Atherosclerosis in C3H/HeJ Mice Reconstituted With Apolipoprotein E–Null Bone Marrow

Weibin Shi, Xuping Wang, Khan Tangchitpiyanond, Jack Wong, Yishou Shi, Aldons J. Lusis

Abstract—Previous studies showed that reconstitution of atherosclerosis-susceptible C57BL/6 (B6) female mice with apolipoprotein E (apoE)-deficient (apoE$^{-/-}$) bone marrow resulted in markedly increased atherosclerosis, despite the fact that plasma lipid levels were unchanged. To determine whether apoE$^{-/-}$ bone marrow would increase atherosclerosis in an atherosclerosis-resistant strain, female C3H/HeJ (C3H) mice were lethally irradiated and reconstituted with bone marrow from either C3H.apoE$^{-/-}$ mice or wild-type C3H mice. Four weeks after transplantation, the mice were fed an atherogenic diet for 12 weeks. We found that reconstitution of C3H mice with apoE$^{-/-}$ bone marrow resulted in a slight reduction in plasma apoE levels and a dramatic reduction in apoE and apolipoprotein B (apoB) in the aortic wall. Plasma apoB and cholesterol levels were unchanged, as were atherosclerotic lesions at the aortic root. These data indicate that reconstitution of C3H mice with apoE$^{-/-}$ bone marrow has no effect on atherosclerosis susceptibility and that apoE promotes accumulation of apoB in the vessel wall. **(*Arterioscler Thromb Vasc Biol*. 2002;22:650-655.)**

Key Words: atherosclerosis ■ macrophages ■ bone marrow transplantation ■ apolipoprotein E ■ mice

Apolipoprotein E (apoE), a 34-kD glycoprotein, is a structural component of chylomicron remnants, VLDLs, IDLs, and some subpopulations of HDLs.[1,2] ApoE mediates the uptake and degradation of chylomicron and VLDL remnants by acting as a ligand for the LDL receptor and the LDL receptor–related protein.[1,3] ApoE deficiency results in severe hypercholesterolemia and diffuse atherosclerotic lesions in humans[4] and gene-targeted mice.[5,6]

The liver produces the vast majority of plasma apoE, but apoE is also synthesized by macrophages in various organs.[7–9] The macrophage-produced apoE has been proposed to exert an antiatherosclerotic effect by promoting cholesterol efflux from macrophages[10,11] and reverse cholesterol transport.[12] Accordingly, low-dose transgene expression of the human apoE3 in macrophages of apoE-deficient (apoE$^{-/-}$) mice did not correct hyperlipidemia but significantly reduced the extent of atherosclerotic lesions.[13] On the other hand, reconstitution of female C57BL/6 (B6) mice with apoE$^{-/-}$ bone marrow had no influence on plasma lipid levels but markedly increased susceptibility to atherosclerosis.[11,14]

Recent studies have shown that genetic backgrounds dramatically influence the effect of the apoE$^{-/-}$ gene on atherosclerosis in mice.[15–17] When the apoE$^{-/-}$ allele of B6 mice was transferred onto the genetic background of strain FVB/NT, aortic atherosclerotic lesions of the mice were reduced up to 9-fold.[15] On the C3H background, we found that the influence was even more dramatic. On a chow diet, the size of atherosclerotic lesions in C3H.apoE$^{-/-}$ mice was reduced >100-fold compared with the lesion size in B6.apoE$^{-/-}$ mice.[17] The insensitivity of endothelial cells to oxidized LDL has been suggested to be responsible for the resistance of C3H mice to atherosclerosis.[17,18] Because endothelial responses to oxidized LDL play a crucial role in the initiation of atherosclerosis,[19,20] we postulated that apoE$^{-/-}$ bone marrow would have little influence on atherosclerotic lesion formation in those mice whose endothelial cells were not responsive to oxidized LDL. To test this possibility, in the present study, female C3H mice were transplanted with apoE$^{-/-}$ or wild-type (apoE$^{+/+}$) bone marrow, and the effect on atherosclerotic lesion formation was determined after the mice were fed an atherogenic diet for 12 weeks.

