Von Willebrand Factor and Occlusive Arterial Thrombosis
A Study In Normal and von Willebrand’s Disease Pigs with Diet-induced Hypercholesterolemia and Atherosclerosis

Timothy C. Nichols, Dwight A. Bellinger, David A. Tate, Robert L. Reddick, Marjorie S. Read, Gary G. Koch, Kenneth M. Brinkhous, and Thomas R. Griggs

The thrombotic response of atherosclerotic arteries to stenosis and injury was studied in 14 pigs, eight normal and six with von Willebrand’s disease (vWD). Atherosclerosis was produced by feeding a 1% to 2% cholesterol diet for 24 weeks. Both groups of pigs developed severe hypercholesterolemia, greater than five times baseline values. Coronary atherosclerosis was detected in all vWD pigs and in all but one normal pig and was not significantly different between groups. At sacrifice under general anesthesia, a Goldblatt clamp (GC) was positioned around the left anterior descending coronary (LAD) and carotid arteries to produce a stenotic segment, which was pinch-injured with needle holders. A 20 MHz Doppler velocity crystal was placed distal to the GC to detect cyclic flow reductions or permanent cessation of flow velocity indicative of occlusive thrombosis. In the phenotypically normal pigs with diet-induced atherosclerosis, occlusive thrombosis was detected in seven of seven LAD and seven of seven carotid arteries. In atherosclerotic vWD pigs, occlusive thrombosis failed to form in six LAD and 10 carotid arteries (p<0.003, Wilcoxon rank sum test). Scanning electron micrographs demonstrated platelet-fibrin microthrombi in both groups of pigs; only phenotypically normal pigs had occlusive thrombi. Von Willebrand factor is essential for the development of occlusive thrombosis and appears to support the progression of a mixed microthrombus to an occlusive thrombus. (Arteriosclerosis 10:449-461, May/June 1990)

Evidence for a role of von Willebrand factor (vWF) in occlusive arterial thrombosis and myocardial infarction has been described in several studies in which normal and von Willebrand’s disease (vWD) pigs were used. Myocardial infarctions were found in five of 24 normal pigs but in none of 14 vWD pigs, despite similar degrees of diet-induced atherosclerosis.1 Occlusive coronary thrombosis was readily induced in normal nonatherosclerotic pigs by superimposed stenosis and pinch injury; in contrast, pigs with vWD failed to thrombose.2 Infusion of a monoclonal antibody against porcine vWF (mAb-vWF) into normal pigs neutralized plasma vWF activity, prolonged bleeding time, and prevented the induction of occlusive coronary thrombosis. This mAb-vWF also caused preformed platelet thrombi to break up.3 Thus, in the absence of plasma vWF in an experimental porcine model, occlusive arterial thrombosis does not occur spontaneously, nor can it be induced by superimposed arterial stenosis and injury.

Whether or not the presence of hypercholesterolemia and atherosclerosis alters the requirement for vWF in occlusive thrombosis is unknown.4 Hypercholesterolemia and atherosclerosis may promote thrombosis by several mechanisms. Both conditions may augment the thrombogenic properties of platelets and clotting factors and reduce the antithrombotic properties of blood vessels.6-8 The frequent finding of thrombi on the surface and in fissures of atherosclerotic plaques suggests that atherosclerotic plaques reduce the native anticoagulant properties of vessel walls.9-17 It is unclear, however, how individual components of an atheroma promote thrombosis.18-25 Atheromas vary in composition and may include lipids, fibrous tissue, smooth muscle cells, and fibrin.18-19,28-27 These different components may induce very different thrombotic responses. It is also conceivable that some plaque constituents have a special affinity for vWF. In addition, turbulent flow and protected vortices due to atherosclerotic plaques may also provide conditions that support thrombosis. Such flow disturbances at areas of plaque-induced stenosis could create shear rates that enhance the thrombotic effects of vWF.25,28,29 On the other hand, these elements may be so thrombogenic as to act by other mechanisms that do not depend upon vWF.

This study extends previous work by determining whether the protection from stenosis and injury-induced occlusive coronary arterial thrombosis conferred by vWD...
in pigs persists in the presence of diet-induced hypercholesterolemia and atherosclerosis.\textsuperscript{2,3} Also in this study, the carotid arteries were subjected to the same protocol to determine if vWD protected arteries other than the coronaries from occlusive thrombosis. The results show that vWD protects both coronary and carotid arteries from induction of occlusive thrombosis and, therefore, that vWF is essential for experimental occlusive thrombosis even in the presence of hypercholesterolemia and atherosclerosis.

