Apolipoprotein E Promotes the Regression of Atherosclerosis Independently of Lowering Plasma Cholesterol Levels

Robert L. Raffai, Samuel M. Loeb, Karl H. Weisgraber

Objective—The mechanisms by which apolipoprotein E (apoE) can promote the regression of atherosclerosis are not well understood. This study examined whether apoE can promote atherosclerosis regression independently of lowering plasma cholesterol levels.

Methods and Results—We studied hypomorphic apoE mice (Apoe<sup>h/h</sup>), which express an apoE<sub>4</sub>-like form of mouse apoE at ≈2% to 5% of normal levels in plasma and are normolipidemic. After 18 weeks of diet-induced hypercholesterolemia, which resulted in advanced aortic atherosclerotic lesions composed of a lipid-rich layer of foam cells covering a fibrotic core, 2 groups of mice were fed a chow diet for 16 weeks. One group continued to express low levels of apoE; the other was induced to express physiological levels of plasma apoE by Cre-mediated recombination of the hypomorphic Apoe allele. In both groups, plasma cholesterol levels fell rapidly to similar levels, and histological analysis at 16 weeks revealed elimination of the foam-cell layer. However, physiological levels of plasma apoE also enhanced the removal of neutral lipids from the fibrotic cores.

Conclusion—These findings demonstrate for the first time that apolipoprotein E promotes the regression of atherosclerosis independently of lowering plasma cholesterol levels. (Arterioscler Thromb Vasc Biol. 2005;25:436-441.)

Key Words: apoE • lipoprotein • atherosclerosis • regression • foam cell
weeks on the chow diet, both groups were euthanized. Atherosclerosis,
the atherosclerotic phenotype. minimize the effect of genetic background as a confounding factor in
mice used in the study. The breeding pairs were constantly mixed to
cholesterol levels, unlike the high-fat Western diet without cholate
cholic acid (ICN) for 18 weeks. This diet provokes very high plasma
an atherogenic diet containing 16% fat, 1.25% cholesterol, and 0.5%
in a barrier facility with a 12-hour light/12-hour dark cycle; and fed
Paraformaldehyde in PBS for 30 minutes on ice, drained, and rinsed
PBS, removed by cutting the aortic root, and further fixed in 3%
Peroxidase with 0.3% hydrogen peroxide in PBS for 30 minutes on ice,
and stained with Sudan IV. Images of each aorta were captured with
isolated, opened longitudinally, pinned out flat on a black wax block,
Morphological Analyses of Atherosclerotic Lesions
with Adobe PhotoShop 6.0 software and Image Processing Tool Kit
and stained with Sirius red to reveal collagen and
were also stained with Movat pentachrome stain to reveal the cellular
lesions in the aortic root was analyzed by staining with oil red O to
arranged 4 sections per slide. The composition of atherosclerotic
from the base of the aortic root, 80 sections were collected and
Statistical Analysis
Differences in plasma cholesterol levels and in the extent of aortic
lesions in induced and noninduced mice were tested with 2-tailed
t tests. To control for differences in cholesterol levels that might
explain differences in atherosclerosis, 1-way analysis of
covariance was performed to test for independent effects of apoE on
atherosclerosis. SAS Statistical Software version 9.0 was used for
statistical computation.
Results
Lipoprotein Profiles and Plasma ApoE Levels
In this study, we capitalized on the rapid reversal of diet-
induced hypercholesterolemia with or without permanently
restoring physiological levels of plasma apoE. Three groups of
Apoεh/hhMx1-Cre mice (Figure 1B) were fed an atherogenic
diet for 18 weeks, which resulted in plasma cholesterol levels
of 1044±179 mg/dL (n=24) and a lipoprotein profile consist-
ing mainly of remnant lipoproteins containing very low-
density lipoproteins (VLDLs), intermediate-density lipopro-
tins, and LDLs (Figure 1C). Plasma apoE levels increased
slightly, reflecting the accumulation of remnant lipoproteins
(Figure 1D). After 18 weeks, 1 group of mice (controls,
n=17) was euthanized, and the aortas were removed for
analysis of atherosclerosis. Mice in the second group (n=17)
were switched to a Chow diet and continued to express low
levels of apoE. Mice in the third group (n=15) were also
switched to a Chow diet but were induced to express normal
plasma apoE levels. Plasma cholesterol levels in these 2