Methods

Mice

Male and female C3H mice were obtained from The Jackson Laboratories, Bar Harbor, Me. C3H.apoE$^{-/-}$ mice were generated in our laboratory by initially crossing B6.apoE$^{-/-}$ mice with C3H/HeJ mice. The resulting heterozygous apoE$^{+/-}$ mice were sequentially backcrossed to C3H mice for 10 generations, followed by brother-sister mating to generate homozygous C3H.apoE$^{-/-}$ mice. The mice were fed a standard rodent chow containing 4% fat (Ralston-Purina Co) or a high-fat high-cholesterol diet containing 15% fat, 1.25% cholesterol, and 0.5% cholic acid (TD 90221, Food-Tek, Inc). All procedures were in accordance with current National Institutes of Health guidelines and were approved by the university Animal Research Committee.

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Bone Marrow Transplantation

Female recipient C3H mice at 8 weeks of age were lethally irradiated with 10 Gy from a cobalt source. Bone marrow was harvested from male C3H.apoE$^{-/-}$ or C3H.apoE$^{+/+}$ mice by flushing femurs and tibias with DMEM containing 10% FBS and 5 U/mL heparin. Red blood cells were lysed with ACK buffer (150 mmol/L NH_4Cl, 61 mmol/L $KHCO_3$, and 1 mmol/L Na_2EDTA, pH 7.3). The remaining cells were washed and suspended in DMEM with 1% bovine albumin. Each recipient mouse was injected with 10^7 bone marrow cells in 0.3 mL through the tail veins.

Four weeks after bone marrow transplantation, mice fasted overnight were bled from retro-orbital veins under isoflurane anesthesia. After centrifugation, the plasma was collected and used for lipid analysis as indicated below. The cell pellet was lysed with the ACK buffer to remove the red blood cells. DNA was extracted from the remaining cells and used to identify engraftment by detecting the presence of the Y chromosome as descibed[21] or genotyping the apoE gene by using a protocol provided by The Jackson Laboratories. The mice that expressed donor DNA were maintained on the atherogenic diet for 12 weeks.

Plasma Lipid Measurements

Mice were fasted overnight, and blood was collected through retro-orbital veins under isoflurane anesthesia. Plasma total cholesterol, HDL cholesterol, and triglyceride levels were measured by enzymatic assays as previously described by Hedrick et al.[22]

Western Blot Analysis for ApoE and ApoB

The presence of apoE and apoB in plasma and the descending thoracic aorta was determined by Western blot analysis. The aorta was washed thoroughly with PBS containing 5 U/mL heparin and 1 mmol/L EDTA through the left ventricle of the heart, cleaned of periadventitial fat and connective tissues, and snap-frozen in liquid nitrogen. The frozen aorta was mechanically broken up, dispersed in lysis buffer containing 10 mmol/L Tris, pH 8, 1 mmol/L EDTA, 2.5% SDS, and 5% mercaptoethanol, and centrifuged at 500*g* for 10 minutes at 4°C, and the supernatant was collected and used for detection of apoE and apoB. One microliter of plasma or 10 μg of aortic protein was separated by electrophoresis on 4% to 12% Tris-polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies for mouse apoE, apoB (BioDesign International), or GAPDH (Chemicon International) for 1 hour and then incubated for 0.5 hour with horseradish peroxidase–conjugated secondary antibodies. The signals were detected by the enhanced chemiluminescence detection method according to the manufacturer's instructions (ECL Western blotting, Amersham). The density of the bands was quantified with a densitometer.

Aortic Lesion Analysis

Methods for the quantification of atherosclerotic lesions in the aorta were the same as those previously reported.[23] In brief, the heart and proximal aorta were excised and embedded in OCT compound. Serial 10-μm-thick cryosections from the middle portion of the ventricle to the aortic arch were collected and mounted on poly-D-lysine–coated slides. In the region extending from the appearance to the disappearance of the aortic valves, every other section was collected. In all other regions, every fifth section was collected. Sections were stained with oil red O and hematoxylin and counterstained with fast green, and the lesion areas were counted by light microscopy.

Immunohistochemical Analysis

The presence of macrophages and apoE in atherosclerotic lesions was determined by immunohistochemical analysis as previously described.[21] In brief, 10-μm-thick cryosections were fixed in acetone and incubated with a rat monoclonal antibody to mouse macrophages (MOMA-2, Accurate Chemicals) or a rabbit polyclonal antibody to mouse apoE, followed by incubation with biotinylated anti-rat or anti-rabbit antibodies. Signals were detected with peroxidase chromogen kits (Vector Laboratories).

