Role of von Willebrand Factor in Mediating Platelet-Vessel Wall Interaction at Low Shear Rate; The Importance of Perfusion Conditions

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We have previously observed that von Willebrand factor (vWF) plays an important role in platelet deposition on subendothelium at low values of wall shear rate (200 to 400 seconds⁻¹). In the present study, we have investigated the mechanism responsible for such a defect in platelet deposition at low shear rates in the absence of vWF. Blood from both normal and von Willebrand's disease (vWD) animals was exposed to de-endothelialized aorta from normal pigs for a range of shear rates (200 to 3,000 seconds⁻¹) and exposure times (three to 30 minutes) in a tubular perfusion chamber. Variations in the method of inhibiting coagulation (none, heparin, citrate, hirudin, and EDTA) and of perfusing blood (in vitro v ex vivo) were compared by determining the influence of wall shear rate and vWF on the deposition of ¹¹¹In-labeled platelets on subendothelium. Whereas platelet deposition was reduced in the absence of vWF for all experimental variations at high shear rates (>850 seconds⁻¹), a defect was observed at low shear rates only when heparinized blood was exposed by means of an ex vivo perfusion system. Maximum sensitivity of the measurement occurs under ex vivo perfusion conditions due to the reduced ability of platelets to deposit in normal blood when recirculated in vitro. Our results indicate that vWF mediates platelet-vessel wall interaction even at low shear rates and that such effect can only be observed in systems where platelet function is minimally affected by the experimental conditions.

A VARIETY of different perfusion techniques have been used for studying the interaction of platelets with the vessel wall in von Willebrand's disease (vWD). Defects in the ability of platelets to adhere to subendothelium and develop into platelet thrombi have been previously reported in pigs with severe vWD. In previous studies performed in our laboratory an ex vivo perfusion system was used to expose homologous (pig) tissues to both nonanticoagulated (native) blood and heparinized blood. Those findings have supported earlier results obtained in vitro that used von Willebrand factor (vWF) containing vessel walls in a heterologous perfusion system (human blood, rabbit vessel), or in homologous systems (human blood, human vessel). However, a novel observation of the studies performed in the ex vivo perfusion system has been the observation of a defect in platelet deposition at low shear rates (212 and 424 seconds⁻¹), a finding that has not been previously observed with in vitro flow systems. The abnormality in platelet deposition at low shear rates may be of some importance with respect to platelet interactions in large and medium size arteries and veins where wall shear rates average several hundred inverse seconds.

Since the present method of perfusion differs in several important features from those used by others, we have investigated these differences concurrently in the same animals in order to determine their potential influence on the interaction of platelets with the vessel wall. We have found that the use of sodium citrate as an anticoagulant and separately the in vitro recirculation of the blood considerably reduce the deposition of platelets in normal animals, but not in animals with vWD. Such conditions reduce the sensitivity for observing differences due to vWF. In addition, the use of nonanticoagulated blood enhances the deposition, especially in the vWD animals. Thus, production of procoagulant factors also reduces the sensitivity of the measurement. We have found that maximal differences between the groups are observed in ex vivo perfusion with anticoagulated (heparinized) blood. Thus, it appears that vWF also mediates platelet deposition at low shear rates and that such an effect can be optimally observed in systems where in vivo platelet function is minimally affected by the experimental conditions.

METHODS

Experimental Model

All procedures performed in this study were approved by the appropriate institutional guidelines and followed the American Heart Association Guideline for animal research.

Normal pigs were obtained from local farmers and pigs with vWD were obtained from the Mayo colony (Dr. E.W., Mayo Institute Hills Farm, Mayo Foundation, Rochester, MN). Pigs with homozygous vWD have an impairment of primary hemostasis and the various other hemostatic abnormalities noted in the severe form of the disease in humans. bleeding times were prolonged from 4 ± 0.7 (normals) to over 15 minutes (vWD). In addition, no vWF is present in the platelet granules or in the vessel wall. Platelet number and platelet size distribution were determined by our previously reported method in a Coulter P 2 60 platelet analyzer. EDTA blood from each animal was diluted 1:1,000 in an isotonic buffered diluent (Thrombopet, Curtis Matheson Scientific, Houston) and counted during the first hours after blood withdrawal. No differences in platelet count were observed between both groups of animals (N = 417 ± 50 x 10⁹/μL; vWD = 415 ± 15 x 10⁹/μL). Hematocrits, measured by the standard microhematocrit technique, were similar in both groups of animals (N = 28.3 ± 1.0; vWD = 27.6 ± 1.9).

