Coronary Atherosclerosis in the Pig
Induced Plaque Injury and Platelet Response

Robert L. Reddick, Marjorie S. Read, Kenneth M. Brinkhous, Dwight Bellinger,
Timothy Nichols, and Thomas R. Griggs

The thrombogenic potential of atherosclerotic diet-induced coronary atherosclerotic plaques was investigated in normal swine and in bleeder swine with homozygous von Willebrand disease. Fourteen paired normal and bleeder swine were placed on a 1% cholesterol diet for 1 to 16 weeks. Serum cholesterol was elevated in all animals at sacrifice. Foam cell deposits developed in all major epicardial coronary arteries, and lesions progressed over time from small subendothelial foam cell deposits to fibrous cap lesions that contained foam cells, elastic fibers, collagen, degenerative material, and smooth muscle cells. Balloon catheter injury resulted in platelet deposition, largely in a monolayer or as small platelet clumps devoid of fibrin. Upld debris was present in injured areas of the subendothelium but did not induce thrombus formation. When the injury involved the media, platelet-fibrin thrombi were formed. Upld was not present in these thrombi. Morphometric analysis of platelet deposition on ballooned atherosclerotic vessels showed similar numbers of platelets in both phenotypes. However, the attached platelets in bleeder pigs showed significantly less spreading than did those in the normal animals. The results show that injury to intimal foam cell and mixed cellular lesions in coronary arteries of cholesterol-fed swine does not promote the development of platelet-fibrin thrombus formation. In contrast, when the injury extended to the media, mixed thrombi were formed. (Arteriosclerosis 10:541-550, July/August 1990)

Vascular lesions in both humans and experimental animals may range from simple foam cell deposits to complicated, highly stenotic proliferative and fibrotic atherosclerotic plaques. In sudden coronary death in humans, plaque-associated thrombosis may occur, and histologic evaluations show that lesions associated with thrombosis are, in general, more advanced. In sudden coronary death in humans, plaques with thrombosis may have necrotic centers or show surface fissures, hemorrhage, medial destruction, nodular foam cell deposition, calcification, and cellular infiltrates involving the fibrous cap and base of the lesion. Falk reported that a thin fibrous cap and prominent collections of foam cells within the plaque were important in the genesis of thrombosis. However, the composition and type of plaque that contributes to thrombogenesis remain unclear.

The purpose of this report is to investigate the relationship of plaque constituents and thrombosis in coronary arteries of cholesterol-fed swine. Normal pigs and swine with von Willebrand's disease (vWD) were fed an atherogenic diet for 1 to 16 weeks. Before sacrifice, the left anterior descending (LAD) coronary artery was balloon-injured. The right and circumflex branches were not ballooned and were used as morphologic guides to evaluate plaque constituents. The results suggest that disrupted foam cell deposits and fibrous cap plaques in the absence of arterial stenosis are no more thrombogenic than is normal subendothelium.

Methods

Von Willebrand Swine Colony

The animals used in these experiments were from the Chapel Hill colony of swine with vWD. The homozygous vWD animals have a severe hemorrhagic diathesis, prolonged bleeding time, reduced levels of Factor VIII procoagulant activity, and less than 1% of normal levels of vWF:Ag and platelet-aggregating von Willebrand factor (vWF) activity, previously designated as platelet aggregating factor (PAF). The animals were housed and cared for following the guidelines set forth in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication #85-23). The use of these animals in this study was approved by the University of North Carolina Institutional Animal Care and Use Committee.

Bleeding Time Determinations

Bleeding times were determined by the ear immersion technique of Mertz. In normal animals, the bleeding time is 2 to 4 minutes, and bleeders characteristically have a bleeding time in excess of 15 minutes.

Experimental Animals

Healthy, male, normal, and bleeder swine at 2 months of age were placed on an atherogenic diet for 1, 2, 4, 8,
and 16 weeks. Blood was drawn from each animal before initiation of the diet for determination of hematologic parameters, and Factor VIII:C, Factor VIII:Ag, and serum cholesterol levels (Blount Laboratories, Burlington, NC). Body weights were obtained at the beginning of the diet, at monthly intervals, and at the time of sacrifice.

