Functional Behavior of Vessels from Pigs with von Willebrand Disease
Values of Platelet Deposition Are Identical to Those Obtained on Normal Vessels

Lina Badimon, Juan J. Badimon, Vincent T. Turitto, Jacob Rand, and Valentin Fuster

Vessels from normal pigs and pigs with severe von Willebrand disease were exposed for up to 30 minutes to both nonanticoagulated and heparinized blood from normal pigs in an ex vivo perfusion system. Shear rates at the vessel surface were varied over a broad physiological range, $\gamma_s = 212$ to 3380 sec$^{-1}$. The deposition of $^{111}$In-labeled platelets was determined by radiometric counting. For all shear rates and exposure times investigated, the levels of platelet deposition on de-endothelialized thoracic aorta of normal and von Willebrand disease pigs were not significantly different. Thus, the functional activity of the vessels correlated with the results obtained previously by Immunofluorescence. Namely, the von Willebrand factor protein in the thoracic subendothelium of normal pigs is significantly diminished or absent and is comparable to the levels observed in von Willebrand disease pigs. (Arteriosclerosis 9:184–188, March/April 1989)

Pigs with von Willebrand disease (vWD), when maintained on a normal or cholesterol-rich diet, develop significantly fewer atherosclerotic lesions of the abdominal aorta than do normal pigs, a finding which may be related to the reduced levels of von Willebrand factor (vWF) in the plasma, platelets, and vessel wall of these animals. A major function of vWF has been related to its ability to support platelet adhesion and platelet–platelet interactions in flowing blood. We have observed reduced platelet deposition when blood from vWD pigs, compared with that from normal controls, is exposed in an ex vivo perfusion system to normal pig aorta. The mechanisms by which vWF helps support platelet adhesion are not entirely clear. Platelet adhesion has been shown to depend on the level of plasma vWF deposited on vessels perfused with plasma. The vascular elements to which plasma vWF attaches are currently unknown, but a role for endogenous vessel wall vWF in this process cannot be excluded. Endogenous vWF has been described in the subendothelium of various species, including humans. Blockage of endogenous vWF in the vessel wall with various antibodies has led to reduced levels of platelet adhesion on the treated vessels, even in the presence of normal amounts of plasma vWF. These studies have supported the idea that vWF in the vessel wall may contribute significantly to platelet adhesion.

From the Divisions of Cardiology and Hematology, Mount Sinai Medical Center, One Gustave L. Levy Place, New York, New York. This work was supported in part by U.S. Public Health Service Grants HL 99849 and 36395 and the New York Heart Research Foundation.

Address for reprints: Dr. Lina Badimon, Division of Cardiology, Amnenberg B-06/Box 1030, The Mount Sinai Medical Center, One Gustave L. Levy Place, New York, NY 10029.

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Recent studies in our laboratory have revealed a deficiency of vWF in the endothelial cells and intima of most of the arterial system in normal pigs, as determined by immunofluorescent techniques in fresh and stored vessels; however, little is known about the functionality of normal and vWD porcine vessels. For example, it is conceivable that small quantities of active vWF available on normal vessels might support platelet adhesion and subsequent thrombus formation. In the present studies, we exposed de-endothelialized aortic vessel wall from normal and vWD pigs to normal pig blood in an ex vivo perfusion chamber. Values of platelet deposition on both types of vessels were not significantly different over a wide range of shear conditions (212 to 3380 sec$^{-1}$). Thus, the results are consistent with the previous immunofluorescent studies, that significantly reduced or absent levels of vWF are characteristic of aortic wall in normal as in vWF pigs.

Methods

Experimental Model

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

Swine with inherited homozygous vWD were used as vessel wall donors and were from the colony kept at the Mayo Clinic by Walter E. J. Bowie (Mayo Institute Hills Farm, Mayo Foundation). No vWF was present in the plasma, platelet granules, or the vessel wall in these pigs. Normal pigs were used as blood and vessel wall donors. Normal swine thoracic aorta did not show positive immunofluorescence to vWF. The pigs were housed at the Animal Research Center pig facilities of our institution. They were individually caged in a light-, temperature-, and humidity-controlled environment, with ad libitum access to water and
controlled food intake (3% wt/wt of the body weight, Purina Laboratories). Table 1 shows the hemostatic parameters of the normal animals used as blood donors in the study determined by previously reported methods.14-17

**Perfusion Chamber**

We used an original perfusion chamber that mimics the tubular shape of the blood vessels.4,7,10,19 In this chamber, a circumferential portion of the tubular flow channel is replaced by the test substrate material, which becomes directly exposed to the blood. Two chambers of different internal diameters (1.0 and 2.0 mm) provide a broad range of wall shear rates on the substrate with moderate change in average blood flow rate.

