Retroviral Gene Therapy in ApoE-Deficient Mice
ApoE Expression in the Artery Wall Reduces Early Foam Cell Lesion Formation

Alyssa H. Hasty, PhD; MacRae F. Linton, MD; Stephen J. Brandt, MD; Vladimir R. Babaev, PhD; Linda A. Gleaves; Sergio Fazio, MD, PhD

Background—Apolipoprotein E (apoE) has long been known to play an important role in the clearance of plasma lipoproteins. More recently, a direct role for apoE in delaying atherogenesis has been proposed. Macrophage production of apoE in the artery wall has been demonstrated to provide protection against atherosclerotic lesion development independently of its role in lipoprotein clearance. However, whether macrophage apoE can affect lesion growth at all stages of atherogenesis remains to be established.

Methods and Results—To evaluate the role of macrophage apoE in different stages of atherogenesis, as well as to establish a novel gene therapy approach to atherosclerotic vascular disease, we used an apoE-expressing retrovirus to transduce apoE-deficient (−/−) bone marrow for transplantation into apoE(−/−) recipient mice. Three weeks after bone marrow transplantation, apoE was expressed from arterial macrophages and was detectable in plasma associated with lipoproteins at 0.5% to 1% of normal levels but did not affect plasma cholesterol levels. We used 2 groups of recipient mice: younger mice with lesions consisting primarily of foam cells and older mice with more advanced lesions. When either the mouse or human apoE transgenes were expressed in mice from 5 to 13 weeks of age, there was a significant reduction in lesion area, whereas no effects were detected in mice that expressed apoE from 10 to 26 weeks of age.

Conclusions—We demonstrate that arterial macrophage apoE secretion can delay atherogenesis if expressed during foam cell formation but is not beneficial during the later stages of atherogenesis. These data also provide evidence that apoE transgene expression from arterial macrophages may have therapeutic applications. (Circulation. 1999;99:2571-2576.)

Key Words: apolipoproteins • atherosclerosis • genes • cholesterol

Apolipoprotein E (apoE) is a 34-kD glycoprotein that circulates on plasma lipoproteins and serves as the ligand for their receptor-mediated clearance. Although the majority of apoE is synthesized in the liver, it is also expressed in a number of other tissues by a wide variety of cell types, including tissue macrophages. In addition, secretion of apoE from the artery wall appears to protect against the development of atherosclerosis. Shimano et al demonstrated that diet-induced atheroma formation could be inhibited by expression of apoE from the artery wall in transgenic mice. Bellosta et al showed that the macrophage-specific expression of the human apoE3 transgene in apoE(−/−) mice results in smaller lesion area than that observed in control apoE(−/−) mice. Conversely, we have recently provided evidence that lack of macrophage-derived apoE promotes atherogenesis. In this study, wild-type mice given apoE(−/−) marrow transplants (which resulted in a macrophage-specific apoE knockout) had a 10-fold greater lesion area than did control mice. Although these studies demonstrate a direct role for macrophage-derived apoE in atherogenesis, they do not indicate the possible mechanism by which macrophage apoE functions, nor do they delineate which stage of atherosclerotic development can be affected by apoE. The present study was designed to express constitutively low levels of apoE from arterial macrophages targeting different stages of atherosclerosis. Our hypothesis was that macrophage apoE exerts a measurable effect only during the macrophage-rich stage of lesion growth and functions less in modulating the later stages of atherosclerosis, when lesions are characterized by smooth muscle cell proliferation, extracellular cholesterol clefts, and areas of necrosis and calcification. To this end, we used an approach of transduction of apoE(−/−) bone marrow with apoE-containing retroviral constructs, followed by transplantation of marrow into apoE(−/−) recipient mice. Here, we report that transplantation of apoE(−/−) mice with apoE-transduced bone marrow leads to the expression of apoE from arterial macrophages and that local low-level expression of apoE in the artery wall...
is capable of delaying foam cell formation but has no effect on later stages of atherosclerosis. These studies support the concept that macrophage apoE secretion is important in the early foam cell formation stage of atherogenesis and that apoE transgene expression from arterial macrophages may have therapeutic applications.

