Platelet adhesion to damaged coronary arteries: Comparison in normal and von Willebrand disease swine
(factor VIII-related activity/coronary arterial injury/platelet activation/hemostasis)

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ABSTRACT The early response to coronary artery injury was investigated in normal swine and in swine with von Willebrand disease (vWD). Thirty minutes after coronary endothelial denudation, a monolayer of platelets was adherent to areas of simple injury in both bleeder and normal swine. The number of adherent platelets was not significantly different in the two phenotypes. Injury involving the media of the vessel produced platelet–fibrin thrombi. Platelet activation, as judged by pseudopod formation and platelet spreading over areas of simple injury, was significantly less in bleeder animals than in normal animals. These studies suggest that chemotaxis and initial contact adhesion of platelets to injured arterial wall is independent of the von Willebrand factor. On the other hand, the spreading and activation of platelets on the subendothelium appear to be dependent on the presence of plasma von Willebrand factor. Through this mechanism von Willebrand factor may contribute to arterial thrombosis and atherogenesis.

von Willebrand disease (vWD) is a hemorrhagic diathesis characterized by bleeding from mucosal surfaces and capillary beds (1). The disease is inherited as an autosomal trait. Homozygotes for the disease have a deficiency of a plasma glycoprotein, von Willebrand factor (vWF), that supports platelet aggregation in the presence of certain exogenous cofactors. The absence of this vWF is probably the cause of the abnormal bleeding. Importantly, vWF in normal people and animals may also contribute to arterial thrombosis and atherogenesis. This possibility has become a topic of broad interest because of the observation that pigs with vWD failed to develop atherosclerosis as rapidly as do normal pigs (2). It is likely that vWF functions in both hemostasis and thrombosis by supporting the response of platelets to vascular disruption; but the mechanism by which this occurs is not known.

Several groups of investigators have studied the role of the vWF in the response of platelets to vessel wall injury (3–6). Most have shown a decrement in the rate of adhesion of platelets to denuded subendothelium in the absence of vWF. Unfortunately, the data accumulated have been from artificial in vitro systems. Many of these study systems have used complicated combinations of animal arterial tissues and human blood and blood products. These results might have been influenced by the species specificity of vWF-platelet reactions (7). Additionally, possible artifacts produced by blood drawing, anticoagulation, and exposure of blood to air or artificial surfaces during these studies could only be excluded by use of a live animal model.

We have used pigs with vWD to study platelet–vessel wall interactions. These animals have a bleeding disease that is essentially similar to that of humans with vWD. The pigs that are homozygous for the disease have undetectable levels of vWF in their plasma, platelets, and endothelial cells. These pigs have been shown to develop diet-induced aortic atherosclerosis at a slower rate than do normal pigs (2, 8). On the other hand, atherosclerotic plaque developing in response to balloon catheter-induced injury of coronary arteries is similar in normal and bleeder pigs (8).

We report here the acute effects of the balloon-injury in coronary arteries. Our observations indicate that at 30 min after delivery of a superficial intimal injury, the number of adherent platelets per unit area of denuded surface was only slightly less in bleeder pigs than in normal pigs; however, the degree of activation of the adherent platelets, as indicated by shape change and pseudopod formation, was retarded in the bleeder pigs. Previous impressions that the vWF plays its primary role in platelet adhesion inadequately explain the multiple functions of this molecule.

MATERIALS AND METHODS
The animals used in this study were from the inbred strain of swine with vWD in the Chapel Hill colony. They were fed a standard diet of pig chow. None had received transfusions before being used in these experiments. Animals were designated as normals (nonbleeders) or bleeders based on the plasma level of vWF (platelet-aggregating factor) (8). Animals homozygous for vWD (bleeders) had vWF levels <1% of normal. Age- and weight-matched normal and bleeder pigs were studied in pairs. The average weight of the animals was 20–25 kg.

Coronary Artery Denudation. The animals were anesthetized with halothane. The external carotid artery and jugular vein were surgically exposed, and a size 4F flow-directed catheter was inserted into the carotid artery. Catheter positions were monitored with fluoroscopy. The catheter was introduced into the left anterior descending coronary artery. The balloon at the end of the catheter was inflated, and the catheter was withdrawn. The procedure was performed three times. The pig was sacrificed 30 min after the ballooning procedure was completed. At sacrifice, fresh 4% formaldehyde or 2% glutaraldehyde at room temperature was perfused into the coronary artery at 100 ml/min for 5 min. The catheter was then withdrawn into the aortic root, and perfusion was continued at a rate of 500 ml/min until the effluent from the jugular vein became clear. The heart was then excised and placed into fresh paraformaldehyde. The coronary arteries were removed and divided into 1-cm segments. These segments were processed for transmission (TEM) and scanning (SEM) electron microscopy.

