CONCISE REPORT

Platelet von Willebrand Factor: Evidence for Its Involvement in Platelet Adhesion to Collagen

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Although it is well established that plasma von Willebrand Factor (vWF), a large multimeric glycoprotein, is synthesized by the endothelial cell that can release it into both plasma and the subendothelium. It is also synthesized by the megakaryocyte, is present in the α-granules of platelets and is released when platelets are stimulated by collagen, thrombin, and adenosine diphosphate (ADP). Whereas plasma and subendothelial vWF clearly play a role in mediating platelet adhesion, the role of platelet vWF is still unclear. Normalization of the bleeding time following 1-deamino-8-D-arginine vasopressin (DDAVP) infusion has been reported in type I von Willebrand disease (vWD) patients with normal but not with low platelet vWF. In addition, in patients with type I vWD, the prolongation of the bleeding time correlates better with the vWF levels in platelets than in plasma. The importance of the α-granule proteins released during platelet adhesion is further illustrated by studies of patients with storage pool deficiency. In order to investigate the involvement of platelet vWF in mediating platelet adhesion to collagen we performed in vitro perfusion experiments using reconstituted platelets and plasma from controls or five patients with severe von Willebrand disease (vWD). In order to investigate the involvement of platelet vWF in mediating platelet adhesion to fibrillar collagen in a parallel-plate perfusion chamber. Reconstituted blood containing RBCs, various mixtures of labeled washed platelets and plasma from controls or five patients with severe von Willebrand disease (vWD), was perfused through the chamber for five minutes at a shear rate of 1,600 s⁻¹. Platelet-collagen interactions were estimated by counting the radioactivity in deposited platelets and by quantitative morphometry. When the perfusate consisted of normal platelets suspended in normal plasma, platelet deposition on the collagen was 24.7 ± 3.6 μg/cm² (mean ± SEM, n = 6). Significantly less deposition (16 ± 2.3) was observed when vWD platelets were substituted for normal platelets. In mixtures containing vWD plasma, significantly greater deposition (9 ± 2.2) was obtained with normal than with vWD platelets (1 ± 0.4) demonstrating a role for platelet vWF in mediating the deposition of platelets on collagen. Morphometric analysis confirmed these data. Our findings indicate that platelet as well as plasma, vWF mediates platelet-collagen interactions at a high shear rate.

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Materials and Methods

Materials. Equine tendon fibrillar collagen was from Hormon-Chemie, Munich, West Germany. Aggregation studies were performed in a Payton Dual-Channel aggregometer; ristocetin was from Lundbeck & Co, A/S, Copenhagen, Denmark. All chemicals purchased from commercial sources were of analytical grade (Fluka AG, Buch, Switzerland; Merck, France).

Patients. Five patients with severe vWD including two from the same family were tested. Studies to characterize these patients were as follows. Bleeding time was measured using a template device (Simple II, General Diagnostics, Morris Plains, NJ). Factor VIII activity (VIII C) was assayed by a one-stage clotting technique; vWF antigen (vWF Ag) by ELISA and ristocetin cofactor activity of vWF (vWF RCo) using formalin-fixed platelets. The concentration of these three parameters was expressed in international units (IU) per deciliter using as reference pooled normal plasma from 20 donors calibrated against the First International Reference Preparation for Factor VIII-related activities in plasma (National Institute for Biological Standards and Control, London). The platelet content of vWF Ag was measured by ELISA in washed platelets lysed by 0.2% Triton X-100. The results of the above studies were essentially the same in all patients. Platelet counts and hematocrit values were in the normal range. The bleeding times were >30 minutes (normal range three to seven minutes), plasma levels of VIII C <5 U/dL, vWF Ag <5 x 10⁻¹ U/mL in plasma (normal >50 U/dL), platelet vWF Ag values <2 x 10⁻³ U/10⁸ platelets (normal >0.2 U/10⁸ platelets). No patient had any inhibitor to vWF at the time of the study.

