Endothelial Healing in the Rabbit Aorta and the Effect of Risk Factors for Atherosclerosis

Hypercholesterolemia

Louise N. Walker and David E. Bowyer

The effect of diet-induced moderate hypercholesterolemia on endothelial healing has been investigated in the rabbit following a narrow superficial injury to aortic endothelium without damage to the media of the vessel. The healing process was compared with that observed in normocholesterolemic animals. The degree of platelet involvement was similar in both normo- and hypercholesterolemic animals. Reendothelialization occurred within 48 hours in both groups of animals, showing that hypercholesterolemia did not delay endothelial healing. It was found that esterase-positive cells, which morphologically resembled monocyte-macrophages, adhered to and penetrated regenerated endothelium only in hypercholesterolemic animals. After reendothelialization in normocholesterolemic animals, there was no increase in the number of cells within the intima of the vessel and no evidence of lipid accumulation. In hypercholesterolemic animals, cells accumulated in the intima in areas of regeneration, and lipid accumulation occurred within both the intima and the media in areas of regeneration. (Arteriosclerosis 4:479–488, September/October 1984)

An essential feature of atherogenesis is the intimal proliferation of smooth muscle cells (SMC). The "response to injury" hypothesis originally suggested that this proliferation was promoted by platelet-derived growth factor (PDGF) released by platelets when they adhered to exposed subendothelial connective tissue in regions of endothelial denudation. It is now recognized, however, that intimal thickening can occur without endothelial denudation and that other factors such as monocytes and lipoproteins are also important in the formation of the fibroproliferative lesion.

In previous studies of mechanical injury to the aortic endothelium in normocholesterolemic rabbits, we have shown that there is no intimal thickening if the injury is confined to the endothelium. Intimal proliferation was observed only when the media of the vessel was also damaged.

In the present study we investigated whether the presence of a risk factor for atherosclerosis, namely hypercholesterolemia, will lead to intimal thickening when injury is confined to the endothelial layer and does not involve the vessel media. Endothelial injury was induced using a nylon catheter containing a nylon filament. In normocholesterolemic animals, this injury is rapidly healed without the involvement of smooth muscle cells, and in this study the model has been used to investigate: 1) whether endothelial repair is delayed in the presence of hypercholesterolemia, and 2) if lipid accumulation and smooth muscle cell proliferation preferentially occurs beneath small areas of regenerated endothelium.

Moderate hypercholesterolemia was induced by feeding rabbits a semisynthetic diet containing 0.1% cholesterol until stable serum cholesterol values of less than 10.4 mmol/liter (400 mg/dl) were obtained. This level is not high enough to induce lesion formation without prolonged duration. The diet was fed for 5 to 6 weeks, when serum cholesterol values had stabilized, making comparisons between individual animals more valid.

Methods

Thirty New Zealand White rabbits (Redfern strain, both male and female) aged 14 to 16 weeks were used in this study. All procedures on animals were
performed under licence in accordance with the United Kingdom's cruelty to animals act, 1876. Animals were divided into three groups as shown in Table 1. Control animals (Group 1) were fed a standard rabbit chow (SG1/V; Dixons, Ware, United Kingdom). All other animals were fed a semisynthetic diet containing 0.1% cholesterol for 5 to 6 weeks.

### Serum Cholesterol

At fortnightly intervals, samples of blood (4 ml) were taken from the marginal ear vein of animals which had fasted for 16 hours. Serum cholesterol levels were determined by using a modification of the Jamieson assay as follows: Toluene-p-sulphonic acid (0.26 mol/liter) dissolved in 25% acetic acid in acetic anhydride was used as the color reagent; the reagent (2.0 ml) was added to the sample (0.20 ml) and allowed to cool; concentrated sulphuric acid (0.16 ml) was added; the tubes were heated at 37°C for 10 minutes. Absorbance was measured at 625 nm within 10 minutes.