Methods

Cloning of Mouse and Human ApoE cDNA Into the pLXSN Retroviral Vector

Mouse apoE cDNA (exons 2 to 4) was amplified by reverse transcription–polymerase chain reaction. Random hexamers were used for the reverse-transcription portion of the reaction. Human apoE3 was amplified from an expression vector driven by the cytomegalovirus promoter.9 Cycling conditions for both the mouse and human apoE consisted of 40 cycles of 97°C for 30 seconds, 62°C for 2 minutes, and 72°C for 2 minutes. The polymerase chain reaction products were used for ligation into the pLXSN retroviral vector and transfected into DH5a cells. Plasmid DNA from colonies was prepared and analyzed for the presence of the 1-κb apoE cDNA insert. Plasmid DNA was prepared with the Plasmid Maxi kit obtained from Qiagen according to kit protocol. The sequence of the apoE cDNA inserts was confirmed by use of the Thermosequenase kit (Amersham).

Preparation of Producer Cell Lines

The retroviral constructs were used to transfect the ectopic packaging cell line Bosc-23, which has been shown to result in transient high-titer production of infectious virus.9 The infectious supernatant from these cells was used to infect the amphotropic packaging cell line PA317.10 The infectious medium from this cell line was then used to infect the ectopic packaging cell line 293 in a transfection or “ping-pong-ping” method for creating high-titer producer cell lines.11 The infected 293 cells were selected for the integration of the provirus with the neomycin analogue G418 (Sigma) at 500 μg/mL. Individual clones were isolated and analyzed for apoE expression and infectious titer. Individual producer cell clones were analyzed for viral titer as well as apoE secretion. Mouse apoE–expressing (pLMESN/2), human apoE–expressing (pL-HESN/2), and parental (pLXSN/2) producer cells were selected for use in bone marrow transplant experiments. The producer cell lines used for bone marrow transplantation (BMT) studies were assayed for the presence of recombinant wild-type virus by the S- L assay and found to be helper virus–free.

Bone Marrow Collection, Transduction, and Transplantation

Bone marrow was collected from apoE(−/−) donor mice by flushing femurs and tibias with RPMI 1640 with 2% FBS and 10 μM heparin (Sigma) added. Cells were washed, counted, and plated in suspension dishes, where proliferation was stimulated by preculturing with interleukin 3 (IL-3) and interleukin 6 (IL-6). IL-3 and IL-6 were produced by transfection of COS-7 cells (ATCC) with pCD-mIL3 and pCD-mIL6.12,13 Bone marrow cells were precultured by plating 5×10^6 cells in 10% FBS, 3% IL-3, 3% IL-6 COS-7 medium for 48 hours. After preculture, bone marrow cells were collected and replated at the same density in the above medium with 6 μg/mL hexadimethrine bromide (Sigma) onto dishes of 80% confluent producer cells for 48 hours. The suspended bone marrow cells were then collected and used to transplant lethally irradiated apoE(−/−) mice as previously described.16

Animal Procedures and Lipid Analyses

All mice used for these experiments were apoE(−/−) at the sixth backcross or higher into the C57BL/6 background and were originally purchased from Jackson Laboratories, Bar Harbor, Me. Mice were maintained in microisolator cages on a rodent chow diet (PMI No. 5010) containing 4.5% fat. All mice were given autoclaved water acidified to pH 2.6. One week before and 2 weeks after BMT, 100 mg/L neomycin (Sigma) and 10 mg/L polymyxin B sulfate (Sigma) were added to the acidified water. Blood samples were collected by retro-orbital venous plexus puncture with heparinized tubes, and serum was separated by centrifugation. Serum cholesterol and triglyceride levels were determined with clinical reagents on a microtiter plate assay, as previously described.17 A 100-μL aliquot of serum from mice was separated by Superose 6 chromatography on a Waters high-performance liquid chromatography system model 600 as described.18 Animal care and experimental procedures were performed according to the regulations of Vanderbilt University’s Animal Care Committee.

