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# Increased atherosclerosis in mice reconstituted with apolipoprotein E null macrophages

**(hyperlipidemia/bone marrow transplantation/foam cells)**

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**ABSTRACT** **Macrophage-derived foam cells express apolipoprotein E (apoE) abundantly in atherosclerotic lesions. To examine the physiologic role of apoE secretion by the macrophage in atherogenesis, bone marrow transplantation was used to reconstitute C57BL/6 mice with macrophages that were either null or wild type for the apoE gene. After 13 weeks on an atherogenic diet, C57BL/6 mice reconstituted with apoE null marrow developed 10-fold more atherosclerosis than controls in the absence of significant differences in serum cholesterol levels or lipoprotein profiles. ApoE expression was absent in the macrophage-derived foam cells of C57BL/6 mice reconstituted with apoE null marrow. Thus, lack of apoE expression by the macrophage promotes foam cell formation. These data support a protective role for apoE expression by the macrophage in early atherogenesis.**

Since the discovery by Basu *et al.* (1, 2) that macrophages synthesize and secrete apolipoprotein E (apoE), the physiologic role of macrophage apoE secretion has been a subject of intense investigation (3). ApoE is a 34-kDa plasma glycoprotein that serves as a ligand for receptor-mediated clearance of remnant lipoproteins (4). Deficiency of apoE results in severe hypercholesterolemia and diffuse atherosclerotic disease in humans (5) and gene-targeted mice (6). The vast majority of apoE in plasma is derived from the liver (7), but apoE is synthesized by a wide variety of tissues (8) and cell types, including the macrophage (1). Atherosclerotic lesions in both humans and experimental animals contain apoE (9), and macrophage-derived foam cells express apoE in atherosclerotic lesions (10).

Because the macrophage-derived foam cell plays an important role in the initiation and progression of atherosclerotic lesions (11), macrophage-derived apoE might have a direct effect on atherosclerosis induction and progression, independent from its effect on plasma lipoprotein metabolism and clearance. ApoE secretion by the macrophage has been proposed to be a protective process, preventing foam cell formation by stimulating efflux of free cholesterol from the cholesterol-loaded macrophage (12) and/or by facilitating reverse cholesterol transport from the artery wall (13). However, an alternative hypothesis suggests that apoE secreted by the macrophage may encourage foam cell formation by associating with the lipoproteins in the extracellular space and promoting increased lipoprotein uptake by the macrophage (14).

Based on the evidence that apoE is synthesized by monocyte/macrophages (1) but not by other leukocytes (15), murine bone marrow transplantation (BMT) has been used as an approach to investigate the role of apoE production by the macrophage *in vivo*. In previous studies, we and others transplanted apoE (−/−) mice with bone marrow from wild-type mice [apoE(+/+)→apoE(−/−)] (16, 17). Reconstitution of the apoE(−/−) mice with apoE(+/+) marrow resulted in the normalization of cholesterol levels and protection from diet-induced atherosclerosis. However, the reduction in serum cholesterol seen in the apoE(+/+)→apoE(−/−) mice obscured the local contribution of apoE secretion by the macrophage in atherosclerosis. Two recent studies of transgenic mice have shown decreased atherosclerosis as a consequence of local expression of the human apoE transgene by macrophages (18) or by other cells in the artery wall (19). However, the question of whether the regulated secretion of native apoE by the macrophage plays a crucial physiologic role in atherogenesis remains unanswered.

## MATERIALS AND METHODS

**Mice.** The female C57BL6/J mice used as recipients for BMT were obtained from The Jackson Laboratory. The apoE(−/−) donor mice (C57BL/6 × 129) (6) were backcrossed nine generations in C57BL/6 background. The ROSA β-geo 26 mice is an engineered strain that shows ubiquitous expression of the *Escherichia coli* β-galactosidase from a *lacZ* gene promoter trap (20), providing a marker for identification of cells of donor origin by flow cytometry (21). The apoE(−/−) mice used in our experiments were crossed with ROSA 26 mice (C57BL/6 × 129 at the ninth backcross in C57BL/6) to obtain apoE(−/−) mice bearing the ROSA 26 marker gene (20). All mice were maintained in microisolator cages, and the following diets were used (all percentages are wt/wt): (1) PMI Autoclavable Rodent Diet (No. 5010), 4.5% fat; this diet was used before BMT, and (2) Butterfat Diet (ICN), 19.5% fat, 1.25% cholesterol, and 0.5% cholic acid; this diet was initiated 8 weeks after BMT. Mice of both groups were healthy throughout the study and showed no differences in feeding pattern. Animal care and experimental procedures involving animals were conducted in accordance with institutional guidelines.

