Intramural Hemorrhage and Endothelial Changes in Atherosclerotic Coronary Artery After Repetitive Episodes of Spasm in X-ray-Irradiated Hypercholesterolemic Pigs

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To assess whether coronary spasm affects the progression of atherosclerosis and results in evolution of myocardial infarction, the role of coronary spasm on the fine structure of conduit coronary arteries was studied morphologically. Göttigen miniature pigs were fed a semisynthetic diet containing 2% cholesterol and 1.1% sodium cholate. One month after being on this diet, the pigs were anesthetized and the endothelium of a branch of the left coronary artery was denuded using a balloon catheter. X-ray irradiation in a dose of 1,500 rad was given twice selectively to the area denuded, after 4 and 5 months of cholesterol feeding. Five months after endothelial denudation, transient (group A) and repetitive episodes (group B) of coronary spasm were provoked by single and periodic (five times every 5 minutes) intracoronary injections of serotonin (10 μg/kg/injection), respectively. The extent of spasm by serotonin at the previously denuded site was 84±4% (n=4) and 90±5% (n=6) narrowing in groups A and B (p=NS between groups), respectively. Forty minutes after the final administration of serotonin, the left coronary artery was relaxed by nitroglycerin, and the heart was isolated and perfuse-fixed under physiological pressure. Intramural hemorrhage was noted at the spastic site in six pigs of group B but not in group A. The average percent luminal narrowing, on cross sections at the spastic site in group B, was significantly greater than in group A (56±7% vs. 23±5%, p<0.01). Scanning electron micrographs revealed that the endothelial lining was intact at the nonspastic site in both groups. In addition to the appearance of intercellular bridges at the spastic site in both groups, squeezing of endothelial cells and adhesion of white blood cells were present at the spastic site exclusively in group B. These findings are consistent with the hypothesis that repetitive spasm may have an important role in the progression of atherosclerosis and/or myocardial infarction. (Circulation Research 1989;65:272-282)

Coronary spasm produces myocardial ischemia at rest in patients with vasospastic angina. Transient coronary obstruction and ischemic ECG-ST changes are provoked by ergonovine or histamine not only in patients with vasospastic angina but also in our animal model of coronary atherosclerosis in Göttigen miniature pigs. Occurrences of myocardial infarction in patients with variant angina have also been reported. The importance of the extent and severity of organic coronary stenosis and unstableness of angina pectoris seem to be factors influencing persistent coronary occlusion. Although myocardial infarction occurred consistently in the area supplied by vessels shown to undergo spasm, the relation between transient coronary spasm and persistent coronary occlusion has not been rigorously examined. These lines of evidence suggest the necessity of an animal model with advanced coronary stenosis, along with inducible severe coronary spasm, which can be used to elucidate the relation between vasospasm and acute myocardial infarction.
The following events have been proposed to explain the mechanisms of acute myocardial infarction: 1) intravascular plugging by blood constituents, 2) vasospasm, 3) embolization, 4) intramural bleeding, or 5) combinations of the above events. Angiographic, angiographic, and pathologic studies revealed that acute myocardial infarction, crescendo angina, unstable angina, and sudden ischemic death were accompanied by plaque fissure and thrombus formation along the respective coronary artery segments. Intramural hemorrhage, which was once considered a pathogenetic factor of coronary obstruction in acute myocardial infarction, has regained interest due to recent evidence demonstrating the presence of dense capillary networks around the atheromatous plaque in coronary lesions in patients who died after ischemic heart disease. Namely, coronary spasm may be an important trigger for initiation of intramural hemorrhage from capillary network; however, this possibility has not been examined rigorously in experimental or clinical studies.

Experimental studies seem appropriate for studying the consequences of vasospasm on the fine structure of the spastic vessel, per se, and for evaluating the relation between coronary spasm and persistent coronary occlusion. For this, the animal model should have advanced organic coronary stenosis and supersensitivity to agonists to reproduce coronary spasm. We studied the pathogenesis of coronary spasm in miniature pigs and dogs. More recently, we have developed advanced atherosclerotic coronary lesions at the denuded area in hypercholesterolemic Gottingen miniature pigs, adding selective x-ray irradiation to the previously denuded area. These are unique with regard to the level of atherosclerosis and the extent of intimal thickening, the presence of neovascularization, and the degree of induced vasoconstrictions compared with the previous one. The goal of the present study was to define whether recurrent coronary constrictions deteriorate the integrity of the endothelial lining and/or subintimal alignment at the spastic portion. We report here the first experimental observation of intramural hemorrhage after repeated provocations of coronary spasm. This evidence along with endothelial alterations may provide important insight into the pathogenesis of acute myocardial infarction and related diseases.

