

Physiological expression of macrophage apoE in the artery wall reduces atherosclerosis in severely hyperlipidemic mice

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Abstract We have previously reported that the introduction of macrophage apoE into mice lacking both apoE and the LDL receptor (apoE^{-/-}/LDLR^{-/-}) through bone marrow transplantation (apoE^{+/+}/LDLR^{-/-}→apoE^{-/-}/LDLR^{-/-}) produces progressive accumulation of apoE in plasma without affecting lipid levels. This model provides a tool to study the effects of physiologically regulated amounts of macrophage apoE on atherogenesis in hyperlipidemic animals. Ten-week-old male apoE^{-/-}/LDLR^{-/-} mice were transplanted with either apoE^{+/+}/LDLR^{-/-} (n = 11) or apoE^{-/-}/LDLR^{-/-} (n = 14) marrow. Although there were no differences between the two groups in lipid levels at baseline or at 5 and 9 weeks after transplantation, apoE levels in the apoE^{+/+}/LDLR^{-/-}→apoE^{-/-}/LDLR^{-/-} mice increased to 4 times the apoE levels of normal mice. This resulted in a 60% decrease in aortic atherosclerosis in the apoE^{+/+}/LDLR^{-/-}→apoE^{-/-}/LDLR^{-/-} compared with the apoE^{-/-}/LDLR^{-/-}→apoE^{-/-}/LDLR^{-/-} controls, (15,957 ± 1907 vs. 40,115 ± 8302 μm² ± SEM, respectively). In a separate experiment, apoE^{+/+}/LDLR^{-/-} mice were transplanted with either apoE^{+/+}/LDLR^{-/-} or apoE^{-/-}/LDLR^{-/-} marrow and placed on a high-fat diet for 8 weeks. In the absence of macrophage apoE, lesion area was increased by 75% in the aortic sinus and by 56% in the distal aorta. These data show that physiologic levels of macrophage apoE in the vessel wall are anti-atherogenic in conditions of severe hyperlipidemia and can affect later stages of plaque development.—Fazio, S., V. R. Babaev, M. E. Burleigh, A. S. Major, A. H. Hasty, and M. F. Linton. Physiologic expression of macrophage apoE in the artery wall reduces atherosclerosis in severely hyperlipidemic mice. *J. Lipid Res.* 2002. 43: 1602–1609.

Supplementary key words apolipoprotein E • low density lipoprotein receptor • hyperlipidemia • bone marrow transplant

Although apolipoprotein E (apoE) of macrophage origin is fully functional in its ability to induce the clear-

ance of plasma lipoproteins, substantial evidence supports the concept of a direct anti-atherogenic effect of apoE in the arterial wall. Strong support of a direct effect of apoE in the artery wall comes from bone marrow transplantation studies (1, 2). We have reported that the lack of macrophage apoE in C57BL/6 mice increases the size of diet-induced arterial lesions by 10-fold in the absence of differences in plasma lipoprotein levels (3). We have also shown that apoE^{-/-} macrophages increase lesion size in apoA-I deficient and apoA-I over-expressing mice (4). In addition, using a retroviral transduction system, we have shown that low level expression of apoE from macrophages has a strong anti-atherogenic effect during foam cell lesion formation, but this effect was lost when the plaques grew larger and more complex, an indication that the protective capacity of apoE is eventually overwhelmed by the severe hyperlipidemia of apoE deficiency (5). Evidence in favor of an anti-atherogenic role of apoE also comes from studies showing that low level expression of human apoE-3 in the arterial wall of apoE^{-/-} mice was accompanied by decreased growth of lesions without affecting plasma lipids (6), and that the expression of low levels of human apoE-3 in the macrophages of apoE^{-/-} mice resulted in decreased atherogenesis independent of a decrease in plasma cholesterol (7). Interestingly, recent studies from our laboratory have shown that retroviral transfer of receptor-binding defective mutant forms of human apoE, such as apoE-2 and apoE-cys142, do not protect apoE null mice against atherosclerosis development, suggesting that the local function of apoE is linked to its ability to bind receptors

Abbreviations: BMT, bone marrow transplant; HSPG, heparan sulfate proteoglycans; LDLR, low density lipoprotein receptor; LXR, liver X receptor; TNF α , tumor necrosis factor α .

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or proteoglycans (8). Although these studies together provide indisputable evidence of the anti-atherogenic action of macrophage apoE, they had the limitation of using sub-physiological apoE expression levels to avoid any influence of this protein on plasma lipoprotein clearance. Thus, the full physiological potential of macrophage apoE in opposing hyperlipidemia-induced atherosclerosis remains to be studied.