At sacrifice at the five different time intervals on the atherogenic diet, the animals were initially sedated with ketamine HCl (15 mg/kg) given intramuscularly. Anesthesia was initiated with 4% halothane and then maintained throughout the study period with this anesthetic at concentrations of 1% to 2%. In each animal, the LAD branch of the left coronary artery was balloon-injured by previously described techniques. At 30 minutes after the balloononing procedure, each animal was given a lethal injection of sodium pentobarbital followed immediately by whole-body pressure perfusion with 4% phosphate-buffered formalin, pH 7.35. The heart and aorta were removed, and the coronary arteries were dissected from the heart and prepared for transmission (TEM) and scanning (SEM) electron microscopy and for light microscopy. Morphologic Classification of Platelet Adherence and Thrombi

The morphologic effects of balloon injury were examined by SEM and TEM. Special attention was paid to identification of the internal elastic lamina, to cellular and matrix components of the vessel wall, and to adherent thrombotic material. We classified the thrombotic material into two categories. The first was superficial platelet adherence. In these areas, platelets were adherent either as single platelets or were arranged in clumps of no more than five or six platelets with only slight overlapping. Occasionally, leukocytes were adherent in these areas, but no erythrocytes or fibrin was present. The second category we refer to as mixed or complex thrombosis. These were macroscopic aggregates composed of degranulated platelets or platelet ghosts, erythrocytes, and leukocytes and strands of electron-dense material consistent with fibrin.

Platelet Counts on Injured Vascular Segments

SEM photographs at 3000× were taken of balloononed vascular segments from both diet- and chow-fed animals for counting of adherent platelets. For consistent analysis, the 0.5 cm portion of the LAD at 3 cm from the common left coronary artery was used. This segment of the LAD has consistently shown the most severe and reproducible morphologic changes after balloon injury. A total of six to eight nonoverlapping photographs from all quadrants of this segment were taken for analysis.

Platelet Spread Analysis

For analysis of platelet spreading, six to eight TEM of platelets present on balloon-injured coronary artery segments were taken at an initial magnification of 3000×. Each platelet was evaluated to determine the extent of spread over the subendothelium. Platelet spread in this report refers to those platelets positioned on the denuded subendothelium so that the long axis of the platelet is in intimate contact with subendothelial structures (See Figure 3.) Nonspread platelets were all other platelets whether or not pseudopodia were present and whether or not there was contact with the subendothelium by the pseudopodia. By using this method, a clear distinction between nonspread and spread platelets could be made. When there was deep injury that involved the media of the vessel wall, segments proximal and distal to the area of deep injury were also used to evaluate the platelet spread.

Control Animals

Two pigs, one normal and one bleeder, were placed on standard pig chow for 4 months. Laboratory data collected on these animals included serum cholesterol levels, hematocrit, white cell counts, platelet counts, and body weight. At the time of sacrifice, the coronary arteries were processed and evaluated as described above.

Statistical Analysis

For each phenotype, means and standard errors were used to describe the data for each laboratory measurement performed. Comparisons between phenotypes were based on the Wilcoxon signed ranked statistical test in a manner that accounted for the pairing of normal and bleeder animals within diet groups. Student's t test was used to evaluate the platelet spread analysis.

Results

Laboratory Studies

Platelet counts and cholesterol values for each pair of animals are shown in Table 1. The serum cholesterol levels were elevated in all of the experimental animals at the time of sacrifice. No significant difference in serum cholesterol level was found between paired normal and bleeder animals. Platelet counts for all animals throughout the study period were within the range of normal for swine in this colony. Values for white and red blood cell counts and hematocrits varied considerably but were within the range of normal for the two groups of animals. All animals showed weight gains throughout the study period.

Coronary Morphology of Pigs on Nonatherogenic Diet

The surface of nonballoononed vessels from chow-fed pigs was free of attached cells, and the endothelium formed a continuous cell layer as revealed by SEM (Figure 1). On TEM, the endothelial cell layer was intact, and the cell surface had occasional villous processes (Figure 2). The subendothelium in both normal and bleeder animals occasionally contained smooth muscle cells, bundles of collagenous fibers, basement membrane material, and elastic fibers. The internal elastic lamina was intact. Smooth muscle cells of the media were normal, however, and within an occasional cell, degenerative cytoplasmic material was evident. The matrix around the smooth muscle cells consisted largely of collagen fibers.

