Aortic Atherosclerosis in Pigs with Heterozygous von Willebrand Disease

Comparison with Homozygous von Willebrand and Normal Pigs

Una Badimon, Peter Steele, Juan-Jose Badimon, E. J. Walter Bowie, and Valentin Fuster

We have reported that pigs with severe homozygous von Willebrand disease (vWd) are resistant to spontaneous and high fat, high cholesterol, diet-induced atherosclerosis. In this study we report the quantitation of aortic atherosclerotic plaques in three groups of pigs fed with a high fat, high cholesterol (2%) diet from age 3 to 9 months. Nine normal pigs (normal factor VIII antigen, VIII R:AG, and ristocetin co-factor, VIII:RWF) had a mean of 21% atherosclerotic involvement of the distal aortic surface and a 4.5% mean involvement of the entire aorta. Five homozygous vWd pigs (undetected VIII R:AG and VIII:RWF) had a mean of 4.2% atherosclerotic involvement of the distal aortic surface and 1.2% involvement of the entire aorta (p < 0.01, rank sum test). Five heterozygous vWd pigs (approximately 35% VIII R:AG and VIII:RWF) had a mean of 25% atherosclerotic involvement of the distal aortic surface and 6% involvement of the entire aorta; the results were not significantly different from those in the normal pigs. We concluded that resistance to atherosclerosis is not found in animals with moderate reduction of VIII R:AG and VIII:RWF. This may have implications for humans, since in human vWd both factors are almost always present.

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There is increasing evidence that platelets, by interacting with a damaged endothelial surface and subsequently releasing a platelet-derived growth factor (or factors), may play a role in the initiation of atherosclerosis. In addition, experimental animals with inhibited platelet function or with thrombocytopenia are resistant to the development of atherosclerosis.

In pigs with homozygous von Willebrand disease (vWd), we have observed a resistance to spontaneous and to high-cholesterol, diet-induced aortic atherosclerosis, which might be related to the impairment of the platelet hemostatic mechanism in these animals. These pigs have a serious, autosomally transmitted bleeding tendency, a long bleeding time, reduced retention of platelet in a glass-beads column, reduced levels of factor VIII coagulant activity (VIII:C), very low levels (0.02% of normal) of factor VIII-related antigen (VIII R:AG), and a lack of ristocetin cofactor in the plasma (VIII:RWF). We have postulated that the absence of VIII R:AG or VIII:RWF may be responsible for the impairment of platelet-arterial wall interaction and the resistance to atherosclerosis in these animals.

The purpose of the present study is to ascertain if swine with heterozygous vWd might also be resistant to high-cholesterol, diet-induced aortic atherosclerosis. The heterozygous animals have no bleeding tendency.
Methods

Swine

The original Poland-China pigs with vWd were crossed with Yorkshire-Hampshire pigs to establish our present colony. Our control pigs were also a mixture of Poland-China and Yorkshire-Hampshire. All pigs were housed at the Mayo Institute Hills Farm, and all were studied concurrently. Procedures followed in this study were approved by the appropriate institutional guidelines on animal and human resources.

High Cholesterol-Induced Aortic Atherosclerosis Study

Twenty-three newborn pigs, 11 normal controls (six female, five male), five heterozygous vWd pigs (three female, two male) and seven pigs with homozygous vWd (three female, four male), were fed maternal milk supplemented with cow’s milk until 3 months of age, at which time they began to receive an atherogenic, high-fat, high-cholesterol diet (500 g/40 kg body weight) that was continued for up to 6 months (Table 1). Fat contributed 48% and essential fatty acids contributed 7% of the total calories of this diet. Two homozygous vWd pigs bled to death at 3 and 4 months of age, and in each instance a control animal was sacrificed.

Hemostatic and Lipid Parameters

The hemostatic data during life and the lipid levels for the three pig genotypes are shown in Table 2. The assay methods used have been previously described. There was no statistically significant difference in lipid levels among the three genotypes. Eating a high cholesterol diet causes a sixfold increase in plasma cholesterol with no change in plasma triglycerides.