Statistical Analysis

Plasma lipid levels were expressed as mean±SEM, with n indicating the number of mice. Atherosclerotic lesion areas were expressed as values of individual mice. The Student *t* test was used to compare differences between the 2 groups in atherosclerotic lesions, apolipoproteins, and plasma lipid levels. Differences were considered statistically significant at $P<0.05$.

Results

Reconstitution of Recipient Bone Marrow

Female C3H mice were lethally irradiated and transplanted with apoE$^{-/-}$ or apoE$^{+/+}$ bone marrow to examine the effect of macrophage apoE deficiency on atherosclerosis in a resistant strain. Because male mice containing XY chromosomes were used as marrow donors for female recipients, we designed primers to amplify a segment of the Y chromosome by polymerase chain reaction (PCR). As shown in Figure 1A, 4 weeks after transplantation, the 250-bp PCR product of the

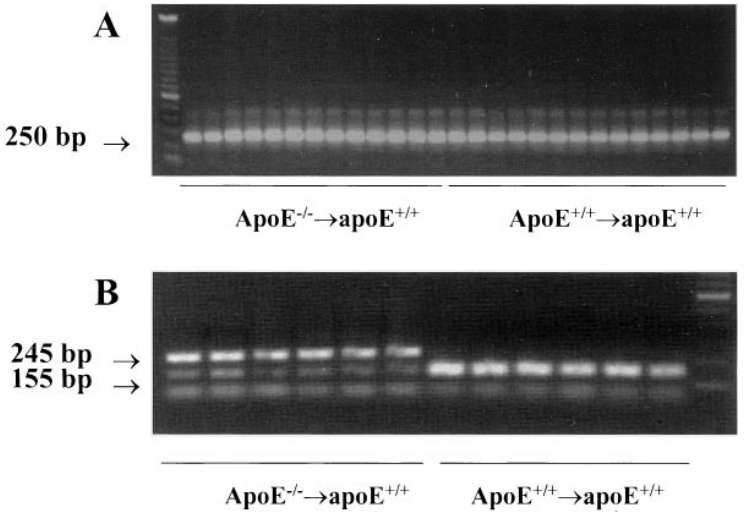


Figure 1. Reconstitution of female C3H mice with male apoE$^{-/-}$ or apoE$^{+/+}$ bone marrow. DNA was extracted from the blood of recipient mice 4 weeks after transplantation and amplified by PCR reactions. A, Presence of a 250-bp sequence of the Y chromosome. B, Presence of the 245-bp apoE-knockout band in mice transplanted with apoE$^{-/-}$ bone marrow.

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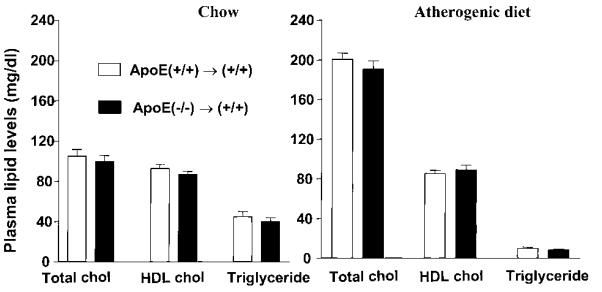


Figure 2. Plasma cholesterol and triglyceride levels of C3H mice after transplantation with apoE$^{-/-}$ or apoE$^{+/+}$ bone marrow. The mice were fasted overnight and bled 4 weeks after transplantation on a chow diet (A) and 12 weeks after initiation of the atherogenic diet (B). Values are mean±SE of 14 or 15 mice. There were no significant differences between the 2 groups.

