Residues Leu261, Trp264, and Phe265 Account for Apolipoprotein E-Induced Dyslipidemia and Affect the Formation of Apolipoprotein E-Containing High-Density Lipoprotein†

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ABSTRACT: Overexpression of apolipoprotein E (apoE) induces hypertriglyceridemia in apoE-deficient mice, which is abrogated by deletion of the carboxy-terminal segment of residues 260−299. We have used adenovirus-mediated gene transfer in apoE$^{-/-}$ and apoA-I$^{-/-}$ mice to test the effect of three sets of apoE mutations within the region of residues 261−265 on the induction of hypertriglyceridemia, the esterification of cholesterol of very low-density lipoprotein (VLDL) and high-density lipoprotein (HDL), and the formation of spherical or discoidal apoE-containing HDL. A single-amino acid substitution (apoE4-[Phe265Ala]) induced hypertriglyceridemia in apoE$^{-/-}$ or apoA-I$^{-/-}$ mice, promoted the accumulation of free cholesterol in the very low-density lipoprotein (VLDL) and HDL region, and decreased HDL cholesterol levels. A double substitution (apoE4[Leu261Ala/Trp264Ala]) induced milder hypertriglyceridemia and increased HDL cholesterol levels. A triple substitution (apoE4[Leu261Ala/Trp264Ala/Phe265Ala] or apoE2-[Leu261Ala/Trp264Ala/Phe265Ala]) did not induce hypertriglyceridemia and increased greatly the HDL cholesterol levels. Electron microscopy (EM) analysis of the HDL fractions showed that apoE4[Leu261Ala/Trp264Ala/Phe265Ala] and apoE2[Leu261Ala/Trp264Ala/Phe265Ala] contained spherical HDL, apoE4-[Leu261Ala/Trp264Ala] contained mostly spherical and few discoidal HDL particles, and apoE4-[Phe265Ala] contained discoidal HDL. We conclude that residues Leu261, Trp264, and Phe265 play an important role in apoE-induced hypertriglyceridemia, the accumulation of free cholesterol in VLDL and HDL, and the formation of discoidal HDL. Substitution of these residues with Ala improves the apoE functions by preventing hypertriglyceridemia and promoting formation of spherical apoE-containing HDL.

ApoE[1] is a very important protein of the lipoprotein transport system that plays an important role in the protection from or the pathogenesis of atherosclerosis, dyslipidemia, and Alzheimer's disease (*1*−*5*).

A well-documented function of apoE is its participation in the clearance of lipoprotein remnants of intestinal or hepatic origin by the liver (*4*−*6*). ApoE at physiological concentrations maintains lipid homeostasis and is atheroprotective (*7*−*13*). ApoE also has been shown to promote cholesterol efflux in vitro (*14*, *15*) and thus may contribute to cell and tissue cholesterol homeostasis and protection from atherosclerosis through different mechanisms (*11*, *12*). Despite the beneficial functions of apoE, it has also been found that overexpression of any of the three apoE isoforms in apoE-deficient mice increases the rate of VLDL triglyceride secretion and is associated with hypertriglyceridemia (*16*−*19*). In humans and mice, plasma apoE levels correlate with plasma triglyceride levels (*6*, *20*). Hypertriglyceridemia was prevented by deletion of the C-terminal region of residues 260−299 of apoE (*16*−*18*, *21*). Site-directed mutagenesis and gene transfer in mice localized the amino acids responsible for hypertriglyceridemia in the region of residues 261−269 of apoE (*22*).

In this study, we investigate the molecular etiology of apoE-induced dyslipidemia. Using adenovirus-mediated gene transfer in apoE- and apoA-I-deficient mice, we found that amino acids Leu261, Trp264, and Phe265 are required for the induction of hypertriglyceridemia. Substitution of these residues with Ala facilitates the clearance of VLDL, promotes the esterification of the cholesterol of VLDL and HDL, and results in the formation of spherical apoE-containing HDL.

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[1] Abbreviations: apoE, apolipoprotein E; EM, electron microscopy; FPLC, fast-performance liquid chromatography; GFP, green fluorescence protein; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; LCAT, lecithin cholesterol acyl transferase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; VLDL, very low-density lipoprotein; WT, wild-type.

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EXPERIMENTAL PROCEDURES

Materials

Materials not mentioned in the Experimental Procedures have been obtained from sources described previously (*23*, *24*).