After balloon injury, platelets were present on the subendothelium of both normal and bleeder pigs (Figure 3). Occasional leukocytes could be found on the denuded subendothelium (not shown). Platelet-fibrin

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Table 1. Laboratory Data

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Figure 1. Control animal, scanning electron micrograph. The endothelial surface is slightly irregular, with raised areas outlining endothelial cell nuclei. The surface demonstrates microvilli and is free of attached cells. × 711

Figure 2. Control animal, transmission electron micrograph. An intact endothelial cell layer is present. The subendothelium is slightly expanded but does not contain cellular debris. × 3450

Thrombi and platelet clumps were absent in areas of balloon injury.

Coronary Artery Morphology after Cholesterol Feeding at 1 to 4 Weeks

The endothelial surfaces of nonballooned arteries from pigs killed at 1 to 4 weeks had large areas of smooth, intact endothelium and focal raised areas. Microvillous processes were also seen. An occasional foam cell was attached to the surface of the endothelial cells. No areas of endothelial disruption were present.

Early lesions in nonballooned vessels consisted of foam cells that were present in the proximal portion of the vessel and around the ostia to small vessels that emanated from the main channel. Smooth muscle cells, calcification, and necrosis were absent. On TEM of nonballooned segments, focal accumulations of foam cells were present beneath an intact, thinned endothelium (Figure 4). The smooth muscle cells had normal morphology and arrangements, and the elastica was largely intact. There was a slight increase in matrix material within the subendothelium in 4-month animals (not shown).

By both SEM and TEM, injured coronary segments had a monolayer of platelets on the denuded subendothelium (Figures 5A and 5B). Leukocytes admixed with platelets were often seen in the proximity of small branch points. Endothelial cells within the orifices of these branches were partially disrupted. Those in other areas were intact.
Figure 3. Comparison of platelet deposition after balloon injury in control normal (A) and bleeder (B) animals. Platelet spread over the subendothelium is pronounced in the normal animal (A). In the bleeder animal (B), individual platelets show almost no spreading. A. × 4746 B. × 4520

Figure 4. Nonballooned coronary artery. An early lesion consisting of focal subendothelial deposits of foam cells in the right coronary artery in a cholesterol-fed animal after 2 weeks on the diet. × 1794

Coronary Artery Morphology at 8 Weeks

The lesions ranged from small deposits of lipid-filled cells within the intima to large foam cell deposits that involved both the intima and superficial media (Figure 6). Fibrous cap lesional development was not seen in these animals. In some areas, extensive cellular debris was present in the media and consisted of both lipid particles and cellular

Figure 5. A. Endothelium after 2 weeks on the diet. Platelets are present on the subendothelium. The subendothelium contains layered smooth muscle cells and fragments of elastic tissue (arrow) × 4757 B. Endothelium after 4 weeks on the diet. The intima is expanded and contains numerous fragments of elastic tissue (arrows). A leukocyte and platelets are present on the intimal surface in areas devoid of endothelium. A portion of an attenuated endothelial cell is present (arrowheads). × 4800
fragments. SEM of the plaques revealed occasional foam cells on the endothelial surface (Figure 7). The endothelial surface was roughened, and numerous microvillous processes were present. Neither endothelial cell loss nor platelets attached to the altered endothelium were seen.

After balloon endothelial denudation, platelets were attached to the subendothelial surface and in some places were admixed with lipid debris (Figure 8). Thrombus formation was absent, although occasionally, small clumps of platelets were present.

**Coronary Artery Morphology at 16 Weeks**

Lesions produced in these vessels consisted largely of foam cell deposits with occasional admixed smooth muscle cells. Some fibrous cap lesions were present, mostly in areas around the ostia. The endothelial cells were attenuated and spread over the surface of the lesions. Lipid droplets were present in the endothelial cytoplasm, and the cell surface contained numerous villous processes. Occasionally surface microvilli of endothelial cells overlapped to form small caveolae. Rough endoplasmic reticulum was prominent. The subendothelial space was widened and contained prominent collections of fragmented basement membrane. Poorly differentiated cells with increased rough endoplasmic reticulum were also present in the subendothelial space. The internal elastic membrane was largely intact, although in some areas, fragments of elastica could be found. In areas where lesions had not developed, the internal elastic lamina was intact.

