ApoE-Deficient Mice Develop Lesions of All Phases of Atherosclerosis Throughout the Arterial Tree

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Abstract
Initial description of apolipoprotein (apo) E-deficient transgenic mice demonstrated the development of severe hypercholesterolemia due to probable delayed clearance of large atherogenic particles from the circulation. Examination of these mice demonstrated foam cell accumulation in the aortic root and pulmonary arteries by 10 weeks of age. In the present study, the animals were fed either chow or a high-fat, Western-type diet and examined at ages ranging from 6 to 40 weeks. Gross examination by dissection microscopy revealed a predilection for development of lesions in the aortic root, at the lesser curvature of the aortic arch, the principal branches of the aorta, and in the pulmonary and carotid arteries. Monocyte attachment to endothelial cells was observed by light and electron microscopic examination at 6 weeks, the earliest time point examined. Foam cell lesions developed as early as 8 weeks, and after 15 weeks advanced lesions (fibrous plaques) were observed. The latter consisted of a fibrous cap containing smooth muscle cells surrounded by connective tissue matrix that covered a necrotic core with numerous foamy macrophages. Mice fed the Western-type diet generally had more advanced lesions than those fed a chow diet. The apoE-deficient mouse contains the entire spectrum of lesions observed during atherogenesis and is the first mouse model to develop lesions similar to those in humans. This model should provide numerous opportunities to study the pathogenesis and therapy of atherosclerosis in a small, genetically defined animal. (Arterioscler Thromb. 1994;14:133-140.)

Key Words • atherosclerosis • apolipoprotein E • transgenic mouse model • lesions of atherosclerosis

Numerous animal species have been used to study the pathogenesis and potential treatment of the lesions of atherosclerosis. 1,2 The most useful animal models have thus far been restricted to relatively large animals, such as nonhuman primates,3-7 swine,8-10 and rabbits.11-13 Hamsters14 and pigeons15 have been used occasionally but present problems peculiar to their species. What has been traditionally lacking is a small, genetically reproducible, murine model of atherosclerosis. Such a model could help to overcome the many problems and deficiencies of the larger animals and, in particular, would permit studies of possible therapies that require relatively large numbers of animals.

Until recently, the only mouse model available for studies of atherogenesis was the C57BL/6 mouse,16-17 which develops lesions with some features of atherosclerosis; lesions in this mouse usually occur only at the root of the aorta and are associated with the cusps of the aortic valve. More recently, apolipoprotein (apo) E-deficient mice,18,19 created by homologous recombination in embryonic stem cells, have been shown to develop severe hypercholesterolemia and lesions of atherosclerosis more characteristic in appearance and distribution to those observed in humans.

The principal purpose of the present study was to analyze in detail the genesis of these lesions, including the nature of the cells involved, the sequence of cellular events, and the anatomic location of specific lesion types with increasing age and time on both a chow diet and a Western-type diet (0.15% by weight cholesterol). This study demonstrates that these animals develop a full range of lesions of atherosclerosis from fatty streaks to fibrous plaques; that the lesions are distributed throughout the arterial tree; and that in their development they contain many features of the specialized, chronic, inflammatory-fibroproliferative response characteristic of atherosclerosis seen in other species.

Methods

Mice
Control and apoE-deficient male mice were second- or third-generation hybrid 129olaxC57BL/6 or 129olaxBALB/c mice derived from brother-sister matings using only apoE-deficient animals that were initially created by homologous recombination in embryonic stem cells.18 Both control and apoE-deficient mice were weaned at 4 weeks of age and maintained on chow for 1 week, at which time they were either maintained on chow (PicoLab Rodent Chow 20), which contained 4.5% fat by weight (0.02% cholesterol), or a Western-type diet (Teklad Adjusted Calories Western-type diet), which contained 21% fat by weight (0.15% by weight cholesterol and 19.5% by weight casein without sodium cholate). Diet and water were provided ad libitum.