Abbreviations: SEM, scanning electron microscopy; TEM, transmission electron microscopy; vWF, von Willebrand factor; vWD, von Willebrand disease.
Preparation and Examination of Tissues. The 1-cm segments of coronary arteries were placed into individual vials of fresh formaldehyde and allowed to fix overnight. The fixed segments were rinsed in phosphate buffer and were then postfixed in 2% OsO₄ for 1 hr. After osmium fixation, the segments of vessels were dehydrated through a graded series of ethyl alcohols. For TEM, small circular sections were taken from each segment and further dehydrated with propylene oxide and then embedded in Epon. From each block, 1-μm thick sections were made. They were stained with toluidine blue and examined by light microscopy for the presence of platelets, for the integrity of the internal elastic lamina, and for evidence of wall disruption. Appropriate areas were then selected for thin-sectioning. They were stained with uranyl acetate and lead citrate and examined with a Zeiss 10A electron microscope.

After alcohol dehydration, sections of coronary artery to be used for SEM were further dehydrated with Freon 113 and were then critical point-dried. Each vascular segment was split along its long axis and the halves were mounted on metal stubs. They were coated with a thin layer of carbon followed by gold palladium and were examined by using an ETEC Autoscan scanning electron microscope.

The sections were examined initially by SEM to determine the extent and location of the injury (Fig. 1). Subsequently, specific areas were examined to determine the depth of injury. Areas where the subendothelial tissues were intact and where fragments of endothelial cells could be discerned were classified as having superficial injury for the purpose of subsequent morphometric analysis. In these areas, single platelets were attached to the subendothelium (Fig. 2). Areas that were shown by SEM to be completely covered by strands of clumped platelets with no visible subendothelial tissues were designated as having "deep injury." Examination by TEM confirmed that in areas where the internal elastic lamina was intact, only individual platelets were attached to the subendothelium (Fig. 3), whereas in areas where the internal elastic lamina was disrupted, platelets were tightly clumped and formed strands (Fig. 4).

Morphometric analyses were conducted on scanning electron micrographs of areas of superficial injury (Tables 1 and 2).

RESULTS
The balloon procedure produced a simple endothelial denudation as well as deep injury in both groups of animals (Fig. 1). In areas of superficial injury, there was loss of endothelial cells...
and mild disruption of the basal lamina. The elastica was intact and the smooth muscle cells of the media had a normal arrangement. In these areas, in both normal and bleeder pigs, a layer of platelets one to two cells in thickness covered the denuded surface. The subendothelium of denuded arteries had a roughened texture. Fragments of endothelial cells could be observed in some locations. Alternating areas of exposed subendothelium and remaining endothelial cells were present in some locations. In such areas, accumulations of white blood cells were present.

Morphometric analysis of adherent platelets in areas of mild injury was performed on scanning electron micrographs taken at a magnification of $\times2,000$. Six micrographs from each animal were analyzed. The results shown in Table 1 were those obtained from four normal and four bleeder animals. Bleeder animals had a slightly lower mean adherent platelet count than did nonbleeder animals. This difference was not significant ($P > 0.25$).

Scanning electron micrographs of areas of mild injury from both normal and bleeder animals were analyzed for evidence of platelet pseudopod formation (Table 2). The results showed that the bleeder animals had significantly fewer pseudopodia per platelet than did normal animals. The platelets in areas of mild injury from bleeder animals were largely oval to disc shaped. Most platelets on mildly injured intima in bleeder pigs had no pseudopodia.

In severely damaged areas where injury extended into the superficial and deep media, large aggregates and columns of platelets admixed with strands of fibrin were present (Fig. 4).

### Table 1. Density of adherent platelets on subendothelium of balloon-injured coronary arteries in normal pigs and pigs with vWD

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Animals, no.</th>
<th>Total platelets counted</th>
<th>(Platelets/µm²) $\times 10^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>4</td>
<td>8,452</td>
<td>180 ± 40</td>
</tr>
<tr>
<td>Bleeder</td>
<td>4</td>
<td>6,586</td>
<td>150 ± 50</td>
</tr>
</tbody>
</table>

Scanning electron micrographs ($\times2,000$) of areas where removal of endothelium had occurred without disruption of the internal elastic lamina were used to quantify platelet adherence. Six separate areas of denuded coronary artery from each animal were photographed, and the platelets were counted. The total number of platelets in the six areas for each normal animal was 1,504, 2,463, 2,615, and 1,870; for each bleeder animal, the values were 2,531, 1,084, 1,425, and 1,578. There was no difference between the density of adherent platelets in the two phenotypes ($P > 0.25$).

* Values are means ± SD.