Smooth muscle cells of the superficial media had altered patterns and contained increased amounts of rough endoplasmic reticulum (Figure 9). The basement membrane surrounding these cells was focally absent. The cells were often in disorganized patterns, and clumps of fragmented elastica could be found adjacent to these cells. Abundant cellular debris and free lipid was present in these areas. At branch points, the lesions were more complex and consisted of mixed foam cell depositions and smooth muscle cells.

Platelet monolayers and small platelet clumps were present on the subendothelium of balloon-injured vessels. In some areas, lipid and cellular debris could be found adjacent to the platelets. Often the plaques were not totally disrupted, although free lipid and matrix tissues were in direct contact with adherent platelets (see Figure 8). Platelet-fibrin thrombus formation was absent in these areas.

When injury to the superficial and deep media occurred, well-formed platelet-fibrin thrombi were present (Figures 10A and 10B). At the periphery of these thrombi, white blood cells were present, and red blood cells could be found in the planes between smooth muscle cells. Small platelet thrombi or platelet-fibrin thrombi were present proximal and distal to the area of medial injury. Leukocytes adhered to the subendothelium and formed monolayers in some areas adjacent to the thrombi. Disrupted foam cell debris was not present within the thrombi.

**Platelet Counts on Injured Vessel Segments**

Morphometric analysis of platelet deposition after balloon endothelial denudation showed platelets on the
Figure 7. Scanning electron micrographs of the endothelial surface in a diet-fed animal. The endothelial surface is irregular (A). × 146 Attached foam cells are shown at a higher magnification (B). × 1168

subendothelium in both normal and bleeder pigs (Table 2). However, the numbers of platelets attached to the injured intima in bleeders and normals were not statistically different (p = 0.23).

Evaluation of Spread and Nonspread Platelets

Balloon injury to coronary arteries of diet-fed swine was followed by platelet deposition in areas of injury. In most areas, a simple platelet monolayer was present. Morpho-

Figure 8. Ballooned segment of coronary arteries at 16 weeks. Lipid and cellular debris are present and are admixed with platelet monolayers. Small clumps of platelets are present. A, × 518; B, × 4144
metric evaluation to determine platelet spread over the injured surface showed that platelets in areas of injury in normal animals were more likely to show a spread configuration than those in bleeder animals (Figure 3, Tables 2 and 3). After diet feeding, the percentage of spread platelets increased over baseline in bleeder animals and remained fairly constant from 2 to 16 weeks (Table 3).

In some bleeder animals, injury to the superficial media occurred during induction of a balloon injury. When these segments were evaluated for evidence of platelet spread, the differences between bleeders and normals was less significant (Table 4, p=0.02). Within these areas, platelet-fibrin thrombi were also present. When animals with deep injury to the coronary artery were excluded from the analysis, significant differences were present (p=0.003).

Discussion

Coronary vascular lesions induced by feeding swine a diet high in cholesterol produced intimal and medial foam cell deposits and occasional fibrous cap lesions. The lesions ranged from foam cell deposits to multilayered lesions and atherosclerotic plaques. Fatty plaques not only developed at branch points and around ostia to small vessels but were also present at other sites within the intima. After balloon injury to the intima, platelet-fibrin throm-
Thrombosis was largely present in areas of plaque formation and stenosis and was felt to be associated with plaque fissuring or ulceration. The events that led to plaque rupture were not clear. In cases of sudden coronary death, Haerem showed that mural platelet microthrombi, defined as platelet masses of 600 μm or less along the largest extension, were present in the coronary arteries of sudden death patients. Fibrin was present at the periphery of these microthrombi.

Stemerman et al. and Richardson et al. showed that injury of previously de-endothelialized vessels with thickened, smooth muscle cell-rich intima resulted in the formation of platelet-fibrin thrombi. The lesions that were present in those animals were described as similar to the preatherosclerotic fibromusculoelastic intimal changes seen in humans and differed from those induced by diet in our study, in which the lesions were either foam cell deposits or mixed plaques. Injury to the fibromusculoelastic lesions was accompanied by the formation of mural platelet thrombi with large amounts of fibrin. In contrast, Stemerman showed that, 10 minutes after injury to rabbit iliac arteries, a single layer of adhering platelets was in contact with the intimal connective tissue. Fibrin was not seen on the intimal surface nor in aggregates of fewer than four platelets. These results are similar to those in our study. In animals fed the diet for 8 to 16 weeks, small platelet aggregates of four platelets or fewer were occasionally present on the intima after balloon injury but were not associated with fibrin. In our study, disruption of diet-induced foam cell lesions within coronary arteries of swine did not produce platelet-fibrin thrombi. The association, however, of deep injury and thrombus formation would suggest that smooth muscle disruption and perhaps flow alterations are important etiological factors in thrombus generation.