Time Schedule of Death and Fixation
Animals from each group were killed at 6, 8, 10, 15, 20, 30, and 40 weeks of age. At each time point, 4 apoE mice and 1 control fed the chow diet and 4 apoE mice and 1 control fed the Western-type diet were killed. Three of the 4 apoE-
deficient mice at each time point were perfusion fixed with 4% paraformaldehyde, methanol-Carnoy's fixative (60% methanol, 30% chloroform, and 10% acetic acid), or 3% glutaraldehyde under intraperitoneal pentobarbital anesthesia (100 mg/kg). One of the 4 was perfused with phosphate-buffered saline under pentobarbital anesthesia, and the heart and aorta were dissected, removed, quick frozen in liquid nitrogen, and mounted in OCT compound and frozen in isopentane cooled in liquid nitrogen. For immunohistochemical analysis, a mouse monoclonal anti-α-actin immunoglobulin (Ig) G (Boehringer Mannheim, Indianapolis, Ind) was used as a specific marker for smooth muscle cells, and the rat monoclonal antibody BM8 (BMA Biochemicals AG, Augst, Switzerland) was used as a specific marker for macrophages. For immunostaining, endogenous peroxidase activity was blocked by incubating each section in 0.3% H2O2 with 1% NaN3. Anti-α-actin was labeled with biotin by using Biotin-LC-Hydrazide (Pierce, Rockford, Ill) and applied to the section in a dilution of 1:10, or applied in a nonbiotinylated form (1:200), which was followed by rabbit anti-mouse IgG (1:2000, Zymed, San Francisco, Calif), a biotinylated secondary antibody. BM8 was used at a dilution of 1:20, followed by biotinylated rabbit anti-rat IgG (H+L) (1:300; Vector Laboratories, Burlingame, Calif). After horseradish peroxidase–conjugated streptavidin (1:5000; Jackson Immunoresearch Laboratories, West Grove, Pa) was applied to the section, antibody binding was visualized with diaminobenzidine (Sigma Chemical Co, St Louis, Mo). Normal mouse IgG (5 μg/mL) and normal rat serum (1:500) were used as negative controls. Sections were counterstained with Harris hematoxylin.

**Morphological Observations**

Specimens fixed with paraformaldehyde or methanol-Carnoy's fixative were used for light microscopic examination. The heart, aorta, and arteries were observed under the dissection microscope and dissected as indicated in Fig 1, left. All segments indicated in the figure were embedded in paraffin, and cut sections were stained with hematoxylin and eosin. Selected sections were also stained with Verhoeff-van Gieson's, Masson's trichrome, and alcian blue–periodic acid–Schiff stains. The unfixed hearts and aortas were cut in a similar manner, and the segments were embedded in OCT compound and frozen in isopentane cooled in liquid nitrogen. Sections were stained with hematoxylin and eosin. Specimens fixed with glutaraldehyde were used for electron microscopic examination. Selected segments were dehydrated in graded ethanol and embedded in epon resin for electron microscopic examination. Semithin sections were stained with toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a transmission electron microscope (JOEL 1200 EX, Tokyo, Japan).

**Immunohistochemistry**

For immunohistochemical analysis, a mouse monoclonal anti-α-actin immunoglobulin (Ig) G (Boehringer Mannheim, Indianapolis, Ind) was used as a specific marker for smooth muscle cells, and the rat monoclonal antibody BM8 (BMA Biochemicals AG, Augst, Switzerland) was used as a specific marker for macrophages. For immunostaining, endogenous peroxidase activity was blocked by incubating each section in 0.3% H2O2 with 1% NaN3. Anti-α-actin was labeled with biotin by using Biotin-LC-Hydrazide (Pierce, Rockford, Ill) and applied to the section in a dilution of 1:10, or applied in a nonbiotinylated form (1:200), which was followed by rabbit anti-mouse IgG (1:2000, Zymed, San Francisco, Calif), a biotinylated secondary antibody. BM8 was used at a dilution of 1:20, followed by biotinylated rabbit anti-rat IgG (H+L) (1:300; Vector Laboratories, Burlingame, Calif). After horse-radish peroxidase–conjugated streptavidin (1:5000; Jackson Immunoresearch Laboratories, West Grove, Pa) was applied to the section, antibody binding was visualized with diaminobenzidine (Sigma Chemical Co, St Louis, Mo). Normal mouse IgG (5 μg/mL) and normal rat serum (1:500) were used as negative controls. Sections were counterstained with Harris hematoxylin.