**BMT.** Recipient mice were housed in autoclaved microisolator cages and were maintained on acidified water (pH 2.0) containing 100 mg/liter neomycin and 10 mg/liter polymyxin B for 3 days before and 14 days after transplantation. Four hours before transplantation, recipient mice were lethally irradiated with 900 rads from a cesium γ source. Bone marrow was harvested from femurs and tibias of donor mice by flushing

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Abbreviations: apoE, apolipoprotein E; BMT, bone marrow transplantation.

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with RPMI medium 1640 (GIBCO) supplemented with 2% fetal bovine serum and heparin 5 units/ml. Bone marrow cells were washed, resuspended in fresh media, and counted. Lethally irradiated recipients received $5 \times 10^6$ bone marrow cells in 0.3 ml by tail vein injection.

**Flow Cytometry.** Bone marrow from C57BL6/J, ROSA 26, apoE(+/+)→apoE(+/+) controls, and apoE(−/−)→apoE (+/+) mice was analyzed by flow cytometry. Red blood cells were lysed with ammonium chloride. Single cell suspensions were washed twice in PBS with 2% BSA. Cells ($1 \times 10^6$) were incubated with phycoerythrin-conjugated mAb for 30 min, washed twice, and one- and two-color flow cytometry analyses were performed on a FACScan (Becton Dickinson). Gating for viable cells was performed using 7-aminoactinomycin D exclusion (Molecular Probes). In each sample, $0.5–1.0 \times 10^4$ cells were analyzed. The antibodies to lineage-specific differentiation antigens were: CD11b (Mac-1) for myeloid cells and B220 for B lymphocytes (PharMingen). Activity of β-galactosidase was measured as intracellular accumulation of fluorescein from the hydrolysis of fluorescein di-β-D-galactopyranoside (FDG; Molecular Probes) by β-galactosidase, as described (21). Cells were loaded with FDG by hypotonic shock: $1 \times 10^6$ cells in 100 μl of staining medium (PBS/4% fetal calf serum/10 mM Hepes, pH 7.2) were incubated with 100 μl of 2 mM FDG in distilled $H_2O$ at 37°C, and the reaction was stopped by adding 1.8 ml of ice-cold staining medium. The cells were incubated on ice before flow cytometry; staining with secondary antibodies was as described above except that all incubations were on ice and centrifugations were at 4°C.

**Serum Cholesterol Determinations.** Nonfasting mice were anesthetized with methoxyflurane, and blood was collected by retro-orbital venous plexus puncture. Serum cholesterol determinations were performed using kit no. 352 (Sigma) adapted for a microplate assay (22). In brief, 10 μl of serum was diluted 1:100 in sterile water, and a 100-μl aliquot (equivalent to 1 μl of serum) was loaded on the microplate well and mixed with 100 μl of freshly prepared cholesterol reagent. After a 20-min incubation at 37°C, the absorbance at 490 nm was read on a Molecular Devices microplate reader. Cholesterol levels on chromatography fractions were determined similarly, except that 100 μl from each tube was loaded directly in the microplate well and mixed 1:1 with the Sigma cholesterol reagent.

**Separation of Lipoproteins.** Mouse lipoproteins were prepared by FPLC analysis of serum using a Superose 6 column (Pharmacia) on a FPLC system model 600 from Waters. A 100-μl aliquot of mouse serum was injected onto the column and separated with a buffer containing 0.15 M NaCl, 0.01 M $Na_2HPO_4$, 0.1 mM EDTA, pH 7.5, at a flow rate of 0.5 ml/min. Fifty fractions of 0.5 ml each were collected, with the lipoproteins being contained within tubes 15–33. Fractions 15–19 = very low density lipoprotein and chylomicrons; fractions 20–26 = intermediate density lipoproteins, low density lipoprotein, and large high density lipoprotein; fractions 27–33 = high density lipoprotein.

**Protein Electrophoresis and Western Blots.** The lipoproteins were extensively dialyzed in 0.15 M NaCl, 0.01 mM EDTA, at 4°C, and then either concentrated using Centricon filters (Amicon) and used for agarose gel electrophoresis, or precipitated using a 50-mg/ml solution of fumed silica (Cab-O-Sil; Sigma) and used for SDS/PAGE. Agarose gels were either stained for lipids using fat red 7B, or immunoblotted using a rabbit anti-rat apoE antiserum that crossreacts strongly with mouse apoE and to a lesser degree with mouse apoAI (gift from K. Weisgraber, Gladstone Institutes, San Francisco). The secondary antibody was a goat anti-rabbit IgG antiserum labeled with peroxidase. Detection of bands was achieved by chemiluminescence, using the ECL kit from Amersham. SDS/PAGE gels were either stained for proteins using Coomassie blue R-250, or immunoblotted using the same procedure described for agarose gels. Autoradiography films were analyzed by computerized densitometry using an imaging densitometer model GS-670 from Bio-Rad.

