The Amino-Terminal 1−185 Domain of ApoE Promotes the Clearance of Lipoprotein Remnants in Vivo. The Carboxy-Terminal Domain Is Required for Induction of Hyperlipidemia in Normal and ApoE-Deficient Mice†

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ABSTRACT: Apolipoprotein E (apoE) promotes receptor-mediated catabolism of apoE-containing lipoprotein remnants. Impairments in remnant clearance are associated with type III hyperlipoproteinemia and premature atherosclerosis. In humans, apoE plasma levels correlate with plasma triglyceride levels, suggesting that excess apoE may also affect plasma triglyceride levels. We have used adenovirus-mediated gene transfer in mice to map the domains of apoE required for cholesterol and triglyceride clearance, in vivo. Adenovirus expressing apoE3 and apoE4 at doses of $(1-2) \times 10^9$ pfu increased plasma cholesterol and triglyceride levels in normal C57BL6 mice and failed to normalize the high cholesterol levels of apoE-deficient mice due to induction of hypertriglyceridemia. In contrast, an adenovirus expressing the truncated apoE 1−185 form normalized the cholesterol levels of $E^{-/-}$ mice and did not cause hypertriglyceridemia. Northern blot analysis of hepatic RNA from mice expressing the full-length and the truncated apoE forms showed comparable steady-state apoE mRNA levels of the full-length apoE forms that cause hyperlipidemia and the truncated apoE forms that do not cause hyperlipidemia. The findings suggest that the amino-terminal residues 1−185 of apoE are sufficient for the clearance of apoE-containing lipoprotein remnants by the liver, whereas domains of the carboxy-terminal one-third of apoE are required for apoE-induced hyperlipidemia.

Apolipoprotein E (apoE)[1] is a major component of the cholesterol transport system, being the ligand that promotes the recognition and catabolism of apoE-containing lipoproteins by cell receptors (*1*−*5*). Mutations in apoE that prevent its binding to the LDL receptor and possibly other receptors and heparan sulfate proteoglycans are associated with type III hyperlipoproteinemia and premature atherosclerosis (*6*−*14*).

Compelling evidence on the role of apoE in cholesterol homeostasis were obtained by studies of human patients and animal models with apoE deficiency or defective apoE forms (*15*−*24*). These studies showed that apoE is required for the clearance of cholesterol ester-rich lipoprotein remnants which float in the VLDL and IDL regions (*17*−*26*). The accumulation of such remnants in plasma is associated with premature atherosclerosis in humans and in experimental animals (*15*, *17*−*19*).

Other studies highlighted the importance of apoE in cholesterol efflux and showed that apoE-containing lipoprotein particles with γ electrophoretic mobility (γLp-E) were very effective in removing excess cholesterol from cholesterol-loaded macrophages, thus contributing to cell and tissue cholesterol homeostasis (*27*−*30*). The involvement of apoE in cholesterol efflux may explain why, when expressed locally in macrophages or endothelial cells, apoE protects from atherosclerosis (*31*−*33*).

Recent studies in humans and in transgenic animal models have indicated that apoE may have other functions relevant to plasma triglyceride homeostasis. Such studies showed that increases in apoE levels inhibit lipolysis of triglyceride-rich lipoproteins, obtained from animals overexpressing apoE (*25*, *26*, *34*−*39*). Lipolysis of VLDL in vitro could be partially restored by the addition of apoCII (*34*, *36*). Overexpression of apoE also stimulates hepatic VLDL triglyceride production in vivo (*39*) and in cell cultures (*34*), possibly by promoting the assembly and/or secretion of apoB-containing lipoproteins. In contrast, lack of apoE was associated with decreased VLDL triglyceride secretion (*40*).

In the current study, we have used adenovirus-mediated gene transfer in normal and apoE-deficient mice ($E^{-/-}$) to map the domains of apoE required for clearance of lipopro-

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[1] Abbreviations: apoE, apolipoprotein E; apoA-I, apolipoprotein A-I; apoCII, apolipoprotein CII; apoA-IV, apolipoprotein A-IV; apoB48, apolipoprotein B48; Ad, adenovirus; BSA, bovine serum albumin; ELISA, enzyme-linked immunoassay; FCS, fetal calf serum; FPLC, fast pressure liquid chromatography; GAPDH, glyceraldehyde acid phosphate dehydrogenase; GFP, green fluorescence protein; HDL, high-density lipoprotein; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; VLDL, very low-density lipoprotein.

