Effects of Moderate Hypercholesterolemia on Rabbit Endothelium

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We investigated the effects of moderate hypercholesterolemia and endothelial regrowth on the function of vascular endothelium. Groups of rabbits were fed either a high or low cholesterol diet and subjected to removal of the aortic endothelium or sham-operated. Endothelial removal caused diffuse thickening of the myointima. The high cholesterol diet did not affect the extent of endothelial regeneration. To test endothelial function, 1 minute before sacrifice each animal was infused with horseradish peroxidase, which will penetrate the intima in areas of increased permeability. After sacrifice, the aorta was removed en bloc and exposed to diaminobenzidine and H₂O₂, which produce a brown stain on reaction with horseradish peroxidase. The lumenal surface of the aorta was examined, and brown-stained areas, indicating increased permeability, were quantitated by stereology. Rabbits fed the cholesterol-rich diet had a greater percentage of brown-stained areas than did rabbits fed the low cholesterol diet; this was true for both intact and regenerated endothelium. The differences were significant at the p < 0.01 level. Scanning electron microscopy of brown areas showed that, although the surface characteristics of the endothelium were altered, endothelial sloughing did not occur. In addition, in brown areas, no platelet attachment to the vessel wall was seen. Therefore, it appears that hypercholesterolemia can be associated with a functional endothelial defect in the absence of endothelial loss.

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Methods
Experimental Protocol

New Zealand white male rabbits weighing 2.5 to 3.5 kg were housed singly in wire bottomed cages and initially fed water and Charles River Rabbit Formula (less than 3.6% cholesterol) ad libitum. During the experiment, the rabbits were divided into two different diet groups and were then subdivided into two subgroups. From Day 0 to sacrifice at Day 42, rabbits in Group 1 (nine animals) were fed about 110 g per day of a mixture of 5.8 g rabbit chow to 1 g powdered whole egg (Dried Whole Egg Solid, Oskaloosa Food Products). These rabbits were later balloon-deendothelialized. Rabbits in Group 2 (nine animals) were fed normal rabbit chow and later balloon-deendothelialized. As controls, rabbits in Group 3 (seven animals) were fed the egg supplemented diet and sham-operated. Rabbits in Group 4 (six animals) were fed normal rabbit chow and sham-operated.

Blood samples were collected from the marginal ear vein after overnight fasting on Day 0 and biweekly thereafter. Sera were promptly harvested and stored at -20°C. Concentrations of total serum cholesterol were determined by the method of Allain et al.9

For removal of the aortic endothelium, the rabbits were anesthetized intravenously with diazepam (5 mg/kg) supplemented with inhaled ether. A No. 4 French thin-walled embolectomy catheter (Edwards Laboratory, Santa Ana, California) with balloon inflated to about 400 mm/Hg was drawn through the aorta as previously described.10

At sacrifice, all rabbits were intravenously anesthetized with 5 mg/kg of diazepam supplemented with inhaled ether. Thirty minutes before sacrifice, rabbits were injected with 4 ml of 0.45% solution of Evans blue (Harvey Laboratories, Philadelphia, Pennsylvania) to enable us to grossly distinguish between that part of the luminal surface covered by smooth muscle cells (indicating nonregeneration of endothelium) and that part covered by endothelium. Nonregenerated areas stained blue, whereas regenerated areas remained white.11 One minute before sacrifice, rabbits were injected with 80 mg/kg of horseradish peroxidase (HRP) (Type II, Sigma) suspended in 10 ml of 0.15 M NaCl. Blood pressure was monitored using a GRASS Model 7B polygraph. Rabbit aortas were then perfused—fixed with fixative for 40 minutes, then rinsed four times with 0.1 M Tris, pH 7.6, and sliced open through the anterior wall to expose the luminal surface. Each aorta was pinned out and photographed grossly using Ektachrome EPT 160. The area between each intercostal artery, from the third to the tenth, was photographed at X16 magnification through a dissecting microscope. Tissue samples were also collected from the kidney and liver.