**Ribonuclease Protection Assay.** We prepared the riboprobe by inserting the 108-bp *Sac*I-*Xho*I fragment of exon 3 of the mouse apoE cDNA into the polylinker region of the vector pBSKS (Stratagene), under the control of both the T3 (sense) and T7 (antisense) promoters. The probe was labeled with [$^{32}$P]UTP using T7 polymerase and the RNA transcription kit from Stratagene. Tissue RNA was prepared by the guanidinium isothiocyanate method (23), followed by CsCl gradient ultracentrifugation. Before tissue collection, mice were extensively flushed by slow injection of 30 ml of ice-cold PBS through the left ventricle (to wash the blood components from tissues). Solution hybridization and RNase protection were carried out with $3 \times 10^5$ cpm of probe and 3 μg of RNA, using the RPAII kit from Ambion (Austin, TX). Control reactions were conducted using a 414-bp fragment of murine hypoxanthine phosphoribosyltransferase cDNA transcribed from a pGEM3Z vector (24). After overnight incubation at 45°C, the samples were separated on 6% polyacrylamide, 8 M urea gels, at 300 V for 2 h. The gels were dried and exposed to autoradiography film. Densitometry analyses were performed on the control lanes to correct for differences in loading, and on the apoE lanes to quantitate the difference in apoE expression.

**Morphologic Analyses.** *Analysis and quantitation of arterial lesions.* ApoE(+/+)→apoE(−/−) and apoE(−/−)→apoE (−/−) mice were started on a 21% fat, 0.2% cholesterol diet 2 months after BMT. After 13 weeks on the diet, the mice were sacrificed and their hearts flushed with saline, embedded in OCT, and snap-frozen in liquid $N_2$. The inferior vena cava was cut to allow the perfusate to exit. Frozen sections of 10 μm thickness were taken in the region of the proximal aorta starting from the end of the aortic sinus and for 300 μm distally, according to the technique of Paigen *et al.* (25). Sections were stained with oil red O and counterstained with hematoxylin. Quantitative analysis of lipid-stained lesions was performed on sections starting just beyond the end of the aortic sinus. The lipid-stained lesions were measured by digitizing morphometry, and reported in $\mu m^2$ per lesion.

*Immunocytochemistry.* Serial cryosections 5 μm thick from the proximal aortic were fixed in acetone and incubated with monoclonal rat antibody to mouse macrophages, MOMA-2 (Accurate Chemicals) (26), or a rabbit antiserum to mouse apoE (Biodesign International, Kennebunkport, ME) diluted in PBS 1:30 and 1:300, respectively. The sections were incubated with biotinylated goat antibodies to rat (or rabbit) IgG (PharMingen) in a dilution of 1:200. Sections were treated with avidin-biotin complex labeled with alkaline phosphatase (Vector Laboratories) and incubated with the fast red TR/naphthol AS-NX substrate (Sigma) and counterstained with hematoxylin. No staining was seen in negative control sections, where the serial sections of the set were treated with nonimmune sera of the same species instead of primary antibodies (not shown). Photomicroscopy was performed on a Zeiss Axiophot with Plan-NeoFLUAR objectives.

*In situ hybridization.* Cryosections were fixed in 4% paraformaldehyde, treated with proteinase K, 5 μg/ml, and incubated overnight with a [$^{35}$S]UTPαS-labeled 108-nucleotide antisense or sense riboprobes for apoE (16). After treatment with 20 μg/ml RNase A, the sections were coated with autoradiographic emulsion (Kodak NTB-2) and exposed for 2–3 weeks. After developing, the sections were counterstained with hematoxylin.

## RESULTS

To examine the role of apoE secretion by the macrophage in early atherogenesis, we transplanted C57BL/6 mice, an inbred

strain that is susceptible to atherosclerosis (27), with bone marrow from apoE(−/−) mice, creating a de facto macrophage-specific knock-out of apoE. After lethal irradiation [9 grays], 8-week-old female C57BL/6 mice were transplanted with $5 \times 10^6$ bone marrow cells from either apoE(−/−) mice ($n = 14$, experimental group) or wild-type apoE(+/+) mice ($n = 11$, control group). All donor mice were on the C57BL/6 background (ninth backcross) and expressed β-galactosidase ubiquitously from the ROSA β-geo 26 *lacZ* promoter trap (20), allowing for easy identification of donor cells by flow cytometry (16, 21). Eight weeks after transplantation, the mice were started on an atherogenic diet to induce foam cell lesions.