Materials and Methods

Animal Preparation

Eleven male Gottingen miniature pigs weighing 15–21 kg (19±2 kg) were housed individually under conditions of controlled room temperature and were fed a semisynthetic diet. Composition of the semisynthetic diet was peanut oil (2.3%), corn oil (0.7%), whole milk powder (53.7%), casein (5.7%), sucrose (21.3%), cholesterol (2%), sodium cholate (1.1%), salt mixture (1.4%), vitamin mixture (3.5%), and cellulose (8.9%). After 1 month on this diet, the pigs were lightly anesthetized with an intramuscular administration of ketamine hydrochloride (12.5 mg/kg) followed by an intravenous administration of sodium pentobarbital (20 mg/kg). They were then intubated and ventilated with room air and supplemental oxygen (Shinano, Tokyo, Japan). The carotid artery was aseptically exposed, and a green Kifa catheter (Kifa, Stockholm, Sweden) was inserted into the orifice of the left coronary artery. The endothelium of the left anterior descending or left circumflex coronary artery was denuded with a balloon catheter (2F embolectomy catheter, Fogarty, Santa Ana, California), under the guidance of fluoroscopy. X-ray was collimated to 5 cm diameter and was irradiated selectively to the denuded site twice, 4 and 5 months after initiating the cholesterol feeding. The dose given each time was 1,500 rad. The concentration of total plasma cholesterol before and 1 and 5 months after the denudation procedure was measured enzymatically.

Experimental Protocol

Five months after the denudation, 10 of 11 pigs were anesthetized with ketamine hydrochloride (12.5 mg/kg i.m.) and sodium pentobarbital (20 mg/kg i.v.) and were randomly allotted to two groups. In four pigs of group A, transient coronary artery spasm was provoked by a bolus intracoronary injection of serotonin (10 μg/kg), and in six pigs in group B, repetitive spasm was induced by five injections of serotonin (10 μg/kg each) given at 5-minute intervals. Heparin (3,000 units) was infused intravenously for anticoagulation. Left coronary catheterization was performed with a preshaped green Kifa catheter inserted from the carotid artery or with a Judkins catheter from the femoral artery. The diameter of the coronary artery was measured by selective coronary arteriography, as previously described. Electrocardiograms were continuously recorded in leads I, II, III, V1, and V4. Arterial pressure was monitored with a strain-gauge manometer. Coronary arteriography was performed 3 minutes after the intracoronary injection of serotonin in group A and after 3 minutes, 13 minutes, and 25 minutes in group B. One of 11 pigs was used for histological study without any provocations of coronary spasm.

Histological Study

Forty minutes after the final provocation of spasm and a bolus administration of nitroglycerin (20 μg/kg i.c.), thoracotomy was performed under positive-pressure respiration, and a polyethylene cannula was inserted into the ascending aorta from the subclavian artery. After exsanguination and ligation of the descending aorta, the coronary artery was perfused via a constant-pressure perfusion system with oxygenated 0.1 M phosphate buffer containing nitroglycerin for 3–5 minutes followed by perfusion of half-strength Karnovsky fixative under physiological perfusion pressure.
The spastic site (shaded area) is further divided into two pieces (B and C). The nonspastic site was taken from the main trunk of the left anterior descending or left circumflex coronary artery, and the other for semithin and ultrathin sections, as shown in Figure 1C. The samples (Figure 1C) for scanning electron microscopy were dehydrated through paraffin, sectioned at 3–5 μm, and stained with hematoxylin and eosin or by the Weigert-van Gieson procedure. In four of six pigs with intramural hemorrhage, serial sections were made and stained with hematoxylin and eosin (B).

Scanning Electron Microscopy and Examination of Semithin Sections

After perfusion fixation, the arteries were opened longitudinally, and one of the parts in the spastic segment was divided for scanning electron microscopy and the other for semithin and ultrathin sections, as shown in Figure 1C. The samples (Figure 1C) for scanning electron microscopy were dehydrated in a graded series of ethanol, immersed in isoamyl acetate, and critical-point dried. The samples were mounted on aluminum studs, coated with gold, and examined with a Hitachi H-430 scanning electron microscope at 20 kV.