Preparation of perfusates. Venous blood from healthy volunteers or patients was collected in 1/10th volume of 0.108 mol/L trisodium citrate. Perfusates consisted either of whole citrated blood perfused immediately following collection or reconstituted blood. Platelets were isolated, labeled with chromium-51 (⁵¹Cr), and washed three times as previously reported. Several control studies were performed to verify that no significant amounts of plasma vWF remained adsorbed on platelets following washing: (1) washed platelets from control subjects did not aggregate in the presence of 1.3 mg/mL ristocetin unless a source of plasma vWF was added; (2) following platelet washing, the last supernatant contained <5 x 10⁻⁴ U/mL of vWF Ag when measured by ELISA; and (3) by the same technique, no or only traces of vWF (5 x 10⁻¹ U/10⁸ platelets) were adsorbed on the platelet membrane.

Washed platelets were suspended in plasma and the final citrate concentration adjusted to 20 mmol/L. Perfusates were reconstituted.
immediately before perfusion by adding washed (three times) red cells to the suspended platelets (hematocrit 40%, platelet count 1.5 $\times 10^{11}$/L). Four types of perfusates were used: (1) control platelets suspended in control autologous plasma; (2) vWD platelets mixed with control plasma; (3) control platelets mixed with vWD plasma; and (4) vWD platelets suspended in autologous vWD plasma. Mixtures contained blood components with similar blood groups.

Perfusion chamber and perfusion procedure. Perfusion studies were performed using a parallel-plate chamber, P1,13 derived from the original device of Sakariassen et al.14 Collagen (30 $\mu$g/cm$^2$) was sprayed onto plastic cover slips (Thermanox TM, Miles Laboratories Inc, Naperville, IL) and the collagen-coated surfaces were perfused at 37°C for five minutes at a shear rate of 1,600 s$^{-1}$ with 20 mL blood samples as previously described.14

Quantitation of platelet-collagen interactions. Following perfusion, cover slips were cut parallel to the flow axis in two equal halves. One half was used for $^{51}$Cr counting in a gamma counter (1260 multigamma II; LKB, Orsay, France). Platelet deposition was expressed as the number of deposited labeled platelets per cm$^2$.15 The other half was used for morphometric evaluation. The percentage surface covered with platelets (platelet adhesion) and microthrombi higher than 2.5 $\mu$m (platelet thrombus formation) were estimated according to Baumgartner and Muggli.16 Fixation, embedding and removal of exposed surfaces from cover slips were performed as previously described.17

Monoclonal antibodies to vWF. Two monoclonal antibodies (MoAbs) to human vWF were used. These MoAbs specifically inhibit vWF binding to its platelet membrane receptors: MoAb H9 inhibits its binding to glycoprotein Ib (GPIb) and MoAb 9 blocks its binding to glycoprotein Iib/IIIa (GPIib/IIIa).18 In some experiments a MoAb to a mammary tumor glycoprotein (MTGP) was used as control.19 IgG fractions were purified from ascites as previously described.20 Per fusates were incubated for five minutes at 37°C with 20 $\mu$g/mL of MoAbs before perfusion.

Statistical analysis. Significance of grouped data was calculated with Student’s t test for paired samples and P values <.05 were considered as significant.

RESULTS

Comparison of platelet adhesion to collagen in whole blood and in reconstituted blood. Perfusion experiments were first carried out to verify that washing the platelets did not modify their adhesion to collagen. Platelet adhesion to collagen was measured using either whole citrated blood or reconstituted blood from ten control subjects and three of the five patients with severe vWD. In controls, the percent surface coverage with platelets was similar in whole or reconstituted blood (Fig 1). As expected, platelet adhesion was markedly decreased in vWD as compared with the control group but no significant difference between whole or reconstituted blood was observed (Fig 1).