### Anesthesia

Anesthesia was achieved by using Hypnorm (Jannssen Pharmaceutica; Crown Chemical Company Limited, Kent, United Kingdom) and Althesin (Glaxo Laboratories, Greenford, United Kingdom), as described previously. Anesthesia was achieved by using Hypnorm (Jannssen Pharmaceutica; Crown Chemical Company Limited, Kent, United Kingdom) and Althesin (Glaxo Laboratories, Greenford, United Kingdom), as described previously.

### Catheterization

A superficial, longitudinal injury was produced along the length of the aorta in animals in Groups 1 and 3 by using a nylon catheter containing a nylon filament. Evans blue dye (0.5% wt/vol in saline) was administered intravenously, 2 ml/kg, 20 minutes after catheterization.

### Fixation

The tissue for electron microscopy was prepared by pressure fixation at 13.33 kPa (100 mm Hg) and 37°C at various times after injury. In brief, the carotid artery was cannulated, and the following solutions were introduced into the aorta without a drop in intravascular pressure: 1) wash solution of sucrose (0.26 mol/liter) buffered with N-2-hydroxyethylpiperazine-N'-ethane sulphonic acid (20 mmol/liter); 2) silver nitrate (10 mmol/liter) in wash solution; 3) wash solution; 4) fixative of 2% glutaraldehyde (25% aqueous solution, EM grade; TAAB laboratories, Reading, United Kingdom) in 0.15 M phosphate buffer (pH 7.4). Fixation was allowed to continue in situ for 1 hour.

Tissue without silver staining was prepared by direct introduction of fixative into the aorta without prior washing.

### Observation of Tissue

Areas of endothelial injury were located by observing the localization of Evans blue dye-albumin complex. The intravenous injection of Evans blue dye at the time of injury allowed the injured area to be precisely located even after restoration of endothelial integrity.

### Scanning Electron Microscopy

Tissue that had been stained with silver nitrate was prepared for scanning electron microscopy (SEM) as described previously. In brief, tissue was sutured onto teflon sheeting, dehydrated in ascending concentrations of AnalR ethanol, and critical-point dried by using liquid carbon dioxide. Tissue that had been fixed without silver staining was prepared by using a modification of the osmium-thiocarboxyhydrate-osmium (OTO) technique. Tissue was sutured onto teflon sheeting and was treated with the following solutions: 1) osmium tetroxide (1%) in 0.15 M phosphate buffer for 2 hours; 2) thio-carboxyhydrate (1%) in deionized water for 30 minutes; 3) osmium tetroxide (1%) in deionized water for 1 hour. Tissue was washed extensively between these treatments. The tissue was then dehydrated in ethanol, and critical-point dried using liquid carbon dioxide. Dried tissue was mounted on aluminum stubs using quick-drying silver paint (Agar Aids, Stansted, England). Mounted tissue was viewed either in a Stereoscan S600 electron microscope at 15 kV, or a Jeol JSM 35CF electron microscope at 20 kV.

### Transmission Electron Microscopy

Tissue for transmission electron microscopy (TEM) was dehydrated in AnalR ethanol, and then embedded in Spurr low viscosity resin. Thin sections were stained with uranyl acetate and Reynolds lead citrate, and were viewed in a Phillips EM300 electron microscope at 80 kV.

### Nonspecific Esterase Activity

Four animals of Group 3 were killed 3, 5, and 7 days after injury for the observation of nonspecific esterase activity. Perfusion fixation was not used because prolonged fixation may interfere with enzyme activity. The method was as follows: The left carotid artery was cannulated, the cannula being connected to a reservoir of Hanks' balanced salt solution (HBSS) (pH 7.4) at 100 mmHg and 37°C. The animals were killed with an overdose (0.7 ml/kg) of Euthatal (May and Baker Limited, Dagenham, Es-
sex) administered intravenously. The left femoral artery was cut to allow the slow outflow of perfusate. When this was clear of blood, the femoral artery was ligated. The aorta was carefully handled as possible, and several segments (3 to 5 cm in length) were excised. These segments were obtained by clamping the aorta with artery forceps above and below the length to be dissected. The piece of tissue containing HBSS was then excised and was placed in fresh HBSS.