Perfusion Chamber

We have used our previously described perfusion chamber that mimics the cylindrical shape of the blood vessels and that has been...
previously characterized. In this chamber, the substrate under study is placed in a lateral position, forming part of the blood channel by which the test surface is directly exposed to the blood. Two chambers of different internal diameter (1.0 and 2.0 mm) permit a broad range of wall shear rates on the substrate with moderate changes in average blood flow rate.

In the present experiments, flow rates were 10 and 20 mL/min in both the small and large chamber. These flows gave theoretically calculated average blood velocities ranging from 5.3 to 42.3 cm/s, and local shear rates form 212 to 3,380 s^-1. These shear rates range from those of large arteries to those of terminal arteries and the microcirculation. At these flow conditions, blood can be considered as having Newtonian fluid properties with constant viscosity. Shear conditions at the vessel wall were calculated from the theoretical expression for shear rate given for a Newtonian fluid in tube flow.

**Preparation of Vessel Segments**

We have studied platelet interaction to mildly damaged normal pig thoracic aorta as previously reported. In brief, the aorta of a deeply anesthetized pig is exposed and all branches ligated. The animal is then euthanized by an overdose of anesthetic and simultaneously the aorta is perfused, by canulation of the aortic arch and the abdominal trifurcation, with phosphate buffered saline, 0.2 mol/L, pH 7.4, containing papaverine (120 mg/L). An air stream is subsequently passed through the aorta at a rate of 1,000 mL/min for ten minutes. The vessel is immediately removed and placed in ice-cold buffer. This procedure induces selective endothelial removal with minimal damage to the underlying basement membrane.

**Experimental Conditions of Perfusion**

Blood was perfused both ex vivo and in vitro for a variety of conditions.

**Ex Vivo Experimental Procedure**

The pigs were sedated with intramuscular PromAce (Acepromazine Maleate; Fort Dodge Lab, Fort Dodge, IA), anesthetized by intravenous (IV) injection of Sodium Pentobarbital (Fort Dodge Lab) and the carotid artery and contralateral jugular vein cannulated. Blood samples were collected to determine hematocrit and platelet count. Blood was collected into various anticoagulants, such as sodium citrate, EDTA, or hirudin for in vitro perfusion in the chamber (described below).

**Native blood studies.** The animal received no anticoagulants (native) and the perfusions were performed by withdrawing the blood directly from the carotid artery through the perfusion chamber for selected flow rates and exposure times. Blood was discarded after passing through the perfusion chamber in order to avoid any possible systemic effects due to the generation of procoagulant material.

**Heparinized blood studies.** The animal was systemically heparinized with an initial bolus of 300 U/kg, (Liquemin 10,000; Roche). The level of heparin was maintained within a normal therapeutic range with IV boosts as needed to maintain the activated partial thromboplastin time test at 1.5 to 2.5 times the baseline levels. Before ex vivo experiments blood (heparinized) was collected for in vitro perfusion experiments (described below). The cannulated carotid artery was connected by polyethylene tubing (20 cm in length, Clay Adams, PE 200, division of Becton, Dickinson and Co, Parsippany, NJ) to the input of the plexiglass chamber. The output of the chamber was connected to a peristaltic pump (model 7013; Masterflex). Blood that passed through the perfusion chamber was recirculated back into the animal by the contralateral jugular vein in animals that were anticoagulated.

**In Vitro Experimental Procedure**

Citrated blood studies. Nonanticoagulated blood from both normal and vWD pigs was collected through the catheter into 90 mmol/L sodium citrate (9 to 1 vol/vol). Citrated blood was distributed into aliquots (30 mL) and was kept at room temperature before exposure to vessel wall (within 60 minutes.). The blood was prewarmed in a water bath at 37°C for at least 5 minutes and then recirculated by a peristaltic pump for a preselected flow rate and a perfusion time of five minutes through the chamber that was prefilled with Vassar-saline and kept at 37°C in a water bath. De-endothelialized vessel segments were previously mounted as described. After exposure to the segments were perfused for 60 seconds with Vassar saline (37°C) under the same flow conditions used during blood exposure.

**EDTA blood studies.** Blood was also collected into EDTA (1%, 0.1%, final blood concentration) and perfused similarly to blood collected into sodium citrate.

