Low Levels of Extrahepatic Nonmacrophage ApoE Inhibit Atherosclerosis Without Correcting Hypercholesterolemia in ApoE-Deficient Mice

Fayanne E. Thorngate, Lawrence L. Rudel, Rosemary L. Walzem, David L. Williams

Abstract—The prevention of atherosclerosis by apolipoprotein E (apoE) is generally attributed to the removal of plasma lipoprotein remnant particles. We developed transgenic apoE-knockout mice expressing apoE specifically in the adrenal gland and found that only 3% of the wild-type plasma level of apoE was sufficient to normalize plasma cholesterol levels in the apoE-deficient mouse. As expected, mice expressing apoE at levels that correct hypercholesterolemia had almost no cholesteryl ester deposition in their aortas. In contrast, their nontransgenic siblings had significant atherosclerosis. Unexpectedly, we found that atherosclerosis was also reduced in 2 transgenic lines expressing too little apoE (<1% to 2% of wild type) to correct their hypercholesterolemia. Gel exclusion chromatographic profiles of plasma lipoproteins and the size distributions of lipoproteins with density <1.063 (low density and very low density lipoproteins), as determined by dynamic laser light scattering, were the same in mice expressing <2 μg/mL plasma apoE and their nontransgenic littermates. We conclude that the antiatherogenic action of low levels of plasma apoE is not due to the clearance of remnant lipoproteins. Thus, low levels of apoE provided systemically, but not made in the liver or in macrophages, can block atherogenesis in the vascular wall independently of normalizing the plasma concentration of atherogenic remnant lipoprotein particles. (Arterioscler Thromb Vasc Biol. 2000;20:1939-1945.)

Key Words: apoE ■ atherosclerosis ■ hypercholesterolemia ■ transgenic apoE-knockout mice

In spite of our expanding knowledge of the causes of coronary heart disease, it remains the leading cause of death in the developed world. Elevated levels of atherogenic cholesteryl ester (CE)-rich lipoproteins are a major risk factor for atherosclerosis. These lipoproteins are believed to act in the artery wall to initiate or propagate complex inflammatory reactions leading to intimal accumulation of cholesterol-laden macrophage foam cells and the progressive development of atherosclerotic plaque. ApoE plays a critical role in plasma cholesterol hometostasis by mediating the hepatic uptake of CE-rich remnant lipoproteins by members of the LDL receptor family. Type III hyperlipoproteinemia and premature atherosclerosis occur in individuals lacking apoE and in a subset of individuals with apoE variants that are defective in binding to receptors. The apoE-knockout (apoE−/−) mouse was the first mouse model to develop massive hypercholesterolemia and the full range of atherosclerotic lesions, even when maintained on a low-fat chow diet. ApoE−/− mice also have increased oxidation-specific autoantibodies and develop stenosis in peripheral arteries, as may result from atherosclerosis in humans.

Previous studies suggest that apoE may have a role in preventing atherosclerosis other than its role in remnant clearance. Watanabe LDL receptor–deficient rabbits given sustained intravenous apoE had reduced progression of lesions without a reduction in total plasma cholesterol (TPC). Also, transgenic C57BL/6 mice overexpressing apoE in the arterial wall had reduced atherosclerosis in response to a high-fat diet, although their plasma cholesterol concentrations and lipoprotein profiles were the same as those of the nontransgenic control mice. However, each of these cases reflects the effects of excess exogenous apoE in addition to the normal endogenous level of apoE.

In apoE−/− mice, a low level of apoE that was expressed either in the liver or in macrophages derived from wild-type bone marrow transplants was sufficient to normalize plasma cholesterol levels and prevent atherosclerosis. Bellosta et al showed that apoE expressed by macrophages, at levels too low to correct hypercholesterolemia in apoE−/− mice, significantly reduced atherosclerosis. They suggested that this protection was due to apoE production by macro-

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phages in the arterial wall, leading to increased efflux of cholesterol from arterial macrophages, thereby inhibiting foam cell development.17

In the present study, we examined apoE−/− mice that express transgenic apoE selectively in the adrenal gland and have very low levels of circulating apoE. ApoE is normally expressed at relatively high levels in the adrenal glands of humans,18 nonhuman primates,19 and rodents.20 Hence, adrenal-specific expression provides a means to test the ability of extrahepatic nonmacrophage apoE to alter the systemic metabolism of plasma lipoprotein particles and the development of atherosclerosis.