Methods

Experimental Animals

Eight phenotypically normal male pigs and six male pigs with vWD were obtained from the closed colony at the Francis Owen Blood Research Laboratory at the University of North Carolina, Chapel Hill. All animals were treated according to the standards set in “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health publication no. 85-23). Porcine vWD, as previously described, is characterized by a prolonged bleeding time, decreased levels of plasma factor VIII coagulant activity (FVIIhC), and less than 1% of normal plasma vWF antigen and activity (See Table 7).\textsuperscript{30-34} One of the vWD pigs included in this study was transfused once with 300 cc of whole blood to treat bleeding from a foot laceration. The hemorrhage occurred 2 months after initiation of feeding the atherogenic diet (vide infra) to the pig (pig 111R, Table 1). This was 3 months before sacrifice. In addition, the atherogenic diet was withheld from this same pig from day 7 to day 14 due to diarrhea. The diet was then resumed without sequelae. No other pig received blood or blood products or required interruption of the diet.

Induction of Atherosclerosis

Administration of Atherogenic Diet

At initiation of the atherogenic diet, the pigs ranged in age from 10 to 15 weeks and weighed between 19 and 32 kg. The atherogenic diet was fed to all pigs for 23 to 26 weeks. At sacrifice, normal pigs weighed 56.4±7.4 kg, and vWD pigs weighed 63.2±12 (p=0.30, Wilcoxon rank sum test). The diet was composed of pig chow (Wayne, T.C. Junior 14/45 Medicated, FCX Pig Starter Feed, or Wayne Pig and Sow Feed) supplemented with 20% beef tallow, 0.75% cholate, and 1% cholesterol except for pigs 145N and 205N, which were fed 2% cholesterol.

Lipid and Hemostatic Profiles

Pigs were weighed, and blood, serum, and plasma samples were obtained before the initiation of the atherogenic diet at monthly intervals while the diet was being administered and at the terminal experiment. Plasma was assayed for vWF activity by the tap-tube macroscopic agglutination test by using formaldehyde-fixed human platelets and porcine plasma.\textsuperscript{31,32,33} Platelet counts, hematocrits, and leukocyte counts were also determined.\textsuperscript{3} Bleeding times were done by the method of Mertz.\textsuperscript{34} Serum cholesterol concentration was determined by Bio Vet Laboratories, Burlington, NC, by the
Table 2. Effect of Phenotype, Stenosis, and Injury on Production of Occlusive Arterial Thrombosis in Coronary Arteries

<table>
<thead>
<tr>
<th>No of pigs per phenotype with CFR and/or PCF</th>
<th>No of injuries</th>
<th>Goldblatt clamp O/S*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>n=7†</td>
<td>7</td>
</tr>
<tr>
<td>Homozygous von Willebrand disease</td>
<td>n=6</td>
<td>0</td>
</tr>
</tbody>
</table>

*p values are from Fisher's exact test.

Goldblatt clamp indicates that the clamp was either open (O) or stenosed (S) when CFR/PCF occurred or the pig completed the protocol. Although eight phenotypically normal pigs were included in the study, pig 1510 did not complete the S1 protocol for the LAD since that vessel was transected during isolation. Pig 205N required two pinch injuries to produce CFR; the other six normal pigs only required one pinch injury. §p values are from Fisher's exact test.

CFR = cyclic flow reduction; PCF = permanent cessation of flow; S1 = stenosis/injury; LAD = left anterior descending coronary artery.

Table 3. Effect of Phenotype, Stenosis, and Injury on Production of Occlusive Arterial Thrombosis in Carotid Arteries

<table>
<thead>
<tr>
<th>No of carotids per phenotype with CFR and/or PCF</th>
<th>No of injuries</th>
<th>Goldblatt clamp O/S*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>n=7†</td>
<td>7</td>
</tr>
<tr>
<td>Homozygous von Willebrand disease</td>
<td>n=10</td>
<td>0</td>
</tr>
</tbody>
</table>

*p values are from Fisher’s exact test.

Goldblatt clamp indicates that the clamp was either open (O) or stenosed (S) when CFR/PCF occurred or the pig completed the protocol. Pig 32R had CFR when the Goldblatt clamp was applied distally in such a way that flow was not impaired. This Doppler crystal was energized by a range-gated pulsed-Doppler unit. The signal was range gated to maximum clarity.

