Effect of Human ApoE4 on the Clearance of Chylomicron-like Lipid Emulsions and Atherogenesis in Transgenic Mice

Bok-Cheng Mortimer, Trevor G. Redgrave, Elizabeth A. Spangler, Judy G. Verstuyft, Edward M. Rubin

Abstract Apolipoprotein (apo) E is a ligand for lipoprotein receptors and mediates the cellular uptake of several different lipoproteins. Human apoE occurs in three allelic forms designated E2, E3, and E4. The E2 isoform is associated with changes in lipoprotein metabolism, and the E4 isoform is associated with Alzheimer's disease and an increased risk of coronary heart disease. In this study transgenic mice were generated to assess the effect of a sustained increase in plasma apoE4 concentration. The transgenic animals had three- to sixfold increases in total plasma apoE, associated primarily with the non–high-density lipoprotein (HDL) fractions of plasma lipoproteins. In response to an atherogenic diet the transgenic mice developed hypercholesterolemia similar to that in nontransgenic mice but did not experience the decrease in HDL cholesterol normally observed in this strain of C57BL/6 mice. The rate of plasma clearance of a lipid emulsion mimicking lymph chylomicros was measured in transgenic mice expressing the human apoE4 gene and compared with the clearance rate in nontransgenic control animals. In animals fed a low-fat diet the emulsion lipids were cleared significantly more rapidly from the plasma of transgenic than control mice. In animals adapted to a high-fat diet, the clearance of chylomicron remnants was slowed markedly in both transgenic and control mice and was not significantly accelerated in transgenic compared with control animals. We also investigated the effect of increasing the plasma concentration of apoE4 on the progression of atherosclerotic heart disease. The extent of fatty streak lesion formation in transgenic mice expressing apoE4 was compared with nontransgenic controls. Fatty streak lesion area in the apoE4 transgenic mice was significantly decreased compared with controls. Thus, an elevated plasma concentration of human apoE4 can attenuate murine atherogenesis by a mechanism perhaps associated with changes in the response of HDL to an atherogenic diet. (Arterioscler Thromb. 1994;14:1542-1552.)

Key Words • chylomicron remnants • apo E isoforms • cholesterol • LDL receptor • triglyceride lipolysis

Apolipoprotein (apo) E is a 299–amino acid, polymorphic glycoprotein that plays a critical role in triglyceride-rich lipoprotein catabolism and cholesterol homeostasis. Present in lymph chylomicros, which transport dietary lipids, and in very-low-density lipoprotein (VLDL), which transports lipids from the liver to other tissues, apoE acts as a ligand for the low-density lipoprotein (LDL) receptor and other receptors. In some species apoE also associates with a minor subclass of high-density lipoprotein (HDL). Clearance of apoE-containing lipoproteins from plasma is mediated by high-affinity binding of apoE to these receptors. Conspicuous defects in the clearance of chylomicron remnants occur in the contexts of human apoE deficiency and in structural changes of apoE that inhibit its interaction with the LDL receptor. ApoE deficiency impairs the clearance of lipoprotein particles that normally contain apoE, resulting in hypertriglyceridemia, hypercholesterolemia, and premature atherosclerosis. On the contrary, recombinant human apoE and rabbit plasma apoE, which rapidly associate with plasma lipoprotein particles, decrease plasma cholesterol levels in cholesterol-fed rabbits after intravenous infusion. Recently, Shimano et al have shown that total plasma lipids, particularly the VLDL-LDL fraction, were significantly reduced in transgenic mice expressing high levels of rat apoE compared with controls after high-fat feeding. The clearances from plasma of injected VLDL, LDL, and chylomicron remnants were also significantly enhanced. In contrast, mice deficient in apoE generated by gene targeting showed spontaneous hypercholesterolemia and atherosclerosis. Taken together, these observations suggest that the rate of clearance of apoE-containing lipoproteins from plasma is influenced by the amount of apoE associated with each particle.

There are three common allelic variants of apoE in humans: E2, E3, and E4. ApoE4 differs from E3 by an amino acid substitution at position 112 (Cys→Arg) and from E2 by a substitution at position 158 (Arg→Cys). The isoforms of apoE also differ in their interactions with the LDL receptor. ApoE3, the predominant isoform, binds to the LDL receptor and mediates the endocytotic process. Human apoE3, when added to canine chylomicros, accelerates clearance of those particles from plasma of normal rabbits. Transgenic mice expressing the apoE3-Leiden gene, a mutant of apoE3 isoform, display hyperlipoproteinemia, which becomes more prominent during cholesterol feeding. ApoE4 also binds normally to the LDL receptor, whereas the binding of apoE2 to the receptor is defective. However, compared with individuals homozygous...
for the E3 isofrom, individuals with apoE4 exhibit high total plasma cholesterol and high LDL cholesterol, and are predisposed to the early development of coronary artery disease. The prevalence of the apoE4 allele is lower in older than in younger people,14,15 suggesting that the apoE4 allele may reduce life expectancy. ApoE4 has also been reported to increase the risk for exercise-induced myocardial ischemia in older men,16 for cerebrovascular disease,17,18 and for Alzheimer’s disease.19,20

In the present study we examine the effect of differing plasma apoE4 concentrations on the plasma lipid profile, clearance of chylomicrons, and the process of atherogenesis. We constructed several lines of transgenic mice expressing the human apoE4 gene. Transgenic mice were created in an inbred strain, C57BL/6, and in an F1 hybrid strain of C57BL/6 x SJL. The effects of increased plasma apoE4 concentrations on chylomicron metabolism were studied in both strains of transgenic and control animals by measuring the clearance of chylomicron-like lipids emulsions after injection. Because emulsions do not contain any apolipoproteins, in particular apoB, receptor-mediated uptake of the remnants derived from the emulsions relies on their association in plasma with apoE.

