Alterations in Endothelial F-Actin Microfilaments in Rabbit Aorta in Hypercholesterolemia

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Abstract—The current study tests whether hypercholesterolemia influences the distribution of endothelial cell microfilaments during the initiation and growth of fatty streak–type lesions. We classified the lesions occurring over a 20-week period into four types based on the location and extent of macrophage infiltration observed microscopically. The earliest lesion was characterized by leukocytes adherent to the endothelial surface. Minimal lesions were characterized by a few cells in the subendothelium. Intermediate lesions consisted of numerous subendothelial leukocytes in a minimally raised lesion. Advanced fatty streak lesions were elevated, with several layers of leukocytes. The organization of peripheral junctional actin (the dense peripheral band) and of central endothelial cell actin microfilament bundles was studied in each of these lesions by using fluorescent microscopy. We found that in the aorta away from branch sites and in areas away from lesions, the central microfilament distribution was unaffected by hypercholesterolemia. The macrophages entered the wall without any identifiable reorganization in the microfilaments. During the accumulation of subendothelial macrophages in minimal and intermediate lesions, stress fibers were initially increased in comparison to lesion-free areas. In advanced lesions, the central microfilaments became thinner and disappeared. However, at flow dividers, where central stress fibers were normally prominent, endothelial cells on the surface of intermediate lesions showed a reduction in central fibers, and peripheral bands became prominent. This finding was associated with changes in cell shape from elongated to cobblestone type. Thus, actin microfilament bundles in endothelial cells underwent substantial changes in distribution during the accumulation of subendothelial macrophages, forming hypercholesterolemia-induced fatty streak–type lesions. These changes may influence endothelial substrate adhesion, permeability, or repair after injury. (Arterioscler Thromb Vasc Biol. 1998;18:52-56.)

Key Words: hypercholesterolemia ▪ actin ▪ endothelium ▪ atherosclerosis ▪ aorta ▪ cytoskeleton

The actin cytoskeleton of endothelial cells is very important in maintaining the structural integrity of the endothelium.1,2 At most sites, endothelial cells in vivo show features of F-actin distribution similar to those seen in endothelial cells forming a confluent, cobblestone, contact-inhibited monolayer in vitro.3-5 The peripheral actin forms a dense peripheral band (DPB) of microfilaments, and the central filaments (stress fibers) are short and randomly distributed but toward the base of the cell. F-actin colocalizes with myosin, tropomyosin, α-actinin, and vinculin,6 suggesting that the microfilaments have contractile properties. The DPB is thought to be important in cell-cell adhesion, thus maintaining the structural integrity of the confluent monolayer and regulating permeability,7,8 while the central microfilament bundles are thought to be important in cell-substratum adhesion, preventing loss of endothelial cells from the wall.9,10

Hemodynamic shear stress is important in regulating endothelial structure and function11-13 and regulates the F-actin microfilament bundle distribution. Elevations in in vivo shear stress result in altered endothelial cell shape, a predominance of prominent long central microfilament bundles, and reduced peripheral actin.3,4 Reduction in in vivo shear stress results in polygonal (cobblestone) cells with reduced stress fibers and enhanced peripheral actin bundles.10 Differences in the distribution of microfilaments in endothelial cells at flow dividers occur over very short distances.14

Experimental atherosclerosis in hypercholesterolemia models is associated with changes in endothelial integrity.15-22 Injured or activated endothelial cells express adhesion molecules on their surface, allowing monocytes and lymphocytes to attach.23 Attached cells may then migrate between endothelial cells into the intima. Monocytes are converted to macrophages in the subendothelium, and some become foam cells with the ingestion of lipids. The advancement of lesions occurs with the accumulation of lipid-laden macrophages and proliferation of smooth muscle cells. Gaps in the endothelium allow platelet attachment and become sites prone to mural thrombi.1 Thus, hypercholesterolemia seems to predispose to altered endothelial-cell–cell and cell-substratum adhesion.

Since actin microfilaments are involved in the regulation of endothelial cell adhesion7,8 to neighboring cells and to the substratum,9,10 we tested the hypothesis that hypercholester-
emia and fatty streak lesion formation influence the distribution of endothelial cell microfilaments at sites of atherosclerosis formation. We classified the lesions occurring over a 20-week period into four types based on the location and extent of macrophage infiltration. The earliest lesion was characterized by leukocytes adherent to the endothelial surface, and minimal lesions were characterized by a few cells in the subendothelium. Intermediate lesions consisted of numerous subendothelial leukocytes in a minimally raised lesion. Advanced fatty streak lesions were raised, with several layers of subendothelial leukocytes. The organization of endothelial cell actin microfilaments in each of these lesion types was studied by using fluorescent microscopy.