Western Blotting and Immunohistochemical Analysis for ApoE

Serum samples were tested for the presence of mouse or human apoE by Western blot analysis as described.19 The primary antibodies used were polyclonal rabbit anti-mouse apoE (BioDesign International) and polyclonal goat anti-human apoE (BioDesign). Similar Western blot analyses were performed on fast protein liquid chromatography samples after concentration of pooled fractions by centrifugation with Microcon-10 columns (Amicon). Immunocytochemical analysis of tissue samples for macrophages and mouse apoE was performed essentially as described.20 Briefly, 5-μm sections were fixed in acetone and incubated with either a rat monoclonal antibody to macrophages, MOMA-2 (Accurate Chemicals), or a rabbit polyclonal antibody to mouse apoE (BioDesign International). Signal was detected with the ABC kit (Vector Laboratories) followed by incubation with the fast red TR/naphthol AS-NX substrate (Sigma). Photomicroscopy was performed on a Zeiss Axioshot with Plan-Fluor objectives.

Primary Macrophage Cultures

Peritoneal cells were collected 2 days after injection of thioglycollate as described.20,21 counted, and plated into 12-well dishes at 1×10^3 cells/well in DMEM+10% FBS. After 2 hours, nonadherent cells were removed. Wild-type, C57BL/6 mouse peritoneal leukocytes collected and plated in the same manner served as a positive control. Medium was collected from macrophages at 48-hour intervals and frozen in 1 mmol/L PMSF for Western blot analysis.

Lesion Area Quantification

Experiments were designed in 2 parts: (1) male 5-week-old apoE(−/−) mice killed at 13 weeks and (2) female 10-week-old apoE(−/−) mice killed at 26 weeks. Atherosclerosis studies in apoE(−/−) mice have not demonstrated any sex differences in extent of lesion formation.22 Frozen sections 10 μm thick were cut from the region of the proximal aorta starting from the end of the aortic sinus and continuing distally, according to the technique of Paigen et al.22 Sections were stained with oil red O and counterstained with hematoxylin. Quantification of lesion area was performed on 15 sections per animal by digitizing morphology and was reported in mean μm²/section. Computer morphology was performed with the KS300 software by Kontron Elektronik on a Compaq Presario 9240. Statistical analyses were performed with both Student’s t test and the Mann Whitney rank sum test comparing control and experimental lesion area in both experiments.

Results

Reconstitution of ApoE(−/−) Mice With Transduced Marrow

Mouse apoE and human apoE were detected in the serum of experimental mice as early as 3 weeks post-BMT and were expressed at levels ~0.5% to 1% and 5% of normal, respectively (Figure 1). Levels of apoE in serum were consistent between animals and over time. ApoE was expressed for as long as 5.5 months post-BMT. Serum cholesterol and triglycerides, measured pre-BMT and at 6 or 8
weeks post-BMT, showed no significant changes from baseline (Table). To evaluate whether recipients of retrovirus-infected marrow developed immunity against the foreign antigen (apoE), we performed a Western blot assay of mouse plasma using the plasma of a retrovirus-infected mouse (pLMESN) as the primary antibody source. No apoE was detected even on undiluted normal mouse plasma when a 1:50 dilution of pLMESN plasma was used as antibody source, suggesting that no anti-apoE immunoglobulins were present in the plasma of retrovirus-infected mice (data not shown).

The distribution of serum lipoproteins in experimental and control mice was similar to that in nontransplant apoE(−/−) mice as determined by gel filtration chromatography (data not shown). Both mouse and human apoE were found to be associated with lipoproteins, with most of the apoE on the large VLDL and on IDL/LDL (Figure 2). ApoE was detected in HDL fractions on longer exposures.