The selected flow rates were 10 and 20 ml/min in the small and large chamber. These flows gave theoretically calculated average blood velocities from 5.3 to 42.3 cm/sec and local shear rates from 212 to 3380 sec⁻¹. At these shear rates, blood can be considered a Newtonian fluid with constant viscosity.20,21 Shear conditions at the vessel wall were calculated from the expression of shear rate given for a Newtonian fluid in tube flow.22

**Experimental Procedures**

The pigs were sedated with Intramuscular PromAze (acepromazine maleate, Fort Dodge Lab, IA)18 followed by anesthesia with intravenous injection of sodium pentobarbital (Fort Dodge Lab). The carotid artery and contralateral jugular veins were catheterized by cutdown, and an in vivo extracorporeal circuit carotid artery-perfusion chamber-jugular vein was established, as previously described.19

**Nonrecirculation Experiments Perfusing Nonanticoagulated Blood**

The carotid artery was connected to the input of the chamber maintained at 37°C, and the output was connected to a variable speed peristaltic pump (Masterflex model 7013) with interchangeable heads calibrated to obtain selected blood flow rates. Test specimens were mounted in the chamber and were perfused with Vasser saline (pH 7.4) at 37°C for 60 seconds. Blood was then perfused over the substrate at a preselected flow rate and exposure time. At the end of the perfusion period, buffer was passed through the chamber for 30 seconds under identical flow conditions. Blood was discarded after exiting the chamber to prevent re-infusion of potentially activated blood.

**Recirculation Experiments**

The animals were intravenously heparinized (300 U/kg, Liquemin 10 000, Roche). The cannulated carotid artery was connected to the input of the Plexiglas chamber, the output of the chamber was connected to the peristaltic pump. Blood that passed through the chamber was recirculated back into the animal by the contralateral jugular vein. The specimens were processed as before. They were perfused with Vasser saline solution at 37°C for 60 seconds. After the perfusion period, blood at a preselected flow rate entered the chamber for various perfusion times. At the termination of the blood perfusion period, the buffer was again passed for 30 seconds under identical flow conditions through the chamber and discarded. The changes from buffer to blood and vice versa were achieved by a three-way valve without the introduction of stasis in the chamber. The extracorporeal circulation produced no changes in hematocrit, platelet count, platelet lysis, or radiolabel uptake by various organ systems during the duration of the experiment, compared with experiments where blood was not recirculated.

**Radioactive Labeling of Platelets**

Approximately 24 hours before the perfusion experiment, autologous platelets were labeled with 111In (tropolone) by a modification20 of our previously described technique in pigs.4 In brief, 111In-tropolone was prepared from 111In-chloride (Medi-Physics, Incorporated, Evanville Company), 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 ACD solution. The isolated pellet of platelets was resuspended in 2 ml of PPP. The 111In-tropolone complex was added to the platelet-rich plasma (2 cc) (PRP) solution, and the mixture was incubated at 37°C for 20 minutes. Free 111In-tropolone was removed by washing with 4 ml of PPP. 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 2 hours. An 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 3×10⁴±2×10⁴ per μl of 111In-labeled platelets (X±SE) were injected in a volume of 4.5 ml of plasma.

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 by using the method we previously described.18 Results were normalized by area of exposed surface.

**Vessel Wall Preparation**

We have studied platelet interaction to mildly damaged normal thoracic aorta of normal and vWD pigs, as previously reported.4 In brief, the aorta of a deeply anesthetized pig was exposed and all branches were ligated. The animal was then euthanized by an overdose of anesthetic, and simultaneously the aorta was perfused by cannulation.

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**Table 1. Blood Values in Normal Pigs**

| Factor VIII (%) | 181±19 |
| VWF (%)        | 87±11  |
| VWF:RCo (%)    | 78±5   |
| Bleeding time (min) | 3.3±0.4 |
| PN (×10⁹/μl)   | 430±50 |
| MPV (μm³)      | 7.1±0.2 |
| Hct (%)        | 32±1   |
| RBC (×10⁹/μl)  | 7.1±0.2 |

VWF—von Willebrand factor, RCo=ristocetin cofactor, PN—platelet number, MPV=mean platelet volume, Hct=hematocrit, RBC=red blood cells.
of the aortic arch and the abdominal truncation with phosphate-buffered saline, 0.2 M (pH 7.4) containing papaverine (120 mg/l). An air stream was subsequently passed through the aorta at a rate of 1000 ml/min for 10 minutes. The vessel was immediately removed and placed in ice-cold buffer. This procedure induces selective endothelial injury without damaging the basement membrane. Absence of endothelium was checked by en face staining with silver nitrate. The vessels stored in Tris 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 was similar in both types of vascular wall.