In a parallel experiment, flow cytometry to detect β-galactosidase due to the ROSA26 marker was used to assess the extent of reconstitution of the recipient mice with donor-derived hematopoietic cells as previously described (16). Six weeks post-BMT, the bone marrow of four C57BL/6 recipient mice, transplanted as described above, was analyzed by dual color flow cytometry for reconstitution of the bone marrow by donor-derived (β-galactosidase+), myeloid (MAC-1+), and lymphoid (B220+) cells. Six weeks post-BMT the Mac-1+ and B220+ cells of the recipient's marrow were 96.6 ± 2.4% and 95.4 ± 3.7% donor-derived, respectively (not shown). At 21 weeks post-BMT, the myeloid cells (MAC-1+) of the bone marrow were 99.0 ± 1.1% donor-derived (β-galactosidase+) in apoE(−/−)→apoE(+/+) mice ($n = 7$) and 99.5 ± 1.1% donor derived in the apoE(+/+)→apoE(+/+) mice ($n = 7$). Thus, mice in both groups were essentially completely reconstituted by myeloid cells of donor origin.

Analysis of serum cholesterol and triglyceride levels at baseline and 6 and 13 weeks after initiation of the atherogenic diet showed no significant differences between the apoE(−/−)→apoE(+/+) mice and the apoE(+/+)→apoE(+/+) mice (Table 1). Examination of the distribution of cholesterol among the serum lipoproteins by gel chromatography demonstrated that the lipoprotein profiles were essentially identical in the two groups on normal chow (not shown) and after 13 weeks on the atherogenic diet (Fig. 1). Serum levels of apoE were determined by comparative dilution analysis on Western blots, and no differences were detected between the two groups (not shown). The distribution of apoE in the lipoprotein fractions was examined by dot blot analysis of pooled FPLC fractions using an anti-rat apoE polyclonal antibody; the majority of the apoE in plasma was found among the serum very low density lipoproteins and intermediate density lipoproteins fractions, and no differences were seen between the experimental and control mice (not shown). At baseline, most of the apoE was associated with the high density lipoproteins in both groups of mice. After 13 weeks on the high-fat diet, both groups of mice showed a similar redistribution of apoE among plasma lipoproteins: about 70% associated with large very low density lipoprotein(s), 20% with smaller very low density and low density lipoprotein(s), and 10% with the high density lipoprotein(s) fraction.

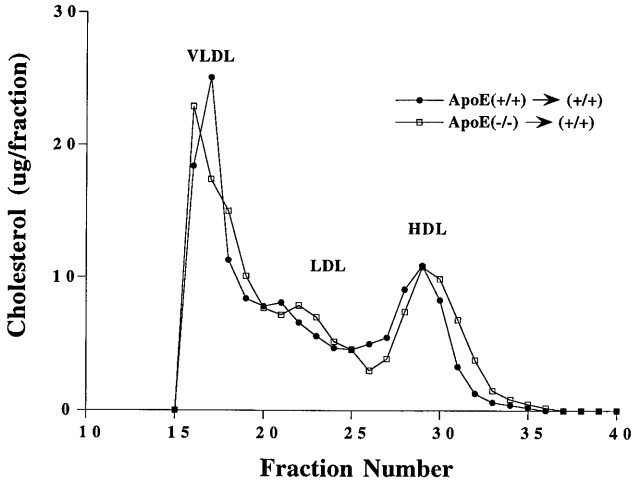


FIG. 1. Distribution of serum lipoprotein cholesterol in apoE(−/−)→apoE(+/+) experimental mice and apoE(+/+)→apoE(+/+) control mice after 13 weeks on the atherogenic diet. Gel filtration chromatography was performed using a Superose 6 column (Pharmacia) on a Waters 600 FPLC system. A 100-μl aliquot of mouse serum was injected onto the column and separated as described above. Cholesterol determinations were done with the Sigma kit 352 in the microplate assay. Data points represent mean values for cholesterol from five control and three experimental mice.

Quantitative analysis of the extent of aortic atherosclerosis was determined on oil-red-O stained sections of the proximal aorta as previously described (16, 25) (Fig. 2). The mean area of aortic lesions was 10-fold greater in the apoE(−/−)→apoE(+/+) mice ($n = 14$) than in the apoE(+/+)→apoE(+/+) mice ($n = 11$) [16,090 ± 3,419 vs. 1,622 ± 458 (in $\mu m^2$ ± SEM); $P = 0.001$ by the Mann–Whitney rank sum test]. Representative oil-red-O-stained sections of the proximal aorta of control and experimental mice are shown in Fig. 3.

Serial sections of the proximal aorta from the 14 experimental and the 11 control mice were examined for staining with antibodies specific for either macrophages or apoE by immunocytochemistry (Fig. 3). The foam cell lesions in each group consisted primarily of macrophages (MOMA+). Intense staining for apoE in the macrophage-derived foam cells was detected in the apoE(+/+)→apoE(+/+) mice. In contrast, the macrophage-derived foam cells in the apoE(−/−)→apoE(+/+) mice showed essentially no staining for apoE, although small amounts of apoE were detected in the extracellular matrix below the macrophages (Fig. 3).

