasthenic platelets spread with difficulty on the subendothelium and did not form aggregates. In contrast with this, platelets from an afibrinogenemic patient have normal adhesion on vessel subendothelium, suggesting a role for GPIIb-IIIa mediating the adhesion of platelets onto damaged vessels.

The binding of plasma von Willebrand factor (vWF) to subendothelial structures and platelet GPIb mediates the primary attachment of platelets to sites of endothelial disruption. The vWF also binds to GPIIb-IIIa. Several investigators have pointed out that this mechanism would have a limited physiologic importance because low concentrations of fibrinogen compete with vWF for binding to GPIIb-IIIa. Further investigations indicate that vWF bound to GPIIb-IIIa may support platelet aggregation in patients with congenital afibrinogenemia. Despite this experimental evidence, little is known about the role of vWF in other disorders of platelet function in which the binding of fibrinogen with GPIIb-IIIa might be initially impaired.

Uremic patients have a bleeding tendency associated with platelet dysfunction. Platelets from uremic patients have a reduced aggregating response to adenosine diphosphate, epinephrine, and collagen. Uremic platelets also have a defective interaction with vessel subendothelium, and radioligand studies indicate an impaired binding of fibrinogen to adenosine diphosphate (ADP)-stimulated uremic platelets. In the present study we have explored the functional relevance of the interaction of vWF with GPIIb-IIIa in uremia. The adhesion of normal and uremic platelets to the subendothelium was investigated in a perfusion system with reconstituted blood in which vWF was the only adhesive protein in the liquid phase. Per fusates consisting of washed platelets and washed red blood cells (RBCs) re suspended in a balanced salt solution containing human serum albumin (5%) and purified vWF (1 U/mL) were circulated through annular chambers containing denuded arterial segments. Specific antibodies to GPIb or the GPIIb-IIIa complex were incorporated in some experiments.

MATERIALS AND METHODS

Patients. The study, approved by the Human Subjects Committee of the Hospital Clinic, was performed according to the principles of the declaration of Helsinki. Informed consent was obtained from all participants.

Seven uremic patients (five men and two women) on maintenance hemodialysis were included in this study. These patients were randomly selected from a group of uremic patients with a previous history of clinical bleeding in whom impairment of platelet function had been demonstrated. Platelet counts and coagulation tests (prothrombin time, partial thromboplastin time, and fibrinogen levels) were, in each case, within the normal range. No patient had received blood-related products for 2 months, and none had taken aspirin or other drugs that affect platelet function for at least 2 weeks.

Monoclonal antibodies (MoAbs). MoAb 6D1 against platelet GPIb blocks ristocetin-induced platelet agglutination and inhibits the binding of vWF to platelet GPIb. This antibody was generously provided by Dr B Coller (Stony Brook, NY).
MoAb EDU3 inhibits GPIIb-IIIa–dependent platelet aggregation tests and inhibits the binding of fibrinogen, vWF, and fibronecin to this GP complex. This MoAb was kindly provided by Dr. R. Vilella (Hospital Clinic i Provincial, Barcelona, Spain).

**Platelet washing.** Platelet-rich plasma (PRP) was obtained by centrifugation of normal anticoagulated blood for 20 minutes at 100g. Platelets from PRP were washed twice with equal volumes of CCD (93 mmol/L sodium citrate, 70 mmol/L citric acid, and 140 mmol/L dextrose) pH 6.5, containing 5 mmol/L adenosine and 3 mmol/L theophylline. The final pellet was reuspended in Hanks’ solution. Washed platelets were always tested for their response to agonists before further manipulation.

**Purification of vWF.** vWF was purified from pooled blood bank cryoprecipitates according to methods previously described. Each batch of purified vWF was tested for ristocetin cofactor activity and found to contain between 100 and 120 U/mg. The multimeric composition was assessed in all batches using agarose electrophoresis in the presence of sodium dodecyl sulfate (SDS). Mutlimers of all sizes were present and the complex multimeric structure was similar to that of plasma vWF. Purity was assessed by SDS-polyacrylamide gel electrophoresis.

**Preparation of perfusates.** To reproduce hematocrit values usually found in uremic patients, perfusates consisted of washed platelets, purified vWF (1 U/mL) suspended in a balanced salt solution containing human serum albumin (5%), and 20% vol of washed RBCs. MoAbs were added at this point, and the perfusates were incubated for 30 minutes in a water bath at 37°C before studies were initiated. The final concentration of platelets in the perfusates was always between 1.5 to 2.5 x 10⁹ cells/µL.

**Perfusion studies.** Perfusion studies were performed at 37°C in perfusion chambers as developed by Baumgartner. Flow was maintained by means of a peristaltic pump. Blood was perfused at 140 mL/min through regular chambers with an effective annular width of 1.2 mm, or at 25 mL/min through small chambers with an effective annular width of 0.35 mm, giving rise to wall shear rates of 800 and 1,600 seconds⁻¹, respectively. α-Chymotrypsin denuded rabbit aortas were used in all experiments in this study. Aortic segments were mounted on plastic rods and rinsed with phosphate-buffered saline for 10 minutes. After 10 minutes of perfusion with reconstituted samples of blood, segments were rinsed with the same phosphate buffer and fixed with 3% glutaraldehyde.