Methods

Construction of Recombinant Adenoviruses Expressing the Wild Type and the Mutant Forms of ApoE4. Three apoE4 mutants were generated [apoE4-mutA (apoE4[Phe265Ala]), apoE4-mutB (apoE4[Leu261Ala/Trp264Ala]), and apoE4-mutC (apoE4[Leu261Ala/Trp264Ala/Phe265Ala])] using the QuickChange-XL mutagenesis kit (Stratagene). The following mutagenic primers were used: apoE4-mutA-f (5′-CGC CTC AAG AGC TGG **GC**C GAG CCC CTG GTG GAA-3′), apoE4-mutA-r (5′-TTC CAC GAC GGG CTC G**GC** CGC GCT CTT GGC GCG-3′), apoE4-mutB-f (5′-GCC TTC CAG GCC CGC **GC**C AAG AGC **GC**G TTC GAG CCC CTG GTG GAA GAC ATG CAG CGC-3′), apoE4-mutB-r (5′-GCG CTG CAT GTC TTC CAC GAC GGG CTC GAA C**GC** GCT CTT G**GC** GCG GGC CTG GAA GGC-3′), apoE4-mutC-f (5′-CAG GCC GAG GCC TTC CAG GCC CGC **GC**C AAG AGC **GC**G **GC**C GAG CCC CTG GTG GAA GAC ATG CAG CGC CAG TGG GCC-3′), and apoE4-mutC-r (5′-GGC CCT CTG GCG CTG CAT GTC TTC CAC GAC GGG CTC G**GC** C**GC** GCT CTT G**GC** GCG GGC CTG GAA GGC CTC GGC GTC-3′). The nucleotides mutated in various codons are in bold and underlined. In both mutagenic reactions, the vector pGEM7-apoE4 (*16*) containing exons II, III, and IV of human apoE was used as a template. In the mutagenic reactions for the generation of the apoE2 mutC, the same vector (pGEM7-apoE4) was initially used as a template to generate pGEM7-apoE3, which was then used as a template to generate pGEM7-apoE2. First, the pGEM7-apoE3 plasmid was generated by introducing the Arg112Cys mutation into the pGEM7-apoE4 plasmid with the mutagenic primers apoE4→E3-f (5′-GCG GAC ATG GAG GAC GTG **T**GC GGC CGC CTG GTG CAG TAC-3′) and apoE4→E3-r (5′-GTA CTG CAC CAG GCG GCC GC**A** CAC GTC CTC CAT GTC CGC-3′). The Arg158Cys mutation was introduced into the pGEM7-apoE3 plasmid with the mutagenic primers apoE3→E2-f (5′-GCC GAT GAC CTG CAG AAG **T**GC CTG GCA GTG TAC CAG GCC-3′) and apoE3→E2-r (5′-GGC CTG GTA CAC TGC CAG GC**A** GTT CTG CAG GTC ATC GGC-3′) to generate the pGEM7-apoE2 plasmid. The pGEM7-apoE2 plasmid was then used as a template to generate the mutant apoE2-mutC with the same primers that were used for the generation of apoE4-mutC. Following 18 cycles of PCR amplification of the template DNA, the PCR product was treated with DpnI to digest plasmids containing methylated DNA in one or both of their strands. The reaction product consisting of plasmids containing newly synthesized DNA carrying the mutations of interest was used to transform competent XL-10 blue bacteria cells (Stratagene). Ampicillin-resistant clones were selected, and plasmid DNA was isolated from these clones and subjected to sequencing to confirm the presence of the point mutations.

The recombinant adenoviruses were constructed as described using the Ad-Easy-1 system where the adenovirus construct is generated in bacterial BJ-5183 cells (*16*, *25*). Correct clones were propagated in RecA DH5α cells. The recombinant adenoviral vectors were linearized with PacI and used to infect 911 cells (*16*, *26*). Following large-scale infection of HEK 293 cell cultures, the recombinant adenoviruses were purified by two consecutive CsCl ultracentrifugation steps, dialyzed, and titrated. Usually, titers of approximately $2-5 \times 10^{10}$ pfu/mL were obtained.

Cell Culture Studies. Human HTB13 cells (SW1783, human astrocytoma) grown to confluence in medium containing 10% fetal calf serum (FCS) were infected with AdGFP-E4 or the adenoviruses expressing the mutant apoE forms Ad-apoE4-mutA, Ad-apoE4-mutB, and Ad-apoE4-mutC at a multiplicity of infection (moi) of 5. Twenty-four hours post-infection, cells were washed twice with phosphate-buffered saline (PBS) and fresh serum-free medium was added. After incubation for 48 h, medium was collected and analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) for apoE expression.

Animal Studies, RNA, and Protein Analyses. Male and female apoE-deficient 8−10-week-old mice were used in these studies. Groups of four to seven mice were injected intravenously through the tail vein with a dose of 2×10^9 pfu. In some experiments, we used a combination of adenoviruses expressing apoE4-mutA (2×19^9 pfu) and human LCAT (5×10^8 pfu) or human lipoprotein lipase (5×10^8 pfu). Blood was obtained from the tail vein after a 4 h fasting prior to the adenoviral injection and 0, 1, 2, 3, and 4 days post-infection. Aliquots of plasma were stored at 4 and
−20 °C. Four or more animals from each group were sacrificed 5 days post-infection, and the mRNA levels in the mouse liver were analyzed by Northern blotting and quantitated by phosphorimaging as described previously (*16*).

Fast-Performance Liquid Chromatography (FPLC) Analysis and Identification of Lipids. For FPLC analysis of plasma samples, 12 μL of plasma was diluted 1:5 with PBS and loaded onto a Superose 6 column in a SMART micro FPLC system (Pharmacia) and eluted with PBS. A total of 25 fractions (50 μL each) were collected for further analysis. Triglycerides and cholesterol were assessed using the GPO-Trinder kit (Sigma) and the CHOL-MPR3 kit (Boehringer-Mannheim), according to the manufacturer's instructions. The triglyceride and cholesterol concentrations of the plasma and the FPLC fractions were determined spectrophotometrically at 540 and 492 nm, respectively, as described previously (*16*).

Quantification of Human ApoE. Plasma at human apoE concentrations was assessed using a sandwich ELISA (*16*, *17*, *21*).

Statistical Analysis. Data are presented as the mean ± standard error. Comparison of data from two groups of mice was performed using the paired-two-sample-for-means *t* test with unequal variance.

Density Gradient Ultracentrifugation. To assess the ability of WT and mutant apoE forms to associate with different lipoproteins, an aliquot of 0.3 mL of plasma from mice infected either with the control adenovirus expressing the AdGFP or with adenoviruses expressing the WT or mutant apoE4 forms was brought to a volume of 0.5 mL with PBS and adjusted to a density of 1.23 g/mL with KBr. This solution was then overlaid with 1 mL of 1.21 g/mL KBr, 2.5 mL of 1.063 g/mL KBr, 0.5 mL of 1.019 g/mL KBr,

and 0.5 mL of saline. The mixtures were centrifuged for 22 h in a SW-41 rotor at 34 000 rpm. Following ultracentrifugation, 10 fractions of 0.5 mL were collected, and the top 9 fractions were analyzed by SDS−PAGE.