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Table 1: Oligonucleotides Used in Overlap Extension PCR[a]

oligo name	oligo sequence
OUTPR1-S	5′-GCT GGG TGC AGA CAC TGT CTG AGC-3′
OUTPR2-A	5′-CGC AGC CGC TCG CCC CAG CAG GCC T-3′
202 Internal-S	5′-GGC CAG CCG ***TGA*** CAG GAG CGG-3′
202 Internal-A	5′-CCG CTC CTG ***TCA*** CGG CTG GCC-3′
185 Internal-S	5′-CCC CTG GTG ***TAA*** CAG GGC CGC GTG-3′
185 Internal-A	5′-GCG GCC CTG ***TTA*** CAC CAG GGG CCC-3′

[a] Boldface underlined bases represent the mutated codon.

tein remnants from the plasma of $E^{-/-}$ mice and determine how apoE induces hyperlipidemia in vivo. The mice were analyzed 4−5 days post-infection for their lipid and lipoprotein profiles, apoE mRNA levels, and the effect of apoE on hepatic VLDL triglyceride secretion. We have found that overexpression of apoE4−185 normalizes the cholesterol levels of $E^{-/-}$ mice. This indicates that the amino-terminal domain 1−185 of apoE contains the determinants required for binding to cells and catabolism of apoE-containing lipoproteins by the liver in vivo. Overexpression of apoE3 or apoE4 in normal C57BL6 mice induces combined hyperlipidemia which is associated with increase in both plasma cholesterol and triglycerides. In addition, hypertriglyceridemia is also induced on top of preexisting hypercholesterolemia in $E^{-/-}$ mice by overexpression of apoE4. Overexpression of apoE4−202 does not affect the plasma cholesterol and triglyceride levels of the C57BL6 mice. These findings indicate that overexpression of apoE alone is responsible for the combined hyperlipidemia of the normal C57BL6 mice as well as for the hypertriglyceridemia of the $E^{-/-}$ mice. The findings also point out that the carboxy-terminal segment of apoE is required for the apoE-induced hyperlipidemia. It is possible that truncated forms of apoE that do not induce hyperlipidemia may find useful gene therapy applications in the future in correcting remnant removal disorders.

EXPERIMENTAL PROCEDURES

Construction of Recombinant Adenoviruses Expressing ApoE3, ApoE4, ApoE4−202, and ApoE4−185. The construction of pUC-apoE3 and pUC-apoE4 cDNA has been described previously (*41*). pUC-apoE4−202 and pUCE4−185 were generated by overlap-extension PCR that resulted in mutagenesis of codons 203 and 186, respectively, to a stop codon (TAA or TGA) using pUC-apoE4 as a template and the four sets of oligonucleotides indicated in Table 1 as primers. The set of external primers OUTPR1-S (sense) and OUTPR2-A (antisense) correspond to nucleotides encoding amino acids 103−111 and 208−215 of apoE, respectively, and contain the restriction sites *Ngo*MI and *Bst*EII, respectively. The set of mutagenic oligonucleotides extending 10 residues 5′ and 10 residues 3′ of codons 203 and 186 have been altered in their sequence to a stop TAA or TGA codon (Table 1). The PCR-based mutagenesis of codon 203 or 186 involved two separate amplification reactions. The first reactions used the 5′ external primer and the antisense mutagenic primer (Internal-A, Table 1) covering codon 203 or codon 186. The second reaction used the 3′ external primer and the sense mutagenic primer (Internal-S, Table 1) covering codon 203 or codon 186. An aliquot of 4% of the volume of each PCR reaction was mixed, and the sample was amplified by the 5′ and the 3′ external primers. The amplified fragment was then digested with *Ngo*MI and *Bst*EII and was used to replace the WT sequence of the pUC-E4 plasmid. To incorporate the PCR-generated mutations to exon IV of the apoE gene, a two-step procedure was followed. The *Eco*RI fragment of the human apoE4 gene, which includes the entire exon IV sequence, was cloned into the *Eco*RI site of the pBS vector to generate the vector pBlue-ExIV. The pUC-apoE4−202 and pUC-apoE4−185 plasmids were digested with *Sty*I/*Bbs*I, and the mutated sequence was exchanged for the WT sequence of the pBlue-E4-ExIV plasmid to generate plasmids pBlue-E4−202-ExIV and pBlue-E4−185-ExIV.