After 1 hour, segments were separated from the rod, dehydrated through a graded series of ethanols, embedded in JB-4 embedding compound (Polysciences, Warrington, PA), thin-sectioned for light microscopy, and stained with methylene blue.

**Morphometric evaluation.** Platelets interacting with subendothelium were evaluated according to Baumgartner and Muggli. An eyepiece with a special reticule was used for the morphometric evaluation. Platelets or groups of platelets were classified as follows: contact (C), platelets that are attached but not spread on the subendothelium; adhesion (A), platelets that are spread on subendothelium or form layers of less than 5 µm in height; and thrombi (T), platelet aggregates of 5 µm or more in height. All of these basic parameters are expressed as a percentage of the total length of the vessel screened. The total covered surface (CS) is obtained by adding the previous basic parameters (C + A + T).

Because the formation of large platelet aggregates is practically nil in the absence of fibrinogen, we have only referred to results of the CS, a parameter that estimates the overall deposition of platelets on vascular subendothelium and C, an estimation of the primary interaction of platelets with subendothelial structures. Spreading of platelets (A) can be easily inferred from the previous parameters. Statistical analysis. Student's t-test was used for statistical comparisons. A P level of .05 was considered statistically significant.

**RESULTS**

**Normal platelets.** Normal platelets in the presence of 1 U/mL vWF interacted with the surface of the denuded vessel (Fig 1A). The total surface of the vessel covered by platelets (%CS) was dependent on the shear rate varying from 23.1% ± 3.6% (mean ± SEM) at 800 seconds⁻¹ to 30% ± 4.3% at 1,600 seconds⁻¹. Most of the platelets were found either in contact with or spread onto the subendothelial surface. Large aggregates were practically absent in these perfusion experiments. As shown in Fig 2A, the percentage of platelets in contact (%C) decreased with increasing shear rate (12.2% ± 1.7% at 800 seconds⁻¹; 9.7% ± 2.1% at 1,600 seconds⁻¹). Conversely, percentages of spread platelets (%A) rose from 10.8% ± 2% (800 seconds⁻¹) to 20.2% ± 3.9% (1,600 seconds⁻¹).

The addition of 6 µg/mL EDU3 (anti–GPIIb-IIIa) to perfusates containing normal washed platelets modified the pattern of platelet interaction (Fig 1B). The total surface of the vessel covered by platelets was markedly decreased (11.1% ± 3.3%) with respect to experiments in which the MoAb was absent (P < .01). The spreading of platelets was almost completely blocked by this antibody. Most of the platelets were found as single platelets anchored on the subendothelium (Fig 1B), and variations in shear rates resulted in slight modifications in the proportions of these contact platelets (10% ± 3.1% at 800 seconds⁻¹; 8.5% ± 1.8% at 1,600 seconds⁻¹).

The presence of 10 µg/mL 6D1 (anti–GPIb) in the perfusates almost completely inhibited the interaction of platelets with the subendothelium.

**Uremic platelets.** The interaction of uremic platelets with the subendothelium was morphologically distinct (Fig 1C). The morphometric results confirmed the defective
interaction of uremic platelets with respect to normal platelets (Fig 2B). The total surface of the vessel covered by uremic platelets (%CS) at 800 seconds⁻¹ was 11.2% ± 2.3%, which represents a significant decrease with respect to values found with normal platelets (P < .01). At this shear rate, the analysis of interacting platelets showed 6.3% ± 0.7% of the surface covered by single platelets in contact with the subendothelium, but only 4% ± 1.3% of this surface was covered by spread platelets. The failure of uremic platelets to spread on the subendothelium was made more evident in perfusions at 1,600 seconds⁻¹ (%CS = 15.7 ± 3.6; %A = 8.6 ± 3). The addition of 6 μg/mL of EDU3 to the perfusates containing uremic platelets resulted in modifications of the pattern of platelet-subendothelium interactions. A decrease in total platelet deposition (%CS) was observed, but in this case differences did not reach the levels of statistical significance. As in those experiments with normal platelets, the spreading was inhibited. Most of the platelets were observed as single, isolated platelets in contact with the subendothelium (9.7% ± 2.1% and 9.4% ± 1.7% at 800 seconds⁻¹ or 1,600 seconds⁻¹, respectively). Only a low proportion of platelets were observed spread on the subendothelial surface (1.7% ± 1% at 800 seconds⁻¹; 1.2% ± 0.8% at 1,600 seconds⁻¹).

The presence of 6D1 (anti-GPIIb) resulted in an almost complete inhibition of the interaction of uremic platelets with the subendothelium.