Excised tissue was fixed by immersion in cold 10% formaldehyde in 0.05 M phosphate buffer (pH 7.4) for 30 seconds. The vessel was rinsed thoroughly in HBSS, opened longitudinally, and sutured onto Teflon sheeting. To outline the endothelial cell borders with silver salts, the tissue was rinsed in N-2-hydroxyethylpiperazine-N'-etohanesulphonic acid (20 mmol/liter) in deionized water (pH 7.4). The tissue was immersed in silver nitrate (10 mmol/l) in sucrose solution (as described above) for 1 minute. The tissue was again washed briefly in sucrose solution and placed in HBSS.

The tissue was stained for nonspecific esterase activity using alpha-naphthyl acetate. The vessels were incubated in a mixture of hexazoitated paraosaniline and alpha-naphthyl acetate at 37°C for 30 minutes. The mixture was prepared by adding, in sequence, 44.5 ml of 0.067 M Sorensen's buffer, 0.5 ml hexazoitated paraosaniline and 3 ml alpha-naphthyl acetate (0.107 mol/liter in ethylene glycol monomethyl ether). Hexazoitated paraosaniline was prepared by combining equal volumes of filtered paraosaniline hydrochloride (0.124 mol/liter in warm 2N hydrochloric acid) and freshly prepared sodium nitrite (0.58 mol/l in deionized water), and allowing the mixture to stand for 1 minute before use. After incubation, the tissue was washed thoroughly with HBSS.

Areas of tissue to be examined were carefully cleaned of adventitia and mounted on glass slides in glycerol-gelatin. The endothelial surface of the vessel was viewed en face in a Leitz Orthoplan light microscope.

**Oil Red O**

Frozen sections (4 μm) were prepared from either fixed or unfixed tissue. Sections from unfixed tissue were fixed in formal saline for 10 minutes before staining. Sections were stained with oil red O and counterstained with Carazzi's hematoxylin.

**Quantitation of Intimal Cell Number**

The number of intimal cells below the site of injury was quantitated as the number of cell nuclei observed per unit length of internal elastic lamina. The internal elastic lamina as observed in cross section was traced onto paper by using a camera lucida (Leitz). The length of internal elastic lamina was then calculated using a Hewlett Packard 9864A digitizer. Cross sections were cut from a minimum of two blocks from each aorta to be studied. Sections were cut 150 to 200 μm apart, distant to side branches, and a minimum of 10 random sections were observed for each time point. The number of intimal cell nuclei was quantitated in animals from Groups 1, 2, and 3 in both uninjured areas and areas of endothelial regeneration. Differences between experimental groups were tested by one-way analysis of variance (ANOVA).

**Results**

**Group 1: Control Animals**

Animals from Group 1 were observed 30 minutes, 4, 30, 48 hours, 3, 5, and 7 days after injury (Table 2).

The injury produced by the nylon filament was of the same dimensions as previously described and was 5 to 8 cells in width. There were no disruptions of the internal elastic lamina or media of the aorta.

The process of endothelial healing was as described previously. Following injury, a sparse monolayer of platelets and isolated leukocytes adhered to the exposed subendothelium. These leukocytes were identified by TEM as mostly polymorphonuclear leukocytes. Endothelial cells adjacent to the injured area became enlarged, and they migrated and divided until endothelial cover was restored. Endothelial integrity was restored at 48 hours, and the injured area was seen as a region of increased cell density. Once endothelial cover had been restored over the denuded area, there was no further interaction of blood cells with the injured area (Figure 1). Comparison of transverse sections of uninjured and injured areas showed that there was no significant increase in the number of subendothelial cells within the intima of the vessel following healing (Table 3).

**Group 2: Diet-Fed, Uninjured**

The mean initial serum cholesterol value for all animals was 2.92 ± 0.92 mmol/liter (± sd); the final mean serum cholesterol was 7.89 ± 2.28 mmol/liter (± sd).