**Measurement of Plasma Cholesterol Levels**

Blood was drawn from the retro-orbital venous plexus when the mice were killed. Plasma levels of total cholesterol were measured by enzymatic assay (Boehringer Mannheim).

**Results**

**Plasma Cholesterol Levels of ApoE-Deficient Mice**

Plasma cholesterol levels of apoE-deficient mice fed a chow diet ranged from 360 to 885 mg/dL with a mean of 606 mg/dL, whereas those of normal control mice fed a chow diet ranged from 101 to 119 mg/dL with a mean of 109 mg/dL. Feeding a Western-type diet to the apoE-deficient mice resulted in much higher levels of cholesterol, ranging from 1085 to 4402 mg/dL. There was greater variability in the cholesterol levels in these groups, and they were always higher than those of...
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animals fed the chow diet (Fig 2). There was no effect of age on total plasma cholesterol levels in mice on either diet (Fig 2). Normal control mice fed the Western-type diet also showed higher levels of plasma cholesterol than their counterparts fed chow, with levels ranging from 154 to 301 mg/dL, but the levels were lower than those of the apoE-deficient mice fed a chow diet.

Atherosclerotic Lesions in ApoE-Deficient Mice

Lesions of atherosclerosis were grossly observed in the apoE-deficient mice on both diets but not in the control mice. The lesions developed not only in the aortic root but throughout the aorta and its principal branches. Sites of predilection are shown in Fig 1, right. The first sites to develop lesions included the aortic root, the lesser curvature of the aortic arch, the branches of the brachiocephalic artery and the right common carotid artery (Fig 3, left), the branches of the superior mesenteric artery, both renal arteries, the aortic bifurcation, and the pulmonary artery (Fig 3, right). In older animals, lesions were detected in the descending thoracic, lower abdominal, proximal coronary, common iliac, and femoral arteries. Lesions first appeared as small yellowish-white nodules (Fig 3, right) and were detected at 10 weeks of age in the chow diet group and at 8 weeks of age in the Western-type diet group. With increasing age, the lesions grew in size. Their appearance was typical of fibrous plaques (Fig 3, left), and they could substantially narrow the arterial lumen, in some cases to the point of 95% occlusion.

Lesion Progression in the ApoE-Deficient Mice on the Western-Type Diet

Histological observations confirmed that lesions occurred only in the apoE-deficient mice and not in the normal control mice. The earliest changes observed were adherence of mononuclear cells to the endothelial surface throughout the arterial tree. Foam cell lesions, or fatty streaks, were subsequently found at the same sites. With increasing age, the lesions progressed to intermediate or fibrofatty lesions containing multiple layers of lipid-filled macrophages and smooth muscle cells and ultimately to fibrous plaques.

At 6 weeks of age, the earliest time examined, mononuclear cell adhesion to the endothelium was observed in the apoE-deficient mice on the Western-type diet (Fig 4, top). Sporadic foam cells were present in the subendothelial space at 6 weeks, suggesting that monocyte adhesion and chemotaxis may have occurred even earlier. Electron microscopic examination of the same region revealed that these cells contained features characteristic of peripheral blood monocytes (Fig 4, bottom). The monocytes had horseshoe-shaped nuclei and many pseudopodia that interdigitated with the endothelial cell surface. Adhesion of mononuclear cells and subendothelial accumulation of foam cells were also observed in apoE-deficient mice fed the chow diet.
after 8 weeks of age. However, the number of attached cells appeared less numerous than in mice on the Western-type diet.