Coronary Artery

After induction of anesthesia and placement of catheters, a 5 mm Goldblatt clamp and 20 MHz Doppler ultrasonic crystal were applied to the left anterior descending coronary artery (LAD) for producing stenosis and measuring blood flow velocity as described.

Carotid Artery

The left and right common carotid arteries were dissected free of surrounding tissue over a 4 cm segment. A 5 mm Goldblatt clamp was placed proximally on the carotid artery, and a 20 MHz Doppler ultrasonic crystal was applied distally in such a way that flow was not impaired. This Doppler crystal was energized by a range-gated pulsed-Doppler unit. The signal was range gated to maximum clarity.

Stenosis and Injury Protocol for Induction of Occlusive Arterial Thrombosis

Coronary and Carotid Artery Stenosis

After a 30-minute period of stabilization subsequent to the surgical preparation, the LAD or carotid arteries had stenosis applied by closing the Goldblatt clamp to a degree sufficient to block reactive hyperemia. A 20-second total occlusion was used to induce reactive hyperemia and to confirm its blockade. With this accomplished, a 30-minute period of observation for cyclic flow reductions (CFR) or permanent cessation of flow (PCF) was undertaken. If either CFR or PCF was seen, indicative of transient or permanent occlusive arterial thrombosis, the portion of the experiment was stopped for that artery. If neither CFR nor PCF occurred, the Goldblatt clamp was released after a second 20-second occlusion confirmed that reactive hyperemia had been blocked throughout the period of observation.

Coronary and Carotid Artery Injury

After stenosis was removed by opening the Goldblatt clamp and blood flow velocity had stabilized, the artery (LAD or carotid) was injured in the area where the clamp was applied. The injury was induced by two or three occlusions of the artery with a spring-toaded forceps (Castroviejo Needle Holders, J. Sklar, Inc., Long Island City, NY). After 10 minutes of observation, the Goldblatt clamp again was partially closed to block reactive hyperemia. If CFR or PCF occurred before or after the clamp was closed, the experiment was stopped for that artery. If, within 30 minutes, neither CFR nor PCF was seen, the clamp was reopened, and after stabilization of flow velocity, the pinch-injury was repeated. If flow velocity remained unchanged, the Goldblatt clamp was tightened a third time sufficient to block reactive hyperemia for a final 30-minute period of observation.

Method of Allain et al. as modified by American Monitor Corporation, Indianapolis, IN, for automated determination.

Terminal Experiment: Arterial Stenosis and Injury Protocol

Anesthetic Administration

All animals were initially sedated with ketamine-HCl (10 mg/kg) intramuscularly. General anesthesia was then established with nitrous oxide, 2% halothane, and oxygen as described. A seven-lead electrocardiogram (ECG) was obtained.

Physiological Monitoring

The femoral artery and vein were isolated and cannulated for infusion of fluids, for blood sampling, and for monitoring arterial pressure and blood gases. Aortic pressure, lead II of the ECG, and Doppler flow velocity (phasic and mean values) were recorded continuously on a model 7 Grass recorder. Sample values of each of these recorded measurements were taken every 5 to 10 minutes and were used for analysis. Ventilator adjustments were made to maintain pH between 7.35 and 7.45.
Figure 1. Coronary atherosclerosis in a normal diet fed pig. A. Photomicrograph of a section of the left anterior descending coronary artery (118Q). Hematoxylin and eosin (H&E) stain. B. A higher magnification of an atherosclerotic plaque in the left circumflex coronary artery from the same normal diet fed pig (118Q) shows foam cells and proliferating smooth muscle cells within the area of intimal proliferation. A fibrous cap is present. H&E stain.

Histological and Ultrastructural Studies

Coronary Artery

The heart and coronary arteries were perfusion-fixed with the Goldblatt clamp in place as described previously except that the right coronary artery was removed before perfusion when the animal was sacrificed and was used in a separate experiment. ²

Carotid Artery

After each pig was killed, the carotid artery segments were removed and were immersion fixed in 1% gluteraldehyde and 4% formaldehyde.
Figure 2. Transmission electron photomicrograph of an atherosclerotic plaque in the left anterior descending coronary artery of a von Willebrand diseased pig (16S). Smooth muscle cell proliferation is present on both sides of the internal elastic lamina. Adherent platelets are present on the luminal surface of the plaque.