To investigate endothelial permeability, each aorta was incubated en bloc with diaminobenzidine (DAB) and H2O2. In areas of increased permeability, HRP collected within the arterial wall, which caused these areas to stain brown on reaction with DAB and H2O2. To prevent spontaneous darkening of the aortic surface, the procedure was performed in the dark. Each aorta was gently agitated for 15 minutes at 20°C with freshly prepared 0.048% 3,3’ dianinobenzidine without H2O2 and then incubated for 15 minutes with 0.048% 3,3’ diaminobenzidine and 0.0095% H2O2 in 0.1 M Tris, pH 7.6. The reaction was stopped by washing the aorta with three 3-minute rinses in 0.1 M Tris, pH 7.6. Once again, the aorta was photographed grossly, and the area between each intercostal artery, from the third to the tenth, was photographed at X16 magnification through a dissecting microscope.

Morphology

After the aortas were exposed to DAB and H2O2, multiple sections from each aorta were obtained by razor blade or punch biopsy and examined by light microscopy, transmission electron microscopy, and scanning electron microscopy. Punch biopsies of brown-stained areas and adjacent white areas were taken using a 1 mm corneal punch biopsy needle. Adjacent white areas were identified for later comparison; no samples were taken from sites near branches. Biopsy samples were dehydrated and embedded in Epon 812, as previously described.10 We cut 1 μ thick sections of these samples and measured the depth of brown stain penetration in Groups 1 and 2 using an eyepiece micrometer. These Epon blocks from all groups were trimmed, and ultrathin sections were cut on a Sorvall MT-2 microtome using a DuPont diamond knife. These thin sections were either stained with uranyl acetate and lead nitrate or left unstained. We viewed all sections with a Phillips 300 electron microscope. At least 10 sections were examined for each animal. Tissues from the kidney and liver were also examined by light microscopy.

For scanning electron microscopy, we cut out 10 × 10 mm areas in the white regions. Brown-stained areas were then outlined by lightly pressing a corneal punch onto the luminal surface,
thereby circumscribing the center of the spot for viewing. Tissues were dehydrated with graded alcohol, critical-point dried, coated with gold, and examined with an AMR-900 scanning electron microscope. We examined at least seven sections per animal.

**Stereology**

To quantitate endothelial regrowth in all groups, we used gross photographic slides of the entire aorta before exposure to DAB and H₂O₂. These slides were projected (×13 magnification) with a Dokumeter No. 1 (Jena Optics, Don Santo, Wellesley Hills, Massachusetts) on a custom-made 4453 bar, 8906 point, coherent multipurpose lattice with horizontal and vertical bars in the shape of a capital “I.” At the places where the bars crossed, about 1500 points per aorta were counted. The points were 2.4 mm apart in the horizontal plane and 4.4 mm apart in the vertical plane in staggered arrays. The percentage of surface area with endothelial regrowth was determined by dividing the number of points falling on white regions by the total number of points (those on blue and white areas) and multiplying by 100.

To determine the percentage of the endothelial-covered surface that was stained brown, we projected the slides (×16 magnification) taken of the aorta after exposure to the reactive agents onto the lattice. We determined the percentage of regions with increased permeability by dividing the number of points falling on brown areas by the total number of points in the white regions and multiplying by 100. About 4000 points were counted in each region. Areas close to nonendothelialized regions in Groups 1 and 2 and branch sites from all groups were not counted. We calculated the differences in the percentage of areas stained brown in different regions in the same animal, within groups, and between groups using Duncan’s test for normally distributed data as determined by histogram and/or the w-statistic; we used the Kruskall-Wallis test if data were not normally distributed.

**Morphometrics**

Cholesterol feeding had no significant effect on the rate of endothelial regeneration. The average percentage of surface area recovered by endothelium on Day 77 was 44.9 ± 1.16 (SEM). Comparison of percent of regeneration showed no difference between Groups 1 and 2.

When the percentage of brown-stained surface area for each group of rabbits was computed, the greatest percentage of surface area deeply penetrated by the brown stain was seen in Group 1 (49.6% ± 4%) and Group 3 (45.6% ± 6.2%). These values were not significantly different. Rabbits from Groups 2 and 4, fed the routine ration, showed staining of 14.4% ± 4.6% and 12.1% ± 5% respectively. These values also were not significantly different. However, Groups 1 and 3 (hypercholesterolemic) were both significantly different (p < 0.01) from Groups 2 and 4 (normal diet) (figure 1). No regional differences were demonstrated.