Electron Microscopy (EM). Aliquots of the five to eight fractions from equilibrium density gradient ultracentrifugation that contain most of the apoE were dialyzed against ammonium acetate and carbonate buffer, were stained with sodium phosphotungstate, were visualized in the Phillips CM-120 electron microscope (Phillips Electron Optics, Eindhoven, The Netherlands), and were photographed as described previously (*27*). The photomicrographs were taken at 75000× magnification and enlarged three times.

RESULTS

Generation of Recombinant Adenoviruses Carrying Mutant ApoE4 Forms and Their Expression in HTB-13 Cells. We have generated recombinant adenoviruses expressing three new apoE4 mutants, where hydrophobic amino acids in the region of residues 261−265 were substituted with Ala. The first mutant, designated apoE4-mutA, carries a Phe265Ala substitution; the second, designated apoE4-mutB, has Leu261Ala and Trp264Ala substitutions, and the third, designated apoE4-mutC, has Leu261Ala, Trp264Ala, and Phe265Ala substitutions. To assess the expression and secretion of the mutant apoE4 forms in comparison to WT apoE4, we infected HTB-13 (human astrocytoma) cells, which do not synthesize endogenous apoE, with the recombinant adenoviruses expressing the WT and the mutant apoE4 forms using a moi of 5. Analysis of the culture media 24 h post-infection showed that the WT and the mutant forms were secreted at comparable levels in the medium (data not shown).

Substitution of Residues Leu261, Trp264, with Phe265 by Ala Prevents ApoE-Induced Hypertriglyceridemia. The adenovirus-mediated apoE gene transfer experiments were performed with viral doses that gave comparable hepatic apoE mRNA levels for WT and the mutant apoE forms. The viral doses that were used did not cause liver damage as determined by the normal serum transaminase levels 1−4 days post-infection. When apoE$^{-/-}$ mice were infected with 2×10^9 pfu of recombinant adenoviruses expressing either WT apoE4 or apoE4-mutA that has a Phe265Ala substitution, cholesterol levels increased and hypertriglyceridemia was induced 3 and 4 days post-infection (Figure 1A,D). FPLC analysis showed that the great majority of cholesterol and all triglycerides were distributed in the VLDL region, whereas the HDL cholesterol peak was very small (Figure 1B,C,E,F). Experiments with apoE4-, apoE3-, or apoE2-expressing adenoviruses gave similar results (*17*, *18*, *28*).

The phenotypes of apoE$^{-/-}$ mice infected with apoE4-mutB, which has two amino acids (Leu261 and Trp264) substituted with Ala, and apoE4-mutC, which has three amino acids (Leu261, Trp264, and Phe265) substituted with Ala, were drastically different. Thus, infection of apoE$^{-/-}$ mice with apoE4-mutB decreased the total plasma cholesterol levels by approximately 40% but induced moderate hypertriglyceridemia (Figure 1G). Fractionation of plasma by FPLC showed that approximately 70% of the total cholesterol was in the HDL region (Figure 1H). The remaining 30% of cholesterol along with the great majority of the triglycerides was in the VLDL region (Figure 1H,I). Infection of apoE$^{-/-}$ mice with adenovirus expressing the apoE4-mutC decreased the plasma cholesterol level by approximately 60% and did not induce hypertriglyceridemia (Figure 1J). More importantly, 85% of the cholesterol was distributed in HDL (Figure 1K). The remaining cholesterol along with low levels of triglycerides was found in VLDL (Figure 1K,L). Infection of apoE$^{-/-}$ mice with the control adenovirus expressing the GFP did not affect significantly their plasma cholesterol and triglyceride levels (Figure 1M). The distribution of lipids in the control mice was in the VLDL/intermediate density lipoprotein (IDL) region as described previously (*4*) (Figure 1N,O).

In ApoE$^{-/-}$ Mice Overexpressing WT ApoE4, Cholesterol Is Not Esterified. Substitution of Leu261, Trp264, and Phe265 with Ala promotes esterification of the cholesterol of the VLDL and HDL fractions. In an attempt to understand the differences in phenotypes produced in apoE$^{-/-}$ mice expressing WT apoE4, which induces dyslipidemia, and apoE4-mutC, which corrects dyslipidemia, we correlated the plasma lipid apoE levels and the lipoprotein composition with the hepatic apoE mRNA levels following infection of mice with the adenoviruses expressing these two apoE forms. It was found that when the expression of WT apoE4 and apoE4-mutC is comparable as determined by the hepatic apoE mRNA levels (Figure 2A,B), WT apoE4 induced dyslipidemia 1−4 days post-infection, which was characterized by severe hypertriglyceridemia (Figure 2C,D). In mice expressing both WT apoE4 and apoE4-mutC, apoE accumulated in plasma at high levels (120−200 mg/dL) (Figure 2E). The majority of the apoE4-mutC was found in the HDL_2 region, whereas WT apoE4 is distributed in all lipoprotein fractions (Figure 2F,G). In mice overexpressing WT apoE4, cholesterol exists mainly as free cholesterol (CE/TC = 0.1) (Figure 2H), whereas in mice expressing apoE4-mutC, cholesterol exists mostly as esterified cholesterol (CE/TC = 0.8) (Figure 2I).

ApoE4-mutA Promotes the Formation of Discoidal HDL and ApoE4-mutC Promotes the Formation of Spherical HDL. The FPLC data of Figure 1A−K suggested that apoE4-mutB and apoE4-mutC had promoted the formation of HDL. To assess the nature of the HDL particles that formed, we have used adenovirus-mediated gene transfer of the apoE mutants in apoA-I$^{-/-}$ mice. The lipid and lipoprotein profiles obtained in apoA-I$^{-/-}$ mice following adenovirus infection were qualitatively similar but not identical to those observed when the same experiments were performed in apoE$^{-/-}$ mice (compare Figure 1D−L with Figure 3A−I). In apoA-I$^{-/-}$ mice expressing apoE4-mutA, plasma cholesterol and triglyceride levels increased (Figure 3A). Triglycerides were distributed in VLDL, whereas cholesterol was distributed in both VLDL (60%) and HDL (40%) (Figure 3B,C). The CE/TC ratio of the VLDL and HDL peak was 0.35 (Figure 3B).