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**Table 2. Number of Animals at Each Time Point**

<table>
<thead>
<tr>
<th>Time point</th>
<th>No diet</th>
<th>Diet-fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 minutes</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>4 hours</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>30 hours</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>48 hours</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3 days</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>4 days</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>5 days</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>7 days</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>8 days</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>11 days</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>
Figure 1. Scanning electron microscopic view of the injured area in a normocholesterolemic animal at 5 days. Regenerated endothelium is at the left. There are no adherent blood-borne cells. Bar represents 10 \( \mu \text{m} \).

Table 3. Quantitation of Intimal Cell Number per 100 \( \mu \text{m} \) of Internal Elastic Lamina

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mean cell number (± SD)</th>
<th>Number of observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. No cholesterol, no injury</td>
<td>0.635 ± 0.160</td>
<td>10</td>
</tr>
<tr>
<td>B. No cholesterol, 7-day injury</td>
<td>0.664 ± 0.352</td>
<td>12</td>
</tr>
<tr>
<td>C. Cholesterol-fed, no injury</td>
<td>0.555 ± 0.108</td>
<td>11</td>
</tr>
<tr>
<td>D. Cholesterol-fed, 8-day injury</td>
<td>5.192 ± 3.505</td>
<td>11</td>
</tr>
<tr>
<td>E. Cholesterol-fed, 11-day injury</td>
<td>6.478 ± 1.008</td>
<td>12</td>
</tr>
</tbody>
</table>

The animals in Group 2 were killed after the same length of time on the diet as the animals in Group 3. No fatty streak lesions were observed in the aorta of these animals by SEM or TEM. Observation of silver-stained tissue by SEM did not show any unusual endothelial morphology such as endothelial cells of altered size or argyrophilic cells. No areas of endothelial cell denudation were observed, and no platelets or leukocytes were seen adherent to the endothelium in any area. SEM of tissue that had not been stained with silver did not show any alteration of endothelial morphology or junctional folds. TEM revealed occasional smooth muscle cells within the intima; the majority of the intima was, however, clear of smooth muscle cells (Figure 2). No leukocytes were observed either adherent to, or beneath, the endothelium. Quantitation of the number of intimal cells showed that there was no significant increase.
as compared with normocholesterolemic animals (Table 3). The endothelial cell ultrastructure was the same as that observed in normocholesterolemic animals.

**Group 3: Diet-Fed, Injured**

Animals of Group 3 were observed 30 minutes, 30 hours, 48 hours, 3, 4, 5, 7, 8, and 11 days after injury (Table 2).

The injury produced by the nylon filament was of the same dimensions as previously described and was 5 to 8 endothelial cells in width; there were no disruptions of the internal elastic lamina or media of the vessel.

After denudation, a sparse monolayer of platelets adhered to the subendothelium, and isolated leukocytes (both polymorphonuclear leukocytes and monocytes) were also observed. The endothelial cells at the edge of the injury became enlarged and migrated over the injured area. At 30 hours after injury, endothelial cells at the edge of the injury had migrated and divided sufficiently to almost cover the denuded area. Complete endothelial integrity was restored over the injured area at 48 hours. The endothelial cells over the injury at this time were raised, spindle-shaped cells, and occasional adherent leukocytes could be seen between these cells. At 3, 4, and 5 days after injury, the injured area was still covered by very narrow, raised endothelial cells with further adherent leukocytes (Figure 3). At 7 days after injury, the injury could be seen as an area of increased cell density with no adherent leukocytes; 11 days after injury, there was little evidence of increased cell density and no adherent leukocytes.