The recombinant viruses were constructed using the Ad-Easy-1 system where recombinant adenovirus construct is generated in bacteria BJ-5183 cells (*42*). The 1507 bp *Msc*I−*Eco*RI fragment of apoE genomic DNA (nucleotides 1853−3360), which contains exons 2 and 3, was cloned into the *Sma*I−*Eco*RI sites of pGEM7 vector, resulting in the pGEM7-apoE-ExII-III vector. The 1911 bp *Eco*RI fragment of apoE3, apoE4, apoE4−202 (that contains the stop mutation at codon 203), or apoE4−185 (that contains the stop mutation at codon 186) gene was then excised from pBlue-E3-ExIV, pBlue-E4-ExIV, pBlue-E4−202-ExIV, or pBlue-E4−185-ExIV vector, respectively, and cloned into the *Eco*RI site of the pGEM7-apoE-ExII-III vector. This generated the pGEM7-apoE3g or pGEM7-apoE4g or the pGEM7-apoE4g-202 or pGEM7-apoE4g-185 vector, respectively, that contains exons II, III, and IV of the apoE gene. The correct orientation of the 1911 bp *Eco*RI insert was checked by restriction digestion with *Not*I and *Xba*I. The entire *Hin*dIII−*Xba*I fragment from pGEM7-apoE3g or pGEM7-apoE4g, or pGEM7-apoE4−202g, or pGEM7-apoE4−185g vector was cloned into the corresponding sites of the pAdTrack-CMV adenovirus shuttle plasmid. The recombinant vector was used to electroporate BJ 5183 *E. coli* cells along with the pAd-Easy-1 helper vector. pAd-Easy-1 contains the viral genome and the long terminal repeats of the adenovirus and allows for the formation by homologous recombination of the recombinant virus containing the gene of interest. The vector also contains the green fluorescence protein gene, which enables detection of the infection of cells and tissues by their green fluorescence. Recombinant bacterial clones resistant to kanamycin were selected and screened for the presence of the gene of interest by restriction endonuclease analysis and DNA sequencing. The viruses expressing WT apoE3, WT apoE4, apoE4−202, or apoE4−185 forms are designated as AdGFP-E3, AdGFP-E4, AdGFP-E4−202, and AdGFP-E4−185, respectively. Correct clones were propagated in RecA DH5α cells. The recombinant vector was linearized with *Pac*I and used to infect 911 cells. The subsequent steps involved in the generation and expansion of recombinant adenoviruses were plaque identification/isolation followed by infection and expansion in 911 cells (*43*). These steps were followed by a purification process involving CsCl ultracentrifugation performed twice, followed by dialysis and titration of the virus. Usually, titers of approximately 5×10^{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-E3 or AdGFP-E4 or with AdGFP-E4−202 or AdGFP-E4−185 at a multiplicity of infection (moi) of 20.

Twenty-four hours post-infection, cells were washed twice with phosphate-buffered saline (PBS) and preincubated in serum-free medium for 2 h. Following an additional wash with PBS, fresh serum-free medium was added. After 24 h of incubation, medium was collected and analyzed by enzyme-linked immunoabsorbent assay (ELISA) and SDS−PAGE for apoE expression.