For this reason, we studied the effect of physiological expression of macrophage apoE in a model that is unresponsive to the cholesterol-lowering effects of apoE, the apoE^{+/+}/low density lipoprotein receptor (LDLR)^{-/-}→apoE^{-/-}/LDLR^{-/-} mouse. In two separate experiments, we developed LDLR^{-/-} mice with the expression of apoE either limited to the macrophage or deleted exclusively from the macrophage. The value of these models lies in the ability to analyze the effects of physiological expression of macrophage apoE in a “cholesterol clamp” environment, given that in either system the severe hyperlipidemia is not affected by macrophage apoE. In addition, the apoE production from these macrophages comes from the natural gene in the natural genomic position, and responds to physiological regulatory stimuli. Our studies show that the LDLR is not essential for the anti-atherogenic effects of apoE in the artery wall, and provide conclusive evidence that macrophage apoE partially overrides the atherogenic influences of the massive hyperlipidemia induced by the combined deficiency of apoE and the LDLR.

MATERIALS AND METHODS

Animal procedures

ApoE^{+/+}/LDLR^{-/-} and apoE^{-/-}/LDLR^{-/-} mice, both at the tenth backcross into the C57Bl/6 background, were originally purchased from the Jackson Laboratories (Bar Harbor, ME) and were maintained on rodent chow diet. Western diet given to apoE^{+/+}/LDLR^{-/-} bone marrow transplant (BMT) mice was purchased from Harlan-Tekland and contained 21% fat and 4.5% cholesterol. Mice were maintained in microisolator cages on a rodent chow diet (PMI No 5010). All mice were given autoclaved acidified (pH 2.6) water. Animal care and experimental procedures were performed according to the regulations of the Institutional Animal Care and Usage Committee of Vanderbilt University.

Experimental design

Two separate BMT experiments were designed as follows: 1) Ten-week-old male recipient apoE^{-/-}/LDLR^{-/-} mice were transplanted with bone marrow from apoE^{-/-}/LDLR^{-/-} (control) or apoE^{+/+}/LDLR^{-/-} donors. Blood samples were collected for lipid analysis at 1 week pre-BMT and at 5 and 9 weeks post-BMT. Animals were sacrificed at 9 weeks post-BMT for lesion analysis. 2) Ten-week-old female apoE^{+/+}/LDLR^{-/-} mice were transplanted with either apoE^{+/+}/LDLR^{-/-} (control) or apoE^{-/-}/LDLR^{-/-} marrow. Four weeks after transplantation, mice were placed on a Western diet. Blood samples were collected at 4 weeks post-BMT, before initiation of the diet, and at 4 and 8 weeks post-diet (8 and 12 weeks post-BMT). Mice were sacrificed after 8 weeks on the diet for lesion area quantitation. Transplanted mice are designated as donor→recipient.

Bone marrow collection and transplantation

Bone marrow was collected from 6-week-old apoE^{-/-}/LDLR^{-/-} and apoE^{+/+}/LDLR^{-/-} donor mice by flushing femurs and tibias using RPMI-1640 media with 2% FBS and 5 U/ml heparin (Sigma), as previously described (9). One week prior to and 2 weeks following transplantation, all recipient mice were given 100 mg/l neomycin and 10 mg/l polymyxin B sulfate (Sigma) in acidified water. Recipient mice were lethally irradiated (9 Gy) from a cesium γ source. Four hours later, the mice received 5×10^6 bone marrow cells in 300 μ l RPMI-1640 media by tail vein injection.

Serum cholesterol and triglyceride analysis

Fasted blood samples were collected by retro-orbital venous plexus puncture and the concentrations of total cholesterol and triglycerides were determined as previously described (5) using Raichem reagent #80015VI and Sigma Reagent No. 339 adapted for microtiter plate assay, respectively.

Lipoprotein separation

Fast performance liquid chromatography analyses using a Superose 6 column from Pharmacia on an HPLC system model 600 (Waters) were conducted on serum samples as previously described (9). Fractions 15–18 contain VLDL; fractions 19–26 contain LDL and IDL; fractions 27–31 contain HDL; and fractions 32–40 contain non-lipoprotein associated proteins.

Western blot analysis of apoE

Serum samples were separated by electrophoresis on 12% SDS polyacrylamide gels, and proteins were transferred from the gel to a nitrocellulose membrane (Gelman). The membranes were incubated with rabbit anti-mouse apoE antibodies (BioDesign International) and then with HRP-conjugated goat anti-rabbit IgG antibody (Amersham). Signal was detected using the ECL kit according to manufacturer's instructions (Amersham).

Quantitation of arterial lesions

Mice were sacrificed and the aorta was flushed with 20 ml cold PBS by slow injection through the left ventricle. The heart with the proximal aorta was embedded in OCT and snap-frozen in liquid N₂. Cryosections of 10- μ m thickness were taken from the region of the proximal aorta starting from the end of the aortic sinus and for 300 μ m distally, according to the method of Paigen et al. (10). Cryosections were stained with Oil Red O and counter-stained with hematoxylin. The images of the aorta were captured with a frame grabber (Vers.3.1 Kontron Elektronik), using a color video camera mounted on an Axioskop micro-