Tissue Sections

Histological sections were taken after at least 24 hours of fixation. Two cross-sections were taken from the carotid, one each from the clamp and Doppler sites. Cross-sections from the LAD and left circumflex (LCX) artery were taken at 1 cm segments. The location of the Goldblatt clamp and Doppler crystal was noted. A special effort was made to ensure that sections were cut perpendicular to the direction of blood flow. The proximal portions of selected segments were processed for transmission electron microscopy by postfixing in osmium tetroxide, dehydrating in a graded series of alcohols, and embedding in Epon. Thin sections were viewed with a Zeiss 109 transmission electron microscope. The middle portion of each section was opened longitudinally and was prepared for scanning electron microscopy (SEM) by dehydration through a graded series of alcohols, was critical-point dried from liquid CO2, and was coated with gold-palladium. Sections were viewed with a Cambridge S-200 SEM. The distal portion of each section was processed for light microscopy. Two sections were cut from each block and stained with either hematoxylin and eosin or Verhoeft-van Gieson (WG) stain. In some instances, sections were stained with van Gieson-Masson instead of WG.

Morphometric Analysis of Atherosclerosis and Stenosis and Injury

The image of each vessel segment was projected onto an automated image analyzer (Zeiss Videoplan, Carl Zeiss, New York, NY), and tracings were made of the external elastic lamina (EEL), internal elastic lamina (IEL), lumen (including thrombus), and thrombus. The areas defined by these tracings were calculated by the computer.

Extent of Atherosclerosis

The extent of coronary artery atherosclerosis was evaluated from three indices calculated from the videoplan tracings: 1) intimal area in mm², 2) percent luminal narrowing by intima, and 3) intimal area as a percent of medial area. The formulas for these indices are given below:

1) intimal area (mm²) = area within the IEL − luminal area

2) luminal narrowing (%) = \(\frac{\text{intimal area}}{\text{area within the IEL}}\) × 100

3) intimal area (as % medial area) = \(\frac{\text{intimal area}}{\text{area within EEL} − \text{area within IEL}}\) × 100

Percent Stenosis

An estimate of the percent stenosis created by the Goldblatt clamp on the LAD was calculated by using the mean of the luminal areas of the sections adjacent to the clamp site as the denominator in the following formula:

\%\text{ stenosis due to clamp} = \left(\frac{\text{luminal area at clamp site}}{\text{mean of adjacent luminal areas}}\right) × 100

Extent of Arterial Injury

The amount of injury to the coronary and carotid arteries at the clamp site was assessed by examination of both light and SEM sections. Individual sections from all animals were examined for the presence or absence of smooth muscle cell damage, medial hemorrhage, disrupted IEL, or endothelial denudation. For both carotid and coronary arteries, smooth muscle cell damage was defined as pale staining cytoplasm and contracted and pyknotic nuclei.

Coronary Artery

The light microscopic cross-section was divided into eight equal segments, and the number of segments that had pale staining smooth muscle cells with or without pyknotic nuclei was recorded. The area of hemorrhage within the media or between the media and adventitia was measured with a Zeiss videoplan automatic analyzer. This area then was expressed as a percent of the total medial area. The degree of disruption of IEL was estimated by measuring the length of vessel lumen where the IEL was absent and expressing this length as the percent of total length of IEL and lumen boundary. The luminal surface area displayed by each SEM section from the clamp sites was measured from photomicrographs using the Zeiss videoplan. The areas covered by 1) thrombus (platelet aggregates, red and white blood cells, and fibrin), 2) exposed subendothelium (completely denuded of endothelial cells with a single layer
Figure 3. Coronary artery stenosis and injury. A. Photomicrograph of a section of the left anterior descending coronary artery (LAD) from a normal pig (32R) from the stenosis and injury site. Note the atherosclerotic plaque (AP), the thrombus (T) contained in the lumen, and the medial adventitial hemorrhage (MAH). Hematoxylin and eosin (H&E) stain. B. In contrast, a photomicrograph of a section of the LAD from a von Willebrand disease pig (112R) shows an advanced AP with calcification, disrupted tunica media, MAH, but no thrombus. H&E stain.

of attached platelets), and 3) intact endothelium were measured and expressed as a percent of total surface.

Carotid Artery

Since the carotid arteries were fixed without pressure perfusion, only the light microscopic cross-sections were used for tabulating the presence or absence of three indicators of injury: 1) smooth muscle cell damage, 2) medial hemorrhage, and 3) disrupted IEL.