The amino acid sequences of the human and murine apoE proteins differ by 30%.21,22 The human E4 allele most closely resembles the murine apoE sequence, especially in the receptor-binding region, and apoE4 has been shown to mediate receptor binding and uptake of lipoproteins by mouse macrophages.23 It is possible to characterize the effects of differing apolipoprotein environments on the metabolism of chylomicrons by intravenously injecting protein-free lipid emulsions that are designed to mimic the size and lipid composition of lymph chylomicrons.24,25 Plasma apolipoproteins associate with the lipid emulsions immediately after injection; subsequent clearance of the emulsion cholesteryl ester measures particle removal as remnants, and clearance of emulsion triglyceride measures lipolysis plus particle removal. Several studies have compared the clearances of lipid emulsions and lymph chylomicrons, leading to the conclusion that both types of particle share similar properties with regard to rates and sites of clearance.26-28

Atherogenesis was studied in C57BL/6 mice, which are susceptible to diet-induced atherosclerosis.29 After consuming an atherogenic diet for 10 to 20 weeks, these mice show a characteristic reduction in plasma HDL cholesterol (HDL-C) concentration and development of extensive fatty streak lesions in the ascending aorta.30 Linkage analysis has identified a single genetic locus, Ath-1, which is thought to be responsible for both of these phenomena.31 With continued consumption of the atherogenic diet, lesions in the ascending aorta acquire many of the characteristics of human atheromatous plaques,32 including the presence of lipid-filled macrophages, cholesterol crystals, necrotic debris, smooth muscle cell proliferation, and a fibrous cap. In the present study we measured the extent of the fatty streak lesion formation in transgenic and nontransgenic controls after 18 weeks’ consumption of the atherogenic diet.

Our results suggest that increasing the plasma concentration of human apoE4 in transgenic mice accelerates the clearance of chylomicron remnants in animals consuming a low-fat diet but not in animals consuming a high-fat atherogenic diet. However, atherogenesis was decreased in apoE4 transgenic mice compared with the control nontransgenic mice after high-fat feeding.

Methods

Transgenic Mice

Transgenic mice were generated as described by Hogan et al.33 Embryos were obtained by breeding C57BL/6 x SJL F1 hybrid parents. DNA was injected into the pronuclei of one-cell embryos at a concentration of 1.5 to 2.0 ng/µL. Injected embryos were transferred to the oviduct of a pseudopregnant female and allowed to develop to term. Transgenic animals were identified by detection of human apoE4 in plasma. Similar studies were done in the C57BL/6 inbred strain of mice.

Preparation of DNA for Microinjection

Plasmid pSVgptAl, which contains the human apoA-I gene, was provided by S. Karathasanas. A 5.2-kb EcoRI-StuI fragment spanning the human apoA-I promoter region, extending from an EcoRI site at -5130 to a StuI site at +69, was inserted into the polyclinker of vector pSP72 (Promega). A cosmid containing the entire human apoE4 gene was isolated from a human placenta library (Stratagene), and a 5.5-kb fragment extending from a BamHII site at -14 to a HindIII site located 1.5 kb 3' to the gene was ligated into the pSP72 polyclinker next to the apoA-I promoter. This construct yields mRNA in which the first (nontranslated) exon of apoA-I is spliced to the second exon of apoE4. The second exon of apoE4 includes the start of translation, so the protein produced is apoE4 with no sequence change.

Injection into mouse embryos a 10.5-kb ClaI-XhoI fragment containing the apoA-I promoter linked to the apoE4 gene was isolated by preparative agarose gel electrophoresis. The fragment was recovered from the agarose gel slice by centrifugation through a 0.22-µm cellulose acetate filter followed by ethanol precipitation. The DNA pellet was resuspended in injection buffer (10 mmol/L Tris, 0.1 mmol/L EDTA, pH 7.5) and filtered (0.22 µm) before injection.

DNA Sequence Analysis

The DNA sequence of a portion of the apoE4 cosmid isolate was determined by using the dideoxynucleotide chain termination method34 as modified for use with Sequenase (US Biochemical Corp). To resolve the sequence of G+C-rich regions, diTTP and 7deazaGTP were substituted for dGTP in the reactions.

Preparation and Analysis of Mouse RNA

RNA was prepared by using an adaptation of the method described by Chomczynski and Sacchi.35 apoE4 transcripts were detected on Northern blots following electrophoresis of RNA samples on agarose gels containing formaldehyde. A 2.2-kb Ace I-HincII fragment isolated from a human apoE cDNA clone was used as a probe. A cytchrome oxidase probe was used as an internal standard when quantifying RNA in the Northern blot analyses. The probes were labeled with 35P to a specific activity of ∼106 cpmp/µg by using the random primer method.36

Analysis of Plasma Lipids and Lipoproteins

Animals were fasted overnight, and ∼400 µL blood was collected from a tail vein into tubes coated with EDTA. Plasma was separated by centrifugation in a microcentrifuge for 5 minutes and was stored at 4°C for up to 1 week before analysis, except for triglyceride measurements, which were made within 4 hours after blood was collected. Plasma lipids were measured colorimetrically by using commercially available enzymatic assay reagents. Triglycerides were measured spectrophotometrically on a plate reader at 540 nm after incubating 25 µL diluted plasma with 100 µL reagent and...
corrected for free glycerol in plasma. HDL-C was measured following selective precipitation of non-HDL lipoproteins with polyethylene glycol (PEG, 8 kD; Polysciences). Plasma was mixed with an equal volume of PEG solution (20% w/v in 0.2 mol/L glycine, pH 10) and centrifuged after standing for 5 minutes at room temperature. The supernatant was assayed for HDL-C. LDL+VLDL cholesterol was derived by subtraction of HDL-C from total plasma cholesterol.