Table 1. Total serum lipid levels in female C57BL/6J mice after transplantation with apoE(−/−) or apoE(+/+) bone marrow

| | | Serum lipid level, mg/dl | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | Atherogenic diet | | | |
| | | Baseline post-BMT | | 6 weeks | | 13 weeks | |
| Donor mouse | *n* | Chol | Trigl | Chol | Trigl | Chol | Trigl |
| apoE(+/+) | 14 | 70 ± 23 | 64 ± 35 | 194 ± 25 | 66 ± 32 | 220 ± 34 | 41 ± 9 |
| apoE(−/−) | 11 | 77 ± 14 | 61 ± 30 | 195 ± 36 | 70 ± 60 | 229 ± 32 | 44 ± 17 |

The baseline values were obtained on a diet of 4.5% fat rodent chow (Rodent Diet 5001, PMI) 8 weeks post-BMT. The atherogenic high fat diet consisted of 19.5% fat, 1.25% cholesterol, and 0.5% cholic acid (Butter Fat Diet, ICN). At the indicated time points, blood was obtained from nonfasting mice. Serum cholesterol (Chol) and triglyceride (Trigl) determinations were done with Sigma kits 352 and 339, adapted for microplate assay. Absorbance was read on a Molecular Devices microplate reader. Values are mean ± SEM. The number of mice in each group is indicated by *n*. Differences between groups were not statistically significant at any time point.

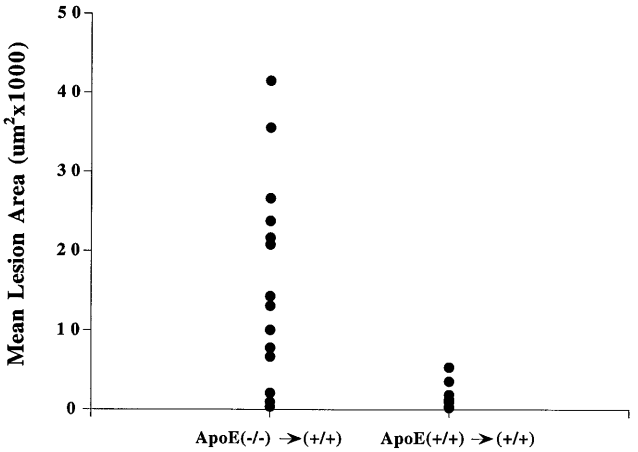


FIG. 2. Mean area of the aortic atherosclerotic lesions per section per animal for the apoE(−/−) →apoE(+/+) experimental mice and apoE(+/+)→apoE(+/+) control mice on the atherogenic diet. The analysis was performed on alternate 10 μm sections starting at the end of the aortic sinus and extending 300 μm distally. Frozen sections (10 μm) were fixed with 4% paraformaldehyde and stained with oil red O. The areas of lipid-stainable lesions were measured by digitizing morphometry in a blinded fashion. Data are from 14 experimental apoE(−/−)→apoE(+/+) mice and 11 control apoE(+/+)→ apoE(+/+) mice after 13 weeks on the atherogenic diet.

The expression of apoE mRNA in the proximal aortic artery wall was examined by *in situ* hybridization. ApoE expression was detected in foam cell lesions of apoE(+/+)→apoE(+/+) mice but not in the larger foam cell lesions of the apoE(−/−)→apoE(+/+) mice (Fig. 4). ApoE expression in the thoracic aorta of the experimental and control mice was examined by RNase protection assay using a $^{32}$P-labeled probe to apoE as previously described (16). Quantitative analysis of the results of the RNase protection assay revealed that the level of total apoE mRNA in aortas of the experimental mice was about half that of control mice (Fig. 5). Because this region of the aorta was not expected to contain atherosclerotic plaques, the decreased apoE mRNA in apoE(−/−)→apoE(+/+) mice might be due to the reconstitution of the adventitial layer with blood monocytes lacking apoE. There were no differences in liver apoE mRNA levels between groups (Fig. 5).

## DISCUSSION

The current study was undertaken to examine the physiologic role of native apoE expression by the macrophage in foam cell formation and early atherogenesis. When challenged with an atherogenic diet, apoE(−/−)→apoE(+/+) mice developed significantly more aortic atherosclerosis than apoE(+/+)→apoE(+/+) mice in the absence of significant differences in the serum lipid levels or in the distribution of cholesterol or apoE among the serum lipoproteins. Thus, apoE-null macrophages are more prone to progress to foam cells than wild-type macrophages in a similar atherogenic environment. This study proves that the absence of apoE secretion by the macrophage is proatherogenic and may directly contribute to the severe atherosclerosis in apoE-deficient mice and humans with apoE deficiency. Based on these observations, we conclude that the normal physiologic role of macrophage apoE in early atherogenesis is to oppose foam cell formation and delay atherosclerosis.