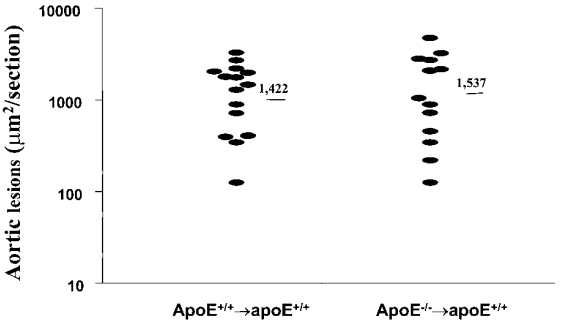


Figure 4. Atherosclerotic lesion areas in cross sections of the aortic root from C3H mice transplanted with apoE$^{-/-}$ (n=14) or apoE$^{+/+}$ (n=15) bone marrow. Each point represents a mean lesion area per section of each individual mouse. The horizontal bars represent mean lesion areas of each group. The mice were fed an atherogenic diet for 12 weeks. Cross sections of the aorta were stained with oil red O and hematoxylin, and the lipid-stained areas were measured by light microscopy.

Y chromosome was detected in the DNA extracted from the blood of recipient mice. Moreover, PRC analysis of the blood DNA showed the presence of the targeted apoE gene (the 245-bp band) in mice reconstituted with apoE$^{-/-}$ bone marrow (Figure 1B). The faint 155-bp band indicated the existence of limited host cells in the mice. In contrast, mice reconstituted with apoE$^{+/+}$ bone marrow only exhibited a 155-bp wild-type band. Those mice that expressed the donor DNA were fed the atherogenic diet for 12 weeks.

Effect of Macrophage ApoE Deficiency on Plasma Lipid Levels

Reconstitution of C3H mice with apoE$^{-/-}$ bone marrow had no significant influence on plasma cholesterol and triglyceride levels (Figure 2). Four weeks after transplantation on chow diet and 12 weeks after initiation of the atherogenic diet, apoE$^{-/-}$→apoE$^{+/+}$, and apoE$^{+/+}$→apoE$^{+/+}$ mice exhibited similar plasma levels of total cholesterol, HDL cholesterol, and triglycerides ($P>0.05$).

Effect of Macrophage ApoE Deficiency on ApoE and ApoB Levels in Plasma and Aortic Walls

ApoE and apoB in plasma and descending thoracic aortas were analyzed by Western blot analysis after the mice were fed the atherogenic diet for 12 weeks. Densitometric scanning of the bands showed that absence of the macrophage-derived apoE slightly but significantly reduced the apoE level in plasma (optical density was 1919±96 in apoE$^{-/-}$→apoE$^{+/+}$ mice versus 2307±139 in apoE$^{+/+}$→apoE$^{+/+}$ mice; $P=0.044$; Figure 3). The apoE level within the aortic wall was dramatically reduced in apoE$^{-/-}$→apoE$^{+/+}$ mice compared with apoE$^{+/+}$→apoE$^{+/+}$ mice (29±18 versus 275±75, respectively; $P=0.013$). We also determined the level of apoB in plasma and the arterial wall by Western blot analysis. The plasma apoB level did not differ significantly between apoE$^{-/-}$→apoE$^{+/+}$ and apoE$^{+/+}$→apoE$^{+/+}$ mice (83.8±11.4 versus 79.3±13.7, respectively; $P=0.81$). However, the amount of apoB was significantly lower in apoE$^{-/-}$→apoE$^{+/+}$ mice than in apoE$^{+/+}$→apoE$^{+/+}$ mice (42±13 versus 307±62, $P=0.003$); in contrast, the level of GADPH in the aorta was not significantly different between the 2 groups (315±52 versus 291±24, respectively; $P=0.69$).