Analysis of Murine and Human ApoE Expression in Transgenic Mice

Human and murine apoE were separated by electrophoresis on 6% polyacrylamide gels containing 18% glycerol and 0.1% sodium dodecyl sulfate. This procedure separates human and mouse apoE into two distinct bands. Proteins were electrophoretically transferred from the gel to a nitrocellulose filter. ApoE bands were detected by Western blots by using the enhanced chemiluminescence (ECL) system (Amersham). Polyclonal antiserum to human apoE (which recognizes both human and murine apoE) was obtained from Biorad, and ECL detection reagent was from Amersham.

Hepatic LDL Receptor Assay

Hepatic LDL receptor in C57BL/6 nontransgenic mice was quantified as described by Szanto et al.² except that the LDL receptor band was detected on x-ray film (Hyperfilm-ECL, Amersham). The rabbit anti-bovine LDL receptor antibody was a generous gift of Dr. P.D. Roach, CSIRO, Australia.

ELISA for Quantification of ApoE in Plasma

Human apoE was measured in plasma of transgenic mice by using an antibody sandwich-type enzyme-linked immunosorbent assay (ELISA).³⁴ Purified goat anti-human apoE immunoglobulin G (100 ng per well) was adsorbed to the solid phase of 96-well microtiter plates. Plates were washed with phosphate-buffered saline (PBS), and active sites were blocked with a solution of 2% bovine serum albumin (BSA), 0.5% casein hydrolysate, and 0.1% Tween-20. Samples in a volume of 100 μL per well were applied at three dilutions in 1% BSA, 0.5% casein hydrolysate, and 0.375% Tween-20. After standing for 2 hours at room temperature, the plates were washed with PBS, incubated with biotin-labeled anti-human apoE for 1 hour, and again washed with PBS. Horseradish peroxidase coupled to streptavidin was added, followed by o-phenylene-diamine dihydrochloride in citric acid-phosphate buffer, pH 5.3. Color was allowed to develop for 20 minutes before stopping with 3 mol/L HCl. Absorbance was measured at 490 nm with a plate reader. Standard curves were linear, in the range of 1 to 60 ng apoE. Reference plasma samples had intra-assay and interassay coefficients of variation of 6.5% and 9.5%, respectively. (The anti-human apoE antibody used in these studies cross-reacted poorly with murine apoE.) Nontransgenic mice gave low measurements with the human antibody (0.5 ± 0.04 mg/dL; n = 10).

Fast Protein Liquid Chromatography Fractionation of Plasma

Lipoproteins were separated from whole plasma on two Superose 12 prep-grade columns connected in series (FPLC Systems, Pharmacia) as described by Jiao et al.²⁷ Seventy 0.5-ml fractions were eluted from the columns. Elution profiles of total protein (represented by absorbance at 280 nm) were generated, and total cholesterol concentration was measured in each elution fraction. Concentrations of apoE were determined in alternate fractions by Western blot analysis. The relative concentrations of murine apoE between fractions was assessed by scanning Western blots. A similar analysis was used to determine relative human apoE concentrations between fast protein liquid chromatography fractions.

Clearance

Lipid emulsions labeled with cholesteryl[9,10(n)-3H]oleate and glycerol tri[1-14C]oleate (Amersham) were prepared by sonicating a mixture of pure triolein, cholesteryl oleate, egg lecithin, and free cholesterol in 0.154 mol/L NaCl and 10 mmol/L HEPES (pH 7.4). Chylomicron-sized particles of diameter 100 to 150 nm were purified from the sonicated mixture by serial ultracentrifugation in a density gradient. Details of the procedure and characterization of the emulsion particles are available.²⁴ ²⁶ ²⁷

For clearance studies in mice, methods developed for rats and rabbits were adopted. Anesthesia was induced by tribromoethanol (Avertin; Aldrich; 0.3 to 0.4 g/kg body weight IP injection), after which exactly 50 μL emulsion was injected via a 30-gauge needle into a tail vein. The emulsion injected contained approximately 250 to 300 μg of total lipid. Blood samples of 100 μL were taken from a retro-orbital venous sinus. Two samples were taken from each animal at either 10, 15, 20, or 30 minutes after injection. Radioactivity in the plasma was measured by liquid scintillation spectroscopy. Individual animals were studied after an interval of 2 to 3 weeks to allow for recovery from the blood sampling.

Diet Study

Groups of control and transgenic mice were fed a high-fat diet containing (by weight), 15% fat (as dairy butter), 1% cholesterol, 0.5% sodium cholate, and 20% casein²⁸ for 3 to 4 weeks. Control groups on a low-fat diet were fed a standard laboratory chow diet containing 4% fat.