**Ultrastructural Analysis of Specimens**

Selected perfused specimens were fixed in a mixture of 3% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4) immediately after removal from the chamber. For scanning electron microscopy, specimens were dehydrated by processing through a series of ethanol solutions and were dried with carbon dioxide by the critical-point method. The dried tissue was coated with 100 Å of gold-palladium and carbon and was examined under a scanning electron microscope.

**Statistical Analysis**

The data were statistically analyzed for the best bivariate data model fitting and repeated measurement analysis of variance. Variance about the means is given as 1SE. The Clininfo and SAS (City University of New York) computer systems were used for all statistical analysis (supported by Grant RR-71 from the Division of Research Resources, National Institutes of Health).

**Results**

Blood from normal pigs drawn directly from the carotid artery either without anticoagulation or after systemic heparinization was exposed to de-endothelialized aorta from normal or vWD pigs for a range of wall shear rate conditions (212 to 3380 sec⁻¹) and times (1 to 30 minutes). The deposition of ¹¹¹in-labeled platelets was determined by radiometric counting.

**Nonanticoagulated Blood Studies**

Platelet deposition with nonanticoagulated blood was not significantly different on normal or vWD aortas at a shear rate of either 212 sec⁻¹ or 1690 sec⁻¹ (Figure 1). Deposition increased with exposure time on both vessels, but comparable levels of deposition were observed.

**Heparinized Blood Studies**

**Wall Shear Rate of 212 Sec⁻¹**

Deposition values in heparinized blood increased with increasing exposure time and gradually approached an asymptotic value of deposition beyond 10 minutes (Figure 2). No significant differences between vessels from normal and vWD pigs were observed.

**Wall Shear Rate of 1690 Sec⁻¹**

Values of platelet deposition with normal and vWD disease pigs were identical over the full range of exposure time (1 to 30 minutes) (Figure 2). An asymptotic level of deposition was reached by 5 minutes, and deposition remained relatively steady at a level somewhat higher than that observed at lower shear rate.

**Wall Shear Rate of 3380 Sec⁻¹**

No significant differences in platelet deposition between normal and vWD vessels were observed at the highest wall shear rate for all exposure times studied (t=1, 3, 5, 10, and 15 minutes). An asymptotic level was reached within 5 minutes of exposure, and little change was observed thereafter (Figure 2).
PLATELET DEPOSITION IN vWD PIGS  Badimon et al.  187

Figure 2. Mean values of platelet deposition (× 10⁶/cm²) (± 1 SE) are plotted vs. perfusion time (min). Heparinized pig blood was perfused at a wall shear rate of 212 sec⁻¹ (A), 424 sec⁻¹ (B), 1690 sec⁻¹ (C), and 3380 sec⁻¹ (D) over de-endothelialized vessel wall from normal (●) and vWD (△) pigs in an in vivo extracorporeal circulation.

Discussion
We have investigated the platelet response to normal and vWD aorta that was denuded of its endothelial lining over a broad range of shear conditions (212 to 3380 sec⁻¹) and exposure times (1 to 30 minutes) in both native and anticoagulated blood. No significant differences between the two types of vessel were seen for all experimental conditions utilized. These conditions included shear rates (212 to 3380 sec⁻¹) for which a defect in platelet-vessel wall interaction has been previously observed when vWD blood was perfused over normal vascular substrate. Thus, the functional activity of the normal vessel wall correlated with the results obtained previously by immunofluorescence, namely, vWF was significantly reduced or absent in the subendothelium of the normal pig, as it is in the vWD pig aorta. These findings do not reflect the relative importance of subendothelial vWF with respect to platelet deposition, since both sets of vessels contain little or no such protein; however, they do indicate that the vessel wall can support platelet adhesion and thrombus formation in the absence of endogenous vWF in the vessel wall.

Pigs develop atherosclerosis of the lower abdominal aorta either spontaneously or in response to a high-cholesterol diet, and we have reported that the vWD animals are protected from developing atherosclerotic lesions in this area of the vasculature. Since we previously found vWF in the abdominal aorta of normal pigs, it is possible that endogenous subendothelial vWF protein is involved in the vascular disease process that typically occurs in this area. Little disease is generally observed in the thoracic region of normal pigs, which also do not show detectable quantities of vWF in the subendothelium. Thus, our present findings do not rule out a role for vascular vWF in atherosclerosis.

The present findings are consistent with a major role for vWF in blood in the deposition of platelets on porcine aortic subendothelium and, presumably, in large vessel thrombosis. The importance of vWF in vessels in the normal pig that do show the presence of vWF by immunological methodology, such as in the microvasculature and the venous and pulmonary circulation, is not clear. Certainly, it is plausible that vascular vWF may play an important role in normal hemostatic function, which is primarily a microcirculatory event. However, its role in large vessels (pulmonary artery, veins, abdominal aorta), which do exhibit differences in the presence of vWF between vWD and normal pigs, is less certain.

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