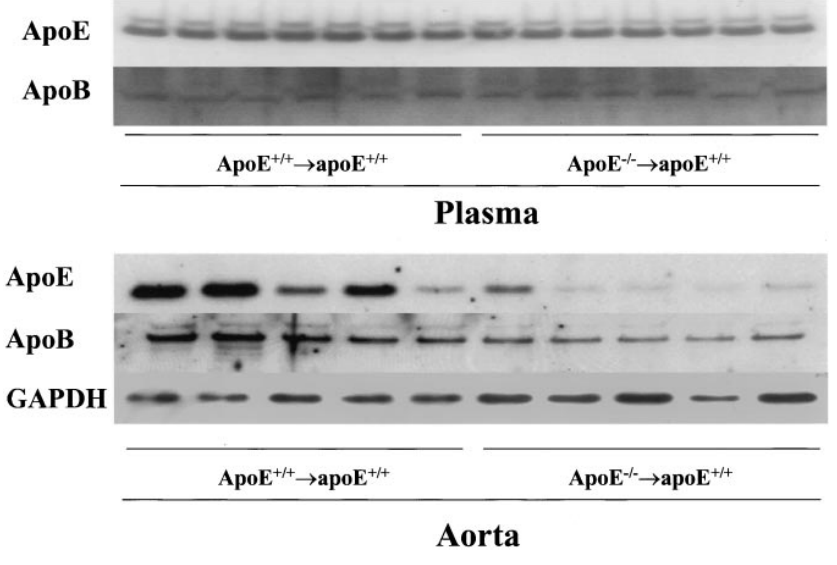


Figure 3. Western blot analysis of apoE and apoB in plasma and of apoE, apoB, and GAPDH in descending thoracic aortic walls of C3H mice transplanted with apoE$^{-/-}$ or apoE$^{+/+}$ bone marrow after 12 weeks on the atherogenic diet. One microliter of plasma or 10 μg of protein from the aorta was electrophoresed on Tris-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with antibodies for the proteins.

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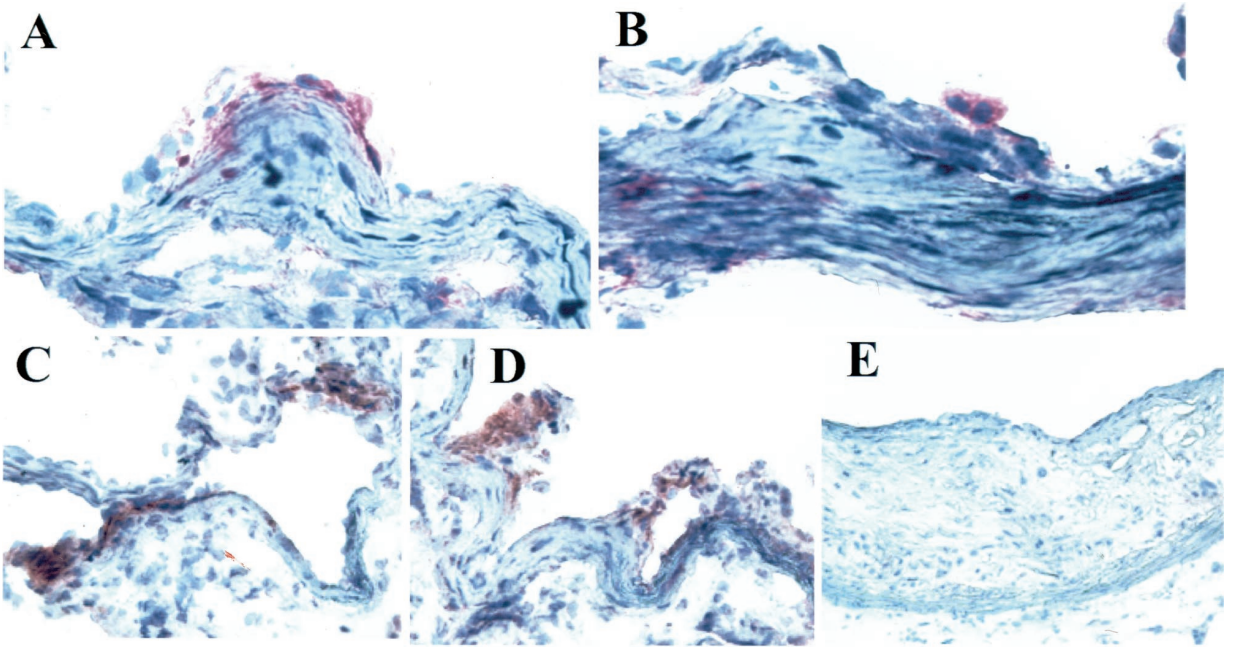


Figure 5. Representative light photographs of immunohistochemical analysis of macrophages and apoE in aortic atherosclerotic lesions of C3H mice transplanted with apoE$^{-/-}$ or apoE$^{+/+}$ bone marrow. Sections were stained with anti-mouse macrophage antibody MOMA-2 (A and B) or an anti-mouse apoE antibody (C, D, and E). A and C, Sections from mice transplanted with apoE$^{-/-}$ bone marrow. B and D, Sections from mice transplanted with apoE$^{+/+}$ bone marrow. E, Negative control from an apoE$^{-/-}$ mouse. Original magnification ×40.