TEM of the injured area at 3 days revealed leukocytes, which resembled monocytes morphologically,
extending pseudopodia between endothelial cell junctions (Figure 4). Occasional smooth muscle cells were observed in the intima of the injured area at 3 days, but these cells rarely showed evidence of lipid accumulation. At 5 days after injury, smooth muscle cells and cells which resembled monocyte-macrophages were seen in the intima of the injured area. Both of these cell types contained large lipid droplets, and occasional smooth muscle cells in the innermost layer of the media also contained some lipid droplets. At 8 and 11 days after injury, the intima of the injured area was still composed of foam cells, smooth muscle cells, and newly synthesized connective tissue (Figures 5 and 6). Quantitation of the number of intimal cell nuclei per unit length of internal elastic lamina showed that there was a significant increase in the number of intimal cells as compared with normocholesterolemic animals and uninjured tissue from hypercholesterolemic animals (Table 3).

**Nonspecific Esterase Activity (Group 3)**

Nonspecific esterase activity (NSEA) was seen as a red-pink cytoplasmic coloration. The tissue at 3 and 5 days after injury showed red-pink staining between endothelial cells over the injured area when viewed en face. This activity had a distribution similar to the pattern of adherent leukocytes observed by SEM. The tissue obtained 7 days after injury did not show staining for NSEA.

**Oil Red O**

In hypercholesterolemic animals, oil red O positive material was observed in the intima and inner media of the injured areas at 3, 5, 7, and 11 days after catheterization. Oil red O positive material was not observed in areas remote from injury. Tissue from normocholesterolemic animals and from uninjured hypercholesterolemic animals did not show oil red O staining in either the intima or media of the vessel.
Figure 4. Transmission electron microscopic view of the injured area in a hypercholesterolemic animal 3 days after injury showing a leukocyte (L) directly beneath the endothelium. Bar represents 1 \( \mu \text{m} \).

Figure 5. Transmission electron microscopic view of the injured area in a hypercholesterolemic animal 11 days after injury. There is an accumulation of cells within the intima, containing lipid droplets. Bar represents 4 \( \mu \text{m} \).
Discussion

The results from this study can be directly compared with the study of endothelial repair in normocholesterolemic animals because the injury was of the same dimensions and depth and an identical experimental procedure was used in both studies.

The responses immediately following endothelial denudation were similar for both injuries, and endothelial integrity was restored over both injuries at 48 hours. This demonstrates that endothelial repair in the rabbit is not delayed in the presence of hypercholesterolemia. In Prescott and Müller's study of a similar injury in the genetically hypertensive rat, they also found that endothelial repair was not delayed.

Although the injured areas were covered by endothelium within the same time in both normo- and hypercholesterolemic animals, the intimal changes that occurred following the restoration of endothelial integrity were markedly different. In the normocholesterolemic animals there were no changes in intimal components after the denuded area had been covered by endothelium; at 7 days it was no longer possible to distinguish the injured area from the normal vessel wall. In hypercholesterolemic animals, however, it was found that leukocytes adhered to the regenerated endothelium and migrated through endothelial cell junctions into the subendothelium. These leukocytes were found to be esterase-positive and they resembled monocyte-macrophages morphologically; it is therefore reasonable to assume that these cells were blood monocytes. Hypercholesterolemic animals also showed an accumulation of smooth muscle cells and intracellular lipid within the intima in areas of endothelial regeneration.

Studies in hypercholesterolemic animals have often demonstrated the infiltration of monocytes into areas of increased permeability and lesion formation. The mechanism by which monocytes are stimulated to adhere to arterial endothelium is unknown. Factors that may be involved include lipoproteins, IgG, growth factors and chemotactic agents, endothelial permeability, and monocyte and/or endothelial function.
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The data from this study show a strong correlation between endothelial cell regeneration and monocyte adhesion in hypercholesterolemic animals. It is unclear why regenerated endothelium in hypercholesterolemic animals should be attractive to monocytes, but one explanation could be the alteration in plasma lipoprotein profile induced by cholesterol feeding. Regenerating endothelium in vitro shows enhanced pinocytosis and altered uptake of low density lipoprotein (LDL), and macrophages have receptors for LDL that has been modified by endothelial cells. Increased plasma LDL levels may play a role in monocyte adhesion to areas of regenerated endothelium in hypercholesterolemic animals.