Evidence of Stem Cell Transduction

The prolonged apoE expression in transplant mice suggests that pluripotent stem cells or early progenitor cells were transduced, because the progeny of late-stage progenitors probably persist in circulation for no more than 3 to 4 months. To investigate whether stem cells had been transduced, a secondary transplantation was performed. Bone marrow was collected from 3 of the murine apoE–expressing mice 16 weeks post-BMT and transplanted into 6 lethally irradiated apoE(−/−) recipient mice. ApoE was detected in serum at 3 months post-BMT in these secondary transplant mice, providing further evidence for stem cell transduction in the original transplants (Figure 3).

Analysis of ApoE Expression by Peritoneal and Arterial Macrophages

ApoE expression in the medium of BMT macrophages was 0.5% that of C57BL/6 macrophages (Figure 4). ApoE was also expressed from other tissues, such as the spleen and the artery wall. Immunocytochemistry analyses showed that apoE was secreted by arterial macrophages of pLMESN/V2 mouse from the 5- to 13-week group (Figure 5) as well as the 10- to 26-week group (data not shown). These apoE-producing macrophages were found throughout the plaques and at the valve cusps, but there was a high degree of variability in apoE expression between individual experimental mice. Of a total of 14 mice in the treated group of the "late

---

![Image 1](image1.png)

**Figure 1.** Immunoblot analysis of murine and human apoE in serum of transplant mice. Aliquots (3 μL) of serum samples were subjected to 12% SDS-PAGE. Lanes 1 through 3 contain 3 μL of diluted normal mouse or human serum as indicated. Lane 4 contains pre-BMT serum, and lanes 5 through 8 contain serum from individual mice at 3, 4, 6, and 8 weeks post-BMT. Top, ApoE in serum from a pLMESN/V2 mouse; bottom, apoE from a pLHESN/V2 mouse.

![Image 2](image2.png)

**Figure 2.** Distribution of serum apoE on lipoproteins of transplant mice. Gel filtration chromatography was performed on serum samples taken at the final time point from both mouse and human apoE-expressing mice to separate lipoproteins. Top, Fractions from a murine apoE-expressing mouse; bottom, fractions from a human apoE-expressing mouse. Lane 1 contains 3 μL of whole serum; lane 2 contains apoE(−/−) serum. Lanes 4 through 13 represent pooled chromatographic fractions: lane 3, fractions 11 through 12 (void volume); lanes 4 and 5, fractions 13 to 14 and 15 to 16 (VLDL); lanes 6 through 8, fractions 17 to 18, 19 to 20, and 21 to 22 (LDL/IDL); lanes 9 and 10, fractions 23 to 24 and 25 to 26 (HDL); and lanes 11 through 13, fractions 27 to 32 (nonlipoprotein fractions).

![Image 3](image3.png)

**Figure 3.** Immunoblot analysis of serum apoE in secondary transplant mice. Bone marrow was collected from 3 mice that expressed murine apoE (16 weeks post-BMT), pooled, and used as donor marrow for 6 lethally irradiated apoE(−/−) recipient mice. Lanes 1 and 2 contain 1 μL of C57BL/6 serum at dilutions of 1:100 and 1:200. Lane 3 contains 3 μL of undiluted apoE(−/−) serum. Lanes 4 through 6 contain 1 μL of undiluted serum from 3 primary BMT donor mice taken at time of death. Lanes 7 through 11 contain 5 μL of undiluted serum from 5 secondary recipient mice at 3 months post-BMT.
study.” 2 showed apoE in 60% to 80% of the arterial macrophages, 5 in 30% to 40%, and 6 in 10% to 20%, and 1 had undetectable apoE. This degree of variability was similar to that observed in the mice of the “early” group, in which significant effects on lesion growth were observed.