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Effect on Aortic Atherosclerotic Lesions

After being fed the atherogenic diet for 12 weeks, the mice were euthanized, and the size of atherosclerotic lesions at the aortic root was quantified by light microscopy. The mean areas of aortic lesions in apoE$^{-/-}$→apoE$^{+/+}$ and apoE$^{+/+}$→apoE$^{+/+}$ mice were 1537±383 μm^2 per section (n=14) and 1422±242 μm^2 per section (n=15), respectively (Figure 4). The difference in lesion areas between the 2 groups was not significant (P=0.80). Immunostaining with MOMA-2 indicated that macrophage-derived foam cells were present in the lesions (Figure 5A and 5B). Because of the small size of atherosclerotic lesions (<0.04 mm in diameter), we could not obtain enough tissues to accurately quantify apoE by Western blot analysis. ApoE expression in atherosclerotic lesions was examined by immunostaining. Atherosclerotic lesions of apoE$^{-/-}$→apoE$^{+/+}$ mice and of apoE$^{+/+}$→apoE$^{+/+}$ mice were intensely stained with the anti-apoE antibody (Figure 5). Because of the semiquantitative nature, immunostaining failed to show differences in the amount of apoE in the lesions.

Discussion

Fazio et al[14] and Van Eck et al[11] showed that transplantation of apoE$^{-/-}$ bone marrow into female B6 mice markedly increased the susceptibility of the mice to diet-induced atherosclerosis in the absence of a significant influence from plasma lipid levels. We have now examined whether transplantation of apoE$^{-/-}$ bone marrow into female C3H mice would increase the susceptibility to diet-induced atherosclerosis in an atherosclerosis-resistant strain. The results showed that the susceptibility of C3H mice to atherosclerosis was not altered by reconstitution with apoE$^{-/-}$ hematopoietic cells.

Bone marrow transplantation leads to replacement of recipient bone marrow and bone marrow–derived cells, including monocytes/macrophages with the donor genotype.[24] In our present study, we confirmed reconstitution of recipient bone marrow by genotyping a segment of the Y chromosome from donor cells. In those mice transplanted with apoE$^{-/-}$ bone marrow, we confirmed the presence of the mutant apoE gene in peripheral blood. These recipient mice also exhibited a faint 155-bp band, which indicates the existence of host cells in the blood. Previous studies have indicated that most of the residual host cells are radiation-resistant long-lived lymphocytes.[25,26] In the present study, we observed that reconstitution with apoE-deficient macrophages resulted in a statistically significant reduction in plasma apoE levels. This finding indicates that macrophages are an important source of plasma apoE, although most of plasma apoE is synthesized by the liver.[11,27] In the descending thoracic aorta, a region that had no atherosclerosis, we observed a dramatic reduction in apoE expression. The lowered plasma apoE level probably contributed to the apoE reduction in the arterial wall. In a recent study, Tsukamoto et al[28] provided evidence that plasma apoE diffuses into vessel walls, inasmuch as expression of the human apoE by the liver but not by macrophages results in substantial apoE retention in the arterial wall of apoE$^{-/-}$ mice. Fazio et al[14] observed apoE mRNA expression in the aorta, which was reduced by the transplantation with apoE$^{-/-}$ bone marrow. Thus, a lower local production may also contribute to the dramatic reduction of apoE in the aortic wall.

ApoE has been proposed to promote reverse cholesterol transport from the arterial wall.[12] However, in those aortas that had a lowered apoE level, we observed a significant reduction in apoB. The apoB reduction in the arterial wall was not due to changes in plasma apoB levels, inasmuch as the 2 groups had a similar plasma apoB level. The connections between apoE and apoB reductions are unknown. However, it is known that apoE binds to cell-surface heparan sulfate proteoglycans in vitro[29] and is colocalized with apoB and proteoglycans in atherosclerotic lesions.[30] Thus, the above findings, together with our present observations, suggest that apoE mediates the binding of apoB to the extracellular matrix. Also, it is known that apoE possesses antioxidant properties,[31,32] which may inhibit LDL oxidation. The oxidized form of LDL can be recognized by scavenger receptors on endothelial cells and macrophages and, subsequently, be taken up and degraded. In those vessels with a lower level of apoE, the accumulated LDL may be readily oxidized and quickly cleared by endothelial cells or macrophages.