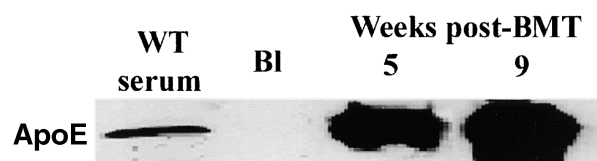


Fig. 1. Immunoblot analysis for apolipoprotein E (apoE) in serum of apoE^{-/-}/low density lipoprotein receptor (LDLR)^{-/-} mice transplanted with apoE^{+/+}/LDLR^{-/-} bone marrow. Immunoblot analysis was performed as described in the Materials and Methods section. Lane 1 contains undiluted normal serum of C57BL/6 mouse. Lane 2 contains the serum of an apoE^{-/-}/LDLR^{-/-} mouse before transplantation. Lanes 3 and 4 contain the serum of apoE^{-/-}/LDLR^{-/-} mouse reconstituted with apoE^{+/+}/LDLR^{-/-} marrow 5 and 9 weeks after transplantation.

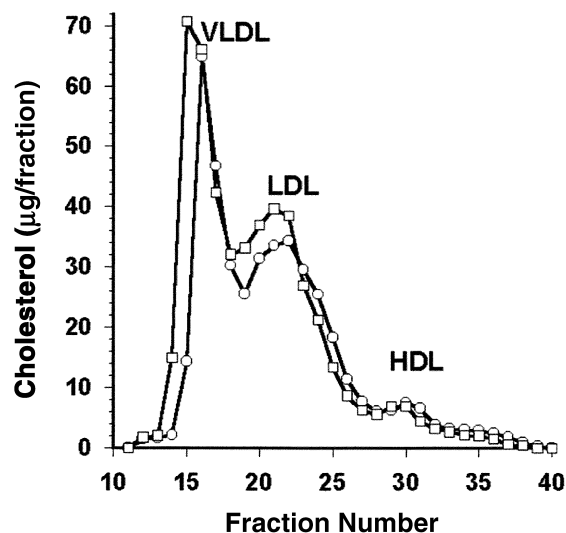


Fig. 2. Lipoprotein distribution in apoE^{-/-}/LDLR^{-/-} mice transplanted with apoE^{-/-}/LDLR^{-/-} or apoE^{+/+}/LDLR^{-/-} marrow (5 weeks after transplantation). Mice were fasted for 4 h, serum collected, pooled, and separated by fast performance liquid chromatography. The cholesterol content of each fraction was assayed for according to the Materials and Methods section. Data are present in µg per fraction. Fractions 15–18 contain VLDL; fractions 19–26 are IDL/LDL; and fractions 27–31 contain HDL. Fractions 32–40 are the bottom non-lipoprotein associated proteins. Open circles indicate the lipoprotein profile of an apoE^{-/-}/LDLR^{-/-} → apoE^{-/-}/LDLR^{-/-} mouse and open squares represent an apoE^{+/+}/LDLR^{-/-} → apoE^{-/-}/LDLR^{-/-} mouse.

scope (Carl Zeiss Inc). Quantitative analysis of lipid-stained lesions was performed using an Imaging system KS300 (Release 2.0, Kontron Elektronik). En face analysis was performed as previously described (11, 12).

Immunocytochemistry

After perfusing mice, liver and spleen were fixed in 4% paraformaldehyde in PBS for 2 days at 4°C and embedded in paraffin. Serial 5-µm liver paraffin sections and cryosections of the proximal aorta were stained for apoE and macrophages as described (13). The sections were pre-treated for 30 min with proteinase K (50 µg/ml; Promega) and 20 min with 10% normal goat serum, then incubated overnight at 4°C with either rabbit antisera to mouse apoE (BioDesign) or with monoclonal rat antibody MOMA-2 (Accurate Chemical and Scientific Corp.). The sections were treated with goat biotinylated antibodies to chicken IgG (Vector Laboratories) or to rat IgG (PharMingen)

for 45 min at 37°C. Then sections were incubated with avidin-biotin complex labeled with alkaline phosphatase (Vector Lab.). Enzyme was visualized with Fast Red TR/Naphthol AS-NX substrate (Sigma Chemical Co.) and counter-stained with hematoxylin. Non-immune rabbit or rat serum in the place of primary antibody was used as a negative control.

In situ hybridization

ApoE mRNA message was detected essentially as described (3). Briefly, ³⁵S-labeled 104-bp antisense and sense riboprobes for mouse apoE were prepared. Cryosection of the proximal aorta and paraffin sections of liver and spleen were treated for 15 min with proteinase K (5 µg/ml), and incubated with riboprobes in hybridization solution (0.3M NaCl, 20 mM Tris, pH 8.0, 5 mM EDTA, 1× Denhardt's solution, 10 mM dithiothreitol, 10% dextran sulfate, 50% formamide) overnight at 55°C. The sections were treated for 30 min with RNase A (20 µg/ml), washed, coated with autoradiographic emulsion (Kodak NTB-2), and exposed for 2–3 weeks. After development, the slides were counter-stained with hematoxylin. The sense probe was used in parallel as a negative control.