Another explanation is that cell replication is initiated by cell injury. Hansson et al. have shown that dead or injured endothelial cells accumulate IgG and that monocytes adhere preferentially to these cells via Fc receptors. Areas of endothelium which showed monocyte adhesion in this study were, however, newly regenerated and there was no evidence of cell injury following initial cell loss. It is therefore unlikely that monocyte adhesion was caused by the presence of dead or injured cells. Growth factors localized at the site of injury, such as platelet-derived growth factor (PDGF), may be involved in promoting monocyte adhesion. However, platelet adhesion in this study was similar in both normo- and hypercholesterolemic animals, as was the length of time required for wound closure, and hence the length of time that the subendothelium was exposed to other circulating factors.

Gerrity et al. have observed that monocytes adhere to specific areas of the vessel wall in diet-fed swine, most particularly in areas that show increased uptake of Evans blue-labeled albumin. We have observed uptake of Evans blue-labeled albumin in areas of regeneration at various times after injury in both normo- and hypercholesterolemic rabbits (unpublished observation) and have observed that areas of regenerated endothelium show increased uptake of dye up to 2 weeks after restoration of endothelial integrity. This agrees with data on tight junction formation in regenerated endothelium and suggests that increased endothelial permeability or loss of tight junction structure is not solely responsible for monocyte adhesion.

Another possibility is that monocyte function is altered in hypercholesterolemia and that this causes increased monocyte adhesion. In this study, however, monocyte adhesion was observed only along the line of injury and not in any other area of the aorta, suggesting that adhesion is not a consequence of monocyte function alone.

Perhaps the most likely explanation is that either endothelial function or the endothelial cell surface is altered following rapid regeneration in the presence of a risk factor, thus promoting monocyte adhesion. Concomitant with the adhesion of monocytes to regenerated endothelium in hypercholesterolemic animals, smooth muscle cell accumulation and intra-cellular lipid accumulation were observed in the intima in regenerated areas. It is not possible from these studies to say whether the accumulation of smooth muscle cells in these areas is due to cell proliferation or cell migration alone, because tritiated thymidine data is not available.

It has been shown in vitro, however, that hyperlipidemic serum stimulates the proliferation of rabbit aortic medial cells and that macrophages produce macrophage-derived growth factor (MDGF) which also stimulates smooth muscle cell growth. Lipid-containing cells beneath regenerating endothelium in hypercholesterolemic animals were identified morphologically as both macrophages (intima only) and smooth muscle cells (intima and media), and were found to contain lipid both by TEM and oil red O staining.

Cholesteryl ester accumulation within macrophages is enhanced in vitro by a lipoprotein formed by a modification of LDL in endothelial cells. The promotion of lipid accumulation in smooth muscle cells may be due to the stimulation of LDL receptor activity. Minick et al. have shown that lipid accumulation occurs preferentially beneath regenerated endothelium in the balloon-catheterized rabbit aorta. This was thought to be a paradoxical result because the "response to injury hypothesis" originally suggested that lipid accumulation was caused by an absence of endothelium. The study described here confirms that lipid may accumulate beneath regenerated endothelium despite a limited exposure of the subendothelium to the blood, suggesting that the function of regenerated endothelium is altered as compared with quiescent endothelium.

In conclusion, areas of superficial endothelial injury to the aorta of moderately hypercholesterolemic rabbits induce lipid accumulation, monocyte infiltration, and a significant accumulation of intimal cells despite rapid restoration of endothelial integrity.

Acknowledgments

The authors thank Mike Pollard and Bob Burgess for animal husbandry and Ken Thurley and Jeremy Skepper of the Department of Anatomy, University of Cambridge, for assistance with electron microscopy.

References


Index Terms: aortic endothelium • atherosclerosis • endothelial injury • hypercholesterolemia • rabbit
Endothelial healing in the rabbit aorta and the effect of risk factors for atherosclerosis. Hypercholesterolemia.
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*Arterioscler Thromb Vasc Biol.* 1984;4:479-488
doi: 10.1161/01.ATV.4.5.479

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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