More recent data would suggest further that significant vascular stenosis must also be present in the area of injury if occlusive thrombosis is to occur after arterial injury. The results in this and previous studies would suggest that smooth muscle contact, vascular wall contents, and stenosis are probably more important than lipid in promoting arterial thrombosis.

The results obtained for platelet spreading on the injured intima agree with our previous findings of reduced platelet spreading on the subendothelium of coronary

| Table 2. Platelet Deposition* after Balloon Injury in Diet-fed Swine. Morphometric Analysis according to Phenotype and Time on Diet |
|------------------|------------------|------------------|
| Phenotype | Weeks on diet | Normal | Bleeder |
| | | Number of platelets (μm²×10⁶) | Number of platelets (μm²×10⁶) |
| Normal | 0 | 34 | 56 |
| | 1 | 46 | 54 |
| | 2 | 56 | 44 |
| | 4 | 54 | 46 |
| | 8 | 25 | 75 |
| | 16 | 44 | 66 |
| | 16 | 52 | 48 |

Values are percentages obtained from two independent observers who evaluated the transmission electron micrographs without knowledge of the phenotype or the extent of injury. The total number of platelets present on the six to eight transmission electron micrographs ranged from 40 to 156.

*Medial injury occurred during the course of inducing a balloon injury and resulted in the development of a microscopic platelet-fibrin thrombus.

| Table 4. Comparative Evaluation of Spread Platelets after Balloon Injury in Cholesterol-fed Swine |
|------------------|------------------|------------------|
| Phenotype | Number of animals | % spread platelets |
| | | (mean ± SD) |
| Excluding coronaries with deep injury | Normal | 7 | 56.3±1.5 |
| | Bleeder | 5 | 35.8±4.3 |
| Including coronaries with deep injury | Normal | 7 | 56.3±1.5 |
| | Bleeder | 7 | 41.0±10.4 |

*p=0.003

*p=0.02
arteries of bleeder pigs. In ex vivo perfusion experiments with blood from normal and bleeder animals, we showed that platelet adhesion to the subendothelium in bleeder animals was dependent upon shear rate. The coronary system is generally considered to be a low shear area. Hence the adhesion of platelets to the subendothelium in bleeder and normal animals should not be significantly different in this system. While there is controversy regarding contact and spreading behavior of platelets from subjects with vWD, most experimental studies show that platelet adhesion is reduced in vWD. It has been suggested that the platelet adhesion defect in vWD is related to a reduced capacity of platelets to attach to the vascular surface rather than to an inability to spread over the subendothelium. Bolhuis et al., however, concluded that the primary defect was one of platelet spread. Both of these investigators used artificially created in vitro systems. Our results were obtained in an in vivo system by using balloon-injured coronary arteries of bleeder pigs. In ex vivo perfusion experiments with blood from normal and bleeder animals, we observed in an in vitro system by using balloon-injured coronary arteries and the blood flow pattern created after injury. In our study, there was no difference in the numbers of platelets present on the denuded subendothelium of normal and bleeder animals. While not conclusive, the reduced platelet spreading and platelet-platelet interaction in this model may in part explain the protection from occlusive thrombosis observed in bleeder pigs.

The results in this study may be summarized as follows. Balloon catheter injury to foam cell deposits, mixed cellular plaques, plaques containing cholesterol clefts, and fibrous cap lesions did not induce thrombogenesis. The results were the same in bleeder and normal swine. In contrast, when the injury involved the media of the vessel, nonocclusive thrombi were formed. Platelet reactivity as judged by platelet spread over the intima was less in bleeder than in normal swine.

References


Index Terms: swine • coronary arteries • atherosclerosis • balloon injury • thrombosis
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doi: 10.1161/01.ATV.10.4.541

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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