Statistical Analyses

The distributions of the measured variables were predominantly described with means ± standard deviations. For variables with skewed distributions, medians, mini-
Table 4. Morphological Evaluation of Coronary Atherosclerosis

<table>
<thead>
<tr>
<th>Pig no and phenotype</th>
<th>Left anterior descending</th>
<th>Left circumflex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intimal area</td>
<td>Intimal area</td>
</tr>
<tr>
<td></td>
<td>% luminal narrowing</td>
<td>% of medial area</td>
</tr>
<tr>
<td>Normal</td>
<td>mm²</td>
<td>mm²</td>
</tr>
<tr>
<td>145N</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>205N</td>
<td>0.07</td>
<td>0.03</td>
</tr>
<tr>
<td>118Q</td>
<td>0.14</td>
<td>0.48</td>
</tr>
<tr>
<td>35R</td>
<td>0.25</td>
<td>0.22</td>
</tr>
<tr>
<td>32R</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>34R</td>
<td>0.15</td>
<td>0.81</td>
</tr>
<tr>
<td>59R</td>
<td>0.19</td>
<td>0.50</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>0.25±0.19</td>
<td>0.71±0.50</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Homozygous von Willebrand disease</th>
<th>Left anterior descending</th>
<th>Left circumflex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intimal area</td>
<td>Intimal area</td>
</tr>
<tr>
<td></td>
<td>% luminal narrowing</td>
<td>% of medial area</td>
</tr>
<tr>
<td>86R</td>
<td>0.06</td>
<td>0.12</td>
</tr>
<tr>
<td>111R</td>
<td>0.20</td>
<td>0.02</td>
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<tr>
<td>112R</td>
<td>0.23</td>
<td>0.54</td>
</tr>
<tr>
<td>22R</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>16S</td>
<td>0.10</td>
<td>0.08</td>
</tr>
<tr>
<td>21S</td>
<td>0.54</td>
<td>0.07</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>0.19±0.12</td>
<td>0.14±0.07</td>
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</table>

*Wilcoxon rank sum test.

Animal 151Q, a phenotypically normal pig, was excluded due to left anterior descending coronary artery transection during isolation.

Results

Increase in Serum Cholesterol in Swine Fed an Atherogenic Diet

Serum cholesterol levels were elevated in all animals during the 6-month period of feeding, with a range from 269 to 1528 mg/dl (Table 1). The 2- to 4-month mean cholesterol levels were 923.5±273.9 mg/dl in normal pigs and 612±224.6 mg/dl in vWD pigs (p=0.055). The mean cholesterol levels were significantly different at the 3-month values, 1006.8±339.9 in normal and 620.7±276 mg/dl in vWD pigs (p=0.025). The levels achieved by both groups, however, during the 2- to 4-month period were at least four times the baseline values. Cholesterol levels at month 5 remained elevated at two to three times baseline in the pigs tested, although data at this time should be viewed cautiously because there were too few to be analyzed formally.

Flow Velocity Changes after Stenosis and Injury

Coronary Arteries

Seven of seven normal pigs had CFR, PCF, or both after stenosis and injury to the LAD (Table 2). In contrast, none of the six vWD pigs exhibited CFR or PCF in the LAD after stenosis and injury. This difference in the incidence of flow velocity changes between the two groups was significant (p=0.005, Fisher's exact test).

Carotid Arteries

In the group of normal pigs, nine carotid arteries were stenosed and injured, seven of which had CFR, PCF, or both (three right, four left, Table 3). In the vWD group of pigs, eleven carotid arteries were stenosed and injured, 10 of which had neither CFR nor PCF (five right, five left). Carotid arteries in two normal pigs and one vWD pig were excluded because the Doppler flow velocity signal was uninterpretable. This difference in incidence of flow velocity changes between groups was significant (p=0.003, Fisher's exact test).

Morphology of Heart

There was no evidence of myocardial infarction in any of the animals before the surgical procedure by electrocardiogram or by direct inspection of the heart.
Table 5. Morphological Evaluation of Coronary Artery at Stenosis/Injury Site

<table>
<thead>
<tr>
<th>Pig no and phenotype</th>
<th>% segments SMD</th>
<th>% MAH</th>
<th>% disrupted IEL</th>
<th>Scanning electron microscopy†</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Thrombus</td>
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<tr>
<td>Normal</td>
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<tr>
<td>145N</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>205N</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>118Q</td>
<td>50</td>
<td>0</td>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td>35R</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>32R</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>100</td>
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<tr>
<td>34R</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>59R</td>
<td>100</td>
<td>60</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Median</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(min,max)</td>
<td>(50,100)</td>
<td>(0,60)</td>
<td>(0,31)</td>
<td>(0,100)</td>
</tr>
</tbody>
</table>