Lipase Assay

Postheparin plasma lipase activity was measured by titrimetric assay at pH 8.5 by using trioctanoin substrate. Mice fed standard laboratory chow or the high-fat diet as described above for 16 days were injected with 250 U/kg body wt heparin (Sigma Chemical Co; 30 U/mL in 0.15 mol/L NaCl solution). Blood samples were taken from a retro-orbital venous sinus 4 minutes after heparin injection. Aliquots (100 μL) of plasma were assayed by titration with 0.1 mol/L NaOH, pH 8.5, at 22°C (Radiometer pH Stat) of free fatty acids released from trioctanoin (99%; Sigma) in NaCl (0.15 mol/L), CaCl₂ (2.0 mmol/L), and albumin (1.5 μmol/L) with apoC-II (0.5 μg/mL) in this system purified lipoprotein lipase (a gift from Dr G. Bengtsson-Olivelcrona) had an activity of 2057 U/mg. Animals not injected with heparin gave measurements of <1 U/mL plasma.

Measurement of Fatty Streak Lesions

Transgenic mice and the nontransgenic siblings from the C57BL/6 strain were fed the high-fat diet for 18 weeks. Mice were anesthetized with Avertin (0.3 to 0.4 g/kg body weight IP) and killed by exsanguination. The heart and aorta were fixed in 10% formalin for at least 5 days and embedded in 25% gelatin. Ten-micrometer sections were prepared, and areas of atherosclerotic lesion were measured.²⁹

Statistics

Groups were compared by ANOVA by using the SYSTAT software package. Statistical significance was accepted at P < 0.05.

Results

Construction of Transgenic Mice and Tissue-Specific Expression

A clone containing the entire human apoE gene was isolated from a cosmid library. The DNA sequence of this isolate in a region of 175 bp spanning the receptor-binding domain (amino acids 110 through 168) was identical to that reported for the apoE4 allele. The construct used to generate transgenic mice, which was derived from...
this cosmid, consisted of a fusion of the human apoA-I promoter to the human apoE gene. Because the fusion site is located 5' to the start of apoE translation, the protein produced from this construct is human apoE4 with no changes in the amino acid sequence.

Transgenic animals were identified by immunoblot detection of human apoE in plasma (Fig 1), and the plasma concentration of human apoE was determined by an ELISA assay (Table 1) by using an antibody specific to human apoE. Several human apoE transgenic founder mice were identified from both the C57BL/6 inbred and the F1 hybrid C57BL/6×SJL strains. Transgenic mice bred from both the C57BL/6 and the F1 hybrid strains were used in the chylomicron clearance studies, but only

![Table 1. Plasma Lipids and Apolipoproteins In Control and Transgenic Mice Fed Low- or High-Fat Diets](image_url)

**Table 1. Plasma Lipids and Apolipoproteins In Control and Transgenic Mice Fed Low- or High-Fat Diets**

<table>
<thead>
<tr>
<th></th>
<th>Low-Fat Diet</th>
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<th>High-Fat Diet</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Transgenic</td>
<td>Control</td>
<td>Transgenic</td>
</tr>
<tr>
<td>C57BL/6 mice</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Human apoE</td>
<td>NA</td>
<td>23±2.1 (10)</td>
<td>NA</td>
<td>10±0.9 (6)</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>88±1.6 (56)</td>
<td>82±2.0 (38)</td>
<td>214±6.6 (58)</td>
<td>242±8.5 (36)</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>68±1.5 (56)</td>
<td>71±1.6 (34)</td>
<td>52±3.1 (56)</td>
<td>74±2.3 (36)</td>
</tr>
<tr>
<td>LDL+VLDL cholesterol</td>
<td>19±1.1 (56)</td>
<td>16±3.4 (36)</td>
<td>185±6.1 (56)</td>
<td>168±9.5 (36)</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>52±3.5 (15)</td>
<td>76±7.0 (11)</td>
<td>33±1.6 (50)</td>
<td>39±2.9 (33)</td>
</tr>
<tr>
<td>Hybrid mice</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Human apoE</td>
<td>NA</td>
<td>8.6±1.1 (2)</td>
<td>NA</td>
<td>5.4±0.7 (4)</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>104±2.6 (19)</td>
<td>104±4.9 (9)</td>
<td>244±14.1 (17)</td>
<td>247±16.1 (9)</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>55±2.0 (7)</td>
<td>64±4.6 (5)</td>
<td>44±1.3 (11)</td>
<td>59±4.5 (5)</td>
</tr>
<tr>
<td>LDL+VLDL cholesterol</td>
<td>49±3.3 (7)</td>
<td>40±6.7 (5)</td>
<td>208±20.6 (10)</td>
<td>214±19.5 (5)</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>109±7.5 (28)</td>
<td>109±11.3 (16)</td>
<td>20±2.5 (13)</td>
<td>26±3.2 (6)</td>
</tr>
</tbody>
</table>

Apo indicates apolipoprotein; NA, not applicable; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and VLDL, very low-density lipoprotein. Data are presented as the concentrations of apoE or lipids in plasma in milligrams per deciliter and are summarized as mean±SEM from (n) animals. Although not shown, immunoblotting with an antibody recognizing human and mouse apoE showed that the concentrations of murine apoE were unchanged in transgenic compared with control animals.
the C57BL/6 mice were used for the atherosclerosis study. Similar results were obtained for both groups of animals with regard to the tissue specificity of expression and apolipoprotein distribution among the plasma lipoproteins in the studies described below.