ApoE secretion by the macrophage has been proposed to play a protective role opposing foam cell formation by promoting cholesterol efflux from the macrophage (12) and facilitating reverse cholesterol transport (13). Alternatively, macrophage apoE has been proposed to promote foam cell formation by enriching lipoproteins with ligand for receptor-mediated uptake by the macrophage (14). Studies in transgenic mice have provided evidence that local production of apoE in the artery wall inhibits atherosclerosis. Shimano *et al.* (19) reported that transgenic mice expressing human apoE3 in the artery wall and in several other tissues, including the liver, developed significantly less diet-induced aortic atherosclerosis than controls, in the absence of significant changes in plasma cholesterol or lipoprotein profiles. Bellosta *et al.* (18) have reported that macrophage specific expression of the human apoE3 transgene in apoE null mice induced a dramatic reduction in atherosclerosis susceptibility compared with cholesterol matched apoE(−/−) littermates. Although supportive of a protective function for macrophage apoE, both of these studies showed the presence of the transgenic apoE on plasma lipoproteins. Despite the lack of changes in lipid levels, it is plausible that human apoE-enriched lipoproteins might *per se* have a different atherogenic action than apoE-deficient lipoproteins or lipoproteins with lower levels of apoE. In addition, apoE expression in these studies was not under the control of its native physiologic promoter. On the other hand, it was recently reported that human monocyte-derived macrophages take up exogenous lipoproteins differently based on their apoE phenotype, with apoE3/3 macrophages incorporating significantly more particles than macrophages expressing the receptor-binding defective isoform apoE2 (28). If this phenomenon occurs *in vivo*, it could be hypothesized that expression of a functional apoE might result in accelerated foam cell formation, and thus favor atherosclerosis development. Our current results support the concept that macrophage production of apoE retards foam cell formation and the growth of arterial lesions, by demonstrating that in response to an atherogenic diet C57BL/6 mice reconstituted with macrophages expressing apoE under the control of its native promoter develop less atherosclerosis than mice reconstituted with apoE null macrophages.

We previously have reported that after transplantation of apoE(−/−) mice with wild-type bone marrow, apoE appears in plasma, associates with lipoproteins, and promotes their clearance (16). Plasma levels of apoE that were only 10% of the levels seen in normal mice were sufficient to normalize the serum cholesterol levels, demonstrating that macrophage derived apoE is functional and efficient in promoting lipoprotein clearance. In the current study, no significant differences in serum lipid levels or lipoprotein profiles were detected between the apoE(−/−)→apoE(+/+) and apoE(+/+) →apoE(+/+) mice on the chow diet or the atherogenic diet. Thus, in the setting of normal hepatic production of apoE, the absence of macrophage-derived apoE does not significantly influence the clearance of plasma lipoproteins.

A number of studies have demonstrated that after bone marrow transplantation the tissue macrophages of the recipient are replaced by macrophages of donor origin (29–31). In the current study, we found that foam cell lesions in the aortas of the apoE(−/−)→apoE(+/+) mice were entirely composed of macrophages that did not synthesize apoE, and were thus of donor origin. This is the first demonstration that bone marrow transplantation results in the reconstitution of the arterial wall with macrophages of donor origin, and that apoE(−/−) monocytes are capable of inducing atherosclerosis. This observation demonstrates the ability of the monocyte to serve as a vehicle for gene delivery to the atherosclerotic plaque. The reduction in apoE mRNA observed in the thoracic aorta (Fig. 5), a region that is normally exempt from atherosclerosis in this model, might be due to the replacement of adventitial macrophages with donor-derived blood monocytes, and suggests that macrophages synthesize a large portion of the apoE in the normal arterial wall. These data also point out that some apoE in the artery wall may derive from cells other than the macrophage. Although our *in situ* and immunocytochemical analyses of lesioned mouse aortas demonstrate that the macrophage is by