Homozgygous von Willebrand disease

|                      | % disrupted IEL |                |                |                 |                     |                     |
| 86R                  | 100             | 0              | 19             | 0               | 100                  | 0                   |
| 111R                 | 100             | 38             | 0              | 20              | 80                   | 0                   |
| 112R                 | 100             | 21             | 27             | 0               | 100                  | 0                   |
| 22R                  | 100             | 0              | 0              | 0               | 100                  | 0                   |
| 16S                  | 100             | 26             | 44             | 0               | 100                  | 0                   |
| 21S                  | 100             | 44             | 59             | 5               | 95                   | 0                   |
| Median               | 100             | 23.5           | 23             | 0               | 100                  | 0                   |
| (min,max)            | (100,100)       | (0.44)         | (0.59)         | (0.20)          | (80,100)             | (0,0)               |

p value               | 0.317           | 0.211          | 0.108          | 0.462           | 0.415                | 0.355               |

*Light microscopy abbreviations: SMD = smooth muscle cell damage, MAH = medial adventitial hemorrhage, IEL = internal elastic lamina. †For scanning electron microscopy (SEM), all values are expressed as percent of luminal surface area covered by thrombus, exposed subendothelium, or intact endothelium measured from SEM photomicrographs. ‡Wilcoxon rank sum test.

Quantification of Atherosclerosis

Coronary Arteries

In both groups, there was a continuum from uninvolved segments of artery to segments with complex lesions containing accumulations of foam cells and smooth muscle cell proliferation (Figures 1, 2, and 3). Some lesions had calcification present as well (Figure 3B). The mean values for the extent of LAD atherosclerosis were somewhat larger for normal pigs than for vWD pigs, but differences between these groups were not statistically significant because of the substantial variability of these measures (Table 4): intimal area in mm² (0.19±0.25 vs. 0.19±0.19, p=0.830), intimal area as a percent luminal narrowing (7.6%±8.8% vs. 5.0%±3.5%, p=0.943), and intimal area as a percent medial area (16.9%±20.5% vs. 8.3%±5.0%, p=0.616). Similar results were obtained for comparisons between the two groups in the extent of atherosclerosis in the LCX (Table 4). Coronary atherosclerosis was evident in all vWD pigs and in all but one normal pig.

Coronary atherosclerosis defined as intimal area of the LAD and LCX was not significantly correlated with the 2- to 4-month mean cholesterol level: r_s = -0.035, p=0.916 and r_s = 0.073, p=0.832, respectively, for the LAD and LCX (Spearman rank correlation coefficient).

Carotid Arteries

There was intimal thickening in the carotid artery of one phenotypically normal pig (59R). This plaque was composed of foam cells several layers thick and was located at the Doppler probe site. Within the media, there were areas of calcification in two carotids from one phenotypically normal pig (35R). Neither intimal thickening nor medial calcification was detected in 10 carotids from the vWD pigs. The difference in the incidence of carotid medial calcification is not significant (p=0.375, Fisher's exact test).

Arterial Stenosis, Injury, and Thrombosis

Coronary Arteries

There was no significant difference in the percent stenosis produced by the Goldblatt clamp on the LAD between normal (n=5) and vWD (n=6) pigs (66.6%±38.5% vs. 66.3%±24.2%, p=0.715). Sections from two normal pigs were not available for analysis (pigs 145N and 205N).

The extent of coronary artery injury in both groups of pigs is tabulated on Table 5. Injury consisted primarily of smooth muscle cell damage. The cytoplasm was pale staining, and the nuclei were often pyknotic. The percent of segments with smooth muscle cell damage was comparable between groups (p=0.317). The percent of medial adventitial hemorrhage and disruption of the IEL...
Figure 4. Scanning electron photomicrograph of the luminal surface of the left anterior descending coronary artery (LAD) at the stenosis and injury site from a von Willebrand diseased pig (111R). A. Exposed subendothelium is present, with adherent platelets and a nonocclusive thrombus. B. and C. Higher magnification of adherent platelets (B) and platelet-fibrin thrombus (C) occurred at the injury site in the LAD of the same atherosclerotic von Willebrand diseased pig.
were lower during surgery but did not differ significantly within normal limits as reported previously. Hematocrits between normal and vWD pigs (p=0.663).