In these transgenic apoE−/− mice, plasma apoE concentrations >2% to 4% of the wild-type level were sufficient to normalize plasma cholesterol levels and, as expected, prevent atherosclerosis. Unexpectedly, atherosclerosis was also dramatically reduced in 2 transgenic lines expressing too little apoE (<1% to 2% of wild type) to correct their plasma cholesterol levels. These data indicate that low levels of apoE in the plasma, not derived from the liver or macrophages, can prevent atherosclerosis independently of a role in maintaining plasma cholesterol homeostasis.

Methods

Transgenic Mice Expressing ApoE in the Adrenal Gland

Mice were maintained on a 12-hour light/12-hour dark cycle with standard rodent chow and water ad libitum. Housing and experimental procedures were approved by the State University of New York at Stony Brook Committee on Laboratory Animal Resources. The DNA fragment used for oocyte microinjections contained ~6 kb of the mouse 21β-hydroxylase promoter,21 attached to the mouse apoE gene as described in Results. This construct was made by use of standard molecular biology techniques22 and was grown in Escherichia coli strain STBL2 (Life Technologies). We coinfected a 2-fold molar excess of the ~3-kb chicken lysozyme matrix attachment region23 to improve specificity of transgene expression.24 Transgenic mice were prepared in the University Transgenic Mouse Facility, with FVB/N mice used as donors and recipients. Positive founder mice were identified by triplex polymerase chain reaction (PCR) with apoE2 background or apoE2/hemizygous for the transgene on apoE2 background.

MgCl2, 0.2 mol/L sucrose, 10 mmol/L Tris-HCl (pH 7.4), 2 mmol/L MgCl2, 0.2 mol/L sucrose, 10 μg/mL leupeptin, 20 μg/mL aprotinin, 5 μg/mL pepstatin, and 5 mmol/L phenylmethylsulfonyl fluoride. Proteins26 (60 μg) were resolved on 10% polyacrylamide–SDS gels; 0.5 μL of C57BL/6 plasma and 2.5 μL of the transgenic mouse plasma were also analyzed. Samples were electrophoretically transferred to nitrocellulose and immunostained as described27 by use of an affinity-purified goat anti-rat apoE (from P. Rohrbach, Department of Physiology, Louisiana State University Medical Center). The secondary antibody was a peroxidase-coupled anti-goat IgG (Sigma Chemical Co). Bands were visualized by enhanced chemiluminescence (Amersham).

mRNA Measurement

RNA was prepared by grinding tissues in RNA STAT-60 (Tel-Test, Inc) following the manufacturer's protocol. Peritoneal macrophages were elicited with thioglycolate broth and plated, and adherent cells were maintained for 2 days.28 RNA was made as described above, and RNA concentration was measured by absorbance at 260 nm or with the RiboGreen fluorescent assay kit (Molecular Probes). Tissue-specific expression was determined by use of an RNA excess solution hybridization assay29 with a mouse apoE–specific probe transcribed from pBSMAE7.30 A reverse transcriptase–PCR protocol based on that of Rexin and Feussner31 was developed for the macrophage RNA with mouse apoE–specific primers.

Lipoprotein Analyses

Mice were fasted for 6 hours, anesthetized with inhaled methoxyflurane, and bled from the tail into heparinized capillaries. Plasma was obtained by centrifugation at 2000g, 4°C, for 20 minutes. TPC was assayed by use of the Cholesterol CII assay (Wako Chemicals USA). Lipoprotein profiles were analyzed by running 200 μL of plasma over a 25-mL Superose 6 column (Pharmacia) in PBS and 1 mmol/L EDTA at 0.5 mL/min; cholesterol was measured in each fraction.