**Gross Morphology**

Gross observations of the aortas before exposure to DAB and H₂O₂ (figure 2 A) showed blue and white regions as previously mentioned. Reaction with DAB and H₂O₂ produced a brown reaction product in the blue areas and brown

![Figure 1. Percentage of surface in regenerated or nondisturbed surfaces spotted brown. There were significant differences between Groups 1 and 2 and between Groups 1 and 4 (p < 0.01). Group 3 was statistically different from Groups 2 and 4. Bars represent standard error. Number of animals in each group is in parenthesis.](http://atvb.ahajournals.org/fig1.png)
spotting in the white areas (figure 2 B). Examination of the luminal surface at x16 magnification showed occasional blue spots within the white regions, but these spots were difficult to see. After reaction with DAB and H₂O₂, brown spots (usually less than 1 mm in diameter) were easily identified in these same white regions (figure 3 B and C). In some places, these spots merged and appeared to cover a large area; however, areas of deep brown could be distinguished. All edges of the white regions in Groups 1 and 2 showed a pattern of mild reaction to the two chemicals, probably due to movement of the HRP from the nonregenerated regions. This effect was consistent wherever blue merged with white or, after reaction with DAB and H₂O₂, brown merged with white. All blue regions were stained deep brown after the reaction.

Additional animals from all groups were sacrificed and their aortas treated in the same fashion as above, except that Evans blue was not given 30 minutes before sacrifice. The staining patterns and distribution of the HRP reaction did not appear affected by the absence of Evans blue. An additional group of animals was treated like Groups 2 and 4 but without sham operation as a further control; the staining pattern and the distribution of HRP were similarly unaffected.
Microscopy

Thick sections were not further stained before viewing by light microscopy, and thus the brown color could easily be seen. In brown areas of Groups 1 (figure 4) and 2, the stain had penetrated about 2 μ into the thickened intima in a cone-shaped pattern. In Groups 3 and 4, the color rarely penetrated the internal elastic lamina. In areas not completely stained brown, the stain was localized to the luminal cells and did not penetrate into the thickened intima. Lipid-laden cells within the thickened intima were typical in Group 1, were occasionally seen in Group 3, but were not seen in Groups 2 and 4; no other differences between cell types in brown and white areas were noted. Neither the liver nor kidney showed evidence of lipid accumulation.

Transmission electron microscopy corroborated and extended these findings (figures 5 and 6). Endothelial cells covered the luminal surfaces of the intima in the white regions, whether or not these regions were spotted brown. In areas not completely stained by reaction with DAB and H₂O₂, some brown could still be seen within the pinocytotic vesicles of the endothelium and penetrating the junctions between cells (figure 5A); brown reaction product was rarely seen beneath the endothelium. Where gross examination showed penetration of the intima, electron microscopy showed that the stain was found not only in the extracellular connective tissue, but also within the pinocytotic vesicles of the smooth muscle cells of the myointima. Lipid-laden cells were seen in both white and brown-spotted areas in rabbits in Group 1, were rare in rabbits in Group 3, and never seen in rabbits from Groups 2 and 4. The endothelium in both stained and unstained areas appeared normal. No platelets were seen in the white or brown-spotted areas by light or electron microscopy.

Scanning electron microscopy (figure 7) provided surface views of the brown spots outlined from adjoining white regions using the corneal punch. The white regions of the luminal surface

Figure 4. Thick section of regenerated region from a Group 1 rabbit showing penetration of brown stain into neointima. Brown spots (arrows) are separated by less permeable areas of regenerated endothelium. Section was not stained after embedding. X 300.

Figure 5. Transmission electron micrographs from a Group 1 animal showing reendothelialized areas deeply penetrated by horseradish peroxidase (HRP) and areas resistant to penetration. A. An area of regenerated endothelium that appeared white and was not spotted by HRP. Penetration of HRP was not beyond the endothelial layer as shown by HRP in pinocytotic vesicles (arrowheads). (c) a lipid-laden cell is shown beneath the endothelium. B. An area of brown. HRP penetrated deeply into neointima and is seen in the pinocytotic vesicles (arrowheads) in smooth muscle cells of the myointima. Photographs are typical of the type and extent of HRP penetration seen in brown and white areas. Lumen is at the right in each photograph. X 10,750.
Figure 6. A higher magnification of the region shown in figure 5 B. Horseradish peroxidase can be distinguished in the pinocytotic vesicles (arrowheads) of the endothelial cell and underlying smooth muscle cell. Lumen is at the right. × 29,000.