The extent of stenosis was not determined in the carotid arteries since they were not pressure perfused.

The luminal surface of the LAD was covered with adherent platelets or mixed microthrombi consisting of platelets, fibrin, and red and white blood cells (Figure 4). Occlusive thrombi were present in two normal pigs by SEM (145N and 32R, Table 5) (Figure 5).

Carotid Arteries

The extent of stenosis was not determined in the carotid arteries since they were not pressure perfused.

The luminal surface of the LAD was covered with adherent platelets or mixed microthrombi consisting of platelets, fibrin, and red and white blood cells (Figure 4). Occlusive thrombi were present in two normal pigs by SEM (145N and 32R, Table 5) (Figure 5).

Hematological Values

The values for bleeding time, vWF activity, vWF antigen, FVIII:C activity, hematocrit, and platelet count are listed on Table 7. Platelet counts decreased in both groups while being fed the atherogenic diet but remained within normal limits as reported previously. Hematocrits were lower during surgery but did not differ significantly between normal and vWD pigs (p=0.663).

Hemodynamics

Normal pigs had significantly higher systolic and diastolic blood pressure values than did the vWD pigs during the open chest portion of the experiment: respectively, systole, 101.4±10.4 vs. 88.4±13.0 mm Hg, p=0.044; diastole, 70.5±10.3 vs. 54.9±11.1 mm Hg, p=0.028. The tendency for higher heart rates for the vWD pigs was nearly significant (normal=95.2±26.8 vs. vWD=117±12.2, p=0.088). The difference between double products (heart rate x systolic blood pressure) for the two groups was not significant (9624±3796.4 vs. 10347.5±1544.8, p=0.197).

Discussion

In this study, hypercholesterolemia and atherosclerotic blood vessels were produced in normal and vWD pigs by feeding an atherogenic diet for 24 weeks, a previously established technique. Coronary and carotid arteries were then subjected to a stenosis and injury protocol for producing occlusive thrombosis. The carotids were included to determine if vWD protected arteries other than coronary arteries from induced thrombosis. While occlusive thrombosis developed readily in the coronary and carotid arteries of normal pigs, occlusive arterial thrombosis failed to develop in the vWD pigs despite the presence of coronary atherosclerosis, severe hypercholesterolemia, and additional stenosis and injury.

Stenosis and pinch injury caused medial damage in almost all arteries. Adherent platelets were noted in both normal and vWD animals at sites of arterial injury as were small amounts of platelet-fibrin microthrombi. Only normal pigs, however, developed occlusive thrombosis. In previous studies, platelet-fibrin microthrombi induced by balloon injury apparently did not progress to occlusive thrombosis. On the other hand, in our experience, stenosis alone has produced occlusive thrombosis in only one artery, a carotid which had been excessively injured during dissection (Table 3). Taken together, these observations suggest that the combination of stenosis, damaged and exposed tunica media, and vWF is required to support the progression of platelet-fibrin microthrombi to occlusive arterial thrombosis.

A vWF-independent thrombogenic effect of tunica media has been suggested by the studies of Reddick et
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Figure 6. Carotid arteries from von Willebrand diseased (vWD) pigs fed an atherogenic diet. A. Photomicrograph of a section taken from where the Goldblatt clamp and injury had been applied (21S). Hematoxylin and eosin (H&E) stain. B. A higher-power magnification from another diet fed vWD pig (112R) shows the presence of smooth muscle cell damage and medial hemorrhage. H&E stain.

In both normal and vWD pigs, deep balloon injury, which exposed tunica media to flowing blood, caused the formation of nonocclusive mixed thrombi containing platelets and fibrin. Superficial disruption of either normal intima or predominantly fatty atherosclerotic lesions in hypercholesterolemic pigs, however, yielded only a single layer of platelets in both phenotypes. Thus, exposure of smooth muscle cells, smooth muscle cell products, or other components of media to flowing blood produced platelet-fibrin microthrombi independent of vWF, hypercholesterolemia, or atherosclerosis.