Methods

Apoεh/hhMx1-Cre Mice
Male and female Apoεh/hhMx1-Cre mice17 were bred to obtain the
mice used in the study. The breeding pairs were constantly mixed to
minimize the effect of genetic background as a confounding factor in
the atherosclerotic phenotype.

Experimental Protocol
Male Apoεh/hhMx1-Cre mice were weaned at 21 days of age; housed
in a barrier facility with a 12-hour light/12-hour dark cycle; and fed
an atherogenic diet containing 16% fat, 1.25% cholesterol, and 0.5%
cholic acid (ICN) for 18 weeks. This diet provokes very high plasma
cholesterol levels, unlike the high-fat Western diet without cholate
that only doubles their plasma cholesterol levels17, which would
likely induce small atherosclerotic lesions only after 6 to 9 months.
The mice were divided into 3 groups (Figure 1B). One group (controls)
was euthanized immediately. One group was switched to a Chow diet,
and 1 group was switched to a Chow diet and induced to
effect normal physiological levels of plasma Arg-61 apoE by 3
intraperitoneal injections of polyinosinic-polycytidylic acid (250 μg;
Sigma) over 2 weeks to activate the Mx1-Cre transgene.17 After 16
weeks on the Chow diet, both groups were euthanized. Atheroscle-
rosis was assessed in all 3 groups.

Plasma Lipoprotein and Cholesterol Determination
Mice were fasted for 4 hours, anesthetized with isoflurane inhalation,
and bled by retro-orbital puncture. Lipoproteins were fractionated by
fast-performance liquid chromatography on a Superoxide 6 column
(Amersham/Pharmacia). Cholesterol levels in plasma and in the
lipoprotein fractions were determined with colorimetric assays
(Spectrum, Abbott and Triglycerides, Boehringer Mannheim,
respectively).

Aortic Lesion Analysis
Mice were anesthetized, perfused with PBS, pH 7.4, and fixed by
perfusion with 3% paraformaldehyde in PBS. The entire aorta was
isolated, opened longitudinally, pinned out flat on a black wax block,
and stained with Sudan IV. Images of each aorta were captured with
a digital camera mounted on a dissection microscope and analyzed
with Adobe PhotoShop 6.0 software and Image Processing Tool Kit
plug-in (ImageReady 3.0) to determine percent lesion areas.

Morphological Analyses of Atherosclerotic Lesions
The heart was perfused with PBS and with 3% paraformaldehyde in
PBS, removed by cutting the aortic root, and further fixed in 3%
paraformaldehyde in PBS for 30 minutes on ice, drained, and rinsed
overnight in PBS containing 20% sucrose. The heart was then
embedded in Tissue-Tek cryosectioning compound (Sakura Finetek),
and tissue blocks were cut into 10-μm-thick sections. Beginning
from the base of the aortic root, 80 sections were collected and
arranged 4 sections per slide. The composition of atherosclerotic
lesions in the aortic root was analyzed by staining with oil red O to
reveal neutral lipids and counterstained with hematoxylin. Sections
were also stained with Movat pentachrome stain to reveal the cellular
and fibrotic components and with Sirius red to reveal collagen
and cellular components. Atherosclerosis was quantified morphometrically
by measuring the extent of surface area covered by neutral lipids
revealed by oil red O staining in a series of 7 sections on immediately
adjacent slides that had a common anatomic location, the coronary
ostium.

Statistical Analysis
Differences in plasma cholesterol levels and in the extent of aortic
lesions in induced and noninduced mice were tested with 2-tailed
2-sample t tests. To control for differences in cholesterol levels that might
explain differences in atherosclerosis, 1-way analysis of
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![Figure 1.](http://atvb.ahajournals.org/.../h9262)
groups were not significantly different at 4 weeks (184±17 versus 168±21 mg/dL, respectively) or 16 weeks (180±32 and 159±30 mg/dL; both P=0.3).