Quantification of Atherosclerosis
In mice that received transplants at 10 weeks of age and were killed at 26 weeks of age, there were no significant differences in the extent or distribution of lesions detected between the experimental (mouse apoE, n = 23) and control groups (n = 21). In contrast, experimental mice given transplants of either murine (n = 11) or human (n = 15) apoE at 5 weeks of age and killed at 13 weeks of age had significantly smaller lesions than control mice (n = 9) (4847 ± 2410, 5294 ± 2868, and 12 837 ± 7166 µm² ± SEM, respectively) (Figure 6). The differences between murine or human apoE–expressing mice and controls were significant at the level of P<0.003 and P<0.002, respectively (Student’s t test and Mann-Whitney rank sum test).

There was no difference between mice that expressed either murine or human apoE. By immunocytochemistry analysis, no major differences in plaque composition were detectable between treated and control groups in either study.

Discussion
In this study, we demonstrate that retroviral infection of bone marrow with an apoE-containing retrovirus leads to expression of the apoE transgene in apoE(−/−) mouse recipients of BMT, resulting in secretion of apoE by macrophages in the artery wall. ApoE was present in the serum of experimental mice for as long as 5.5 months after transplantation, at levels of ~0.5% to 1% of normal, and was associated with lipoproteins. ApoE was expressed by both splenic and peritoneal macrophages, as well as by macrophages within the artery wall of transplant mice. Finally, both human and mouse apoE were effective in reducing foam cell formation when expressed during early atherogenesis but had no effect during later stages of atherogenesis.

In the first experiment, mice received transplants at 5 weeks of age and were followed up until 13 weeks. When normal marrow is used to reconstitute apoE(−/−) mice, apoE is not detected in serum until 2 weeks post-BMT. Therefore, mice in the present study expressed apoE only from 8 to 13 weeks of age (monocyte adhesion and foam cell formation stages of atherogenesis). Although plasma lipid levels were not affected by the intervention, mice that expressed either human or mouse apoE had significantly smaller lesion area (2- to 7-fold) than controls (P<0.003, mouse apoE versus controls, and P<0.002, human apoE versus controls). These data demonstrate that low-level apoE expression from arterial...
macrophages is able to modulate early atherogenic events even when expressed for only a few weeks. The second group received transplants at 10 weeks and were allowed to progress until 26 weeks. The apoE in these mice was therefore expressed at the beginning and well into the intermediate lesion formation stage. In these mice, low levels of mouse apoE were expressed for a longer time than in the younger group but had no apparent effect on lesion formation. These data support the concept that apoE expression from macrophages in the artery wall is important in the early stages of atherogenesis but is not beneficial when delivered to more advanced lesions. The difference in atherosclerosis between studies cannot be ascribed to variations in apoE expression level or duration in transplant mice. Dot blot assays using all the samples of all time points from all mouse groups confirmed that in every instance, plasma apoE levels were ≥0.5% of normal (not shown). In addition, secondary transplants using the marrow from retrovirus-infected mice from the late experiment show that apoE was detectable in the plasma of apoE null recipients of marrow from primary transplant mice, proving that long-lived stem cells were transduced during the original procedure (Figure 3). Although we cannot exclude the possibility that the lack of apoE effect on atherosclerosis in the mice of the late experiment was specific for the female sex, previous work from other investigators shows that progression and composition of arterial plaques in apoE null mice is not significantly different between sexes.21,24

Because no macrophage-specific promoter was used in this study, it is possible that other bone marrow–derived cell lines may have contributed to plasma apoE levels in reconstituted mice. The question of which cell type expressed apoE in the retrovirus-infected mice was indirectly addressed in a series of experiments comparing apoE levels in plasma and in macrophage-conditioned medium of either the retrovirus-infected apoE null mice (pLMESN) or apoE null mice that received 2 different dilutions (5% and 10%) of C57BL/6 marrow in apoE null marrow. Because the macrophage is the only apoE-producing cell line derived from normal unpelilated bone marrow, we used the ratio between the densitometric readings of plasma and macrophage medium apoE in normal transplant mice as a comparative marker of the contribution of macrophages to plasma apoE in retrovirus-infected mice. The data (not shown) are compatible with the notion that ≈70% of apoE in the plasma of these mice is derived from macrophages, whereas only 30% derives from other leukocytes.