Determination of LDL+VLDL Particle Size Distributions

Mice from line 619 were fasted, tail vein blood was collected, and plasma was prepared as described above. Plasma from 9 male mice carrying the transgene was pooled, as was plasma from 8 of their apoE−/− siblings. The LDL+VLDL lipoprotein fraction (density <1.063) was isolated by KBr density ultracentrifugation.32 The isolated fractions were dialyzed against 0.15 mol/L NaCl, 5 mmol/L EDTA, and 0.05% sodium azide, and lipoprotein particle diameters were determined by dynamic light scattering.33

Mouse ApoE ELISA

A mouse apoE sandwich ELISA, as previously described for human apoE,34 used the affinity-purified goat anti-rat apoE described above as the capture antibody. For the secondary antibody, an anti-mouse apoE antiserum (BioDesign International) was affinity-purified and biotinylated with sulfo-NHS-LC-biotin (Pierce). This was followed by streptavidin–horseradish peroxidase conjugate (Life Technologies). An apoE–glutathione-S-transferase fusion protein, expressed from pGEX-4T-1 (Pharmacia Biotech) containing the coding region for amino acids 72 to 311 of mouse apoE, was used as the standard in the ELISA. ApoE−/− plasma was added to the standard in equal volume to the plasma samples. The final mass determination was corrected for the percentage of the fusion protein that is apoE.

Measurement of CE Deposition in the Aorta

After ~1 year on a standard chow diet, mice were anesthetized with ketamine HCl (80 mg/kg) and xylazine (1.2 mg/kg) administered intraperitoneally. Blood was drawn by cardiac puncture into a syringe rinsed with 0.2 mol/L EDTA, and plasma was obtained by centrifugation at 4°C. With the heart attached, the aorta was removed down to the ileal bifurcation and fixed in 10% neutral buffered formalin and 5% sucrose at 4°C. After removing the adventitia, aorta CE concentration was determined as previously described.35 For visual evaluation, aortas were fixed, cleaned, opened longitudinally, and stained with Sudan IV.13 Aortas were pinned out on a dissecting microscope and photographed with a Leaf digital camera attached to a dissecting microscope. Pin shadows were removed by using the airbrush function of Adobe PhotoShop set to adjacent tissue color.

Statistical Analysis

Statistical significance was determined by the Student t test if the data distributions were gaussian or a Mann-Whitney U test if not. A value of P<0.05 was considered significant. All values are presented as mean±SEM.
Results

Transgenic Mice Expressing ApoE in the Adrenal Gland

An adrenal-specific transgene was made with the promoter for 21-ß-hydroxylase, followed by the mouse genomic apoE sequence. 21-ß-Hydroxylase expression is unique to the adrenal gland and is highly tissue specific in transgenic mice when driving ß-galactosidase expression. We removed a 1.2-kb mouse retroviral remnant, containing a putative androgen response element, from the promoter region by PCR-mediated mutagenesis to prevent a sex bias in expression. Microinjected oocytes were implanted in 2 FVB/N females, resulting in 10 pups with the transgene in the germ line. These founders were crossed for 2 generations with apoE heterozygous mice (C57BL/6 × 129) to generate mice producing apoE only from the transgene.

Detection of ApoE Protein in the Adrenal Gland and Plasma of the Transgenic Knockout Mice

Western blotting of adrenal lysates showed wide variation in apoE expression among the transgenic lines (Figure 1A). Line 621 had only a trace of signal. Line 624 also had low adrenal expression, whereas most of the other lines made more apoE in the adrenal than did wild-type C57BL/6. Because the transgenic lines had robust adrenal expression, we assayed plasma samples for apoE by Western blotting (Figure 1B). The lines had various levels of plasma apoE that were all considerably less than the levels in C57BL/6. Except for line 616, the females had dramatically lower levels of plasma apoE than did the males (and lower levels of adrenal apoE, data not shown), suggesting that there is still an active androgen response element in the 21-ß-hydroxylase promoter of the transgene. Because of this difference, males were used in subsequent studies.