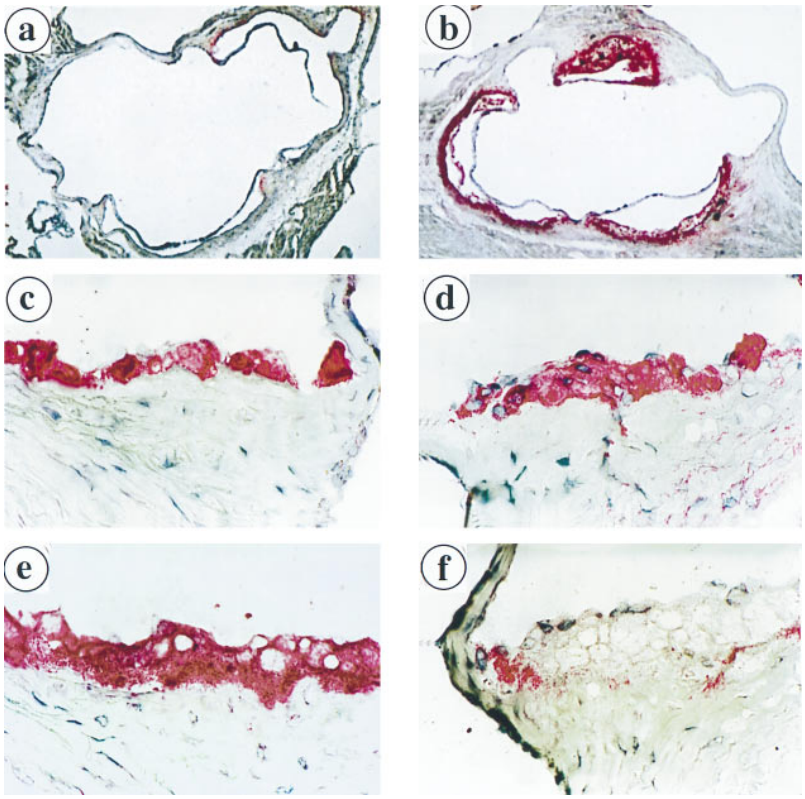


FIG. 3. Increased aortic atherosclerosis in C57BL/6 recipients of apoE(−/−) marrow. (*a* and *b*) Comparison of the extent of lipid staining by oil red O in representative sections of the proximal aorta of control apoE(+/+)→apoE(+/+) and experimental apoE(−/−)→apoE(+/+) mice after 13 weeks on the atherogenic diet (10×). Cryosections 10 μm thick were fixed with 4.0% paraformaldehyde, stained with oil red O, and counterstained with hematoxylin. (*c* and *e*) Serial sections from control animals. (*d* and *f*) Serial sections from apoE(−/−)→apoE(+/+) mice. (*c* and *d*) Immunocytochemical staining for macrophages reveals that foam cell lesions consist primarily of macrophages in experimental and control mice (63×). (*e* and *f*) Immunocytochemical staining of serial aortic sections for apoE (63×). ApoE staining in control mice colocalizes with macrophages, but the macrophages in the experimental mice do not stain with apoE.

far the largest producer of apoE, it is plausible that the relative contribution of other cell types to apoE synthesis in the artery wall might become more significant when fewer macrophages are present.

The striking absence of apoE staining in the arterial wall macrophages of apoE(−/−)→apoE(+/+) mice indicates that most of the apoE in the foam cell lesion derives locally from macrophages, rather than from the plasma compartment. In addition, this observation also proves that macrophage apoE plays a direct role in preventing or delaying foam cell formation, and that lack of macrophage apoE has deleterious repercussions on the induction and progression of atherosclerosis despite the abundance of apoE in plasma and on lipoproteins. There are several potential mechanisms by which apoE secreted by macrophages may be related to arterial lesion development: (*i*) It may influence monocyte recruitment to the lesion area. Although a