Methods

Thirty-two male New Zealand White adult rabbits, 3 kg body weight, were used. Animals were randomly divided into the control (C, n=12) or experimental (E, n=20) group and fed semisynthetic test diets (Teklad Inc) modified from Rosenfeld et al (1987). 17,18 The control diet (No. TD88070) contained 16% corn oil (unsaturated fat), and the experimental atherogenic diet contained 15% beef tallow (saturated fat), 1% corn oil, and 0.4% cholesterol by weight. Both diets contained 20% protein in the form of casein. Total plasma cholesterol was measured enzymatically (CHOD-PAP method, kit 236691, Boehringer-Mannheim). Initial mean serum values (±SE) were 121 mg/dL at 12 to 14 weeks (6E/5C), or 15 to 20 weeks (5E/2C), 130 mg/dL at 5 to 6 weeks (4E/2C), 8 to 10 weeks (5E/3C), 39 mg/dL at 15 to 20 weeks. Control values did not change significantly over time.

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Rabbits were fed standard Purina rabbit chow for 2 days and then received increasing amounts of the diets during a “pump” period lasting 5 to 7 days. The rabbits were then started on the full test diet of 100 g/d. Daily food intake was recorded and body weight measured, and these values were similar in control and diet-fed rabbits. Groups of rabbits were maintained on test diets for periods of 5 to 20 weeks before killing at 5 to 6 weeks (4E/2C), 8 to 10 weeks (5E/3C), 12 to 14 weeks (6E/5C), or 15 to 20 weeks (5E/2C).

All animals were treated in accordance with CCAC (Canadian Council on Animal Care) guidelines.

Preparation of Tissue for Fluorescence Microscopy

Animals were killed and perfusion fixed as previously described. After blood samples were drawn, 1000 U of heparin sodium (anticoagulant) was injected via an ear vein catheter and allowed to circulate for 1 minute, followed by 1 mL of T-61, a euthanasia solution, consisting of 200 mg N-[2-(m-methoxymethyl)-2 ethylbutyl-(1)-]-γ-hydroxybutyramide, 50 mg 4.4’-methylene-bis(cyclohexyl) trimethylammonium iodide, and 5 mg tetracaine hydrochloride (Hoechst). An aortic catheter was then introduced into the descending thoracic aorta and advanced about 2 cm. The superior mesenteric and celiac arteries were tied off. A small catheter connected to a water manometer was placed in the left femoral artery to monitor perfusion pressure. After a flush of 60 mL PBS, the aorta was pressure fixed at 100 mm Hg with 3% paraformaldehyde in 0.1 mol/L phosphate buffer with Ca2+ and Mg2+ for 20 minutes. Perfusate was vented, using a catheter placed in the right ventricle. After a pressurized PBS wash of 15 minutes, the aorta was permeabilized with a 0.2% solution of Triton X-100 (30 mL in 15 seconds). After another PBS rinse under pressure for 10 minutes, the endothelium was stained for F-actin, using the fluorescent dye rhodamine-phalloidin (750 mL), and nuclei were stained using bisbenzimide Hoescht 33258 (200 mL). The stains were diluted together in a flask containing 100 mL of distilled water and infused into the aorta via a peristaltic pump (flow rate 4.3 mL/min) followed by a 10-minute pressurized PBS rinse. The aorta was then gently excised from the midthoracic region to the iliac arteries, including major arterial branches, and placed in a covered dish containing 3% paraformaldehyde.