Animal Studies. Female apoE-deficient mice 20−25 weeks old were used in these studies (*44*). Groups of mice were formed based on their plasma cholesterol and triglyceride levels before initiation of the experiments, to ensure similar mean cholesterol and triglyceride levels in each group. The mice were injected intravenously through the tail vein with doses ranging from 5×10^8 to 1×10^{10} pfu of AdGFP (control adenovirus), AdGFP-E3, AdGFP-E4, AdGFP-E4−202, or AdGFP-E4−185 virus, as indicated. Each group contained 8−10 mice. Blood was obtained from the tail vein or retroorbital plexus after a 4 h fast preceding adenoviral injection. On the indicated days after injection (0, 4, 5, 8, and 12), blood was collected into a CB300 or CB1000 blood-collection tube (Sarstedt). Aliquots of plasma were stored at 4 and −20 °C. One or more animals from each group were sacrificed on each of the indicated days SO that mRNA expression in the mouse liver could be analyzed.

FPLC Analysis. For FPLC analysis of serum samples, 12 μL of serum were diluted 1:5 with PBS. The sample was loaded onto a Separose 6 column in a SMART micro FPLC system (Pharmacia), and eluted with PBS. A total of 25 fractions of 50 μL volume each were collected for further analysis.

Triglyceride and Cholesterol Analysis. Ten microliters of serum sample were diluted with 40 μL of phosphate-buffered saline (PBS), and 7.5 μL of the dilute sample were analyzed for triglycerides and cholesterol using the GPO-Trinder Kit (Sigma) and CHOL-MPR3 kit (Boehringer-Mannheim), according to the manufacturers' instructions. Triglyceride and cholesterol concentrations were determined spectrophotometrically at 540 and 492 nm, respectively. Triglyceride analysis of the FPLC fractions was performed using the TG-Buffer (Sigma), and concentrations were determined spectrophotometrically at 492 nm according to the manufacturers' instructions. Cholesterol analysis of the FPLC fractions was performed as described above for the serum samples.

Quantification of Human ApoE. Serum human apoE4 concentrations were measured by using sandwich ELISA (*45*). Affinity-purified polyclonal goat anti-human apoE antibody was used for coating, and the same affinity-purified polyclonal goat anti-human apoE antibody conjugated to horseradish peroxidase (HRP) was used as the secondary antibody. After incubation of the plates with the secondary antibody (conjugated to HRP), detection was done by the immunoperoxidase procedure using tetramethylbenzidine as substrate. Pooled plasma from healthy human subjects with known apoE levels was used as a standard.

RNA Isolation and Hybridization Analysis. Total RNA was isolated from liver samples of the infected mice using RNA Easy Solution (RNA Insta-Pure, Eurogentec Belgium) according to the manufacturers' instructions. For Northern blot analysis, RNA samples (15 μg) were denatured and separated by electrophoresis on 1.0% formaldehyde−agarose gels. RNA was stained with ethidium bromide to verify the integrity and equal loading and then transferred to Gene-Screen Plus (DuPont NEN). RNA was cross-linked to the membrane by UV irradiation (Stratalinker, Stratagene) at 0.12 J/cm^2 for 30 s. Probes, prepared by random priming, were used as described previously, and 2.0×10^6 cpm/mL of ^{32}P-labeled DNA was employed.

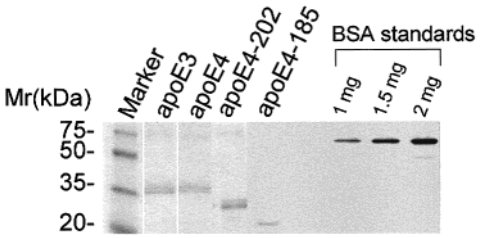

FIGURE 1: SDS−PAGE analysis of culture medium of HTB-13 cells infected with adenoviruses expressing apoE3, apoE4, apoE4−202, and apoE4−185. Fifteen microliters of culture medium were analyzed. "Marker" indicates protein markers of different molecular mass, as indicated in the figure.