To observe capillaries microscopically, the other segments (Figure 1C; LM) were cut into small blocks after fixation and treated with half-strength Karnovsky fixative for 4 hours at 4°C. After postosmication, the specimens were dehydrated and embedded in epoxy resin. Semithin sections were made on an ultramicrotome (Porter-Blum MT-2), and these were stained with toluidine blue for light microscopy.

Transmission Electron Microscopy

For transmission electron microscopy, segments of C in Figure 1 were cut into small pieces after perfusion fixation and treated with half-strength Karnovsky fixative for 4 hours at 4°C. After postosmication and en bloc staining with 2% uranyl acetate, the specimens were dehydrated and embedded in epoxy resin. Ultrathin sections were made on an ultramicrotome (Porter-Blum, MT-2, Ivan Sorvall, Norwalk, Connecticut), stained with uranyl acetate and lead citrate, and examined in a JEOL 100-CX transmission electron microscope at 80 kV.

Data Analysis

Areas of the thickened intima and of the lumen circumscribed by the internal elastic lamina were measured by a computer-aided image analyzer (Cosmozone, Nikon, Tokyo, Japan). Percent area stenosis was calculated as follows: percent area stenosis equals area of spastic segment divided by area of spastic segment circumscribed by internal elastic lamina × 100%. The number of leukocytes adhering to the luminal lining was determined on scanning electron micrographs. All results are expressed as mean±SEM. The statistical significance of the difference between groups was evaluated by Student’s t test. A probability of less than 5% was considered indicative of statistical significance.

Results

Baseline Condition

After 1 month of feeding the semisynthetic diet, the plasma cholesterol level increased from 48 ± 4 mg/dl to 484 ± 41 mg/dl. A branch of the left coronary artery was then denuded by a balloon catheter. At the end of 5 months of feeding the semisynthetic diet, body weights were increased from 19 ± 2 kg to 33 ± 1 kg and to 31 ± 2 kg in groups A and B, respectively, and levels of plasma cholesterol were 275 ± 20 mg/dl in group A and 284 ± 26 mg/dl in group B. These chronological changes in body weight and cholesterol level were similar between groups A and B.

Hemodynamic and Angiographic Findings

Heart rate and arterial pressure at the baseline state in the anesthetized pigs were 116 ± 7 beats/min and 98 ± 4/70 ± 3 mm Hg (systolic/diastolic) in group A, and 105 ± 8 beats/min and 98 ± 3/71 ± 2 mm Hg in group B, respectively (not statistically significant between groups). Absolute coronary diameters of the left anterior descending and left circumflex arteries were similar in groups A and B. Representative angiograms at control and after serotonin (10 μg/kg
i.e.) are shown in Figure 2. Wall irregularities were noted angiographically along the denuded portion at the control state, but the percent diameter narrowing in vivo was less than 20%. Luminal diameter reduction at the spastic site after intracoronary injection of serotonin, as assessed by in vivo angiography, was not different between the groups (84±4% in group A and 90±5% in group B). Serotonin-induced percent narrowings in coronary arterial diameter at the non-spastic site were 30±6% and 35±4% in groups A and B, respectively. Significant ischemic ECG-ST elevation was noted in four of four pigs in group A and in six of six pigs in group B.

**Light Microscopy**

Intramural hemorrhage was noted at the spastic sites of all pigs in group B but not in group A. Typical location of the intramural hemorrhage was selected from serial sections for light microscopy and is depicted in Figure 3. Intramural hemorrhage was present inside the thickened intima in all six pigs and also extended into the media in one of six pigs, as shown in Figure 3D. Rupture of the fibrous cap, a fresh thrombus, or histological communication between the lumen and extravasation was never evident.

Luminal stenosis, as assessed histologically on cross sections of three locations, such as at the site most severely spastic and the sites 5–10 mm proximal and distal to the spasm is summarized in Table 1. The percent area of the intimal thickening to the

**FIGURE 2.** Left coronary angiograms (left anterior oblique projection) or control (A) and repetitive provocations (B) of serotonin-induced coronary spasm. Subtotal stenosis was noted along the left circumflex coronary artery. Arrow indicates the severe spastic site. During coronary spasm, ECG-ST elevation was recorded.