**DISCUSSION**

Results of the present study indicate that the primary process of attachment of uremic platelets on subendothelial surfaces is perfectly preserved. However, uremic platelets have an intrinsic anomaly characterized by a defective spreading that is related to functional anomalies of the GPIIb-IIIa complex.

Our experiments were conducted so that vWF was the only adhesive protein present in the liquid phase of the perfusates. Under this condition, normal platelets did interact with denuded vessels. However, the interaction of platelets with subendothelium was almost completely blocked by an antibody to GPIb or in perfusion experiments performed in the absence of exogenous vWF (data not shown). These data indicate that the attachment and spreading of normal platelets can be supported by the mere presence of exogenous vWF. These findings would also explain why other investigators have observed near to normal platelet adherence on SE in studies performed with blood from afibrinogemic patients. The active role that GPIIb-IIIa plays during platelet spreading is well documented.²²⁻²⁵ It has been recently suggested that the interaction of fibrinogen with GPIIb-IIIa is not required for platelet adhesion onto subendothelium at high shear rates.²⁶ Our experimental data support this evidence. Moreover, they confirm previous findings on exogenous vWF supporting platelet interactions with subendothelium at low shear rates²⁷ or in the absence of other plasma-adhesive proteins.²⁸

Uremic platelets showed a defective interaction with vessel subendothelium that confirms an intrinsic anomaly of uremic platelet independent of an additional plasmatic interference. In comparisons with controls, uremic platelets displayed a lower surface coverage and a higher proportion of platelets in contact. The spreading defect of uremic platelets was more evident at high shear rate. However, no differences were found between normal and uremic platelets when an antibody to GPIIb-IIIa was added to the perfusates, which suggests that the primary attachment of uremic platelets to subendothelial structures is well preserved.

A defective binding of fibrinogen with GPIIb-IIIa of ADP-stimulated platelets has been described in uremia.¹⁴ However, immunocytochemical studies indicate that the presence and distribution of GPIb and GPIIb-IIIa in the
plasma membrane of unstimulated platelets from uremic platelets does not basically differ from that found in normal subjects. In this respect, Gralnick et al. have recently shown that the platelet binding sites for vWF are normal in uremic platelets. Although our results on the interaction of vWF with GPIb-IIIa seem to differ, it should be emphasized that the methods used are basically different. In our experiments, GPIb-IIIa integrity is explored as a function of its ability to promote platelet spreading under flow conditions, while previous investigators have explored the binding of radiolabeled vWF to platelets stimulated by thrombin in suspension.

Recent reports indicate that vWF released from the α granules avidly binds to the surface of platelet stimulated in plasma membrane of unstimulated platelets from uremic subjects. In this respect, Gralnick et al. have recently shown that the platelet binding sites for vWF are normal in uremic platelets. Although our results on the interaction of vWF with GPIb-IIIa seem to differ, it should be emphasized that the methods used are basically different. In our experiments, GPIb-IIIa integrity is explored as a function of its ability to promote platelet spreading under flow conditions, while previous investigators have explored the binding of radiolabeled vWF to platelets stimulated by thrombin in suspension.

Recent reports indicate that vWF released from the α granules avidly binds to the surface of platelet stimulated in suspension. This process cannot be blocked by the presence of antibodies to GPIb or to the GPIIb-IIIa, suggesting that the binding of platelet-released vWF may occur within the platelet canalicular system. This membrane-bound vWF would facilitate platelet interactions with damaged vessels. Uremic platelets have low levels of vWF in their α granules. Theoretically, a decrease in surface-bound platelet vWF may help to explain the impairment of spreading we have observed in uremic platelets. However, in our experiments an antibody to GPIIb-IIIa effectively blocked the spreading of platelets. It is very unlikely that this antibody could have reached the deep membranes of the structures of the open canalicular system. Thus, it seems conceivable that under our experimental conditions, the spreading of platelets on the subendothelium would be mainly dependent on the binding of the exogenous vWF to the externally available GPIIb-IIIa.

Data obtained in our study suggest that the process of stimulus response coupling is intrinsically defective in uremic platelets. In all probability, the transmission of the original signal triggered by the primary interaction GP1b/vWF/subendothelial components is abnormal or not sufficiently amplified in uremic platelets. Platelets from severe uremic patients have shown a smaller increase in cytoplasmic Ca2+ after stimulation. Under these circumstances, changes in the conformation of platelet cytoskeleton that result in platelet spreading or platelet internal contraction might be compromised.

To conclude, our data confirm the physiologic significance of GPIIb-IIIa mediating platelet spreading. The interaction of GP1b with vWF, responsible for the anchorage of platelets to subendothelial components under flow conditions, is perfectly normal in uremic patients. Uremic platelets have a functional impairment of the externally available GPIIb-IIIa receptor, which is demonstrated by a limitation of the platelet-spreading capabilities.

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