After the aorta was gently cleaned of adventitia, it was cut into the following segments: thoracic aorta, including 4 to 6 intercostal orifice pairs; celiac, superior mesenteric, and right and left renal arteries and their aortic ostia; abdominal aorta; aortic bifurcation; and common iliac arteries. Thoracic and abdominal aortic segments were opened lengthwise along the ventral side, and other tissue segments were opened in different ways to allow for optimal viewing of branch points and flow dividers. Usually, branches were cut along lateral margins into ventral and dorsal sections. The opened aortic segments were then examined visually under a 2X objective of a dissecting microscope, and the lesions were assessed. Segments were then mounted in 50% glycerol in PBS, orientation of blood flow was noted, and samples were examined and photographed using a Zeiss Photomicroscope III with epifluorescence optics. Zeiss filter sets 47714 and 487702 were used for rhodamine-phalloidin and Hoescht 33258, respectively. Thus, patterns of F-actin microfilament distribution were colorized with nuclear staining patterns in areas of distinct aortic blood flows for each animal. Two independent observers viewed the tissue and assessed microfilament distribution in the endothelial cells. Photographs were taken of representative lesions at all sites to show colorization of actin staining with nuclear staining of the same cells. For microfilaments, five representative photographs each, at 40X magnification, from normal and advanced lesions in nonbranching areas and at bifurcations were counted to quantify the percentage of endothelial cells with and without central microfilaments. After examination by fluorescence microscopy, aortic segments were dismounted, trimmed, and placed in 10% formalin to be processed for paraffin embedding. Sections at 4 μm were examined histologically after staining with hematoxylin-eosin and Movat pentachrome stains.

Results

Lesion Localization

Lesions, identified grossly and histologically, were not observed in control animals over the 20-week period. All but 2 of the 20 experimental rabbits fed an atherogenic diet showed some degree of raised white patches on the intimal surface that were confirmed to be fatty streak lesions by histopathology. As previously reported in several studies,17,18 small focal lesions first appeared in the thoracic and abdominal segments either as patches just downstream from intercostal flow dividers or as small random lesions. Lesions then appeared directly on flow dividers oriented as thin streaks. Later, the majority of intercostal and small branches in the abdominal aorta had distinct U-shaped lesions located downstream of the flow divider. As disease progressed, these divider lesions extended over and upstream of the anatomical flow divider. As disease became extensive, thoracic aortic lesions were frequently continuous and almost encompassed the entire central dorsal intercostal area, while abdominal lesions became large and diffuse and encompassed all branch orifice areas, often covering one third of the abdominal aorta. All major branch dividers were affected.

We classified the lesions occurring over a 20-week period into four types based on the location and extent of macrophage infiltration. The first step in the formation of hypercholesterolemic fatty streak lesions was characterized by leukocytes adherent to the endothelial surface. Minimal lesions were characterized by a few cells in the subendothelium. Intermediate lesions consisted of numerous subendothelial leukocytes in a minimally raised lesion. Advanced fatty streak lesions were raised, with several layers of leukocytes in the subendothelial space.
Microfilament Distribution in Control Animals
The pattern of microfilament distribution in endothelial cells of the thoracic and abdominal aorta and at flow dividers in normal rabbits has been previously described. Briefly, cells away from flow dividers have a band of microfilaments at the cell periphery and a few short central stress fibers, while cells at flow dividers have prominent central stress fibers and fewer peripheral bands. We also described transitional zones, which are present adjacent to flow dividers and are characterized by cells with both peripheral bands of microfilaments and prominent short central microfilament bundles.

Microfilament Distribution Away From Flow Dividers
Areas of endothelium without lesions (Figs 1, 2A, and 2B) or with only adherent macrophages in the thoracic and abdominal aorta showed normal microfilament distribution (Fig 1C and 1D).

In localized minimal lesions with macrophages beneath the endothelium, endothelial cells showed a few thin long central stress fibers. Raised intermediate lesions, occurring in thoracic and abdominal aorta away from branches, showed a decrease in peripheral staining and prominent long central microfilament bundles that were thin and wavy and had more random orientations (Fig 2C and 2D) than were found in normal cells (Fig 2A and 2B). Sometimes isolated thick bundles of microfilaments were also present. In advanced raised lesions with several layers of macrophages, endothelial cells were cobblestone in shape, with thin peripheral bands and no or very few central actin microfilaments (Fig 2E and 2F). In these advanced lesions, 34 ± 4.2% (mean ± SD) of the endothelial cells had no visible central microfilaments, compared with 2 ± 1% (mean ± SD) in normal animals.

Microfilament Distribution at Flow Dividers
At major branch flow dividers, early lesion formation occurred in areas where the organization of microfilaments showed prominent central microfilament patterns, as in the lip areas at the upstream tips, at the upper half of the divider limbs, and at the sides close to the apex (Fig 3A and 3B; see reference 14 for flow divider terminology). At later time points, occasional macrophages were also located beneath elongated transitional...
type endothelial cells with prominent central stress fibers (Fig 3C and 3D).