**FIGURE 3.** Diagrams of cross sections of the spastic coronary artery accompanied with intramural hemorrhage in six pigs (A–F) of group B. Internal elastic laminae are shown by a thin circle. The intima is the portion between the luminal lining and the internal elastic lamina. Hemorrhagic mass is depicted as a dotted area.
Electron Microscopy

Scanning electron micrographs of the luminal surface of nonspastic and spastic sites fixed after nitroglycerin infusion are represented in Figure 6. Endothelial cells of both groups were spindle shaped and arrayed longitudinally at the nonspastic site and at the spastic site induced by a single provocation of spasm (Figures 6A and 6B). Intercellular bridges were noted at the spastic but not the nonspastic site in both groups (Figures 6B, 6C, 6D, and 6E). Longitudinal folds produced hills and valleys at the spastic site in all six pigs in group B and were mostly located around the plaque (Figure 6C). Platelet adhesion was not observed. There were occasionally a few gaps between endothelial cells, where leukocytes adhered to the endothelium, as shown in Figures 6D and 6E. The number of leukocytes adhering to the luminal lining was significantly larger at the spastic site in group B than that at the nonspastic site in group B or the spastic site in group A (23±5%; 10-32%) (p<0.01).

Alterations noted around the valley seen on the scanning electron micrograph of the spastic site were reexamined on semithin sections by conventional light microscopy and on ultrathin sections by transmission electron microscopy (Figure 7). Although the spastic site in group B was fully dilated by nitroglycerin, as confirmed by the distended shape of medial smooth muscle cells and stretch of the internal elastic laminae, the endothelial cells over the valley remained squeezed (Figures 7A–7C), and the nuclei were moved toward the protruded site of cytoplasm (Figures 7B and 7C) as compared with that of the site denuded and x-ray irradiated but not spasm provoked (Figure 7D). Focal detachments of the endothelial cells from the underlying tissue were not observed, and junctions between endothelial cells remained intact (Figure 7C).
area was augmented, and neovascularization was invariably present in the thickened intima and the media of the spastic segment in both groups (Figure 5). Nevertheless, in pigs given a single injection of serotonin, a transient severe coronary spasm did not produce intramural hemorrhage or bizarre alterations of endothelial cells. Thus, recurrent torsional burdens on fragile capillaries inside the atheromatous plaque were conditions necessary for the evolution of intramural hemorrhage.

Pathophysiology of Intramural Hemorrhage

Important events after x-ray irradiation are vasculitis and interstitial inflammation involving polymorphonuclear leukocytes. Increases of neutrophil chemotactic activity were also demonstrated after x-ray irradiation. These neutrophil chemotactic agents may accelerate atherosclerotic processes at the previously denuded area. Atherogenic effects of x-ray irradiation may stimulate angiogenesis because capillaries were observed at the site in denuded and x-ray-irradiated segments.

Lee et al demonstrated that among 28 Yorkshire pigs fed an atherogenic diet and x-ray irradiated twice, 24 developed myocardial infarcts 4.5–28 weeks (14.2 ± 0.8 weeks) after the first x-ray. All had advanced coronary atherosclerosis along the small vessels, and...
Despite the frequent involvement of experimentally, coronary spasm of 75% stenosis caused occlusive thrombus formation between recurrence of spasm and structural disarrangement such as hemorrhage and the resultant progression of coronary stenosis. Increment of luminal narrowing at the hemorrhagic site may be potentially hazardous and result in either the persistence of myocardial ischemia or a more disastrous chain of events such as plaque rupture, luminal thrombosis, and acute myocardial infarction.

A recent pathological study of 103 ruptured plaques revealed that crack of the plaque surface always was accompanied by the formation of thrombus around the plaque. Rupture of atherosclerotic lesions in excess of 75% stenosis caused occlusive thrombus formation, with increasing frequency. Coronary spasm has often been considered an initiating event for sudden rupture of the plaque surface and/or capillaries. However, events in a dynamic process relating to rupture of the fibrous cap, dissection of the intima by bleeding per se, and/or thrombus formation have not been confirmed either clinically or experimentally. Despite the frequent involvement of intimal hemorrhage at the site of advanced thrombosis, the clinical implication of pure intimal hemorrhage for the initiation of thrombi has been debated. Constantinides examined 17 thrombi causing acute myocardial infarction in serial sections and noted that hemorrhage in the plaque could be traced to an entry into the plaque from the lumen. On the contrary, applying careful serial sections, Wartman described seven patients who died as a result of intrinsic intramural hemorrhage but not of thrombosis. Accordingly, a long history of debate concerning whether the luminal thrombosis is a primary or a secondary phenomena has not been settled.