Figure 7. Scanning micrographs from Group 1 animals. These are representative of findings in the other three groups. A. Low magnification of a region circumscribed by a corneal punch needle. × 70. B. Center of such a region in a white area. This view shows a region from a brown area. Endothelium is broader and somewhat flattened; linear pattern is not as consistent as in figure C. No platelets are seen; no areas of endothelial loss can be found. × 300. C. View of area not stained brown in regenerated region from a Group 1 animal. Endothelium is linear without distortion. Similar changes were seen in rabbits in Groups 2, 3, and 4. × 300.
indicated endothelia in linear arrangement with centrally located nuclei. This linear pattern was interrupted in brown-spotted areas; cells were smaller and somewhat flattened and appeared to be rotated from the linear axis. There were no areas of endothelial loss or signs of endothelial detachment or swelling. No platelets or leukocytes associated with the spots were seen.

Discussion

In this study, enhanced endothelial permeability, rather than widespread loss, appeared to occur when serum cholesterol in rabbits was raised to moderately high levels by a cholesterol-rich diet whether nonballooned endothelium or regenerated endothelium in ballooned aortas were examined. The lack of endothelial loss may have been due to the relatively moderate level of hypercholesterolemia, the fixation of the arteries before experimental manipulation that might otherwise have damaged the endothelium, and the use of an in vivo marker of permeability that made tissue manipulation unnecessary until fixation was complete. Perfusion fixation of the arteries at the outset also provided increased morphologic detail and minimized distortion.

Dislodging the endothelium exposes the underlying subendothelium and usually results in platelet attachment and occasional fibrin formation. We specifically looked for morphologic evidence of these two events, but saw none. This finding further indicates that endothelial detachment due to hypercholesterolemia did not occur. However, higher levels of serum cholesterol or different sources of dietary cholesterol may induce more severe or different endothelial changes.

A primary function of endothelial cells is maintenance of a permeability barrier. Since there was no endothelial loss, it seems likely that increased permeability in localized areas represents endothelial dysfunction. It is as yet unclear what causes this increased permeability and whether it is active or passive. Transmission electron microscopy showed no morphologic changes in the intercellular structure of the endothelial cells overlying these areas, and the intracellular junctions seemed unaffected. Some evidence suggests that the increased permeability may be associated with endothelial replication: Davies and Ross have shown that endothelial pinocytosis is proportional to endothelial proliferation. The increased permeability may therefore be caused by accelerated pinocytosis due to increased endothelial turnover.

In our study, endothelial regeneration at sacrifice was not associated with an increase in the area of heightened permeability. The percentage of areas with increased permeability were comparable in parallel groups (i.e., Group 1 and 3 rabbits fed the supplemented diet; Group 2 and 4 rabbits fed the routine ration). Thus, it appears that removal of the aortic endothelium and the subsequent regeneration were not, by themselves, responsible for increased permeability.

In addition, increased permeability was found in all experimental animals wherever regenerated endothelium overlapped the myointima (the border between the blue and white regions). Since this held true for Group 2 rabbits, which were fed only the routine ration, it must be independent of cholesterol feeding. Rapid movement of HRP from the blue into the white regions may explain this phenomenon, since the blue regions showed little resistance to HRP movement.

The localized, spotted pattern of the areas of increased permeability may provide further insight into the distribution pattern of the atherosclerotic plaque. Widespread endothelial loss would likely lead to widespread and rapid narrowing of vessels. General endothelial dysfunction due to hypercholesterolemia would likely lead to uniform thickening of the entire intima. In contrast, localized dysfunction would more probably produce focal intimal lesions and might explain the localized pattern of atherosclerotic thickening. Increased permeability probably allows lipids to enter the vessel wall in greater concentrations. In addition, it may promote proliferation of smooth muscle cells by permitting substances such as growth-promoting factors, or growth hormones, to enter in increased concentrations. Even though platelet attachment at the sites of increased permeability is lacking, platelet-derived growth materials or other growth-promoting factors that may be circulating due to circumstances other than local platelet attachment would have ready access to the vessel wall at sites of increased permeability.

In summary, our findings indicate that moderate hypercholesterolemia in the rabbit is not associated with endothelial cell loss but does provoke increased endothelial permeability. In addition, we have shown that endothelial regrowth is not impeded by elevated levels of hypercholesterolemia, endothelium does not show a uniform permeability defect for a prolonged period after regrowth, and this new endothelium is not more readily injured by hypercholesterolemia than is undisturbed endothelium. The exact mechanism of this endothelial dysfunction needs further investigation but our study indicates that the concept of endothelial injury should be broadened to include subtle functional derangements.

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References


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