Foam cell lesions were observed in apoE-deficient mice fed the Western-type diet after 8 weeks of age and in mice on the chow diet after 10 weeks of age. As shown in Fig 5, foam cells accumulated in the subendothelial space with no obvious changes in the underlying media. Several nonfoamy mononuclear cells were also observed adhering to the surface of the endothelial lining or intermingled with foam cells within the lesions. After 20 weeks, lesions containing only foam cells were no longer observed in apoE-deficient mice fed the Western-type diet but were still present at 30 weeks in mice on the chow diet.

Subsequent to early foam cell formation (10- to 15-week-old mice), intermediate lesions consisting of a mixture of spindle-shaped cells (presumably smooth muscle) and foam cells were observed. The spindle-shaped cells were intermingled with the foam cells or tended to form a cap at the top of the lesion. Deposition of a small amount of connective tissue matrix was also observed in these lesions.

By 15 weeks of age, early fibrous plaques were observed in apoE-deficient mice on the Western-type diet. These lesions contained small necrotic cores together with a few foam cells that were covered by a well-formed fibrous cap containing smooth muscle cells surrounded by elastic fibers and collagenous tissue (Fig 6, left panels). The spindle-shaped cells that made up the fibrous cap were shown to be smooth muscle cells by immunohistochemical staining for α-actin (Fig 6, upper right). Further immunohistochemical analysis demonstrated that the foam cells in the neointima stained positively with the monoclonal antibody BM8, suggesting that they were of macrophage lineage (Fig 7). In older mice (20 to 40 weeks of age), the fibrous plaques appeared more advanced, with larger necrotic cores and abundant fibrous tissue (Fig 8). Electron microscopic analysis of these lesions revealed large, lipid-laden, macrophage-derived foam cells together with numerous lipid-containing smooth muscle cells, identified by their myofilaments, dense bodies, and surrounding basement membrane (Fig 9). Also abundant in these advanced lesions was deposition of extracellular matrix, consisting of collagen, elastic fibers, proteoglycans, and extracellular lipid deposits. Fig 9 also shows an adherent, flattened mononuclear cell attached to the endothelial cells with numerous pseudopodia, demonstrating the continuing influx of mononuclear cells. In some of the advanced lesions, there was partial destruction of underlying medial cells and calcification in the fibrous tissue. In the apoE-deficient mice on the chow diet, fibrous plaques appeared after 20 weeks and were smaller than those in mice fed the Western-type diet.

Lesion Progression in the Coronary Artery

Lesions of atherosclerosis appeared somewhat different in the coronary arteries than in the other parts of the arterial system. At early ages, the coronary arteries were not involved with atherosclerosis, though lesions
were present in the root of the aorta at the base of the valves near the ostia of these arteries. After 20 weeks, the apoE-deficient mice fed the Western-type diet contained lesions in the coronary arteries, presumably by direct invasion of the lesion from the aortic root. The lumen of the coronary artery may be extremely narrowed, as seen in Fig 10. Although no obvious myocardial infarctions were observed, areas of myocardial fibrosis were observed in two animals at 40 weeks of age (Fig 11).

**Lesion Progression on the Western-Type Diet**

Although there was some variability in lesion severity among the mice at any given time on the diet, the histology of the lesions was remarkably similar among the animals in each group of four that was sampled. Lesions at various stages of evolution could be observed in a single animal. For example, after 15 weeks on the Western-type diet, sites numbered 1, 2, 3, and 5 (Fig 1, right) had advanced fibrous plaques, whereas earlier stages of lesion development were observed at sites such as the lower abdominal aorta and the lower thoracic aorta. The mice fed the Western-type diet generally contained more advanced lesions at each stage of development compared with mice fed chow (Fig 12).