In the present study, we observed that absence of the macrophage-derived apoE had no influence on plasma lipid levels of C3H mice. This finding was consistent with previous observations of B6 mice.[11,14] ApoE in the plasma of a normal mouse is far above the level required to maintain normal plasma lipid levels. Indeed, bone marrow transplantation studies in apoE$^{-/-}$ mice have indicated that a small percentage (10%) of the total plasma apoE in normal mice is sufficient to maintain normal plasma lipid levels.[21,27]

ApoE$^{-/-}$ bone marrow has been shown to markedly increase susceptibility of female B6 mice to atherosclerosis, independent of influences on plasma lipid and apoE levels.[11,14] In contrast, we found that apoE$^{-/-}$ bone marrow had no influence on the susceptibility of female C3H mice to atherosclerosis. Lipid accumulation in the arterial wall is unlikely to account for the different alterations of the 2 strains in susceptibility to atherosclerosis, inasmuch as the mice with the decreased level of proatherogenic lipoproteins in the vessel wall did not exhibit a reduction in lesion formation. Recently, we found that endothelial cells isolated from the aorta of B6 mice exhibited a dramatic induction of monocyte chemotactic protein-1, macrophage colony–stimulating factor, vascular adhesion molecule-1, and heme oxygenase-1 in response to minimally modified LDL, whereas endothelial cells from C3H mice showed little induction.[17,18] LDL oxidation and inflammatory gene induction have been considered to play a key role in atherogenesis.[19,20] Indeed, monocyte chemotactic protein-1, macrophage colony–stimulating factor, and vascular adhesion molecule-1 are primarily associated with the recruitment and differentiation of monocytes. Therefore, if endothelial cells of C3H mice were unable to recruit monocytes to the artery wall and promote their differentiation into macrophages, it might be expected that atherogenic monocytes would have no chance to exert their effect on atherosclerosis. Although there are macrophages in atherosclerotic lesions of C3H mice, the number is much smaller than the number in the lesions of B6 mice.[23]

Plasma HDL levels of recipient mice on an atherogenic diet could influence the effect of apoE$^{-/-}$ macrophages on atherosclerosis. Hayek et al[33] reported that apoE on HDL rather than apoE within the macrophage was responsible for cholesterol efflux. On the atherogenic diet, B6 mice but not C3H mice exhibit a significant reduction in plasma HDL levels. The decreased plasma HDL levels would result in a reduction of HDL-associated apoE in the arterial wall, which could lead to impaired cholesterol release from macrophages. In contrast, in C3H mice, because there is no reduction in plasma HDL levels, HDL-associated apoE in the arterial wall would remain relatively stable; thus, cholesterol release from macrophages could remain unaffected.

In a recent study, Boisvert and Curtiss[34] reported that elimination of macrophage-derived apoE reduced atherosclerosis in male B6 recipient mice. The reasons for the conflicting data between male and female mice are unknown. One plausible explanation is that male B6 mice, which are resistant to atherosclerosis (10-fold more resistant, regarding the size of the atherosclerotic lesion), could be resistant to the proatherogenic effect of apoE$^{-/-}$ bone marrow. Another explanation is that mice in the studies of Fazio et al[14] and Von Eck et al[11] were fed the atherogenic diet for $<$13 weeks, whereas mice in the study of Boisvert and Curtiss were fed the atherogenic diet for 16 weeks. Male B6 mice develop gallstones more readily than do their female counterparts when fed the atherogenic diet.[35] Gallstones can markedly elevate plasma lipid levels and, consequently, influence atherosclerotic lesion formation.

The present study examined the physiological effect of native apoE expression by macrophages on cholesterol metabolism and early atherogenesis in the C3H strain. The results clearly indicate that macrophage-derived apoE promotes the accumulation of apoB in the arterial wall, suggesting that apoE may have proatherogenic as well as antiatherogenic properties. Although apoE$^{-/-}$ bone marrow has been shown to increase the susceptibility of female B6 mice to diet-induced atherosclerosis, it had no effect on the susceptibility of female C3H mice to atherosclerosis. The present study provides further evidence that genetic background modulates the effect of apoE$^{-/-}$ bone marrow on atherosclerosis.

Acknowledgments

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