Statistical analyses

Statistical analyses were performed using the Student's *t*-test.

RESULTS

The first set of BMT experiments was performed in apoE^{-/-}/LDLR^{-/-} mice. Ten-week old-male mice were lethally irradiated and reconstituted with bone marrow from either apoE^{+/+}/LDLR^{-/-} or apoE^{-/-}/LDLR^{-/-} donor mice, thus the experimental LDLR^{-/-} animals expressed apoE from macrophages while control LDLR^{-/-} animals were completely apoE deficient. As expected from our previous studies (13), the transplantation of wild-type marrow into the double knockout recipients resulted in the accumulation of large amounts of apoE in plasma, up to 10-fold above wild-type levels (Fig. 1). It should be noted that the transplanted mice have a severe hyperlipidemia compared with the wild-type control mouse, thus the amount of apoE per lipoprotein particle is only slightly higher in these mice than in wild-type mice, as we previously reported (13). Despite the accumulation of apoE in plasma of the apoE^{+/+}/LDLR^{-/-} → apoE^{-/-}/LDLR^{-/-}, plasma lipid levels and lipoprotein profiles were not different between experimental and control mice (Fig. 2 and Table 1).

In order to determine the localization of apoE, immu-

TABLE 1. Total serum cholesterol and triglyceride levels in male apoE^{-/-}/LDLR^{-/-} mice before and after transplantation with apoE^{-/-}/LDLR^{-/-} or apoE^{+/+}/LDLR^{-/-} bone marrow

Group	n	Serum Lipid	Baseline Pre-BMT	5 Weeks Post-BMT	9 Weeks Post-BMT
apoE ^{+/+} /LDLR ^{-/-} → apoE ^{-/-} /LDLR ^{-/-}	11	Cholesterol	685 ± 28	532 ± 34	481 ± 45
		Triglycerides	189 ± 22	161 ± 14	154 ± 17
apoE ^{-/-} /LDLR ^{-/-} → apoE ^{-/-} /LDLR ^{-/-}	14	Cholesterol	666 ± 39	505 ± 26	523 ± 36
		Triglycerides	188 ± 19	194 ± 19	171 ± 16

Values are in mg/dl (mean ± SEM). The number of animals in each group is indicated by n. Differences between groups were not statistically significant at any time point. BMT, bone marrow transplant; LDLR, low density lipoprotein receptor.

ApoE^{-/-} → ApoE^{-/-}/LDLR^{-/-} ApoE^{+/+} → ApoE^{-/-}/LDLR^{-/-}

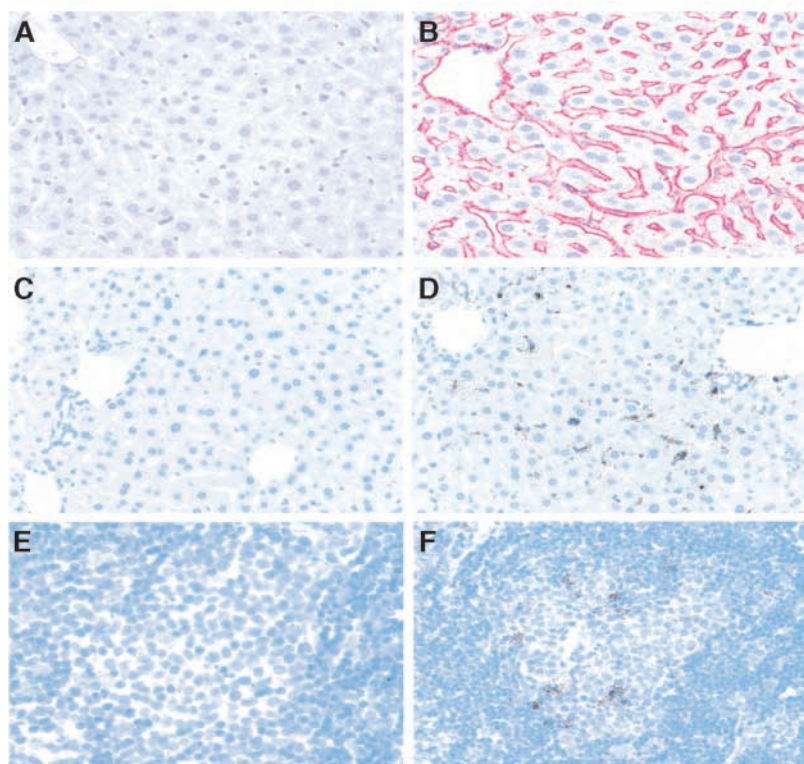


Fig. 3. Detection of apoE protein and mRNA in liver and spleen of transplanted mice. A, C, and E are tissue samples from apoE^{-/-}/LDLR^{-/-} → apoE^{-/-}/LDLR^{-/-} and B, D, and F are from apoE^{+/+}/LDLR^{-/-} → apoE^{-/-}/LDLR^{-/-}. A and B are liver sections stained for apoE as described in the Materials and Methods section. Mouse apoE mRNA level was detected in both liver (C and D) and spleen (E and F) by in situ hybridization technique as described in the Materials and Methods section. Magnification: A–D ×40; E and F ×20.