In comparison to normal endothelial cells (Fig 4A and 4B), the endothelial cells covering minimal lesions were also elongated and had prominent long central microfilament bundles. However, endothelial cells at intermediate lesions on the lips of flow dividers had changed shape from elongated to cobblestone. The shape change was accompanied by a redistribution of microfilaments. Regions normally characterized by prominent central fibers and no peripheral actin now displayed less of the prominent long central stress fibers and more peripheral bundles (Fig 4E and 4F). The central microfilaments were very thin and were not all distributed in the direction of flow. In advanced lesions, the overall staining for actin microfilaments was reduced. Central microfilaments were absent in 23±5.5% (mean±SD) of the cells compared with no cells in normal animals or markedly reduced and characterized by wavy microfilaments similar to those found in endothelial cells in intermediate and advanced lesions in lesions away from flow dividers (Fig 4G and 4H). There were no discernible differences in microfilament distribution in endothelial cells of lesions among the several different bifurcations (flow dividers) examined.

Discussion

In this study, we have observed alterations in the pattern of microfilament distribution and organization in endothelial cells during the development of atherosclerotic fatty streak–type lesions in hypercholesterolemic rabbits. Away from flow dividers, where central stress fibers are not prominent in endothelial cells, central microfilaments became more prominent in the early stages of lesion development and then disappeared once the lesion was raised and contained several layers of macrophages. Thus, central microfilaments undergo dynamic changes during lesion formation. At flow divider sites, where prominent central microfilaments are normally present, the microfilaments persisted after the attachment of a few monocytes, but they became thinner and eventually disappeared in many cells as the lesion progressed. Thus, the actin microfilament bundles undergo changes that initially may promote cell-substratum adhesions, but eventually further changes occur that are likely to result in dysfunction of cell-substratum adhesion due to a reduction in central actin microfilaments.

The response of endothelial cells under different types of stressful conditions indicates that microfilament organization is dynamic in nature. Endothelial cells exposed to low shear conditions have fewer central stress fibers than cells under normal conditions. In cells exposed to high shear conditions, the central microfilaments become both thicker and longer, while the peripheral band of microfilaments becomes disrupted. The latter is reversible if normal shear is reinstated.

The mechanisms underlying the reorganization of actin microfilaments in response to hypercholesterolemia are not known and were not examined in our study. It is possible that these changes are a result of the expansion of the lesion, resulting in stretching of the endothelial cells. Similarly, acute hypertension results in an increase in stress fiber formation thought to be due to stretching of cells. Subendothelial macrophages could have an effect on endothelial responses through the release of cytokines or through disruption of endothelial-substratum adhesion, especially at cell-matrix adhesion complexes. In addition, changes in the substratum and/or associated endothelial cell adhesion molecules may alter microfilament distribution.

It is interesting, however, that in the initial stages of lesion development, there are prominent stress fibers present, and it is only once the lesion is elevated that central microfilaments are reduced. These findings favor the hypothesis that mechanical factors impinging on the endothelial cells as the lesion expands may be more important than hypercholesterolemia per se. However, it has been shown that oxidized LDL delayed endothelial cell migration. Since migration is dependent on actin microfilament organization, it is possible that oxidative stress may play a role in actin microfilament organization, possibly through an interaction at endothelial focal adhesion sites.

Mediators present at sites of injury, such as thrombin, which may be present at the site of elevated lesions, could have an effect as well, as shown by the presence of microthrombi on
the surface of prominent fatty streak lesions in nonhuman primates. Thrombin treatment of confluent monolayers of endothelial cells in culture causes a reduction of the DPB and an increase in central microfilament bundles. In single endothelial cells, thrombin promotes cell spreading and an increase of central microfilaments. Thrombin could be involved in F-actin microfilament reorganization in vivo. Platelet-derived growth factor, released at sites of microthrombi, induces cytoskeletal reorganization in skeletal muscle and mesenchymal cells and could therefore be involved as well.

The effects on the actin cytoskeleton could be mediated through effects on actin-binding proteins, which regulate the equilibrium of the G-actin (globular) form and F-actin (filamentous) form. The endothelial cytoskeleton is subject to rapid polymerization and depolymerization, allowing it to respond to a given stimulus, and it thus plays a critical role in maintaining endothelial integrity and repair mechanisms. The changes we have observed in the cytoskeleton in the development of hypercholesterolemic fatty streak–type lesions are part of the cellular response to injury or stress. The loss of the DPBs may induce an increase in endothelial permeability and thus promote further leakage of lipids and transmigration of monocyte/macrophages into the vascular wall. The eventual loss of the central microfilament bundles is associated with the growth of atherosclerotic plaque and may be associated with endothelial loss, microthrombus formation, and further increases in permeability.

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References
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