Furthermore, the areas of the lesions were wider and medial destruction was more severe in the group fed the Western-type diet. Complications such as calcification in the lesion were recognized only in mice fed the Western-type diet.
Discussion

Extent and Severity of Lesion Formation in ApoE-Deficient Mice and Other Genetic Mouse Models

This study, in contrast to the currently available mouse models of atherosclerosis, showed that the homozygous apoE-deficient mouse demonstrates all of the known phases of atherogenesis. The cellular interactions involved in the early inflammatory responses that characterize the development of fatty streaks in nonhuman primates, rabbits, and humans were also observed in this study of the apoE-deficient mouse. As shown in Figs 5 and 6, adherent monocytes were observed on the surface of the endothelium in the thoracic aorta and in other arteries before the development of foam cell–rich fatty streaks at these same anatomic sites. Immunocytochemical analysis demonstrated that the fatty streaks primarily consisted of lipid-laden, monocyte-derived macrophages with, initially, few or no smooth muscle cells. As the lesions continued to progress, smooth muscle cells appeared, many of which contained lipid deposits. Ultimately, fibrous caps rich in smooth muscle cells formed over the foam cell–rich areas. The spindle-shaped cells that made up the fibrous caps of the developing fibrous plaques and advanced lesions were identifiable as smooth muscle cells. As the lesions continued to progress, cholesterol clefts and necrotic areas appeared within the core regions of the fibrous plaques. The lesions continued to increase in size and increasingly occluded the lumen of the affected artery.

In contrast to the apoE-deficient mouse, other mouse models of atherosclerosis lack the progressive series of atherogenic events seen in humans. The most commonly used mouse model is diet-induced atherosclerosis of the Western type diet. The diagram shows the time periods over which the indicated lesion types were observed.
Lesion Formation in the ApoE-Deficient Mouse and Chronic Inflammatory Responses

The lesions of atherosclerosis that form in the apoE-deficient mouse bear striking similarities to those that develop in markedly hyperlipidemic rabbits (including fat-fed and Watanabe heritable hyperlipidemic rabbits), \textsuperscript{11,12} monkeys, \textsuperscript{3-6} and swine.\textsuperscript{8,9} In each of these models, all of the phases of the inflammatory fibroproliferative response can be observed. Whatever factors induce changes in the endothelium, the interactions between endothelium and circulating monocytes (and possibly T lymphocytes), and subsequent interactions within the intima, these models appear to be histologically similar. Many different factors play roles in the genesis and progression of atherosclerosis in these models. The details of the adhesive cellular interactions, chemotactic factors, growth-regulatory molecules, and cytokines that participate in the atherogenesis of the apoE-deficient mouse remain to be determined.

Western-Type Diet and Atherogenesis

The apoE-deficient mice that were fed a Western-type diet showed striking increases in their cholesterol levels, particularly in the very-low-density lipoprotein and intermediate-density lipoprotein fractions.\textsuperscript{18} There were also large increases in low-density lipoprotein. These mice showed markedly accelerated atherogenesis. Although the sites of the advanced lesions of atherosclerosis were the same in the apoE-deficient mice on the Western-type diet as those on the chow diet, the lesions in the mice on the Western-type diet were more advanced, occurred much earlier, and contained more lipid than those in the mice on the chow diet. Thus, the Western-type diet appeared to markedly aggravate as well as accelerate the process of atherogenesis, suggesting another similarity with humans. This observation provides an opportunity for analysis of those components of the diet that may be particularly critical in this process and may provide opportunities for pharmacological intervention.

ApoE-Deficient Mouse Lesions and Human Lesions

Lesions and their development in the apoE-deficient mouse bear a striking similarity to the process of atherogenesis we have come to understand in humans. As in the experimental animal models, there is ample evidence to support adhesive interactions between leukocytes and the lining endothelium, entry and chemotaxis of these leukocytes into the subendothelial space, and conversion of monocytes into scavenger cells and, ultimately, to foamy macrophages.\textsuperscript{20,24-27} The similarities in comparison with human lesion formation suggest that this particular mouse model may provide a fertile source for further investigations of the process. In addition, further genetic recombinations, including models of diabetes, hypertension, and other factors that can be genetically studied in the mouse, offer an opportunity to examine the multiple interactions of factors associated with the pathogenesis of atherosclerosis.

References


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Arterioscler Thromb Vasc Biol. 1994;14:133-140
doi: 10.1161/01.ATV.14.1.133

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

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