Rate of VLDL Triglyceride Production in Mice Infected with Different ApoE Forms. To determine the effects of the apoE truncations on VLDL triglyceride synthesis, apoE-deficient mice were infected with a dose of 2×10^9 pfu of AdGFP-E4 and AdGFP, and with 1×10^{10} pfu of AdGFP-E4−185. Five days post-infection, when the expression of the apoE transgene was maximum, mice were fasted for 4 h and then injected with Triton WR1339 at a dose of 500 mg/kg of body weight, using a 15% solution (w/v) in 0.9% NaCl. Triton WR1339 has been shown to completely inhibit VLDL catabolism (*46*). Then, serum samples were isolated 20, 40, and 60 min after injection with Triton WR1339. As control, serum samples were isolated 1 min immediately after the injection with the detergent. Serum triglyceride levels were determined as described above, and a linear graph of serum triglyceride vs time was generated. The rate of VLDL triglyceride secretion, expressed in miligrams per deciliter per minute, was calculated from the slope of the linear graph for each individual mouse. Then, slopes were grouped together, expressed relative to the AdGFP control, and reported in a bar graph as mean ± standard deviation.

RESULTS

Expression and Secretion of ApoE by HTB-13 Cell Cultures Infected with Control and Recombinant Adenovirus Containing the Full-Length and the Truncated ApoE Forms. HTB-13 cells that do not synthesize apoE were infected with recombinant adenoviruses expressing apoE3, apoE4, apoE4−202, and apoE4−185, at a multiplicity of infection of 20. Analysis of the culture medium by sandwich ELISA showed that the full-length and truncated apoE forms are secreted efficiently in the culture medium at comparable levels in the range of 60−70 μg of apoE/mL, 24 h after infection. A similar quantitative assessment was obtained by SDS−PAGE analysis of the secreted protein using known BSA standards (Figure 1).

The Amino-Terminal 1−185 Domain of ApoE Suffices for the Clearance of Lipoprotein Remnants from the Plasma of ApoE-Deficient Mice. ApoE$^{-/-}$ mice are characterized by high plasma cholesterol and low plasma triglyceride levels. Infection of E$^{-/-}$ mice with 2×10^9 pfu of the control virus AdGFP did not alter the plasma lipid levels of these mice.

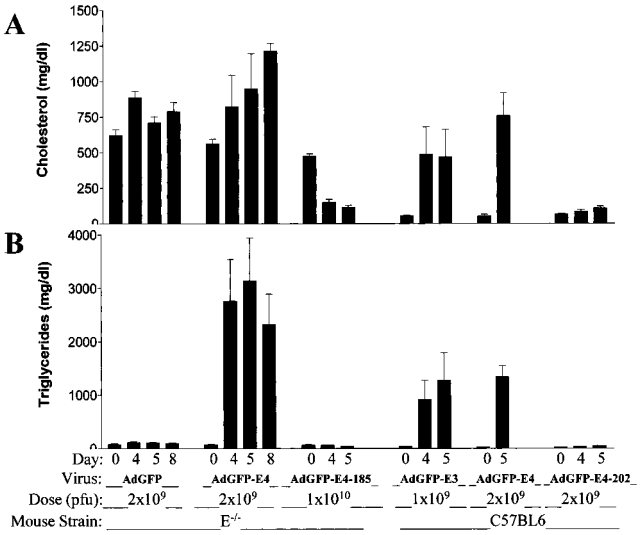

FIGURE 2: Cholesterol and triglyceride levels of apoE-deficient and C57BL6 mice infected with the control adenovirus AdGFP, or recombinant adenoviruses expressing wild-type and truncated apoE forms. Mice were infected in triplicate with the indicated doses of the different recombinant adenovirus, and serum samples were isolated and analyzed for cholesterol (panel A) and triglyceride levels (panel B) on the indicated days after infection as described under Experimental Procedures.

The plasma cholesterol levels of the $E^{-/-}$ mice could be normalized by infection with 1×10^{10} pfu of apoE4−185-expressing virus (Figure 2A,B, left side). This finding indicates that the amino-terminal domain 1−185 of apoE contains all the determinants required for clearance of the lipoprotein remnants which accumulate in the plasma of the $E^{-/-}$ mice. Infection of mice with 2×10^9 pfu of the apoE4-expressing adenovirus failed to clear the cholesterol-rich lipoprotein remnants from the plasma of the $E^{-/-}$ mice and also increase plasma triglyceride levels (Figure 2A,B, left side).