In apoA-I$^{-/-}$ mice expressing apoE4-mutB, the level of cholesterol was increased and mild hypertriglyceridemia was observed (Figure 3D). However, 90% of the cholesterol was found in HDL (Figure 3E), and the remaining 10% of the cholesterol along with all the triglycerides was found in VLDL (Figure 3E,F). The CE/TC ratio of the VLDL and HDL was 0.6 (Figure 3E).

In mice expressing apoE4-mutC, there was a small increase in the level of total plasma cholesterol that could be ascribed

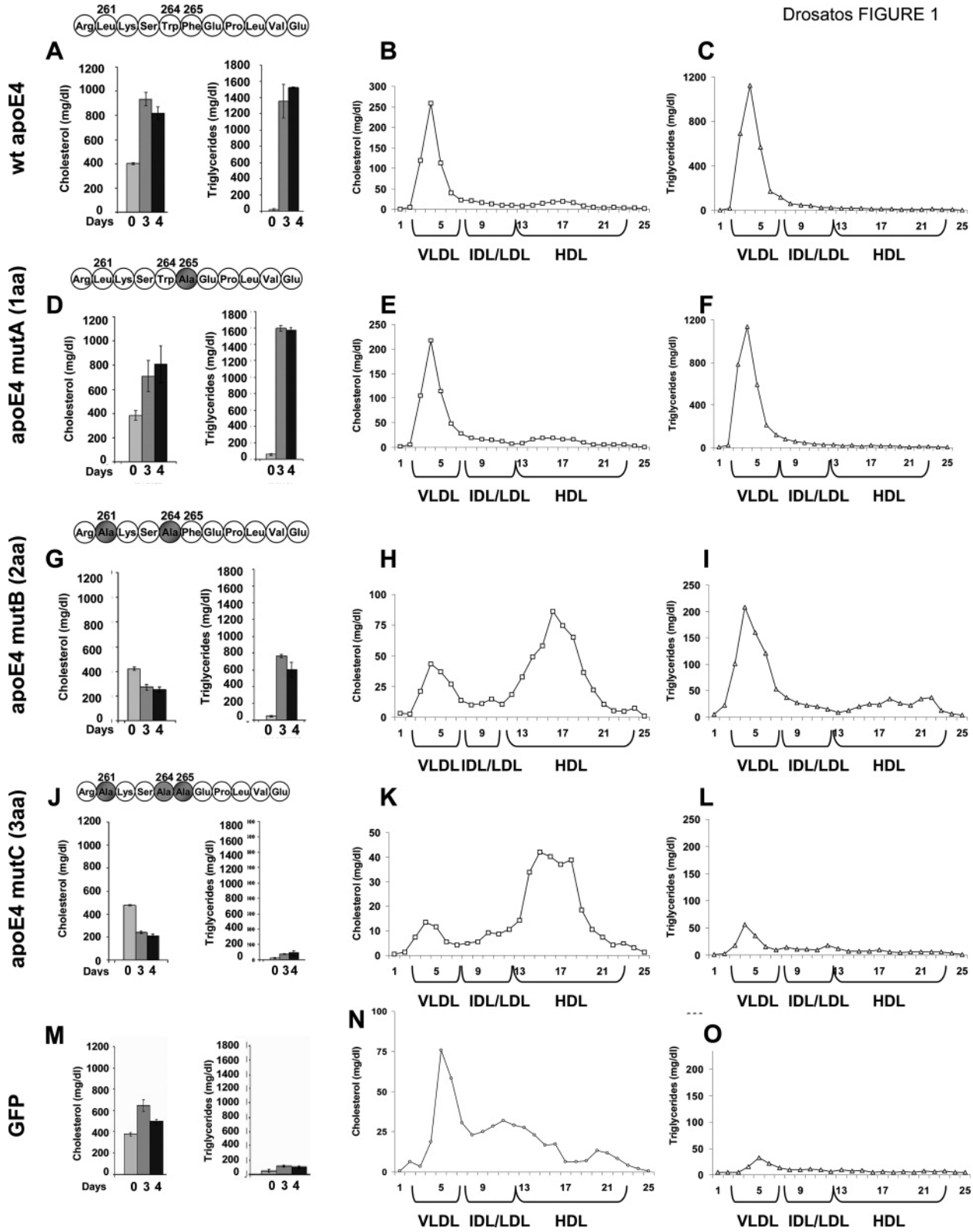

FIGURE 1: Plasma cholesterol and triglyceride levels and FPLC profiles of apoE$^{-/-}$ mice infected with recombinant adenoviruses expressing WT apoE4, apoE4-mutA, apoE4-mutB, apoE4-mutC (Panels A−L) or the control adenovirus expressing the green fluorescence protein (Panel M) or apoE4mutA in combination with human LCAT or lipoprotein lipase (Panels N and O, respectively). Mice were infected with 2×10^9 pfu of recombinant adenovirus, and serum samples were isolated 4 days post infection. Serum samples were analyzed for total plasma cholesterol and triglyceride levels as indicated in panels A, D, G, J, and M as well as for the distribution of cholesterol (B, E, H, K, and N) and triglycerides (C, F, I, L, and O) following FPLC fractionation. The sequence of apoE4 and apoE4 mutants in the region of residues 261 to 265 is indicated at the top of panels A, D, G, and J, and the amino acid alterations in apoE4 mutants are highlighted in gray.

to the increase in the level of HDL cholesterol, whereas the level of plasma triglycerides was low (Figure 3G−I). The CE/TC ratio of VLDL and HDL was 0.85 (Figure 3H). Similar results were obtained with apoE2-mutC, indicating that the ability of the triple apoE mutant to promote formation of HDL is independent of the apoE phenotype (Figure 3J−