**Integrity of Endothelial Cell Lining**

Factors related to arterial occlusion are 1) protrusion of the thickened intima toward the lumen due to increases in intraplaque pressure resulting from an accumulation of foam cells, cholesterol clefts, and blood infiltration across the injured endothelial barrier; 2) rupture of the very thin cap of fibrous tissue due to drag generated through hemodynamics or vasmotion; 3) endothelial ulceration related to free radicals and/or inflammation; 4) hemorrhage in an atheromatous lesion, with disruption of tissue and release of thromboplastic substances; 5) roughening of the endothelium over the degenerated area, providing a suitable site for platelet deposition; and 6) increased coagulability of the blood associated with lipids. These factors are intimately related to alterations of endothelial function; however, none of the above hypotheses has been rigorously tested, either experimentally or clinically.

Bridges between endothelial cells have been speculated as physiological structures with the function of preserving the regular alignment of endothelial folds and also of preventing the excessive opening of intercellular junctions. We observed bridges at the spastic but not at the nonspastic site in both groups. Our findings suggest that the bridges are a remnant of acute structural changes related to abnormal cell-to-cell contact due to spasm, which accords well with observations by Joris and Majno.

In the present study, morphological changes of endothelial cells after repetitive episodes of spasm...
**FIGURE 6.** Scanning electron micrographs of the luminal surfaces of coronary arteries. A: Non spastic site. B: Spastic site after a single provocation of spasm. C: Spastic site after five episodes of coronary spasm. D: Leukocyte adhesions at the spastic site after five repetitive provocations. E: A higher magnification of D. Numerous fine bridges are seen between endothelial cells in B, C, and D. In E, white arrows indicate gaps between endothelial cells, and black and white arrows are endothelial bridges. Bar represents 50 μm in A–D and 25 μm in E.
for 30 minutes were found even after nitroglycerin, as shown in Figures 6C and 6D. The presence of hills and valleys and the intercellular bridges between endothelial cells coincide well with those produced after severe vasoconstriction by superfusion of L-norepinephrine to muscular arteries of rats. Because the squeezing of endothelial cells evolved at the spastic site in Group B, repetitive constrictions by serotonin may reduce dynamic stability of the endothelial cells. Multiple reperfusions after repetitive coronary obstruction resulted in the partial detachment of endothelial cells from the underlying tissue. However, such phenomena were not observed in the present study. Leukocyte adherence around the disrupted portions of endothelial junctions was demonstrated after 1–5 minutes of hypertension in a canine model. We also documented leukocyte adherence preferential to the endothelial gap along the spastic site in group B. Thus, leukocyte adherence seems to be a sign of endothelial injury caused by mechanical burdens such as spasm and hypertension.

The present observations concerning the structural alterations of endothelial lining as a result of severe vasomotion suggest the presence of a hierarchy of endothelial cell damage. The bridges between endothelial cells may be changes encountered immediately after spasm because these alterations were noted in both groups. Signs of advanced lesions are hills and valleys, gap formation, and
adhesions of white blood cells because these findings were observed at the spastic portion in group B. To elucidate the effects of intramural hemorrhage on structural alterations of endothelial cells, chronological observations of the endothelial lining after intramural hemorrhage have to be made.

Clinical Implications

The present results suggest that severe and repetitive spasm alters vascular integrity, especially along the endothelial lining, and that intramural hemorrhage evolves exclusively at the spastic site along the atherosclerotic coronary arteries. Persistent arterial constriction induced mechanically on surgically exposed canine coronary arteries by a suture string resulted in severe longitudinal folding and endothelial desquamation with extensive platelet deposition on the exposed subendothelium. In these studies, the vessels were surgically exposed, and endothelial cells were fixed after arterial constriction that was maintained for 1 hour. However, there were no platelets on the altered endothelium in our experiments, in which the coronary spasm was induced in the closed-chest condition and was repeated every 5 minutes for 30 minutes. Therefore, the shorter duration of vasoconstriction and the absence of adventitial dissection may explain why platelets did not adhere to the surface of the spastic vessel in the present study.

Intramural hemorrhage will increase intraplaque pressure and volume and enhance luminal narrowing. Thus, spasm and related immediate changes of vascular structure not only function as a trigger for the vicious cycle of myocardial ischemia and infarction but also accelerate the progression of atherosclerosis. Some relation of coronary spasm to atherosclerosis was also speculated from observations made on patients with variant angina. These results suggest the importance of immediate and thorough antispastic treatment to prevent the progression of organic changes of the coronary arterial lesion. The present animal model may pave a way to elucidate the cause-and-effect relation among coronary spasm, intramural hemorrhage, endothelial damage, adhesions of blood cells, coronary thrombosis, and progression of atherosclerosis.

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