To characterize the tissue specificity of human apoE4 gene expression in the transgenic mice, RNA was prepared from a variety of tissues and assayed for the presence of human and murine apoE transcripts by Northern blot. In transgenic animals, human apoE transcripts were present at a very high level in the liver, at a moderate level in the kidney, and at a very low level in the testis (Fig 1). Murine apoE transcripts were present predominantly in liver, and at a much lower level in all other tissues examined (data not shown).

Plasma Apolipoproteins, Lipids, and Lipoproteins

To examine the distribution of human and murine apoE on murine lipoprotein particles, plasma samples from six transgenic and six nontransgenic control C57BL/6 animals were pooled and fractionated by fast protein liquid chromatography. Whole plasma was used in this experiment to avoid the dissociation of apoE from murine lipoprotein particles that was found to occur during ultracentrifugation. The positions of lipoprotein peaks were determined by measuring cholesterol in each fraction, and apoE was detected in the fractions by Western blot. Murine apoE was localized entirely in the LDL+VLDL-containing fractions (Fig 2). In transgenic mice total plasma apoE was localized primarily in the LDL+VLDL-containing fractions; only a small amount was detectable in the HDL fraction. Comparisons of the endogenous murine apoE plasma concentrations in transgenic and control animals by Western blot analysis suggested that expression of human apoE4 in the transgenic mice did not affect the expression of endogenous murine apoE. Table 1 shows the concentrations of human apoE4 measured with an ELISA assay. When animals were fed a low-fat diet (standard chow), the plasma human apoE4 ranged from 9 mg/dL in the hybrid transgenic mice to 23 mg/dL in the C57BL/6 transgenic mice. When the animals were adapted to a high-fat diet the plasma content of human apoE decreased. The concentration of human apoE4 was significantly decreased (P<.001) when C57BL/6 transgenic mice were fed the high-fat diet compared with the chow diet (Table 1). A similar but nonsignificant trend was apparent in the hybrid mice (P<.1). Despite the decrease, human apoE4 concentrations in both groups of transgenic animals fed the high-fat diet still ranged between 5 and 10 mg/dL.

The results of plasma lipid analyses of transgenic and control mice are summarized in Table 1. In either transgenic line, total plasma cholesterol, HDL-C, and LDL+VLDL cholesterol (calculated as the difference between total cholesterol and HDL-C) did not differ significantly between the transgenic and control animals when both groups consumed a low-fat chow diet. Due to a much lower content of LDL+VLDL cholesterol (P<.001), plasma total cholesterol levels in transgenic and control C57BL/6 mice were lower than in transgenic and control hybrid mice. In both strains of transgenic mice, after 3 to 4 weeks' consumption of a high-fat diet, plasma total cholesterol increased by about 25-fold in control and transgenic animals (P<.001) due to a fivefold to 10-fold increase in LDL+VLDL cholesterol. In both C57BL/6 and hybrid mice on the high-fat diet the plasma HDL-C also decreased significantly (P<.05) in the control but not in transgenic mice, in which the HDL-C was significantly higher than in control mice (P<.005). Plasma triglycerides on a low-fat diet were higher in hybrid mice than in the C57BL/6 strain, and with the C57BL/6 strain but not with hybrid animals triglycerides were significantly higher in transgenic than control animals (P<.01). Plasma triglycerides decreased markedly (P<.001) after the high-fat diet to similar low concentrations in both control and transgenic mice.

Clearance Studies

Figs 3 and 4 show the patterns of clearance of lipids in C57BL/6 and hybrid mice after injection of chylomicron-like emulsions labeled with radioactive cholesteryl oleate and triolein when the recipient mice were fed low-fat and high-fat diets. On low-fat diets the clearance of cholesteryl oleate, representing remnant particle clearance (A panels), was rapid, with about 70% of label removed from plasma by 10 minutes after injection. The clearance of cholesteryl oleate was significantly more rapid in both groups of transgenic mice than their controls on the low-fat diet (P<.001) by
Chylomicron Clearance and Atherogenesis in Mice Overexpressing ApoE4

A. Cholesteryl Oleate Clearance

- Non-transgenic low-fat diet, n=8; for transgenic, low-fat diet mice, n=4 to 7; for nontransgenic, high-fat diet mice, n=7 to 9; and for transgenic, high-fat diet mice, n=5 to 7.

B. Triolein Clearance

- Non-transgenic low-fat diet, n=10 to 40; for transgenic, low-fat diet mice, n=4 to 15; for nontransgenic, high-fat diet mice, n=3; and for transgenic, high-fat diet mice, n=4 to 8.

Two-way ANOVA). Removal of emulsion cholesteryl oleate was markedly slowed after switching to the high-fat diet in both control and transgenic groups. Clearances of emulsion cholesteryl oleate on the high-fat diet were no longer significantly different between transgenic and the respective nontransgenic control mice. Clearance of emulsion triolein, representing particle clearance plus lipolysis (B panels), was faster than clearance of cholesteryl oleate in all animals. On the low-fat chow diet the clearance rate of triolein, like that observed for cholesteryl oleate, was faster in transgenic mice compared with their nontransgenic controls (P<.001 by two-way ANOVA). After switching to a high-fat diet, the clearance of triolein in nontransgenic C57BL/6 animals was faster than when these animals were fed a low-fat diet, whereas in transgenic C57BL/6 animals clearance was unchanged. The clearance of triolein in both the transgenic hybrid and the nontransgenic hybrid control animals was more rapid after being fed a high-fat than a low-fat diet.