Tissue Specificity of Transgenic ApoE Expression

Lysates (100 μg protein) of kidney, liver, lung, spleen, adrenal gland, and testis were assayed for apoE by Western blot. In the 2 lines having the highest plasma levels of apoE, lines 614 and 616, we observed a very faint signal in the liver and the testis in males. We did not detect apoE in the liver or ovary in females from these lines, only adrenal apoE (data not shown). The other lines had detectable apoE expression only in the adrenal gland. To determine the tissue specificity of expression from the transgene with more sensitivity, we used an RNase protection assay to determine the amount of apoE mRNA in the liver, adrenal gland, brain, and testes. We did not detect brain apoE expression in any of the lines. Lines 619, 620, 621, and 630 had no detectable apoE mRNA expression in the liver or the testes. The levels of apoE mRNA in the adrenal glands in the various lines roughly correlated with the observed protein levels in the Western blots. Line 614 had the highest apoE expression (494 pg apoE mRNA per microgram total RNA), and line 619 had the lowest adrenal expression. For transgenic lines 619 and 620, in which apoE expression was detected only in the adrenal gland, line 619 males had 1.6 pg apoE mRNA per microgram total RNA, and line 620 males had 36.9 pg apoE mRNA per microgram total RNA. Wild-type mice averaged ~10 pg apoE mRNA per microgram total adrenal RNA, similar to the level in the adrenal glands of rats. We did not detect apoE mRNA in peritoneal macrophages from even the high apoE-expressing lines with the use of a very sensitive reverse transcriptase–PCR assay. In contrast, a control housekeeping message, cyclophilin, was readily amplified from the same reverse transcription reactions (data not shown).

Correlation of Plasma ApoE and Plasma Cholesterol Levels

Although the levels of apoE in the plasma of the transgenic knockout mice were low, we determined TPC levels in 2 of the highest expressing lines to determine whether there was any reduction relative to the nontransgenic knockout littermates. As an estimation of wild-type levels, we used littermates without the transgene but heterozygous for the normal mouse apoE allele. For line 614 with the transgene, TPC was 123.2 ± 12.1 mg/dL (plasma apoE 2.20 ± 0.15 μg/mL, n = 5). Line 614 without the transgene had a TPC of 843.1 ± 137.4 mg/dL (n = 6, P = 0.0005), and mice heterozygous for normal mouse apoE had a TPC of 83.8 ± 5.9 mg/dL (n = 4, P = 0.0013). Similarly, for line 615 with the transgene, TPC was 132.2 ± 12.3 mg/dL (plasma apoE 1.96 ± 0.28 μg/mL, n = 5), and without the transgene, TPC was 792.5 ± 164.1 mg/dL (n = 4, P = 0.0013). The knockout mice carrying the transgene have plasma cholesterol levels that are almost normalized to the levels of their littermates heterozygous for wild-type mouse apoE (C57BL/6 mice typically have TPC levels ranging from 70 to 100 mg/dL.)

Because the various transgenic lines showed a gradient of plasma apoE, the relationship between TPC and plasma apoE concentration was determined with age-matched males from each line, and from the parental apoE heterozygous mice, by using an ELISA for mouse apoE. The data shown in Figure 2 demonstrate an inflection point in the curve at ~2 μg/mL of apoE; above this concentration, TPC was similar to that in wild-type mice. Between 2 μg/mL and 0 μg/mL of plasma apoE, an inverse linear correlation between apoE concentration and cholesterol (r = −0.819, P < 0.0001) was seen. These data show that only ~3% of wild-type plasma apoE was sufficient to correct the hypercholesterolemia of apoE heterozygous mice.
Reduction in Atherosclerosis

With such a dramatic effect of low levels of apoE on plasma cholesterol concentration, we tested whether a similar reduction would be seen in atherosclerosis in the transgenic knockout lines. For these experiments, we used lines of mice that had almost normalized TPC. Lines 614, 615, and 616. They were maintained on a standard chow diet for 9 months to 1 year. (Complex fibrous lesions are observed in apoE−/− mice by 8 months.) We used age-matched knockout siblings as controls. Aortic CE deposition was used as a measure of atherosclerosis, because studies with apoE-deficient mice have shown that aortic CE deposition provides as good a measure of aortic atherosclerosis as measurements of surface area covered by lesions. We have also observed a strong correlation ($r^2=0.8528$, $P<0.0001$) between aortic CE and lesion surface area in the aortas of LDL receptor–deficient human apoB transgenic mice (J.K. Sawyer, L.L. Rudel, unpublished data, 1999). As seen in Figure 3, there was an almost complete suppression of aortic CE deposition in the transgenic mice, in striking contrast to their knockout siblings ($P<0.0001$), who showed a range of atherosclerosis typically seen in apoE−/− mice. Thus, plasma apoE concentrations as low as 2 μg/mL were sufficient to normalize plasma cholesterol and prevent atherosclerosis.