**Hirudin blood studies.** Blood was collected in hirudin to attain a concentration of 20 U/mL (H-7016; Sigma Chemicals, St Louis) and perfused similarly to blood collected into sodium citrate.

**Radioactive Labeling of Platelets**

Approximately 24 hours before the perfusion experiment, autologous platelets were labeled with 111In-(tropolone), by a modification of our previously described technique in pigs. In brief, 111In-tropolone was prepared from 111In-chloride (Medi-Physics, Inc, Emerville, CO), by the addition of 50 μg of tropolone dissolved in 50 μL of saline to 500 μCi of 111In-chloride. This solution was mixed with 1 mL of platelet-poor plasma (PPP). Platelets were harvested from 43 mL of blood, collected by venipuncture into 7 mL of modified citric acid-sodium citrate-dextrose (ACD)-solution. The isolated pellet of platelets was resuspended in 2 mL of PPP yielding a concentrated platelet-rich plasma (PRP). The 111In-tropolone complex was added to the PRP solution and the mixture incubated at 37°C for 20 minutes. Free 111In-tropolone was removed by washing with 4 mL of PPP. The average efficiency of platelet labeling in plasma was of 55.5% ± 3.3%. The final pellet of labeled platelets was resuspended in 4.5 mL of PPP and injected into the animal after a low spin centrifugation to remove any microaggregates. The labeling procedure required approximately two hours. The average Indium plasma activity of 3.7% ± 0.7% (X ± SE) was measured just before injection of the platelet concentrate. The injected activity was 212 ± 12 μCi (X ± SE), and 3 x 10^10 ± 2 x 10^10/μL of 111In-labeled platelets (X ± SE) were injected in a volume of 4.5 mL of plasma. This
method of platelet labeling does not affect platelet aggregation to adenosine diphosphate (ADP) and collagen as previously reported.2

Evaluation of Vessel Segments

The perfused segments were fixed and counted in a gamma-well counter for quantitation of deposited platelets.

The number of platelets deposited on each specimen was calculated from the platelet count and the 111In-activity on the perfused area and in blood, using the method previously described.4,19 Results were normalized by area of exposed surface.

Absence of endothelium was checked by "en face" staining with silver nitrate. The vessels, stored in Iris-buffer with antibiotics (penicillin and streptomycin) at 4°C were used within 1 to 3 weeks of harvesting. The residual release of prostacyclin by the vascular wall was assayed at the time of its exposure in the perfusion chamber and there were no significant differences in quantities of released material by vessels used in any group (range, 27 to 37 pmol/g/cm²). All harvesting. The residual release of prostacyclin by the vascular wall was assayed at the time of its exposure in the perfusion chamber and there were no significant differences in quantities of released material by vessels used in any group (range, 27 to 37 pmol/g/cm²). All

Statistical Analysis

Results were statistically analyzed for the best bivariate data model fitting, Student t test, factorial analysis of variance (ANOVA), and repeated measurement analysis of variance as required. Variance about the means is given as 1 SE. For data analysis we used SAS (Statistical Analysis System; SAS Institute, Cary, NC) via City University of New York (CUNY) and Stat. View 512° (Brain Power, Inc, Calabasa, CA).

RESULTS

Blood from both normal and vWD animals was exposed in a laminar perfusion chamber for selected times (from three to 30 minutes) to blood flowing at wall shear rates of 212, 424, 848, 1,690, and 3,380 seconds⁻¹. Blood from the same experimental animal was treated with a variety of anticoagulants (including none) and exposed in a perfusion chamber either ex vivo in an extracorporeal circuit or in vitro in a recirculation perfusion system. Hematocrit did not differ among the different perfusion conditions or perfusion times nor between both genotypes (ANOVA).

Comparison of Normal and vWD Animals Using Anticoagulated Blood

Ex Vivo Perfusion With Heparinized Blood

As reported previously by our group, when heparinized blood was drawn directly from a catheterized vessel and exposed to de-endothelialized aorta from normal pigs in the perfusion chamber, values of platelet deposition in vWD animals were significantly reduced (P < .001) when compared with normal control animals. This defect in platelet deposition was observed for all values of wall shear rate (212, 424, 1,690, and 3,380 seconds⁻¹) and exposure times studied (five to 15 minutes in the present experiments). The maximum defect, seen at 1,690 seconds⁻¹ appears from Fig 1 to be related primarily to the increase in deposition observed in normal animals at this condition, rather than any decrease in vWD animals. Wall shear rate had a significant effect on platelet deposition (P < .05) in normal animals, but no dependence on shear values was observed in vWD pigs.