At euthanization, the noninduced mice had plasma apoE levels similar to those of Apoe<sup>h/h</sup> mice fed a chow diet (2% to 5% of wild-type); the induced mice had levels identical to those of wild-type mice (Figure 1D). Both groups had similar lipid profiles, consisting mainly of high-density lipoproteins (HDL); noninduced mice had slightly more LDL-sized particles (Figure 1C).

Effect of Plasma Lipid Levels on Aortic Atherosclerosis

After 18 weeks of elevated plasma cholesterol levels, the control group had advanced atherosclerotic lesions covering 6.6±1.5% of the aorta, predominantly in the aortic arch (Figure 2). Morphological assessment of the lesions by staining aortic root sections with Movat pentachrome stain revealed advanced lesions consisting of a layer of foam cells covering a fibrotic core containing proteoglycans, collagen, and cells (Figure 3). Staining the sections with Sirius red revealed that collagen was abundant in the fibrotic core but scarce in the foam cell layer (Figure 4). This stain also revealed foam cells deep in the fibrotic core. Staining the sections with oil red O showed that the foam cell compartment was rich in neutral lipids (Figure 3). Immunohistochemical analysis of lesions in control mice revealed apoE mainly in the foam cell layer (not shown). This apoE could have been produced locally by macrophage and smooth muscle cells, or it could have infiltrated the plaques along with circulating lipoproteins. Ongoing studies are currently aimed at localizing the source and cellular location of this lesion-associated apoE.

Effect of Plasma Lipid Lowering on Atherosclerosis Regression

After 16 weeks of lipid lowering, sudanophilic aortic lesions were 40% smaller in noninduced than in control mice (3.9±1.5% versus 6.6±1.5%, P=0.0004). Moreover, sudanophilic aortic lesions were 30% smaller in induced than in noninduced mice (2.7±1.3% versus 3.9±1.5%, P=0.01; Figures 4 and 5). After adjustment for the nonsignificant difference in plasma cholesterol levels between the noninduced and induced groups (mean, 21 mg/dL), the induced group had smaller lesions at all cholesterol levels (mean absolute difference, 1.1%, P=0.05; Figure 6A).

In both induced and noninduced mice, but not in controls, morphological analysis revealed an almost complete loss of the foam cell layer and collagen accumulation resulting in a fibrous cap (Figure 4), a feature of stable lesions. Both groups also had lower levels of apoE in the lesions than the controls (data not shown). The dynamics of apoE levels and expression in lesions undergoing regression in Apoe<sup>h/hMx1-Cre</sup> mice are currently being investigated as part of an ongoing study. Despite the loss of the foam cell layer in the lesions of both groups, morphometric measurements showed a 3-fold greater loss of neutral lipids from lesions of induced mice (P=0.002) (Figure 3), consistent with the en face measurements (Figure 5). After adjustment for the nonsignificant difference in plasma cholesterol levels, the induced group had a significantly lower neutral lipid content in lesions at all cholesterol levels (mean absolute difference, 0.6, P=0.004; Figure 6B).

Discussion

In this study we determined whether apoE could promote the regression of atherosclerosis independently of lowering
plasma cholesterol levels, using our novel Apoe<sup>h/hMx1-Cre</sup> mouse model. This model is sensitive to diet-induced hypercholesterolemia, resulting in atherosclerosis that can be reversed by diet change alone while maintaining 2% to 5% of normal plasma apoE levels or by permanent restoration of physiological apoE levels. Our study demonstrates that physiological levels of apoE expression promote the regression of atherosclerosis independently of lowering plasma cholesterol levels. Lowering plasma cholesterol levels in mice expressing apoE at 2% to 5% of normal plasma levels eliminated the foam cell layer beneath the endothelium. However, induction of physiological levels of apoE expression in addition to cholesterol lowering also enhanced the removal of neutral lipids from the fibrotic component of lesions.