nocytochemical analyses of liver sections revealed an abundant presence of apoE in the space of Disse of apoE^{+/+}/LDLR^{-/-} → apoE^{-/-}/LDLR^{-/-} mice (Fig. 3A). In situ hybridization, however, showed apoE mRNA in a small percentage of non-parenchymal cells in liver and in numerous cells in the spleen (Fig. 3C, E). Control apoE^{-/-}/LDLR^{-/-} → apoE^{-/-}/LDLR^{-/-} sections showed no evidence of apoE protein or mRNA in either tissue (Fig. 3B, D, F). In agreement with our previous report (13), hepatic apoE was exclusively found in the extra-cellular space between columns of hepatocytes and the endothelial lining of acinar capillaries. This is most likely due to the inability of extra-hepatic apoE to activate lipoprotein uptake and clearance in mice lacking both hepatic apoE and LDLR expression.

The proximal aorta showed a 60% reduction in atherosclerotic lesion area in the mice that received apoE^{+/+}/LDLR^{-/-} marrow (15,957 ± 1907 μm²/section) compared with those that received apoE^{-/-}/LDLR^{-/-} marrow (40,115 ± 8302 μm²/section, Fig. 4). Immunocytochemistry studies of aortic sections demonstrated the presence of apoE in and around macrophage clusters only in the mice receiving apoE^{+/+}/LDLR^{-/-} marrow (Fig. 5A–D). In addition, apoE mRNA was abundantly expressed in the artery wall of apoE^{+/+}/LDLR^{-/-} → apoE^{-/-}/LDLR^{-/-} mice but not controls (Fig. 5E–F).

In a different set of experiments, female apoE^{+/+}/LDLR^{-/-} mice were transplanted with bone marrow from either apoE^{+/+}/LDLR^{-/-} or apoE^{-/-}/LDLR^{-/-} donor mice. In this case, hepatic and systemic apoE production

was normal, and the deletion of macrophage apoE did not influence serum apoE or lipid levels. Four weeks after transplantation the mice were placed on a Western-type diet until the end of the experiment (8 weeks). The

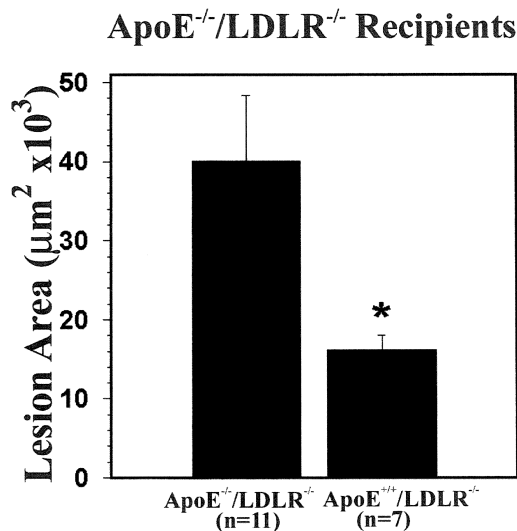


Fig. 4. Quantification of atherosclerotic lesion area in the proximal aorta of apoE^{-/-}/LDLR^{-/-} mice transplanted with apoE^{-/-}/LDLR^{-/-} or apoE^{+/+}/LDLR^{-/-} bone marrow. The atherosclerotic lesions were quantified 9 weeks post-bone marrow transplant (BMT) using Oil-Red O-stained sections and computer-assisted video imaging according to the Materials and Methods section. Data represent the average of mean (μm² ± SEM) lesion area per group measured in 15 sections per mouse.