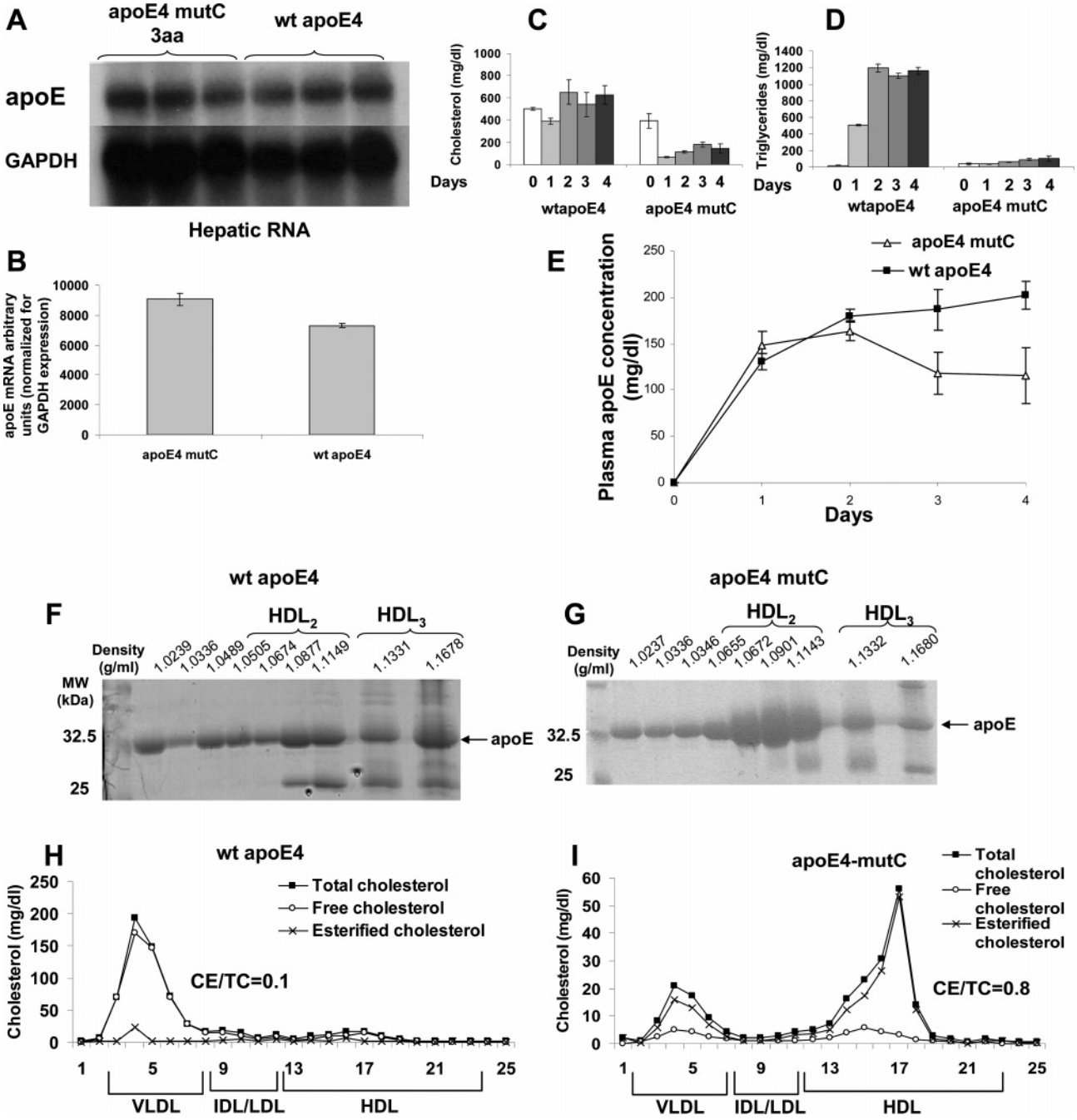

FIGURE 2: Correlation of hepatic apoE mRNA levels, plasma apoE levels, and triglyceride levels of apoE4 and apoE4-mutC carrying three amino acid substitutions (L261A, W264A, and F265A). (A and B) Hepatic apoE mRNA levels of individual apoE$^{-/-}$ mice expressing WT apoE4 or apoE4-mutC. (C−E) Plasma cholesterol, triglyceride, and apoE levels of apoE$^{-/-}$ mice expressing WT apoE4 or apoE4-mutC. (F and G) Distribution of apoE following density grandient ultracentrifuation and SDS−PAGE analysis of the plasma of mice expressing WT apoE (F) or apoE4-mutC (G). (H and I) FPLC profiles of free, esterified, and total cholesterol of apoE$^{-/-}$ mice expressing WT (H) apoE4 or apoE4-mutC (I).

L). The data of Figure 3M−O are explained later. To determine the type of particles which accumulate in the HDL region, plasma obtained from apoA-I$^{-/-}$ mice 4 days post-infection was fractionated by density gradient ultracentrifugation and analyzed by SDS−PAGE (Figure 4A−C,G−I). HDL fractions 5−8 obtained by this fractionation that contained most of the apoE were analyzed by EM (Figure 4D−F,J−L). This analysis showed that apoE4-mutA promoted the formation of discoidal HDL (Figure 4D). In contrast, apoE4-mutC and apoE2-mutC promoted the formation of spherical HDL (Figure 4F,J). Finally, apoE4-mutB promoted the formation of mostly spherical and few discoidal HDL particles (Figure 4E).