In Vitro Perfusion With Heparinized Blood

When blood from normal pigs was collected from a systemically heparinized pig and exposed in a recirculatory perfusion system, levels of platelet deposition on vessel segments were significantly lower (P < .001), as seen in Fig 2, than when measured in ex vivo perfusion systems, Fig 1. The decreased deposition was observed for all shear conditions (424 to 3,380 seconds⁻¹), and exposure times (five to ten minutes) investigated (P < .01). However, with blood from vWD pigs, values of deposition were slightly, but not significantly, reduced when comparing in vitro ex vivo perfusion with heparinized blood at perfusion time of five minutes and significantly reduced at perfusion time of ten minutes. Results obtained in the typical laboratory in vitro setting with normal and vWD blood were compared by two-factor analysis of variance (factor A, genotype; factor B, wall shear rate). A significant effect of genotype (P < .001) was found; however, when independently compared, the two lower shear rates (424 and 848 seconds⁻¹) resulted in values of platelet deposition for normal and vWD pigs that were not significantly different. Values reached a statistically significant difference at the greater values of wall shear rate.

In Vitro Perfusion With Citrated Blood

Perfusions with citrated blood (9 mmol/L final concentration in whole blood) were conducted at wall shear rates ranging from 424 to 3,380 seconds⁻¹ and an exposure time of five minutes, as seen in Fig 3. Platelet deposition with citrated blood from normal pigs was compared with heparinized blood in vitro and found not to be statistically different.
In vWD animals, a significant reduction in platelet deposition with citrated compared with heparinized blood perfusion in vitro was observed (two factor, genotype x shear rate, ANOVA; significant effect found for genotype \([P < .01]\) but not for shear rate). Both normal and vWD animals showed highly significant reductions in platelet deposition \((P = .003\) and \(.05\), respectively\) when compared with ex vivo \((\text{heparin})\) results (Fig 1).

Two-factor analysis using ANOVA showed a significant effect of genotype on platelet deposition in citrated blood \((P = .002)\); however, analysis by individual shear rate values indicated a significant difference in platelet deposition between normal and vWD animals only at wall shear rates of 1,690 and 3,380 seconds\(^{-1}\).

**In Vitro Perfusion With EDTA Blood**

Blood was collected into 1% EDTA (0.1% final concentration, 2.6 mmol/L EDTA) and perfused for five minutes, at wall shear rates of 848 and 1,690 seconds\(^{-1}\) (Fig 3). A significant reduction in platelet deposition in the absence of vWF was observed by two-factor ANOVA at the two shear rates studied \((P < .004)\). Platelet deposition was reduced by the stronger chelation of divalent cations with EDTA in normal and vWD pigs compared with sodium citrate. However, the difference in deposition between these two treatments was not statistically significant when analyzed by three-factor ANOVA (genotype, shear rate, and perfusion).

**Comparison of Normal and vWD Animals With Nonanticoagulated Blood**

Platelet deposition in an ex vivo perfusion system that used nonanticoagulated animals was obtained for shear rates of 212 and 1,690 seconds\(^{-1}\) and an exposure time of three and five minutes (Fig 4). A significant inhibition in the absence of vWF was observed by two-factor ANOVA, \(P < .001\); however, this difference, as determined by the Student \(t\) test was statistically significant only at 1,690 seconds\(^{-1}\). As can be seen from Fig 5, values of platelet deposition in normal animals are comparable for the two ex vivo treatments, nonanticoagulated and heparinized. However, deposition results are significantly decreased in the presence of an anticoagulant in the vWD pigs.

**DISCUSSION**

In the present study we have explored the nature of the defect in platelet deposition in vWD at low shear rates (212 to 424 seconds\(^{-1}\)). We have considered two factors in our present perfusion system that differ substantially from other
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Shear rate. When blood from vWD animals was perfused, deposition results were lower and comparable with those obtained at low shear rates ex vivo; there was no marked difference between the in vitro and ex vivo perfusion results in the vWD animals (Figs 1, 2, and 5). This lack of difference helps explain the normal levels of platelet deposition at low shear rates noted in in vitro perfusion conditions. It is plausible that recirculation of relatively small volumes of blood (30 mL) through the in vitro circuit results in a partial depletion of fully active platelets; this deactivation is more pronounced in normal platelets than in vWD platelets, possibly due to the abnormality in platelet-surface interactions associated with the latter. It may also be that in the ex vivo system the newly arriving normal platelets are able to locally secrete mediators for platelet-vessel and/or platelet-platelet interactions, such as platelet vWF, that are missing in the vWF-deficient newly arriving platelets.