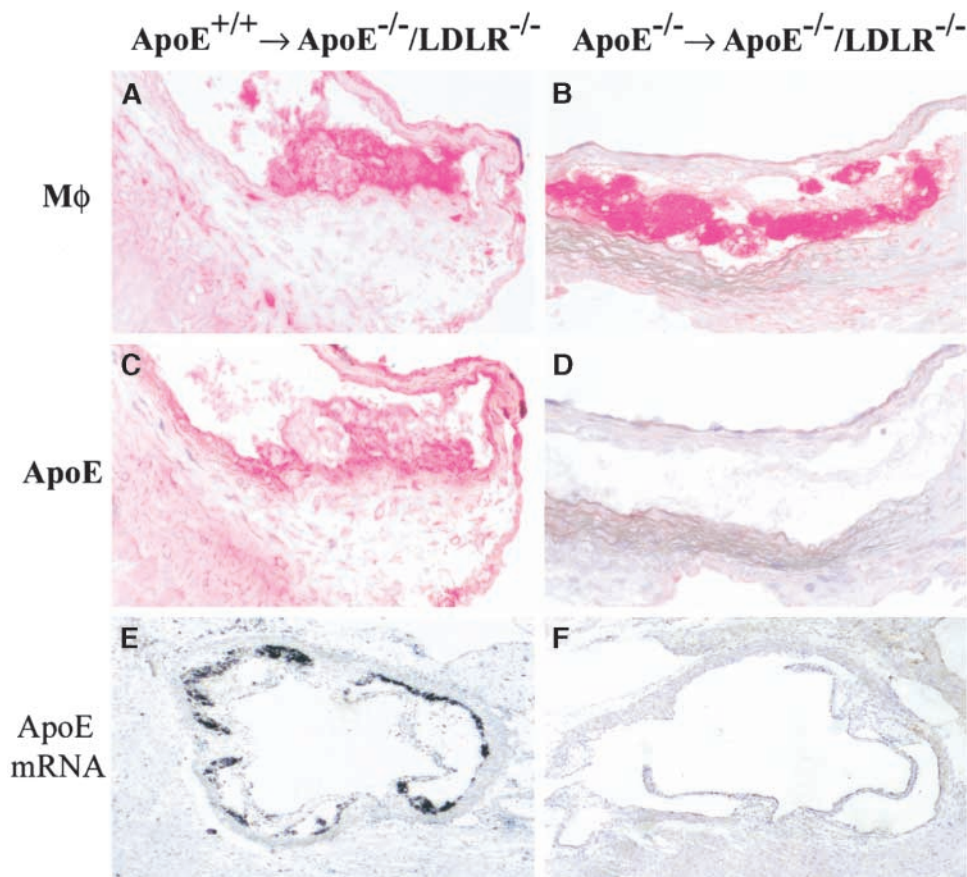


Fig. 5. Detection of apoE protein and mRNA message in the proximal aorta of transplanted mice. A–D are aortic sections stained by immunocytochemistry for apoE as described in Materials and Methods. Note that the apoE expression is detectable in the apoE^{+/+}/LDLR^{-/-}→apoE^{-/-}/LDLR^{-/-} mouse and co-localizes with markers for macrophages. Proximal aorta sections in panels E and F were treated by in situ hybridization for the detection of apoE mRNA expression. Note a high level of apoE mRNA expression in atherosclerotic lesions of aorta of the apoE^{+/+}/LDLR^{-/-}→apoE^{-/-}/LDLR^{-/-} mouse. Magnification: A–D ×63 and E, F ×20.

atherogenic diet induced increases in serum cholesterol and triglyceride levels in both mouse groups, without any significant differences at any time point between groups (Table 2). Lipoprotein profiles remained unchanged between groups over the course of the study (data not shown). As expected, apoE was produced by arterial macrophages in the apoE^{+/+}/LDLR^{-/-}→apoE^{+/+}/LDLR^{-/-} but not in the apoE^{-/-}/LDLR^{-/-}→apoE^{+/+}/LDLR^{-/-} mice, and there were no detectable differences in plasma apoE levels (data not shown). In agreement with our previous results in C57Bl/6 mice (3), the absence of apoE in-

creased lesion area by 75% in the aortic sinus (Fig. 6A) and by 56% in the distal aorta (Fig. 6B).

DISCUSSION

In this paper, we report that macrophage-derived apoE has direct protective effects on atherosclerotic lesion formation in mice that do not express the LDLR and in plaques that are more advanced than foam cell lesions. Using two separate models, we have shown that: 1) physio-

TABLE 2. Plasma cholesterol and triglyceride levels from apoE^{+/+}/LDLR^{-/-} mice transplanted with apoE^{-/-}/LDLR^{-/-} or apoE^{+/+}/LDLR^{-/-} marrow and placed on a Western-type diet for 8 weeks

Group	n	Serum Lipid	Baseline Post-BMT	4 Weeks Post-Diet	8 Weeks Post-Diet
apoE ^{+/+} /LDLR ^{-/-} → apoE ^{+/+} /LDLR ^{-/-}	12	Cholesterol	323 ± 28	624 ± 141	789 ± 76
		Triglycerides	124 ± 42	310 ± 32	317 ± 23
apoE ^{-/-} /LDLR ^{-/-} → apoE ^{+/+} /LDLR ^{-/-}	12	Cholesterol	310 ± 39	611 ± 117	724 ± 86
		Triglycerides	125 ± 35	344 ± 42	322 ± 39

Values are in mg/dl (mean ± SEM). The number of animals in each group is indicated by n. Differences between groups were not statistically significant at any time point.