Dyslipidemia Induced by ApoE4-mutA Is Corrected by Treatment with Human Lecithin Cholesterol Acyl Transferase (LCAT) or Lipoprotein Lipase. The data of Figure 3A suggested that the activity of the endogenous lipoprotein lipase was insufficient to hydrolyze the triglycerides of the VLDL, which accumulates in the plasma of apoA-I$^{-/-}$ mice expressing apoE4-mutA. In addition, the data of Figures 3B and 4D suggested that the activity of the endogenous LCAT was insufficient to esterify the cholesterol of VLDL

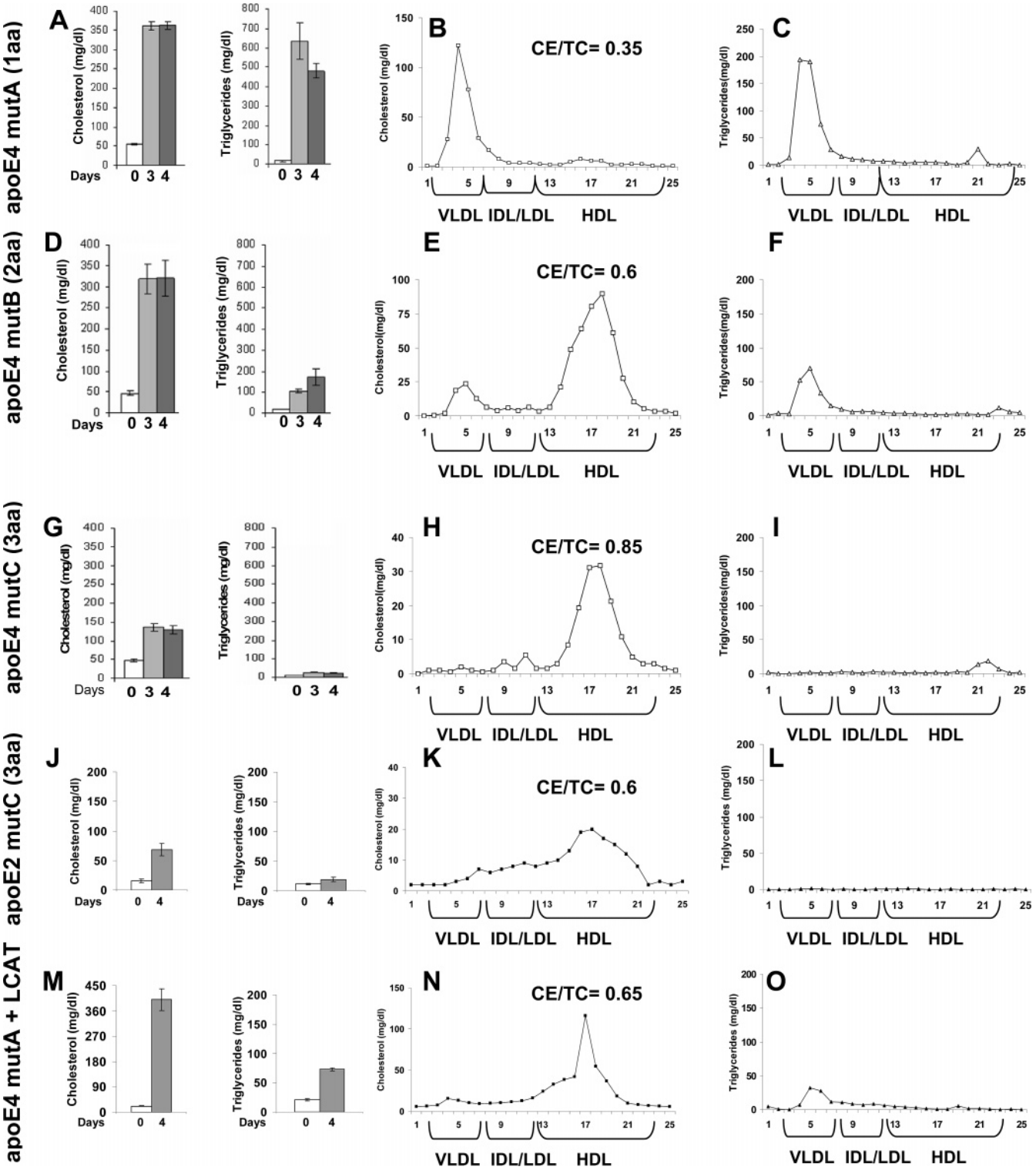

FIGURE 3: Plasma cholesterol and triglyceride levels and FPLC profiles of apoA-I$^{-/-}$ mice infected with recombinant adenoviruses expressing apoE4-mutA, apoE4-mutB, apoE4-mutC, apoE2-mutC, and apoE4-mutA in combination with a low dose of an adenovirus-expressing LCAT. Mice were infected as described in the legend of Figure 1. Serum samples were isolated 4 days post-infection and analyzed for total plasma cholesterol and triglyceride levels as indicated in panels A, D, G, J, and M as well as for the distribution of cholesterol (B, E, H, K, and N) and triglycerides (C, F, I, L, and O) following FPLC fractionation.

and HDL in apoA-I$^{-/-}$ mice expressing apoE4-mutA. This question was addressed by treating the apoA-I$^{-/-}$ mice with a combination of adenoviruses expressing both apoE4-mutC and either human LCAT or human lipoprotein lipase. The treatment of apoA-I$^{-/-}$ mice with the combination of adenoviruses expressing apoE4-mutA and LCAT corrected the apoE4-mutA-induced hypertriglyceridemia (Figure 3M−O). This treatment also caused a dramatic increase in the magnitude of the HDL cholesterol peak, and the CE/TC ratio of HDL increased to 0.65 (Figure 3N). Hypertriglyceridemia was also corrected by treatment of apoA-I$^{-/-}$ mice with a combination of adenoviruses expressing apoE4-mutA and