Having observed nearly complete protection from atherosclerosis development in mice with >2 μg/mL apoE, we measured atherosclerosis in transgenic lines that have too little apoE to normalize plasma cholesterol (~0.5 to 1.5 μg/mL apoE). These mice all are hypercholesterolemic and have plasma lipoprotein profiles indistinguishable from those of their apoE−/− siblings on Superose 6 gel exclusion chromatography (Figure 4A and 4B). In addition, as seen in Figure 4C, we observed no significant differences in LDL+VLDL particle sizes or distributions between line 619 versus apoE−/− littermates as assayed by dynamic light scattering.

Figure 2. TPC vs plasma apoE concentration for the independent transgenic apoE−/− lines. Male mice from the indicated lines were fasted for 6 hours and tail-bled, and TPC and plasma apoE concentrations were determined as described in Methods. Each point indicates value from an individual mouse. There were 2 mice for the apoE−/− parent line. Between 0 and 2 μg/mL plasma apoE, there is an inverse linear correlation between plasma apoE concentration and TPC ($r=-0.819$, $P<0.0001$).

Figure 3. CE deposition in aortas from transgenic apoE−/− lines with sufficient plasma apoE to normalize TPC compared with their nontransgenic siblings. Male mice were fed standard mouse chow for 9 months to 1 year. Aortas were removed and fixed, and CE was determined as described in Methods. Each point indicates value from an individual animal. Horizontal bars indicate mean CE concentrations (0.80 ± 0.33 μg/mg aorta protein for mice with the transgene and 25.05 ± 4.16 μg/mg aorta protein for siblings without the transgene, n=15 for each group). The difference between the 2 groups was significant, as assayed by Mann-Whitney U test ($P<0.0001$).

Figure 4. Plasma lipoprotein profiles and particle size distributions. A, Representative fast protein liquid chromatography profile is from an apoE−/− male mouse, with the typical high VLDL cholesterol. B, Representative profile is from a line 619 male mouse expressing the transgene. C, Mice from line 619 with the transgene (n=9) or their apoE−/− siblings (n=8) were fasted for 6 hours and tail-bled. Plasmas for each type were pooled, and lipoproteins with density <1.063 were assayed by dynamic light scattering to determine the percentage of particles of each diameter from 3.2 to 6541 nm. No particles <21.5 nm or >205 nm were observed. Each sample was assayed 3 times, and the mean±SEM values are shown.

Figure 5. Aortas prepared en face and stained with Sudan IV. Male mice were maintained on standard mouse chow for 1 year. TG indicates transgene. Aortas were removed from the aortic arch to the renal arteries. Dark red areas indicate neutral lipid deposits in the artery. Lines 619 and 620 have low levels of plasma apoE (~1 μg/mL and 1.2 μg/mL, respectively), which are not sufficient to normalize TPC (see Results).
scattering. The mean particle diameter was 40.67±0.66 nm for the line 619 transgenic particles and 40.03±0.78 nm for the nontransgenic particles. There were no significant differences between the sets in any of the size divisions. Thus, the level of apoE in line 619 was too low to alter the distribution of plasma lipoprotein remnants.