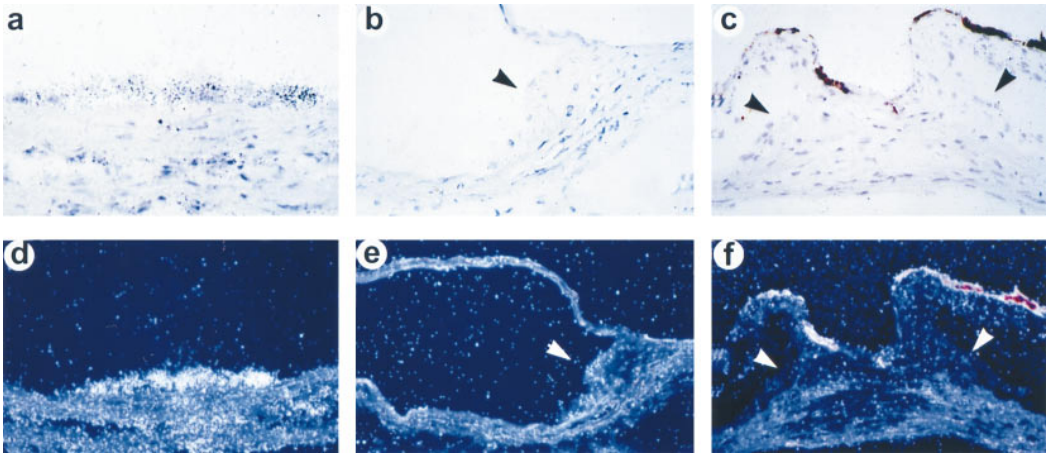


FIG. 4. Decreased apoE gene expression in the aortic root of apoE(−/−)→apoE(+/+) mice by *in situ* hybridization. The hybridization signals of the apoE antisense probe appears as black grains located in aortic foam cell lesion of apoE(+/+)→apoE(+/+) mice on bright field (*a*, 40×), or as white dots on dark field (*d*, 20×). (*b* and *e*) The sense probe did not produce hybridization with foam cell lesions under the same conditions on bright field (40×) or dark field (20×), respectively. (*c* and *f*) Absence of apoE expression in foam cell lesions of the proximal aorta in an apoE(−/−)→apoE(+/+) mouse after hybridization with the antisense probe on bright field (40×) and dark field (20×). The intense coloration of the borders of the aortic valve stumps visible in *c* and *f* is an artifact not related to hybridization-specific silver grain exposure.

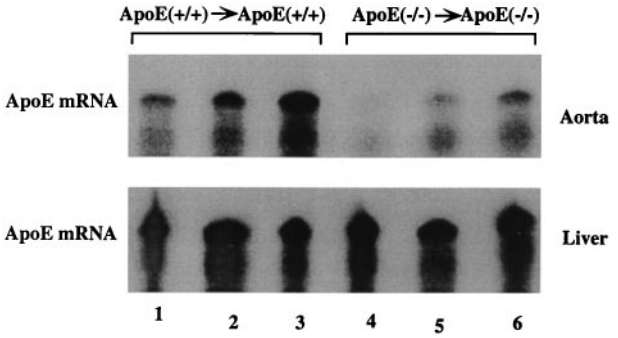


FIG. 5. Ribonuclease protection assay of total RNA extracted from ascending and descending aorta and from liver of C57BL/6 mice 13 weeks after transplantation with either wild-type C57BL/6 or apoE(−/−) marrow (apoE(+/+)→(+/+), and apoE(−/−)→(+/+), respectively. Solution hybridization and RNase protection were carried out with $2 \times 10^5$ cpm of probe and 5 $\mu$g of RNA, using the Ambion RPA II kit. There were seven mice in the control group [apoE(+/+)→(+/+)] and nine mice in the experimental group [apoE(−/−)→(+/+)]. The RNase protection experiment was repeated three times using different 5-$\mu$g aliquots of the same RNA preparations. After electrophoresis, the gels were dried and incubated in a PhosphoImager 445 SI from Molecular Dynamics. Quantitative comparison of radioactivity in the protected bands showed a 47% reduction in counts in the apoE(−/−)→(+/+) mice relative to the controls. Mean ± standard error was 256,860 ± 43,389 counts for the seven apoE(+/+)→(+/+) mice and 135,262 ± 49,003 counts for the nine apoE(−/−)→(+/+) mice ($P = 0.054$ at the Mann–Whitney rank sum test). No differences were observed in liver samples between experimental and control mice. This figure reports data for three mice in each group. Lanes 1–3, control apoE(+/+)→(+/+) mice; lanes 4–6, experimental apoE(−/−)→(+/+) mice.

significantly larger lesion area was occupied by macrophages in apoE(−/−)→apoE(+/+) mice, it is difficult to establish whether this reflected a difference in monocyte recruitment rate or in the amount or nature of the atherogenic stimuli; (*ii*) it may lead to differences in cholesterol efflux. Cell culture studies have shown that internally produced or exogenously added apoE acts as a strong acceptor of cellular cholesterol (12), and by this mechanism may facilitate reverse cholesterol transport (13); (*iii*) it may affect lipoprotein uptake by reducing the rate of apoE-mediated removal and increasing the rate of removal through the scavenger receptor. Local enrichment of remnants with apoE secreted by the hepatocyte is thought to be necessary for the capture of remnant lipoproteins mediated by the chylomicron remnant receptor, or low density lipoprotein-receptor related protein (LRP) (32, 33). A similar mechanism of "secretion-capture" of lipoproteins may result from macrophage apoE production (33), targeting arterial lipoproteins to the low density lipoprotein-receptor or the LRP, which both bind apoE (34, 35). ApoE has been demonstrated to have antioxidant properties *in vitro* (36), and oxidized lipoprotein epitopes are found in arterial foam cell lesions of apoE-deficient mice (37). According to this hypothesis, lack of apoE secretion by the macrophage results in increased uptake of oxidized lipoproteins by the scavenger receptor (38), and production of intracellular cholesterol pools unavailable for efflux. Further studies will be required to elucidate the molecular mechanism of the antiatherogenic effect of apoE secretion by the macrophage.

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