Previously, the primary model used to investigate the role of apoE in the regression of atherosclerotic lesions was adenovirus-mediated expression of apoE in Apoe<sup>−/−</sup>/H11002 mice.6,25,26 This approach results in sustained lipid lowering and significant remodeling of aortic root lesions, characterized by collagen deposition and elimination of foam cells. However, limitations associated with adenovirus-mediated gene transfer in mice complicated the assessment of the role of apoE in promoting the regression of atherosclerosis. For example, plasma apoE levels are well above the physiological level and vary throughout the period of the experiment.6,25 Moreover, the use of immunocompromised mice to prolong adenovirus-mediated apoE expression may markedly change the inflammatory reaction in atherosclerotic lesions, complicating the interpretation of the results.25 Lastly, variations in apoE expression levels among transfected mice make comparative studies difficult to control.

In another model, Apoe<sup>−/−</sup> mice are lethally irradiated and transplanted with wild-type bone marrow as a source of apoE.27 Unlike adenovirus-derived apoE, which is produced principally by the liver and promotes atherosclerosis regression in Apoe<sup>−/−</sup> mice, macrophage-derived apoE expression did not promote atherosclerosis regression in Apoe<sup>−/−</sup> mice, even though it lowered plasma lipid levels and halted atherosclerosis progression.27

Although both models resulted in lower plasma lipid levels and enrichment of lesions with apoE, only adenovirus-mediated expression of apoE led to lesion regression. These results suggest that the source of apoE is important for atherosclerosis regression, with liver-derived apoE playing a critical role in this process, rather than lesion-associated apoE.

**Figure 4.** Sirius red staining of aortic root sections reveals loss of foam cells and increased deposition of collagen after lipid lowering. A, Representative section from a control mouse showing collagen (red) surrounding areas of foam cells. Most foam cells are on the surface of the lesion (black arrow) covering a fibrotic layer rich in collagen (green arrow), but many pockets of foam cells are buried in the fibrotic component (yellow arrows). B and C, Representative section from noninduced and induced mice, respectively, shows loss of the foam cell layer and accumulation of collagen in the arterial wall and in the fibrotic component of the lesion, resulting in a fibrous cap (black arrows). Original magnification, ×20.

**Figure 5.** Regression of atherosclerosis caused by lowering plasma lipids for 16 weeks in Apoe<sup>h/hMx1-Cre</sup> mice. Lesions were assessed by staining with Sudan IV (abdominal aorta) or oil red O (aortic root). Induction of normal levels of plasma apoE enhanced the regression of lesions in both anatomic locations, P<0.01 and P=0.002, respectively.

**Figure 6.** Scatter plot analysis of percent lesion coverage in the abdominal aorta (A) and area of the aortic root (B) plotted as a function of plasma cholesterol levels. In each anatomic location, induced mice had more regression than noninduced mice; the slopes of the regression lines were not significantly different, as shown by analysis of covariance (A, P>0.35; B, P=0.96).
derived from infiltrating plasma lipoproteins or expressed by infiltrating macrophages.

However, the recently recognized role of radiation-induced injury in altering atherosclerosis phenotypes in mice, causing less atherosclerosis to develop in the thoracic aorta and more in the aortic root,\(^28\) may also affect the reversibility of lesions and account for the absence of regression in lethally irradiated Apoe\(^{-/-}\) mice transplanted with wild-type bone marrow. Lesion regression may require that macrophages upregulate expression of genes involved in cholesterol efflux, such as ATP-binding cassette transporter A-1.\(^29,30\) Egress of macrophages from the arterial intima after loss of their cholesterol stores may also be important, as was recently demonstrated in the aortic transplant mouse model of atherosclerosis regression.\(^31\) Radiation injury might have disrupted both processes, preventing foam cells from upregulating genes involved in cholesterol efflux and downregulating genes involved in egress, such as adhesion molecules. Alternatively, the regression period might have been too short,\(^27\) the concentration of apoE in the artery wall may have been too low,\(^27\) or the expression of apoE by macrophages may be required for foam cells to rapidly unload lipids and egress from atherosclerotic lesions.\(^31\) Future comparative studies of these different and complementary mouse models of atherosclerosis regression will allow clarification of the importance of the source of apoE expression for effective lesion regression in response to sustained plasma lipid lowering.