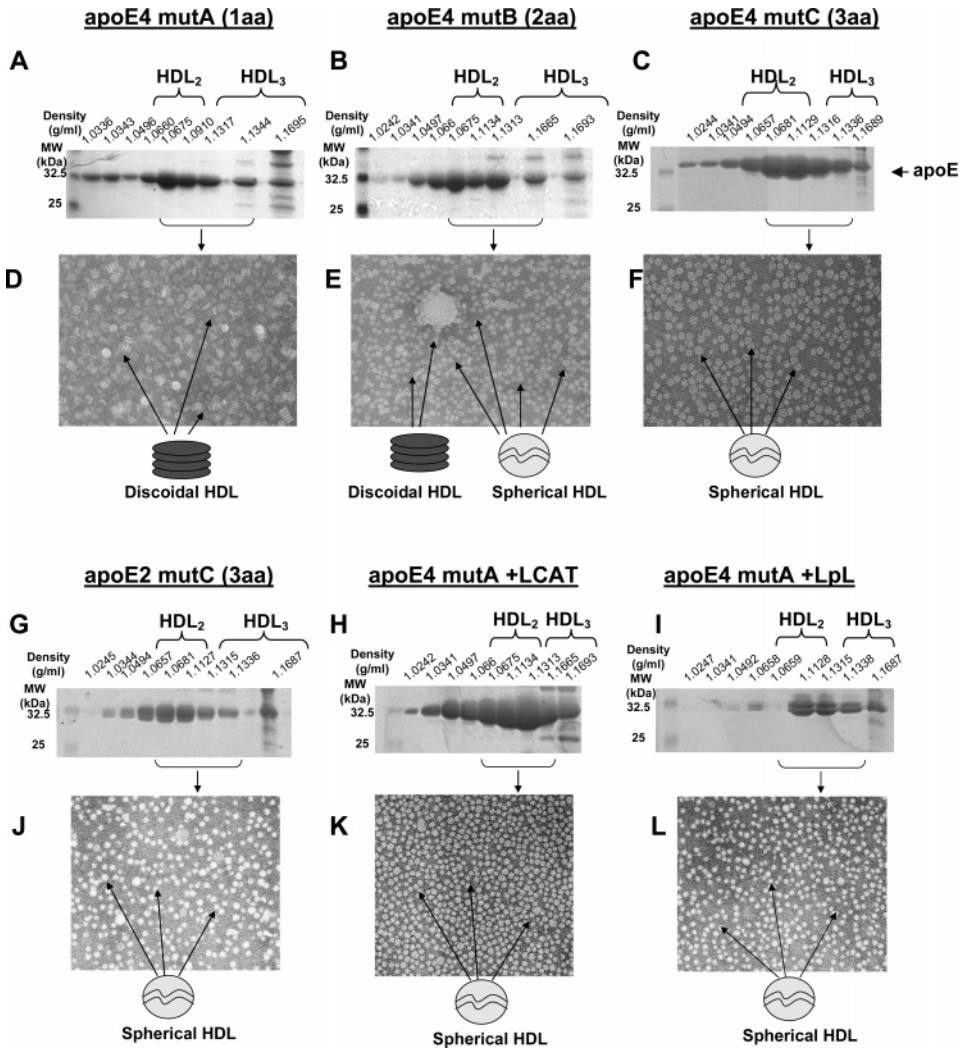

FIGURE 4: Distribution of apoE following density gradient ultracentrifugation and SDS−PAGE analysis of plasma (A−C and G−I) and EM analyses of HDL (D−F and J−L). ApoA-I$^{-/-}$ mice were infected with 2×10^9 pfu of recombinant adenoviruses expressing apoE4-mutA (A and D), apoE4-mutB (B and E), apoE4-mutC (C and F), apoE2-mutC (G and J), and a combination of apoE4-mutA (2×10^9 pfu) with LCAT (5×10^8 pfu) (H and K) or with lipoprotein lipase (5×10^8 pfu) (I and L). Samples were collected 4 days post-infection and analyzed as described in Experimental Procedures.

human lipoprotein lipase (data not shown). As expected, the treatment of the apoA-I$^{-/-}$ mice with the adenovirus expressing apoE4-mutA with either human LCAT or lipoprotein lipase also converted the discoidal HDL into spherical HDL (Figure 4K,L).

DISCUSSION

The Triple ApoE Mutant in either an ApoE4 or ApoE2 Background Promotes Exclusively the Formation of Spherical ApoE-Containing HDL, and a Single ApoE4 Mutant Induces Hypertriglyceridemia and Promotes the Formation of Discoidal ApoE-Containing HDL. This study had two objectives. The first was to determine how the apoE mutations influence the formation of apoE-containing HDL, and the second was to identify individual amino acids or groups of amino acids that are capable of inducing hypertriglyceridemia and to find the etiology of apoE-induced hypertriglyceridemia.

Previous studies have shown that overexpression of N-terminal apoE forms extending from residue 1 to residues 185, 202, 229, and 259 can clear cholesterol of apoE-deficient mice without induction of hypertriglyceridemia (*16*−*18*, *21*). However, full-length apoE forms, when expressed at similar levels, increased further plasma cholesterol levels, induced severe hypertriglyceridemia, and affected the formation of HDL (*16*−*18*, *21*). The FPLC lipid profiles of apoE$^{-/-}$ or apoA-I$^{-/-}$ mice infected with adenoviruses expressing the WT and the three apoE mutants showed an inverse correlation between the VLDL triglyceride peak and the HDL cholesterol peak, suggesting that hypertriglyceridemia may interfere with the biogenesis of HDL. To clarify this question, we performed adenovirus-mediated gene transfer of the apoE mutants in apoA-I$^{-/-}$ mice, which cannot synthesize apoA-I-containing HDL (*28*). It was found that in mice expressing apoE4-mutB, apoE4-mutC, and apoE2-mutC cholesterol was found in the HDL region and was mostly esterified. In contrast, in mice expressing apoE4-mutA, the magnitude of the HDL peak was diminished and the cholesterol was mostly unesterified. Determination of the morphology and composition of the HDL particles formed in apoA-I$^{-/-}$ mice by the various apoE mutants by EM showed that apoE4-mutA, which is associated with hypertriglyceridemia, formed discoidal HDL and apoE4-mutC and apoE2-mutC, which are associated with normal triglyceride

levels, formed spherical HDL particles. ApoE4-mutB, which is associated with moderate hypertriglyceridemia, formed a mixed population of mostly spherical and few discoidal particles. Since apoA-I$^{-/-}$ mice expressing GFP form very few lipoprotein particles (data not shown) (*29*), we conclude that the observed discoidal and spherical HDL particles formed in mice expressing the various apoE mutants represent apoE-containing HDL.