The half-lives for clearance of emulsion lipids calculated from the data of Figs 3 and 4 were estimated from the fractional clearance rates obtained after fitting biexponential curves using the SIGMAPLOT curve-fitting routines (Jandel Corp). On the low-fat diet the estimated half-lives of chylomicron remnants traced by emulsion cholesteryl oleate were shorter in transgenic mice (5 to 6 minutes) compared with control mice (8 to 11 minutes). After feeding high-fat diets in both nontransgenic and transgenic mice, the estimated half-lives of cholesteryl oleate were much longer (18 to 54 minutes). The estimated half-life of emulsion triolein was always shorter than that of cholesteryl oleate in the same animal group and reflected the changes described in the previous paragraph in transgenic animals and in response to diet.

LDL Receptor

Using a polyclonal antibody against the LDL receptor followed by a secondary antibody conjugated to horse-radish peroxidase as described in “Methods,” a band was clearly observed by ECL detection (Fig 5). Mice fed the high-fat, high-cholesterol diet (lanes 5 through 7) showed about a 44% (P<.025) reduction in LDL receptor mass compared with the control diet (lanes 1 through 4). A limited number of transgenic mice were also tested for LDL receptor mass following high-fat and control feeding, with results showing a tendency for receptor reduction (=19%) in the high-fat group.
Lipase Assay

Table 2 shows the lipase activity in plasma 4 minutes after heparin injection in C57BL/6 mice fed the control diet or the high-fat diet as described above. Lipase activity increased about 40% in the female and 46% in the male mice after 16 days of feeding the high-fat diet.

Fatty Streak Lesion

Fig 6 compares the areas of lipid-staining fatty streak lesions in aortic sections of the transgenic mice versus the nontransgenic siblings from the same parents. Measurements were made without the observer knowing which samples were from transgenic animals, and the data were subsequently analyzed by using a nonparametric method for comparison since they were not normally distributed. The lesion areas in the transgenic mice were significantly less than in controls (P=.015 by the Mann-Whitney U test). The median lesion size area was 301 \( \mu \text{m}^2 \) for the transgenic and 1267 \( \mu \text{m}^2 \) for the control mice.

Discussion

In humans, the e4 allele of apoE4 is associated with increased risk of coronary artery disease, ischemic cerebrovascular disease, Alzheimer's disease, and reduced life expectancy. In an effort to analyze and identify the pathogenic role of apoE4 in the development of disease, transgenic mice overexpressing human apoE4 were generated for the study of plasma lipid profiles and chylomicron catabolism, atherogenesis, and changes in lipid metabolism when mice were challenged with an atherogenic diet. To ensure the liver-specific production of apoE in the mice, the construct contained the entire apoE gene fused to the human apoA-I promoter in the first (nontranslated) exon. The DNA sequence of 175 bp spanning the receptor-binding domain (amino acids 110 through 168) was determined and found to correspond to the apoE4 allele. The same construct was also used by Fazio et al.43 with insertion of appropriate mutations, to produce liver-specific expression of a human apoE variant gene.

In this study, high expression of the apoE gene was observed in the liver of the transgenic mice (Fig 1A). In the plasma, human apoE associated mainly with the VLDL and LDL fractions, indicating the expression of apoE4. In previous studies of mice transgenic for human apoE genes,43,46 the human apoE associated with murine lipoproteins, although in some studies43,45,46 human apoE was produced in the kidney (Table 3). These results are consistent with the present studies, in which we found similar distributions of human apoE4 and murine apoE on the plasma lipoproteins of the transgenic mice (Fig 2). Allelic variants of human apoE differ in their distribution among lipoproteins. The presence of a positively charged residue at position 112 is a factor determining the distribution of human apoE primarily to the VLDL fraction.47 In transgenic mice overexpressing rat apoE,8 the distributions of both rat and murine apoE on lipoprotein particles were different from what we observed, with >60% of apoE associated with HDL. Variations in apoE sequences between strains of mice

**TABLE 2. Postheparin Lipase Activity in Mouse Plasma**

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6 Female Mice</th>
<th>C57BL/6 Male Mice</th>
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<tbody>
<tr>
<td></td>
<td>Low Fat</td>
<td>High Fat</td>
</tr>
<tr>
<td>Lipase activity, U/mL plasma</td>
<td>4.0±0.13</td>
<td>5.6±0.36*</td>
</tr>
</tbody>
</table>

Data are mean±SEM for six mice in each group. Mice were fed control (low-fat) or a high-fat diet for 16 days. Blood samples were taken from a retro-orbital venous sinus 4 minutes after heparin injection. Plasma from animals not injected with heparin was subjected to gel electrophoresis and transferred to nitrocellulose. The LDL receptor band was detected with a polyclonal antibody against the LDL receptor and the enhanced chemiluminescence system.

**Fig 6.** Plot showing area of lipid-staining fatty streak lesions in mouse aortic sections. Mean lesion area was determined in the proximal aorta by quantification of internal lipid accumulation from oil red O-stained sections using a calibrated eyepiece.
and between rat and human apoE possibly account for the different findings between these studies.