The beneficial effect of apoE in the artery wall may be a result of one of several mechanisms: (1) the promotion of cholesterol efflux from the cholesterol-loaded macrophage25; (2) the facilitation of reverse cholesterol transport from the artery wall26; (3) the induction of remnant uptake through apoE-dependent pathways (as explained by the secretion-capture hypothesis)27; or (4) its local effects on inflammatory processes such as platelet aggregation,28 leukocyte activation,29,30 or growth factor sequestration (see Reference 31 for review). It is interesting to note that the level of human apoE in serum was higher than that of mouse apoE (Figure 1), but still no reduction in serum cholesterol levels was observed. Although human apoE3 binds to the murine LDL receptor with lower affinity than mouse apoE, it was nonetheless as effective as mouse apoE in reducing lesion area in transplant mice. These data suggest that the mechanism of action of macrophage apoE in the artery wall is not completely explained by its receptor-binding properties.

Gene therapy has been considered a promising alternative for treatment of cardiovascular diseases, such as restenosis and atherosclerosis.32,33 The majority of studies performed to date have focused on eliminating smooth muscle cell proliferation in vascular injury models of restenosis. Several different approaches have been taken using adenoviral vectors to deliver many different categories of genes, including oligonucleotides against proto-oncogenes,34 suicide genes,35 and genes affecting cellular proliferation.36 Although these studies have clearly demonstrated the potential for reducing smooth muscle cell proliferation after angioplasty procedures, they do not address the question of whether gene therapy for atherosclerosis might be possible. Endothelial cells, smooth muscle cells, and macrophages all contribute to atherogenesis and are potential target cells for gene therapy approaches. Because monocytes/macrophages are derived from bone marrow progenitors, can migrate into the vessel wall, and are involved in the very early events in atherogenesis, they are attractive targets for cell-mediated gene therapy of atherosclerosis.

This study clearly demonstrates that macrophage-derived apoE has a significant impact on foam cell formation but does not play a role in the later stages of atherogenesis, in which macrophages are no longer the principal cell type involved in the lesion formation. In addition, this study demonstrates the feasibility and efficacy of transduction of bone marrow followed by transplantation as a way to express transgenes from macrophages in the artery wall with potent effects on atherogenesis. ApoE represents only one of the many potential genes with therapeutic effects on atherosclerosis. Other genes related to lipoprotein metabolism, fibrinolysis, thrombosis, cell proliferation, and oxidation have a potential application for gene therapy of atherosclerosis if expressed from macrophages in the artery wall.

Acknowledgments
This work was supported in part by American Heart Association Grant-in-Aid 95011450 and by National Institutes of Health grants HL-53989 and HL-57986. Alyssa Hasty was supported in part by an NIH Vascular Biology Training Grant. Dr Babaev was supported in part by an NIH Diabetes Research Training Grant. Dr Linton was partially supported by a Clinical Investigator Development Award from the NIH (HL-02925). Drs Fazio and Linton are Established Investigators of the American Heart Association.

References
Macrophiage ApoE Delays Foam Cell Formation


Retroviral Gene Therapy in ApoE-Deficient Mice: ApoE Expression in the Artery Wall Reduces Early Foam Cell Lesion Formation

Alyssa H. Hasty, MacRae F. Linton, Stephen J. Brandt, Vladimir R. Babaev, Linda A. Gleaves and Sergio Fazio

*Circulation.* 1999;99:2571-2576
doi: 10.1161/01.CIR.99.19.2571

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/99/19/2571

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation* is online at:
http://circ.ahajournals.org/subscriptions/