Atherosclerosis in these very low apoE–expressing mice was evaluated by en face examination after staining with Sudan IV to reveal lipid-laden lesions (Figure 5). Unexpectedly, we saw almost no lesion staining in the transgenic mice, in spite of their persistent hypercholesterolemia. One small lesion in the line 620 transgenic male is evident in the aortic arch, but there are no lesions in the 619 transgenic male. This is in marked contrast to line 620 nontransgenic males, who have lesions throughout the aorta. Aortas from line 619 mice not carrying the transgene also have lesions throughout the aorta (data not shown). CE deposition was measured in aortas from transgenic mice without the transgene also have lesions throughout the aorta (data not shown). CE deposition was measured in aortas from transgenic mice without the transgene also have lesions throughout the aorta (data not shown). CE deposition was measured in aortas from transgenic mice without the transgene also have lesions throughout the aorta (data not shown).

Figure 6. CE deposition in aortas from transgenic mice without normalized TPC compared with their nontransgenic siblings. Each symbol represents an individual mouse. Aortic CE was determined for line 619 and 620 mice from 7.5 months to 2 years of age. A, Aortic CE for lines 619 and 620 with or without the transgene vs age. The line shows the correlation between age and aortic CE for mice without the transgene ($r^2=0.53, P=0.0021$). B, TPC vs age for the mice in panel A.

Levels of Plasma ApoE Too Low to Affect the Hypercholesterolemia of ApoE−/− Mice Still Prevent the Deposition of CE in the Aorta

<table>
<thead>
<tr>
<th>Mice</th>
<th>Plasma Cholesterol (mg/dL)</th>
<th>Aortic CE (μg/mg protein)</th>
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<tbody>
<tr>
<td>Transgenic (n=10)</td>
<td>564.6±69.43</td>
<td>5.17±1.66*</td>
</tr>
<tr>
<td>Nontransgenic (n=10)</td>
<td>626.3±31.82</td>
<td>42.42±9.53</td>
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Values are mean±SEM. *$P<0.0008$ by Mann-Whitney $U$ test. Five mice from line 619 and 5 mice from line 620 were used in each case.

Discussion

Our results showed that mice with levels of plasma apoE too low to correct the hypercholesterolemia of their apoE−/− background had almost complete suppression of atherosclerotic lesion development. The apoE in our mice came from neither the liver nor macrophages and seems to be protective by a mechanism independent of plasma cholesterol concentration or remnant particle clearance.

The mechanism by which low levels of systemic apoE protect against atherosclerosis is unknown. One possibility is that the low levels of apoE allow for the selective clearance of a minor, but highly atherogenic, remnant lipoprotein subfraction. To test this possibility, we examined the size distribution of remnant lipoproteins in mice expressing low levels of apoE compared with their nontransgenic littermates. Gel exclusion chromatography showed no difference in the size profile due to low-level apoE expression (Figure 4A and 4B). A more sensitive analysis of size differences was provided by dynamic laser light scattering of remnant particles in the LDL+VLDL size range (density <1.063 g/mL). This analysis also failed to detect a difference in the LDL+VLDL particle size distribution due to low-level apoE expression (Figure 4C). This method would detect a change of as little as 2.5% of the total remnant population if it occurred at a specific size range, because the maximum analyzed in any sample bin, in the midrange of particle sizes, is $\approx 10\%$ of the total population. The sensitivity of the analysis is increased at the extremes of particle size. Nevertheless, we detected no significant difference due to low-level apoE expression in any bin within the entire profile. These data argue strongly that low-level apoE expression did not perturb remnant metabolism. The mean particle diameters
observed (40.67 ± 1.66 nm for line 619 plus the transgene and 40.03 ± 0.78 nm without the transgene) were very similar to those previously found for VLDL in apoE<sup>−/−</sup> mice (41.1 ± 11.0 nm, mean ± SD<sup>43</sup>). These results strongly suggest that the antiatherogenic action of low-level apoE expression in line 619 is not due to the removal of a minor fraction of highly atherogenic remnant lipoproteins.