Total plasma lipids were not affected by the expression of apoE4 in transgenic mice. When mice were fed the chow diet, concentrations of plasma cholesterol, triglyceride, HDL-C, and VLDL+LDL cholesterol were not significantly different between transgenic and nontransgenic control animals (Table 1). When mice were fed a high-fat diet, hypercholesterolemia occurred equally in transgenic and nontransgenic mice, although HDL-C remained significantly higher in both strains of the transgenic mice compared with nontransgenic controls. Most of the increase in plasma total cholesterol on the high-fat diet was due to the increase in LDL+VLDL cholesterol, but plasma triglycerides did not reflect this increase. Plasma triglycerides decreased when inbred strains of mice were fed an atherogenic diet, and plasma HDL also decreased. Our study confirmed the decrease in plasma triglycerides in both control and transgenic mice, but HDL-C concentrations in transgenic mice were significantly higher than in controls. The decrease in plasma triglycerides, which was associated with an apparent increase in LDL+VLDL cholesterol, was consistent with the enrichment of VLDL with cholesterol described by Breckenridge et al. Major increases of plasma concentrations of apoB-48 have been reported in mice fed high-fat diets.

In contrast to the present studies with human apoE transgenes, Shimano et al report reduced plasma lipid, VLDL, and LDL levels in transgenic mice expressing rat apoE under the control of the inducible metallothionein promoter. Similar findings in plasma lipids have also been reported in transgenic mice overexpressing the human apoE3 gene. Moreover, transgenic mice overexpressing human apoE3 or rat apoE are protected from diet-induced hypercholesterolemia. Thus, human apoE4 not only differs in its effects on murine plasma lipoproteins from the human apoE3 and rat apoE genes expressed in transgenic mice, but also exerts different effects on plasma cholesterol when challenged with a high-fat diet.

In these experiments we examined the effects of human apoE4 on murine chylomicron catabolism by using lipid emulsions designed to mimic natural chylomicrons. Clearance of chylomicrons and remnants from the plasma of mice has been studied by Hussain et al and Choi et al, who used lymph chylomicrons from a different animal species. The chylomicrons in previous clearance studies carried their own complement of apolipoproteins, which possibly mediate metabolism of the lipid particles. In contrast, the clearances in our study reflect the endogenous apolipoproteins of the recipient animal because the emulsions contain no proteins before their injection into the bloodstream.

Our results show that clearances of chylomicron cholesteryl ester and triglyceride were accelerated by the increased plasma concentrations of human apoE4 when the transgenic mice consumed a low-fat chow diet. This finding suggests that the availability of apoE may be rate-limiting for the clearance of chylomicron remnants. It is likely that remnant uptake by the liver is mediated when several apoE molecules on a remnant particle interact with several receptor molecules. Increased availability of apoE may facilitate the interaction with multiple receptor molecules to initiate receptor-mediated endocytosis. This finding is consistent with those of Shimano et al, who found enhanced clearance of chylomicron remnants by using an oral retinyl palmitate-loading test in apoE transgenic mice, and with an increased clearance rate of labeled canine chylomicrons.
in mice transgenic for human apoE. For comparison, the effects on plasma lipid profile and catabolism of chylomicrons in mice overexpressing various apoE genes are summarized in Table 3.

Contrary to the effects of human apoE4 on chylomicron clearance in mice fed a chow diet, clearance rates for remnants, traced as cholesteryl oleate label, were significantly slower in all groups of animals on the high-fat diet. Additionally, in contrast to the human apoE effect on the low-fat diets, on the high-fat diet remnant clearance was not significantly accelerated in the transgenic mice compared with controls. The decreased remnant removal in animals fed a high-fat diet was probably due to reduced transcription of the murine LDL receptor. As a result, there is a reduced number of LDL receptors, consistent with observations in rodents fed high-fat diets. Jackie et al. found that an atherogenic diet decreased the uptake of remnants by perfused rat livers to 54% of control uptake, consistent with a decreased number of LDL receptors. Under these dietary conditions, decreased numbers of LDL receptors are likely to be a limiting factor in remnant removal. In this study (Fig 5), LDL receptor mass decreased by about 44% (P < .025) after feeding C57BL/6 mice with the atherogenic diet.

Ishida et al. report a sixfold increase and Reue et al. a threefold increase in apoE levels while the apoE mRNA remained unchanged when mice were fed high-fat diets containing 1.25% cholesterol with cholic acid and 4.5% cholesterol without cholic acid, respectively. In the present study, the plasma concentrations of human apoE4 decreased when animals were switched from low- to high-fat diets (Table 1). The apoA-I promoter used in these studies, when linked to the apoA-I gene, was not associated with decreased expression when animals containing this transgene were placed on a high-fat diet. The mechanism for the decrease in plasma human apoE4 levels when animals were fed high-fat diets is as yet unclear. It is possible that apoE4 mediates increased receptor uptake of the surplus fatty remnant particles in an effort to get rid of the extra fat when mice are fed a high-fat diet. Gregg et al. report that in humans the in vivo metabolism of apoE4 is abnormal, apoE4 being catabolized faster than apoE3, resulting in a decreased plasma apoE concentration. Other studies have showed that apoE4 is associated with greater sensitivity to dietary manipulations. Thus it is also possible that apoE4 was catabolized faster when mice were fed a high-fat diet. Finally, as the dietary cholesterol content differed in the various studies discussed, the amount of cholesterol, as well as the inclusion of cholic acid, in the atherogenic diet may also be important in the regulation of apoE.