Overexpression of the Full-Length ApoE3 and ApoE4 Causes Hypercholesterolemia and Hypertriglyceridemia (Combined Hyperlipidemia) whereas Overexpression of the Truncated ApoE Form, ApoE4−202, Does Not Alter the Plasma Lipid Levels of C57BL6 Mice. The hypertriglyceridemia induced in $E^{-/-}$ mice by overexpression of the human apoE4 mice could be the consequence of the underlying hypercholesterolemia. Alternatively, full-length apoE could by itself elicit high plasma lipid levels. To differentiate between these two possibilities, we infected C57BL6 mice with $(1-2) \times 10^9$ pfu of adenoviruses expressing apoE3, apoE4, or apoE4−202 forms. This analysis showed that overexpression of either apoE3 or apoE4 was sufficient to induce combined hyperlipidemia (high cholesterol and triglyceride levels) in normal C57BL6 mice whereas overexpression of apoE4−202 had no effect on the plasma lipid levels of the C57BL6 mice (Figure 2A,B, right side). The findings indicate that overexpression of full-length apoE suffices to cause combined hyperlipidemia in normal C57BL6 or $E^{-/-}$ mice. Furthermore, the induction of hyperlipidemia requires the carboxy-terminal region of apoE. It is possible that domains of apoE within the carboxy-terminal 203−299 region contribute directly to the secretion of lipoprotein particles. These particles apparently cannot be removed efficiently by the liver, thus resulting in high plasma cholesterol and triglyceride levels.

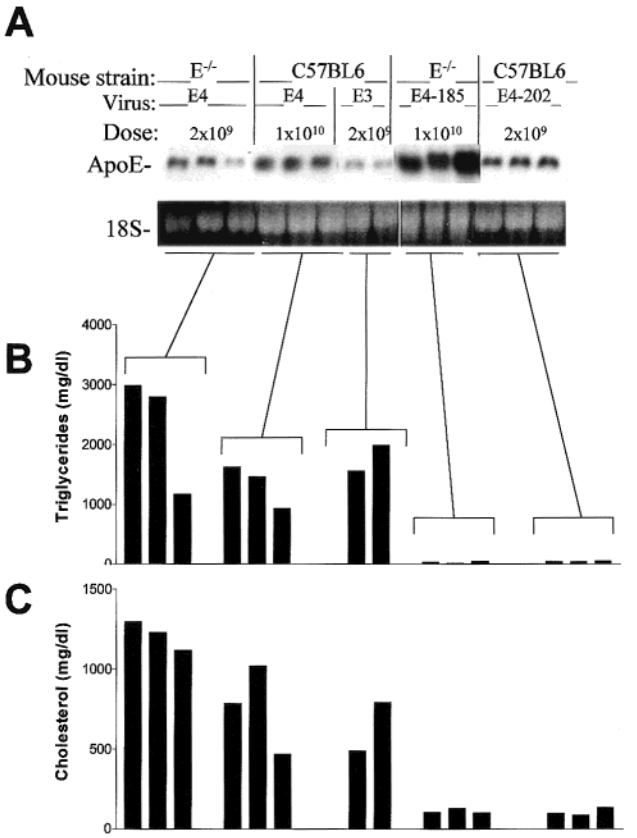

FIGURE 3: Representative mRNA analysis of mice infected with the indicated dose of control adenovirus AdGFP or recombinant adenoviruses expressing wild-type and truncated apoE forms. Total RNA was isolated from livers of infected mice on the indicated days after infection and analyzed by Northern blotting for the expression of apoE mRNA. Panel A: Representative autoradiograms of Northern blot analysis of RNA from the livers of C57BL6 or $E^{-/-}$ mice infected with adenoviruses expressing different forms of apoE, as indicated. The strain of the mouse used and the dose of apoE-expressing adenovirus for each form used are shown. Ethidium bromide staining of the gel for the 18S ribosomal RNA is shown, as marker of total RNA loaded, and RNA integrity. Panel B: Triglyceride levels of the individual mice expressed in mg/dL. Panel C: Cholesterol levels of the individual mice expressed in mg/dL.