In agreement with our results, Hasty et al<sup>42</sup> observed no differences in the gel exclusion chromatographic profiles, nondenaturing gel electrophoresis, or lipoprotein clearance rates that were due to the low levels of apoE when they expressed apoE in macrophages via a bone marrow transplantation protocol. They observed a reduction in the fraction of total lipoproteins of <30-nm diameter via negative-stain electron microscopy of total plasma from mice with low-level apoE expression. We observed no differences in particle diameters between low-level apoE-expressing mice and apoE<sup>−/−</sup> mice by dynamic light scattering with particles in the LDL+VLDL size range (Figure 4C). Similarly, in a single analysis of the <1.063-g/mL density fraction via negative-stain electron microscopy (measuring diameters of 800 to 900 particles for each population), we did not detect significant differences in particle diameters due to low-level apoE expression (R.H. Hamilton, F.E. Thorngate, D.L. Williams, unpublished data, 1999). Thus, in transgenic mice and their nontransgenic littermates with equivalent hypercholesterolemia, low-level apoE expression provided protection against atherosclerosis without altering the total level, size distribution, or mean particle size of remnant lipoproteins in the LDL+VLDL fraction.

The response to retention hypothesis states that a key event in the initiation of atherogenesis is the subendothelial retention of atherogenic particles within the artery wall,<sup>43</sup> thereby initiating a cascade of events that lead to macrophage recruitment and intimal foam cell formation. In a recent report, freeze-etch electron microscopy showed an accumulation of remnant lipoproteins associated with the intimal extracellular matrix of aortas in apoE<sup>−/−</sup> mice as early as 3 weeks of age.<sup>44</sup> This event precedes the appearance of adherent monocytes and intimal foam cells and may be the initial event in atherosclerosis. One hypothesis derived from the present results is that low levels of systemic apoE suppress this early remnant lipoprotein retention. Other possibilities are that apoE acts at downstream steps to suppress the upregulation of endothelial vascular cell adhesion molecule-1<sup>45</sup> or to suppress the action of chemokines that promote monocyte migration and macrophage foam cell formation.<sup>46</sup> Each of these is an early event that precedes significant lipid accumulation in the artery wall. It is interesting to note that apoE has a variety of hormone- or cytokine-like effects in steroidogenic cells, platelets,<sup>47,48</sup> and lymphocytes.<sup>49</sup> Thus, there is significant precedent to speculate that cytokine-like effects of apoE in the artery wall may underlie its atheroprotective actions.

Another potential antiatherogenic effect of low levels of apoE is to promote cholesterol efflux from macrophages in the arterial wall, as suggested by Bellota et al<sup>46</sup> on the basis of their results with transgenic mice expressing apoE in macrophages. If this mechanism is operative, the present results with adrenal-specific apoE expression indicate that low levels of systemic apoE can produce this effect without the need to have apoE expressed by the macrophage itself. The lines of transgenic mice described in the present study can be used to test each of the above hypotheses.

These studies demonstrated that only 2 μg/mL of plasma apoE is sufficient to maintain normal TPC on an apoE-knockout background. Between 2 μg/mL and 0 μg/mL of plasma apoE, an inverse linear correlation between apoE concentration and cholesterol (r = -0.819, P < 0.0001) was seen. This result suggests a simple dose-response relationship between plasma apoE concentration and the clearance of remnant lipoproteins. A similar result was seen by Hasty et al<sup>42</sup> when apoE was expressed by macrophages derived from bone marrow transplants. Our data as well as those of Hasty et al yield the surprising result that only ∼3% of wild-type plasma apoE was sufficient to correct the hypercholesterolemia of apoE<sup>−/−</sup> mice. On the basis of the results with our transgenic lines 621 and 630, in which liver apoE expression was not detected, it appears that this low level of plasma apoE is sufficient to mediate remnant lipoprotein clearance even when the apoE is made in a peripheral tissue and not in either the liver or macrophages. An interesting question arising from these results is why apoE is normally expressed in mice at levels greater (≥30-fold) than are necessary to prevent atherosclerosis and to maintain normal remnant particle clearance. One possibility is that there are other actions of apoE that are less sensitive to apoE concentration and that have dose-response relationships shifted to higher plasma apoE concentrations. Such possibilities could include the effects of apoE on ovarian or adrenal steroid production,<sup>47,48</sup> platelet aggregation,<sup>49</sup> lymphocyte activation,<sup>50</sup> or activation of hepatic lipase.<sup>51</sup> Additional studies will be required to test these possibilities.

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