The combination of decreased LDL receptors and reduced apoE is probably sufficient to account for slower remnant clearance after a high-fat diet in the transgenic mice. Thus human apoE4, although accelerating chylomicron clearance when animals were fed a low-fat diet, showed little protective effect against hypercholesterolemia and did not enhance chylomicron remnant clearance after high-fat feeding. In contrast, a high-fat atherogenic diet accelerated triglyceride removal in all groups of animals (Figs 3 and 4). This acceleration of triglyceride removal mirrored the decrease in plasma triglyceride contents associated with the high-fat diet. The retarded remnant removal suggests that the increased rate of triglyceride removal was probably due to increased lipolysis. We found that postheparin lipase activity was decreased by the atherogenic diet (Table 2). Hepatic lipase has been found to be more active against substrates rich in apoE. In rabbits hepatic lipase activity is increased by cholesterol feeding, whereas Sultan et al. found that the activity of hepatic lipase in rats was decreased by cholesterol feeding.

The C57BL/6 inbred strain of mouse is susceptible to diet-induced arterial disease, and mice in which the apoE gene is disrupted by gene targeting develop extensive fatty streak lesions after a short period of consuming an atherogenic diet. In this study, fatty streak lesions in transgenic C57BL/6 mice were compared with their nontransgenic siblings. Results (Fig 6) indicate that the expression of human apoE4 was protective against atherosclerosis but not totally preventive. The specific mechanism by which apoE4 acts to reduce murine atherogenesis is not clear. It is possible that the antiatherogenic effect is a result of maintaining a high HDL-C level. However, there are several other mechanisms by which apoE might influence atherogenesis. Increased apoE may activate removal of excess cholesterol from the peripheral vasculature, and an increase in the number of molecules of apoE per lipoprotein particle may enhance cholesterol uptake by the liver.

It is also possible that apoE specifically accelerates the clearance of smaller atherogenic lipoprotein particles such as intermediate-density lipoprotein and LDL. Accelerated catabolism of apoB-containing remnants occurred when normolipidemic or hypercholesterolemic animals were injected with apoE. Shimano et al. also demonstrated a two- to threefold faster clearance of VLDL and LDL in transgenic mice overexpressing rat apoE. Each of these possibilities would limit the accumulation of cholesterol in macrophage-derived foam cells and thus reduce the formation of atherosclerotic lesions. Moreover, it is also possible that within the time frame of the experiments, the cholesteryl oleate in emulsion does not fully reflect the behavior of endogenous remnant particles, which are produced abundantly after high-fat feeding, and that differences in size and composition of remnant particles over time may also contribute to the varying vessel wall changes in the two groups of animals.

The frequency of apoE4 increased significantly among patients with sporadic and familial late-onset Alzheimer's disease, suggesting the involvement of apoE4 in the pathophysiological processes leading to the disease. ApoE is produced in the brain and binds avidly to βA4, a 39- to 42-amino acid peptide that aggregates to form amyloid in the senile plaques that are characteristic of Alzheimer's disease. βA4 is also produced by normal individuals and by cultured cells. Since βA4 binds to the lipoprotein-binding domain apoE, apoE could mediate the uptake of βA4 by astrocytes and neurons via the receptor pathways. Internalization of the βA4-apoE complex into cells would lead to its incorporation into primary lysosomes and pH-dependent dissociation, mediating clearance of βA4 from the neuropil. Although the single amino acid change in the receptor domain of apoE responsible for the E4 isoform does not affect its ability to bind receptors, in vitro binding of apoE4 to βA4 differs remarkably from apoE3. Rebeck et al. speculate that
the apoE4 isoform might have a decreased ability to clear βA4 from the neuropil, while Strittmatter et al. show a more rapid binding of βA4 to apoE4 than apoE3. Thus the possible biological link between apoE4 and Alzheimer’s disease needs to be explored much further. Mice transgenic for human apoE4 may provide a useful model for in vivo studies of interactions between apoE4 and βA4 and the uptake of this complex by receptors.

In conclusion, the increased levels of human apoE4 in the plasma of transgenic mice facilitated the clearance of chylomicron remnants during consumption of a low-fat chow diet. When the mice were fed a high-fat diet no acceleratory effect was found. Consequently, the accumulation of chylomicron remnants in plasma following a high-fat diet leads to hypercholesterolemia. Further verification is needed to determine if the reduced capacity of apoE4 to promote the clearance of lipoprotein remnants, compared with apoE3 or rat apoE, contributes to the adverse effects of the apoE4 allele.

Acknowledgments

This research was supported by the Arnold Yeldham and Mary Raine Medical Research Foundation and a project grant from the Australian National Health and Medical Research Council (Drs Redgrave and Mortimer) and by a grant from the National Dairy Promotion and Research Board (Dr Rubin) and was conducted in part at the Lawrence Berkeley Laboratory through the US Department of Energy under contract No. DE-AC03-76SF00098. Dr Mortimer thanks the International Atherosclerosis Society for a Visiting Fellowship to support this work at the Lawrence Berkeley Laboratory. Purified human apoE was the generous gift of Elaine Gong. Technical assistance was provided by Dianne Beveridge, Deepa Kumar, and Pat Blanche.

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B C Mortimer, T G Redgrave, E A Spangler, J G Verstuyft and E M Rubin

Arterioscler Thromb Vasc Biol. 1994;14:1542-1552
doi: 10.1161/01.ATV.14.10.1542

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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