Morphological Studies

Animals were sacrificed with an overdose of pentobarbital. In all pigs, the entire aorta from the aortic valve to the abdominal trifurcation was removed and preserved in 10% formalin solution. All aortas were opened longitudinally and carefully inspected for lesions.
sions. They were analyzed blindly by three observers at the same time. Raised atherosclerotic plaques were identified and quantitated by gross and microscopic examination as previously described. 6,7 In brief, gross quantitative determinations were done by a template method with transparent acetate paper; the extent of the atherosclerotic lesion was expressed as a proportion of the total aortic surface. Because the atherosclerotic plaques involved primarily the distal abdominal aorta between the origin of the renal arteries and the aortic trifurcation, the quantitation of the atherosclerotic plaques of the distal aortic region was of major interest. The aortas were studied later under Sudan IV staining. 20

For microscopic examination, a full-thickness segment of the aortic wall, = 5 mm² in surface, was excised from each atherosclerotic plaque and normal neighboring areas. The tissues were cut as accurately as possible perpendicular to the surface. They were stained with hematoxylin and eosin, with Heidenhain's Weigert-Van Gieson stain and with Sudan IV. Histologic sections were photographed at a magnification of 160 to 400 x. From these pictures, the thickness of the intima from the luminal surface down to the internal elastic membrane was measured. In each section, the thickness was measured at ten equally separated levels, and the average result was obtained.

**Results**

On gross examination, two types of plaques were seen. One was a raised fatty plaque consisting of a soft, yellow elevation with predominantly fat-staining material as detected microscopically; the other was a raised fibrous plaque consisting of a firm, gray, very prominent elevation with predominantly collagen and elastic bundles. The fatty plaques stained homogeneously with Sudan IV, whereas the fibrous plaques stained more prominently in the periphery.

Table 3 gives the results obtained for the three genotypes by the template method of quantitation. All control pigs developed raised atherosclerotic plaques. They showed a mean of 21% involvement of the distal aorta and a 4.5% involvement of the entire aorta. These plaques tended to be large, elongated, and very irregular. The range of intimal thickening over the plaques was 309 μm to 3500 μm, with an average of 1300 μm (normally areas are 80 μm to 400 μm, Table 4).

Likewise, in the five heterozygous vWd pigs the mean involvement of the distal aorta was 25% and 6% of the total aortic luminal surface, which was not significantly different from the control group. The range of intimal thickening over the plaques was 603 μm to 1400 μm, with an average of 811 μm.

In the five homozygous vWd pigs, the mean involvement of the distal aorta was 4.2% and 1.2% of the entire aorta. This decreased incidence of atherosclerotic plaque in these animals when compared to the control pigs and to the heterozygous vWd pigs was statistically significant (p < 0.01, rank sum test). The range of intimal thickening over the few plaques found in these animals was 440 μm to 1780 μm, with an average of 560 μm. This was not significantly different from the thicknesses obtained in the heterozygous or control pigs.

**Discussion**

This study has shown a pronounced difference in the extent of atherosclerotic lesions between normal and heterozygous vWd pigs compared with homozygous vWd pigs when all were fed a high-fat high-cholesterol diet. In homozygous vWd pigs there was no detectable factor VIII:RWF and less than 3% (of normal) of factor VIII R:AG; there was also an impaired platelet-vessel wall interaction as demonstrated by a bleeding time longer than 15 minutes.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Pig no.</th>
<th>Distal aorta (%)</th>
<th>Total aorta (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1</td>
<td>6.0</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.0</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9.0</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>34.0</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>32.0</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>41.0</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>34.0</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>20.0</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>12.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

(Mean ± SE) 21.0 ± 5 4.5 ± 1.0

Homozygous vWd

<table>
<thead>
<tr>
<th>Pig no.</th>
<th>Distal aorta (%)</th>
<th>Total aorta (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26.0</td>
<td>4.0</td>
</tr>
<tr>
<td>2</td>
<td>36.0</td>
<td>9.0</td>
</tr>
<tr>
<td>3</td>
<td>15.0</td>
<td>3.0</td>
</tr>
<tr>
<td>4</td>
<td>39.0</td>
<td>11.0</td>
</tr>
<tr>
<td>5</td>
<td>8.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

(Mean ± SE) 25.0 ± 6.0 ± 2.0

Homozygous vWd

<table>
<thead>
<tr>
<th>Pig no.</th>
<th>Distal aorta (%)</th>
<th>Total aorta (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.0</td>
<td>0.7</td>
</tr>
<tr>
<td>2</td>
<td>0.9</td>
<td>0.2</td>
</tr>
<tr>
<td>3</td>
<td>8.0</td>
<td>2.0</td>
</tr>
<tr>
<td>4</td>
<td>8.0</td>
<td>3.0</td>
</tr>
<tr>
<td>5</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

(Mean ± SE) 4.2 ± 1.7 1.2 ± 0.6

vWd = von Willebrand disease; SE = standard error of the mean.