Effect of platelet and plasma vWF on platelet-collagen interactions using reconstituted blood from controls and patients with severe vWD. The perfusion experiments were performed with six control subjects and the five patients. One patient was tested on two occasions and identical results were obtained. The results of the four types of mixtures were compared for one patient and one control subject on each occasion under the same experimental conditions. When vWD platelets were suspended in normal plasma, platelet deposition was significantly less than when normal platelets were suspended in autologous plasma (Table I) and this difference suggests that normal platelet vWF plays a role in platelet-collagen and platelet-platelet interactions. This was confirmed by results obtained with the last two mixtures: when normal platelets were suspended in vWD plasma, platelet deposition was significantly higher than when vWD platelets were reconstituted in vWD plasma (Table I). Results obtained by morphometric evaluation were similar to those using counting of radioactivity. Maximal platelet adhesion to collagen was obtained with mixtures of normal plasma and normal platelet vWF in normal plasma and normal platelet vWF (Table I). In mixtures containing control plasma the percent surface coverage with platelets (adhesion) was significantly less than vWD platelets than with control platelets. Further evidence that platelet vWF mediates platelet adhesion was obtained in studies using vWD plasma. In these studies the mean percent surface coverage with platelets was significantly higher with normal platelets than with vWD platelets (Table I). The percent surface coverage with thrombi $>2.5\mu$m showed parallel results to those of surface coverage with platelets in each of the tested mixtures (Table I).

Effect of monoclonal antibodies to vWF on platelet adhesion mediated by plasma and/or platelet vWF. The effect of MoAbs to vWF (H9 and 9) was tested in three perfusion experiments using mixtures containing normal
platelets and either normal or vWD plasma. In the mixture containing normal blood components both MoAbs markedly inhibited platelet adhesion to collagen (Fig 2). This effect was specific as a control MoAb was without effect (Fig 2). In the mixture containing normal platelets and vWD plasma, both MoAbs also markedly inhibited platelet adhesion (Fig 2).

DISCUSSION

vWF mediates platelet adhesion to the subendothelium and to collagen at high wall shear rates.\(^4\)\(^{,}\)\(^{14}\) Considerable progress in understanding the structure-function relationship of the vWF molecule in plasma has been achieved recently. At least two platelet membrane receptors, GPIb and GPIIb/IIIa, as well as three functional domains of vWF are required for optimal interactions between platelets, vWF and collagen.\(^2\)\(^{,}\)\(^{12}\) The question whether platelet vWF can functionally substitute for plasma vWF in mediating adhesion of platelets to subendothelium is yet unanswered. Evidence for the hemostatic properties of platelet vWF is provided by clinical studies of patients lacking plasma but not platelet vWF.\(^4\)\(^{,}\)\(^{9}\) Recently, Bowie et al\(^2\)\(^3\) transplanted normal bone marrow into a pig with severe homozygous vWD and concluded that the platelet vWF alone partially corrected the abnormal hemostatic tests in severe porcine vWD. Our study on the respective influence of human plasma or platelet vWF on the interaction of blood with collagen-coated surfaces in a flow system is consistent with these studies. Under conditions of relatively high wall shear rates (1,600 s\(^{-1}\)) we confirmed that platelet adhesion to collagen is considerably impaired in vWD blood when both platelet and plasma vWF are absent. The defect in platelet adhesion was corrected to a considerable extent when normal plasma was substituted, but complete correction was not achieved. Similarly, with vWD plasma, both platelet deposition and surface coverage with platelets and with thrombi were greater with normal than with vWD platelets. Our results strongly suggest that platelet vWF plays a role in mediating platelet-collagen interactions at high shear rates. In these experimental conditions both platelet and plasma vWF are necessary for optimal platelet adhesion. In the absence of plasma vWF, platelet vWF can partially mediate platelet-collagen interactions.

We have previously shown,\(^5\) as others have,\(^6\)\(^{,}\)\(^{24}\) the involvement of both GPIb and GPIIb/IIIa in platelet adhesion to collagen and subendothelium mediated by vWF. In our experimental conditions, this function was blocked by two MoAbs to vWF, H9 and 9, directed against the vWF binding domain to GPIb and to GPIIb/IIIa, respectively. In the present study we tested the effect of these MoAbs to vWF on platelet adhesion suppressed interactions mediated by platelet vWF in the absence or in the presence of plasma vWF. Using vWD plasma, that is when vWF was only provided by control platelets, the inhibition of the percent surface coverage with platelets by either MoAb is an indication that platelet vWF acts in mediating platelet adhesion to collagen through the binding to the two same glycoproteins that are required for plasma vWF mediated platelet-collagen and platelet-platelet interactions.

In conclusion, platelet vWF is involved in vitro in platelet adhesion to collagen and probably also in vivo when platelet-released vWF accumulates in large quantities at the site of vessel wall injury.

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