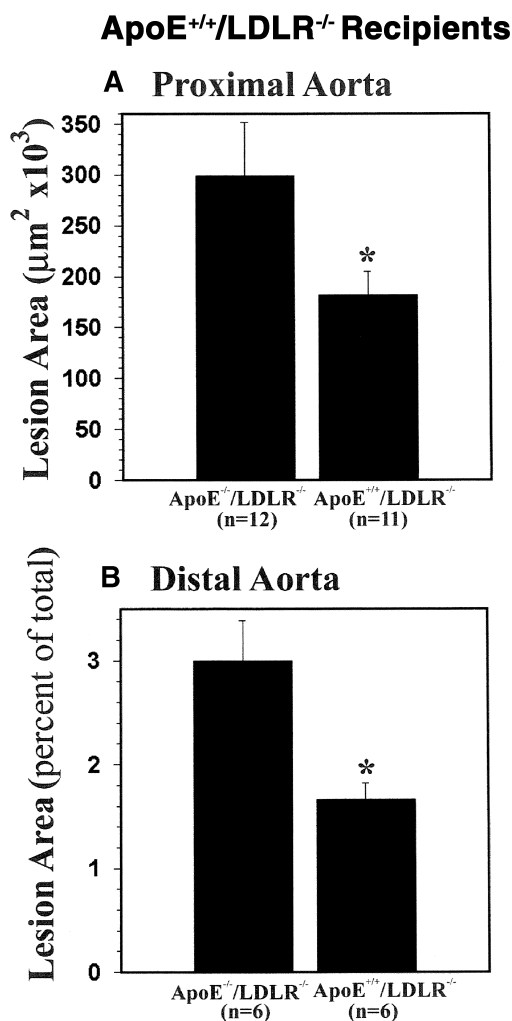


Fig. 6. Quantification of atherosclerotic lesion area in the proximal and distal aorta of apoE^{+/+}/LDLR^{-/-} mice transplanted with apoE^{-/-}/LDLR^{-/-} or apoE^{+/+}/LDLR^{-/-} bone marrow. A: The atherosclerotic lesions were quantified after 8 weeks of feeding a Western-type diet. Data represent the average of mean ($\mu\text{m}^2 \pm \text{SEM}$) lesion area per group measured in 15 sections per mouse. The atherosclerotic lesions were quantified after 8 weeks of feeding a Western-type diet. B: En face lesion area quantitation was performed as described in the Materials and Methods section. Lesion area is presented as a percent of the total aortic area. Error bars represent SEM. * $P < 0.01$.

logical levels of macrophage-derived apoE in the hypercholesterolemic environment of the double mutant apoE^{-/-}/LDLR^{-/-} mouse model significantly reduce atherosclerosis; and 2) the exclusive deletion of macrophage-apoE in LDLR^{-/-} mice increases lesion area without affecting serum lipid levels or circulating apoE. These studies provide definitive evidence that macrophage-derived apoE does not require the LDLR for its anti-atherogenic effects, and that macrophage apoE is atheroprotective when expressed at a physiological level on a hyperlipidemic background.

The observation that apoE has a functional role in the artery wall, apart from its more systemic effects on lipoprotein clearance, has been reported in many studies. We, and others, have used the approach of bone marrow trans-

plantation to express apoE from macrophages in the artery wall of apoE^{-/-} mice. ApoE levels only 10% of wild-type caused the normalization of lipoprotein profiles and a 50-fold reduction in atherosclerotic lesion formation (9, 14, 15), but the reduction in plasma lipids did not allow for conclusions to be made regarding a direct effect of macrophage apoE in the artery wall. To answer this question, we expressed very low levels of apoE from retrovirus-transduced macrophages in apoE null mice recipients of bone marrow transplantation. This approach resulted in a decrease in lesion area during the early stages of atherosclerosis, but the effect was lost in advanced lesions (5). Moreover, using transgenic mouse models, Shimano et al. and Bellosta et al. have shown that low levels of apoE expression in the artery wall can lead to delayed atherosclerotic lesion formation (6, 7). The limitation of all these studies was in the need of using low level apoE expression as a way to avoid the cholesterol lowering effect of larger amounts of apoE. The importance of the present study is in the demonstration that physiologically normal expression of macrophage apoE has anti-atherogenic effects that are not limited to the foam cell lesion but extend into the more advanced stages of plaque growth on a setting of hyperlipidemia.

The current study provides a model of a “hyperlipidemic clamp” in which apoE gradually accrues but does not have any measurable effect on lipoprotein clearance. In this setting, it is possible to study and contrast the harmful effects of hyperlipidemia and the protective effects of apoE in atherosclerosis. The results of this experiment are unequivocal: the presence of macrophage-derived apoE for about 6 weeks in the severely hyperlipidemic apoE^{-/-}/LDLR^{-/-} mice reduced atherosclerosis by 60% compared with controls. In fact, the extent of lesion area in the apoE-expressing, hypercholesterolemic mice was 15,000 μm^2 per section, a level that corresponds to the extent of lesion area that is commonly found in 13-week-old irradiated apoE^{-/-} mice (5), an indication that from the moment apoE began to accumulate, progression of atherosclerosis was halted. Evidence in support of the protective effect of systemic apoE in the presence of dyslipidemia comes from studies in which apoE was over-expressed in the livers of LDLR^{-/-} mice by adenoviral infection (16, 17). In these studies, a tremendous over-expression of apoE by the liver was acutely and transiently induced, and this human apoE accumulated in addition to the pre-existing endogenous apoE, thus producing a true supra-physiological systemic accumulation. In contrast, in our system the production of apoE occurred at physiological rate and from a physiological source, and because the study animals were apoE deficient and hyperlipidemic, macrophage apoE accumulated in VLDL particles in concentrations within the physiological range (13). This suggests a local effect of physiological levels of macrophage apoE capable to counter the strong atherogenic pressure of severe hypercholesterolemia.