The thrombotic response to injury of atherosclerotic lesions has been studied in a variety of animal models. Injury to artificially created neointima in rabbits caused fibrin deposition and the formation of platelet-fibrin microthrombi in contrast to the simple platelet monolayer seen with injury to normal intima. Changes in the amount, type, and relative distribution within the arterial wall of proteoglycans and other subendothelial matrix constituents such as collagen during atherogenesis have been implicated in the alteration of the response of blood components to the injured proliferative lesion. In our present study and those of Reddick et al., the atherosclerotic arteries had not been mechanically injured before feeding the atherogenic diet, an experimental technique which induces growth of neointima. In contrast, platelet-fibrin microthrombi were only found when the tunica media was exposed or damaged in normal and vWD pigs. Injury of neointima, then, induces a thrombotic response, which is similar to that seen with injury to normal media. These observations are consistent with the hypothesis that the thrombotic response of flowing blood in vivo varies with the composition of the exposed vessel surface and is augmented by stenosis.

Neither the stenosis and injury nor the balloon injury study addresses quantitatively the contribution of altered patterns of blood flow or shear rates at individual atherosclerotic lesions. It is possible that the apparent altered thrombogenicity of atherosclerotic arteries is the result of subocclusive or mural thrombosis or disruption of normal laminar blood flow producing protected vortices in the area of the obstructive lesion. The known functions of vWF that are shear-rate-dependent directly relate to this hypothesis. In ex vivo systems, vWF is required for maximal platelet adhesion to subendothelium at high shear rates and for buildup of platelet thrombus at lower shear rates. In addition, vWF has been shown to affect the rate of adhesion but not the number of adherent formaldehyde-fixed washed platelets to fibrilar collagen. The results of our in vivo studies are consistent with those done ex vivo. Attached platelets were present in similar numbers on balloon-injured arteries of both phenotypes of pigs, but vWF was required for occlusive thrombosis to occur after stenosis and injury.

Cholesterol levels were significantly higher in the normal pigs at 3 months and nearly so for month 4 (Table 1). Additionally, there was a suggestion that there was a greater fall in cholesterol levels after the third month in the vWD group. These findings suggest that there may be a difference in lipid metabolism between normal and vWD pigs.
bleeder pigs. A tendency for normal pigs to have higher levels of diet-induced hypercholesterolemia than do vWD pigs has been noted in previous studies. This issue has never been studied systematically.

Previous studies have shown that VD pigs develop less aortic atherosclerosis in response to a high fat and cholesterol diet than do normal pigs. Our experience has been, however, that coronary atherosclerosis can be fairly reliably produced in both the normal and the vWD pigs from the Chapel Hill colony. The degree of lesion development in the pigs in this study was similar to that found in these previous studies. This allowed us to pursue the primary goal of the study, which was to examine the effect of VWF on inducibility of thrombosis in atherosclerotic arteries. A detailed discussion of the several studies of diet-induced atherosclerosis was to examine the effect of VWF on inducibility of thrombosis in atherosclerotic arteries. A detailed discussion of the several studies of diet-induced atherosclerosis in VWF swine is presented in reference 4.

In conclusion, VWF is essential for occlusive thrombosis even in the presence of hypercholesterolemia and coronary atherosclerosis. Von Willebrand’s disease protects both coronary and carotid arteries from experimentally induced occlusive thrombosis. The thrombotic response of flowing blood in vivo to exposed subendothelium of atherosclerotic vWD pigs consists of adherent platelets and nonocclusive platelet-fibrin microthrombi. The progression of microthrombi to occlusive thrombosis appears to be dependent on VWF in this model.

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References

Table 7. Hematological Values

<table>
<thead>
<tr>
<th>No of pigs per phenotype</th>
<th>Bleeding time (min)</th>
<th>vWF (%)</th>
<th>vWF:Ag (%)</th>
<th>FVIII:C (%)</th>
<th>Hematocrit (%)</th>
<th>Platelet count (mm³ x 10⁹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>n=8</td>
<td>2.9±0.7</td>
<td>105±15.6</td>
<td>123±67.3</td>
<td>121±27.1</td>
<td>30±3.4</td>
</tr>
<tr>
<td>Homozygous vWD disease</td>
<td>n=6</td>
<td>&gt;10</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>10±3.4</td>
<td>28.8±3.8</td>
</tr>
<tr>
<td>p value*</td>
<td>0.001</td>
<td>0.001</td>
<td>0.002</td>
<td>0.002</td>
<td>0.663</td>
<td>0.153</td>
</tr>
</tbody>
</table>

*Wilcoxon rank sum test. Descriptive statistics are means±SD. Values for the hematocrit and platelet counts are taken from those obtained at the terminal experiment.

vWF=von Willebrand factor, Ag=antigen, FVIII:C=factor VIII coagulant activity.
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