<table>
<thead>
<tr>
<th>Pig group</th>
<th>Mean ± SE</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1300 ± 323</td>
<td>309-3500</td>
</tr>
<tr>
<td>Heterozygous vWd</td>
<td>811 ± 120</td>
<td>603-1117</td>
</tr>
<tr>
<td>Homozygous vWd</td>
<td>560 ± 290</td>
<td>440-1780</td>
</tr>
</tbody>
</table>

No statistically significant difference between the three groups. vWd = von Willebrand disease.
The heterozygous vWd pigs had detectable levels of both factor VIII R:AG (35%) and factor VIII-RWF (32%) and a normal bleeding time; it is therefore reasonable to assume a normal platelet-vascular wall interaction. In fact, these pigs developed atherosclerotic lesions to the same extent as the control pigs. A study of induced aortic atherosclerosis by Holman et al.20 also showed no difference between normal and heterozygous for von Willebrand disease pigs in the percentage of total aortic area covered by fibrous plaque.

The interaction between platelets and damaged vessels that stops bleeding may furnish an important clue to the origin of arteriosclerosis. In experimental arteriosclerosis induced by removing the endothelium, platelets initially adhere to denuded subendothelial tissue and undergo intracytoplasmic degranulation;21 this sequence of events is followed by release of platelet material into the intima and by intimal migration and proliferation of smooth muscle cells.30 In homozygous vWd, the platelets exhibit a defect in their ability to adhere or degranulate when exposed to damaged endothelial surfaces32 which depends upon the flow conditions.33 If platelet/vessel wall interaction plays a main role in the initial development of atherosclerosis, the hemostatic defect in homozygous animals may preserve them from that triggering factor. Heterozygous and normal pigs with a normal bleeding time have available von Willebrand factor, a normal platelet/vessel wall interaction, and, therefore, they are susceptible to arteriosclerosis. These results support the previously proposed role of the von Willebrand factor as favoring the initial platelet/vessel wall interaction.5,24-28

The data also showed that the susceptibility or resistance to atherosclerosis correlates with the presence or absence of von Willebrand factor,6,7,9 suggesting an important role for the platelet in atherosclerosis.

Reports showing that the major effects of von Willebrand factor during in vitro testing occur under high shear-rate conditions25 not existing in the aorta suggest that the von Willebrand factor deficiency could play a protective role in the long-term induction of atherosclerosis by a different mechanism. However, the development and localization of the plaque appears to be intimately related to hemodynamic stresses at branching points during physiological flow.20 In our pigs, the raised atherosclerotic plaques involved mainly the bifurcation of the abdominal aorta where turbulences of the blood flow modify the low shear stress of the thoracic aorta. At the aortic bifurcation, two geometric risk factors have been described: branch angle variations and an offset, flow-divider tip.30

Even though the extension or luminal surface involvement of the atherosclerotic plaques was different and significantly lower in the vWd animals, the thickness of the lesions was not statistically different in the three groups. One possible explanation is that the capacity for intimal proliferation exists in von Willebrand-deficient vessels, as shown by our aortic transplantation study3 and other reports.31,32 Another factor may be the variability between animals, which in slightly different parameters requires studies with larger numbers of animals to show any significance.

There are some reports describing atherosclerosis development in humans with von Willebrand disease.33,34 However, this is not totally unexpected, since these patients have been treated with plasma and cryoprecipitates to correct the hemostatic defect. Also, the occurrence of the severe type of von Willebrand disease is rare in humans35 but most humans with von Willebrand disease have detectable levels of normal or abnormal Willebrand factor in their plasma. In our study, heterozygous swine with intermediate levels of von Willebrand factor were not resistant to atherosclerosis.

There may be in this long-term, noninvasive study other factors influencing the difference in the susceptibility to arteriosclerosis between homozygous pigs and those with von Willebrand factor.

References
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