Using Apoe\(^{abh}\)Mx1-Cre mice, a model of reversible hyperlipidemia and apoE expression that is free of the limitations associated with currently used models, we found that neutral lipids responded differently to apoE-mediated regression, depending on their location within the plaque (Figures 3 and 4). In the foam cell layer immediately below the arterial endothelium, plasma lipid lowering rapidly eliminated neutral lipids irrespective of plasma apoE levels. Within the fibrotic core, however, elimination of neutral lipids, which were either associated with buried foam cells or extracellular, required normal plasma levels of apoE.

Several mechanisms may underlie the cholesterol-independent apoE-mediated removal of neutral lipids from different compartments of atherosclerotic lesions. One possibility, suggested by the plasma lipoprotein profile, is that the HDL in the induced mice are more potent at accepting cholesterol from foam cells in the lesions, promoting the reverse cholesterol transport pathway.\(^32\) A class of HDL containing exclusively apoE, called γLP-E, may play such a role.\(^33–35\) Elevated levels of plasma apoE might have also increased nitric oxide production\(^36\) in the arterial wall of the induced mice, which could have reversed endothelial dysfunction more rapidly than in noninduced mice. Alternatively, the slightly elevated levels of plasma LDL in noninduced mice may have inhibited the removal of neutral lipids from the fibrotic component of the lesions.

The enhanced regression of atherosclerotic lesions in the induced mice could also have resulted from apoE produced by macrophages that infiltrated the lesions after plasma lipid lowering\(^1\) or by the expression of apoE in endothelial cells. Induction of the Mx1-Cre transgene has been reported to recombine genes flanked by loxP sites in the marrow.\(^37,38\)

Within lesions, apoE from newly infiltrating macrophages could help reduce inflammation, endothelial dysfunction, and lipid oxidation and promote cholesterol efflux from foam cells.\(^16,39–41\) Indeed, a recent study has shown evidence for an antioxidant role of apoE in promoting the regression of atherosclerosis in the presence of hyperlipidemia.\(^45\) Although apoE can infiltrate atherosclerotic plaques from circulating lipoproteins in the plasma,\(^6\) locally derived apoE secreted by macrophages is important in controlling atherosclerosis progression even in the presence of hypercholesterolemia.\(^43,46\) Perhaps apoE-rich HDL in the plasma and macrophagederived apoE in the lesion are both required to enhance lipid efflux from foam cells trapped deep in the fibrotic core or from extracellular lipid deposited in the artery wall, whereas apoE-poor HDL alone are sufficient to enhance cholesterol efflux from foam cells that are just beneath the arterial endothelium. Because we did not investigate the effects of restored apoE expression in circulating macrophages or in lesion-associated macrophage foam cells immediately after induction, we cannot speculate on the mechanism responsible for the enhanced regression of neutral lipids.

Future studies in the Apoe\(^{abh}\)Mx1-Cre model will make it possible to study the dynamics and mechanisms of atherosclerosis regression and to identify the lipid-independent role of apoE in this process. For example, it will be possible to compare the changes in gene expression in cells of the arterial wall in response to dietary lipid lowering and restored apoE expression levels. Such studies will clarify the roles of plasma HDL,\(^47\) cholesterol efflux,\(^30,32\) apoptosis,\(^48\) lipid oxidation,\(^49,50\) and egress of foam cells from the arterial intima.\(^31\) In addition, when Apoe\(^{abh}\)Mx1-Cre expressing wild-type mouse apoE becomes available, the role of apoE4 domain interaction\(^51\) in atherosclerosis regression can be studied by comparing lesions in mice expressing either Arg-61 or wild-type mouse apoE.

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References


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