### Table 2. Comparison of degree of activation of platelets adherent to subendothelium of balloon-injured coronary arteries in normal pigs and in pigs with vWD

<table>
<thead>
<tr>
<th>Normal</th>
<th>Bleeder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal no.</td>
<td>Pseudopodia per platelet, no.</td>
</tr>
<tr>
<td>1</td>
<td>0.92</td>
</tr>
<tr>
<td>2</td>
<td>1.07</td>
</tr>
<tr>
<td>3</td>
<td>0.88</td>
</tr>
<tr>
<td>4</td>
<td>1.19</td>
</tr>
<tr>
<td>5</td>
<td>1.29</td>
</tr>
</tbody>
</table>

The degree of activation of platelets adherent to denuded subendothelium was determined by counting the average number of pseudopodia projecting from the body of each platelet. Scanning electron micrographs ($\times4,000$) of areas where endothelium had been removed without disruption of the internal elastic lamina were used. Micrographs of five separate areas of denuded coronary artery from each pig were examined. The number of pseudopodia associated with each platelet was assessed, and that number was used to assign a rank of 0, 1, or 2, where 0 equals no pseudopodia, 1 equals one pseudopodium, and 2 equals two or more pseudopodia. The number shown reflects the mean of this ranking for all of the platelets counted. The total number of platelets examined in each of the normal pigs was 37, 28, 18, 86, and 76; the total number of platelets examined in each of the bleeder pigs was 42, 28, 23, 69, and 70. The differences in degree of formation of pseudopodia of platelets in the two phenotypes are significant when analyzed by the Wilcoxon sum rank test ($P = 0.01$).
This was true in specimens from both phenotypes. There was clear degranulation of platelets, and platelets were present in the deep media of these arteries. Leukocytes and erythrocytes could also be demonstrated in these thrombi.

**DISCUSSION**

We have compared the effects of balloon-induced injury to the coronary artery intima in normal pigs and pigs with vWD. The most striking difference was in the morphology of the adherent platelets. The platelets in bleeder pigs appeared larger and more rounded and had fewer pseudopodia than those in the vessels of the normal pigs. These morphologic characteristics suggest that the platelets in the pigs without vWD are less activated. Previous studies of the role of the vWF in platelet–vessel wall interactions have highlighted the importance of vWF in the acceleration of adhesion of platelets. However, a function of vWF in the activation of platelets on denuded arterial surfaces has been suggested by several authors (5, 6, 9, 10, 11). The most recent of these studies showed that (i) radiolabeled human factor VIII in a perfused medium was bound to renal artery subendothelium within 2 min, (ii) adhesion of platelets proceeded at a faster rate in the presence of the bound factor VIII and at higher shear rates, and (iii) in the absence of the bound factor VIII–vWF, the adherent platelets were less spread and had fewer pseudopodia (10).

The effect of shear rate has been noted in many of the *in vitro* studies. Weiss *et al.* demonstrated that native blood samples from normal subjects and patients with vWD showed essentially equal platelet adhesion to rabbit subendothelium in an annular perfusion chamber when shear forces were \(<1,300 \text{ sec}^{-1}\) (11). Similarly, Baumgartner *et al.* (12) showed that the inhibition of platelet adhesion in this system by anti-factor VIII antibodies was evident only when the shear forces were \(>1,300 \text{ sec}^{-1}\). Additionally, these investigators showed that the rate of adhesion was directly proportional to shear rates between 1,300 and 5,200 sec\(^{-1}\). These shear forces are expected in small arterioles and capillaries but not in coronary arteries. Therefore, our observations show that the vWF supports platelet activation in large arteries where shear forces are low—a function not suggested by *in vitro* studies. This represents important documentation that the vWF is involved in the response of platelets to superficial vascular injury in arteries that commonly develop atherosclerosis.

Activation of adherent platelets by vWF might occur by direct or indirect mechanisms. The vWF can promote platelet aggregation and release (13). This process involves aggregation mediated by the vWF followed by release of adenosine diphosphate and other aggregating agents. The exact mechanism by which the vWF precipitates release and the subsequent associated events has not been well defined. Whether this process of *in vitro* aggregation mediated by vWF is similar to the activation of adherent platelets at an injured vessel wall is unknown.

The presence of vWF may indirectly influence activation of adherent platelets by affecting the rate of adhesion. This would mean that at 30 min, platelets that had become adherent in the absence of vWF would have been at the vessel wall for a shorter period of time than would those that had become adherent at a more rapid rate in the presence of vWF. More likely is the possibility that adhesion involves more binding sites on the platelet in the presence of vWF than in its absence; one effect of this might be to distort the platelet membrane and promote further activation.

The implication of our findings is that the role of the vWF in atherogenesis is to promote activation of the platelet, which leads to release of the platelet-derived growth factor. This mechanism, rather than failure of adhesion, would probably best explain the limited resistance to development of aortic atherosclerosis that pigs with vWD exhibit. The absence of this protection in balloon-injured coronary arteries of the bleeder pigs in our previous studies was probably a result of the fact that our balloon procedure causes both superficial denudation of endothelium and, at other areas, deeper medial injury.

Our observations suggest that the vWF supports the activation and spreading of platelets that are adherent at a site of vascular intimal injury. This function of the vWF may be a critical factor in the initiation of arterial thrombosis and promotion of atherogenesis. The chemotaxis of platelets to the injury site and their initial contact and adhesion to the damaged surface occurs in the absence of vWF.

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