ApoE is a strong physiological ligand for the LDLR, and also binds to other membrane receptors such as the LDLR-related protein and heparan sulfate proteoglycans

(HSPG). We have demonstrated that macrophage expression of human apoE-2, a common isoform of apoE defective in binding to the LDLR, does not affect atherosclerotic lesion formation in setting of hyperlipidemia, whereas apoE-3 reduces foam cell lesion size by about 40% (8). Similarly, apoE-cys142, a rare human apoE variant defective in the binding to HSPG, did not reproduce the beneficial effects of apoE-3 in the vessel wall and actually appeared to increase lesion size (8). These data suggest that the local function of apoE may derive from its physical engagement with membrane receptors, and may be related to its effects on lipoprotein internalization by the macrophage or on cellular cholesterol efflux.

The second set of experiments presented here also supports the idea that macrophage apoE can significantly modulate the biology of arterial lesions in the presence of massive hyperlipidemia. In this study, deletion of apoE exclusively from the macrophages of LDLR^{-/-} mice fed a Western-type diet increased aortic atherosclerosis by approximately 70% without affecting plasma lipids or systemic apoE levels. We previously reported that the removal of macrophage apoE increases lesion size in C57BL/6 mice on a high-fat diet (3), and have recently shown that this intervention is deleterious in both apoA-I^{-/-} and apoA-I over-expressing mice (4, 18). However, in all these models the hyperlipidemia is modest and atherosclerosis is limited to the very early stages of foam cell formation. The present study shows that the atherogenic potential of the lack of apoE in the artery wall is fully measurable in advanced atherosclerosis and in severely hypercholesterolemic LDLR^{-/-} mice. Of note is that the macrophage-rich lesions of apoE^{-/-}/LDLR^{-/-}→apoE^{+/+}/LDLR^{-/-} did not stain for apoE (not shown), confirming our previously reported data in C57BL/6 mice indicating that most of the local apoE in the arterial wall has a macrophage origin (3).

Several recent studies have shed light on potential mechanisms by which macrophage apoE may be protective. Control of macrophage apoE expression appears to be related to cellular cholesterol loading (19), and recently two distal enhancers that specify apoE gene expression from macrophages have been identified (20) and found to contain conserved liver X receptor (LXR) response elements (21). Of interest is the recent observation that macrophage apoE and ABCA1 (both LXR-regulated genes) may have a non-cooperative role in cholesterol homeostasis. Mazzone et al. have shown that the absence of apoE from macrophages is accompanied by increased levels of ABCA1 protein, and that the expression of apoE reduces ABCA1 levels (22). Considering that apoE deficient macrophages have significantly reduced cholesterol efflux (19, 23), these data are compatible with a system where apoE may be the primary motor of cholesterol efflux from the macrophage in a pathway that not only is independent from ABCA1 but cannot be corrected for by the counter-regulatory over-expression of ABCA1. In support of this, Joyce and co-workers found that whereas ABCA1 over-expression was protective against atherosclerotic lesion development in C57BL/6 mice, the protection was lost in

the absence of apoE (24), indicating that apoE is a stronger modulator of atherogenesis and cholesterol efflux than ABCA1 mediated mechanisms. Our own recent observations that the transgenic or retrovirus-based expression of apoA-I from the macrophage corrects the increased atherosclerosis induced by apoE deficiency (4, 18) support the notion that enhanced cholesterol efflux is an important mechanism for the effect of apoE in the arterial wall.

In addition to its effects on cholesterol accumulation in macrophages, apoE can also modulate immune and inflammatory responses and exert anti-oxidant properties. ApoE decreases lymphocyte responses in vitro (25, 26) and apoE^{-/-} mice have higher concentrations of circulating tumor necrosis factor α (TNF α) following challenge with bacterial pathogens (27–29). In addition, apoE suppresses microglial cell activation and inflammatory responses, including the secretion of TNF α and nitric oxide (30, 31). Although an antioxidant effect of apoE is certainly visible at higher concentrations (32), we were unable to show a difference in free F2 isoprostanes in apoE^{-/-}/LDLR^{-/-} mice transplanted with apoE^{+/+}/LDLR^{-/-} compared with those transplanted with apoE^{-/-}/LDLR^{-/-} marrow (data not shown). Therefore, oxidative stress is unlikely to represent an important mediator of the vascular effects of apoE.

In conclusion, we have provided definitive evidence that the physiological expression of apoE from macrophages is a powerful regulator of atherogenesis under conditions of extreme hypercholesterolemia, complicated arterial plaques, and absence of the LDLR. The knowledge that apoE gene regulation is within the control of the LXR axis raises the possibility of exploiting macrophage apoE expression through pharmacologic interventions as a means to prevent and cure atherosclerosis. **FIG**

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