These experiments indicate that, although gross platelet count is not significantly reduced over the course of in vitro perfusion, the platelet reactivity may be altered. In fact, in in vitro perfusions (experiments not shown) we have been able to detect platelet lysis directly related to the average blood flow and perfusion time. Thus, experiments designed with a significant extent of recirculatory flow should be evaluated with concern for reduced platelet function over the course of perfusion. It is interesting to note in the literature that in vitro studies have often used exposure times of up to 40 minutes with flow rates as high as 160 mL/min. Our results are, on the other hand, in agreement with the well-recognized fact that platelets are highly sensitive to manipulation.

The inhibition of the coagulation system does not seem to have been directly responsible for the defect in platelet deposition observed at low shear rates. In normal animals, deposition results in heparinized blood are quite comparable with those observed in native blood (Figs 2 and 4); further, results obtained with either heparin, hirudin, or citrate are identical when compared in vitro experiments (Fig 3, Table 1). Sodium citrate, which is a known inhibitor of platelet-vessel and platelet-platelet interactions, reduces significantly the deposition of vWD platelets on the vessel wall below levels obtained with heparinized blood. Extensive depletion of Ca** concentration with EDTA further suggests the influence of these cations on platelet deposition.

The GP IIb and GP IIIa, receptor site for vWF, may form heterodimeric complexes in the presence of Ca** and these complexes can bind Ca** possibly through GP IIb that represents the major calcium binding site on the platelet surface. In the present study, Ca** chelation has inhibited platelet deposition in flowing blood. It is of special interest to observe that such an effect was significantly magnified in the absence of vWF indicating an important role for vWF in platelet interaction with the vessel wall at physiological levels of calcium. As such, our results differ from those recently presented by Harfenist et al who reported in in vitro aggregation testing that vWF does not support platelet aggregation at physiological level of Ca** concentrations and hence in vivo.

**Platelet deposition (x10^6 platelets/cm^2)**

Fig 4. Mean deposition values (±SE) of 111In-labeled platelets (expressed as platelets/cm^2 x 10^6) obtained when native (non-anticoagulated) blood from normal (clear bars) or vWD (hatched bars) pigs is exposed for three or five minutes in an ex vivo perfusion system to subendothelium obtained from normal pig aorta. Number of perfusion runs are indicated in the figure. Shear rates at the vessel wall are 212 (A) and 1.690 (B) seconds^-1.

Fig 5. Mean deposition values (±SE) of 111In-labeled platelets (expressed as platelets/cm^2 x 10^6) obtained when native (- - -) or heparinized (---) blood is exposed for five minutes in an ex vivo perfusion system to subendothelium from normal pig aorta. Also shown are results obtained with heparin in vitro (-----) for comparison. Number of perfusion runs are indicated in the figure. Shear values at the vessel wall are indicated.
Our finding that platelet deposition with hirudin is comparable with that obtained in heparinized blood suggests that under these conditions, where the level of platelet deposition ($5 \times 10^9 \text{/cm}^2$) are indicative of a mono- or bi-layer of platelet coverage, thrombin does not significantly mediate platelet-de-endothelialized wall interaction at either low or high shear rate.

Several other factors have been considered as potentially contributing to the low shear rate defect in platelet deposition. One obvious possibility is that vWD in pigs is different with respect to its sensitivity to local shear conditions than the human disorder or that produced by antibodies to vWF, for which such a defect has not been previously reported. However, the results obtained with the in vitro perfusion system using both heparin and citrate are quite comparable with respect to those in vitro studies reported with human blood. Namely, the platelet defect in vWD was only observed under high shear rate conditions. Thus, species difference is not a likely explanation.

Another difference that could contribute to the defect observed at low shear rate may be related to the components of the vessel wall. The normal pig aorta used in the present experiments contains no vWF, as determined by immunofluorescence. The vWF in vessel walls has been shown to contribute to the levels of adhesion observed on vascular surfaces. We do not feel that the absence of vascular vWF contributes to the differences observed between normal and vWD animals at low shear rates in the present experiments. Similar vessels were used in all experiments, and, in all but the $\alpha$-vivo experiments, these vessels were relatively low and they highlight the importance of vWF as a ligand for platelet function at a much wider range of shear conditions than previously reported.

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