Substitution of Leu261, Trp264, and Phe265 with Ala Prevented the Induction of Hypertriglyceridemia in ApoE$^{-/-}$ Mice. The analysis of three apoE4 mutants carrying one, two, or three substitutions of hydrophobic amino acids with Ala in the region of residues 261−265 showed that apoE4-mutC that has three amino acid substitutions and has the ability to clear the VLDL cholesterol of apoE$^{-/-}$ mice without induction of hypertriglyceridemia. Similarly, apoE4-mutC or apoE2-mutC did not induce hypertriglyceridemia in apoA-I$^{-/-}$ mice. In this respect, these mutants behave like the truncated apoE forms that lack the C-terminal domain (*16*−*18*, *21*). These findings indicated that residues Leu261, Trp264, and Phe265 are required for the apoE-induced hypertriglyceridemia and that the development of hypertriglyceridemia is not influenced by the apoE phenotype. The induction of hypertriglyceridemia requires some form of combined action of all three residues, since neither residue 265 alone nor a combination of residues 261 and 264 is sufficient to prevent the induction of hypertriglyceridemia. Previous studies showed that expression of another apoE4 mutant in apoE$^{-/-}$ mice, where the hydrophobic residues Trp276, Leu279, Val280, and Val283 were substituted with Ala, did not correct hypercholesterolemia and induced hypertriglyceridemia, thus suggesting a unique role for residues 261, 264, and 265 in the induction of hypertriglyceridemia (*22*).

The Clearance of VLDL Cholesterol in Mice Expressing the Triple ApoE4 Mutant Is Associated with an Increase in the Magnitude of the HDL Cholesterol Peak and an Increase in the CE/TC Ratio of HDL. In apoE$^{-/-}$ mice with similar levels of expression of WT apoE4 and the "triple" apoE4 mutant, the plasma apoE levels were high. It is noteworthy that when the plasma apoE levels were identical 1 and 2 days post-infection, mice expressing WT apoE4 developed hypertriglyceridemia whereas mice expressing apoE4-mutC did not. The increased level of plasma apoE4-mutC, which accumulates predominantly in the HDL_2 region, accounts for the increase in the magnitude of the HDL cholesterol peak. As explained, the biogenesis of this type of apoE-containing HDL is promoted by the triple apoE mutation. In contrast to apoE4-mutC, WT apoE4 was distributed rather uniformly in all lipoprotein classes. Thus, in this case, the increase in the level of plasma apoE could reflect both an increased level of synthesis of apoE-containing HDL and a defective clearance of apoE-containing triglyceride-rich lipoproteins.

LCAT and Lipoprotein Lipase Can Rescue the Dyslipidemic Phenotype of ApoE4-mutA. In apoE$^{-/-}$ mice expressing WT apoE4, the VLDL contained mostly free cholesterol (CE/TC = 0.1). In contrast, the VLDL and HDL peak of mice expressing apoE4-mutC contained mostly esterified cholesterol (CE/TC = 0.8). The observed differences in the free cholesterol of VLDL suggest that the esterification of the cholesterol of VLDL is inhibited in mice overexpressing the apoE4 but not in mice overexpressing apoE4-mutC. Similar observations were made in apoA-I$^{-/-}$ mice expressing apoE4-mutA. It has been suggested recently that apoE facilitates esterification of cholesterol of apoB-containing lipoproteins by activating LCAT (*30*). Thus, inhibition of LCAT in vivo could explain the increased cholesterol content of VLDL and HDL. When apoA-I$^{-/-}$ mice were treated with a combination of adenoviruses expressing apoE4-mutA and either LCAT or lipoprotein lipase, hypertriglyceridemia was corrected and spherical HDL particles were formed. This indicated that in mice expressing apoE4-mutA the activity of these enzymes in plasma was rate-limiting. The ability of lipoprotein lipase to correct the apoE-induced hypertriglyceridemia has also been demonstrated in a previous study using WT apoE4 (*22*). The conversion of the discoidal to spherical particles in vivo by treatment with LCAT indicates the central role of LCAT in the biogenesis of spherical apoE-containing HDL particles.

ApoE Has Dual Functionality in That It Participates in Remnant Clearance as Well as in the Biogenesis of ApoE-Containing HDL. Taken together with the known function of apoE (*1*, *4*, *5*), the data are consistent with the following events. ApoE secreted by the liver and other tissues can be incorporated into triglyceride-rich lipoproteins, and following hydrolysis of the triglycerides by lipoprotein lipases, apoE directs the clearance of the lipoprotein remnants via the LDL receptor (*6*, *18*). Under conditions of overexpression of WT apoE or certain apoE mutants, the apoE induces hypertriglyceridemia that can be treated with either lipoprotein lipase or LCAT. Here we show that WT and mutant apoE forms can participate in the de novo biogenesis of apoE-containing HDL particles following a pathway similar to that described for the apoA-I-containing HDL particles (*31*) and that specific apoE mutations promote the formation of spherical apoE-containing HDL. The dual functionality of apoE in remnant clearance and the formation of apoE-containing HDL may account at least partially for its anti-atherogenic properties (*4*, *5*, *16*, *32*, *33*). This study also establishes that substitutions of Leu261, Trp264, and Phe265 with Ala in either the apoE4 or apoE2 background improve the biological functions of apoE in two different ways: the mutant apoE forms can correct the high cholesterol levels of apoE$^{-/-}$ mice without induction of hypertriglyceridemia, and they can promote formation of spherical apoE-containing HDL. Bioengineered apoE variants with improved biological functions may find future therapeutic applications in the correction of remnant removal disorders and in atheroprotection.

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