The Human ApoE mRNA Levels in Mice Infected with the Wild-Type and Mutant Forms of ApoE Are Comparable. To assess the steady-state apoE mRNA levels in mice infected with adenoviruses expressing the full-length and the truncated apoE forms, at least two to three infected mice from each group were sacrificed on day 5 post-infection, and their livers were collected. Total RNA was isolated from these livers and analyzed for apoE mRNA levels by Northern blotting. Ethidium bromide staining of the gel for the 18S ribosomal RNA was used as an index of equal loading and integrity of the RNA (Figure 3A). In agreement with the cell culture data of Figure 1, the apoE mRNA levels in C57BL6 mice infected with doses of $(1-2) \times 10^9$ pfu of adenoviruses expressing the full-length apoE3, apoE4, or the truncated apoE4−202 are comparable (Figure 3A). In addition, the mRNA levels of $E^{-/-}$ mice expressing apoE4−185 are greater than the mRNA levels of the full-length apoE4 (Figure 3A). However, apoE4−185 and apoE4−202 still do not cause hyperlipidemia, as opposed to the full-length apoE forms which trigger combined hyperlipidemia, characterized

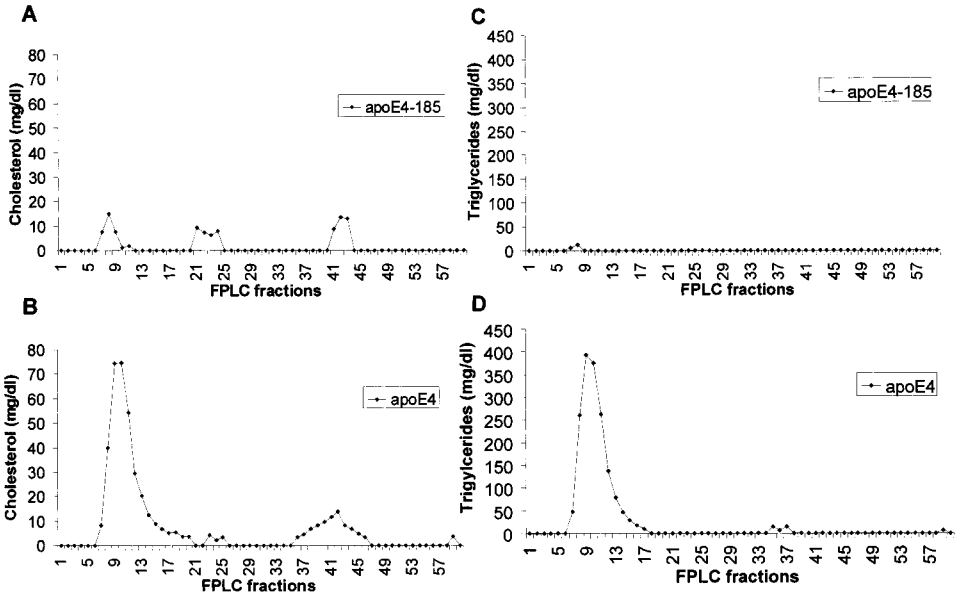

FIGURE 4: FPLC profiles of cholesterol and triglyceride of mice infected with the apoE4−185 (panels A, C) or apoE4 (panels B, D) expressing adenovirus. Serum samples obtained from mice infected with 2×10^9 pfu of the recombinant adenovirus expressing AdGFP-E4 or 1×10^{10} pfu of the recombinant adenovirus expressing AdGFP-E4−185, on day 5 post-infection, were fractionated by FPLC, and then the cholesterol and triglyceride levels of each FPLC fraction were determined as described under Experimental Procedures.

by high cholesterol and triglyceride levels (Figure 3B,C). Thus, the different effects of the full-length and the truncated apoE forms on hyperlipidemia most likely are not due to differences in the expression between these two apoE forms (Figure 3A−C).

Cholesterol and Triglyceride FPLC Profiles of Plasma Isolated from $E^{-/-}$ Mice Infected with AdGFP-E4−185, AdGFP-E4, or the Control Virus AdGFP. FPLC analysis of plasma from mice infected with AdGFP-E4−185 showed that cholesterol was very low and distributed in the VLDL, LDL, and HDL at a ratio of approximately 2:1:1 (Figure 4A). The triglyceride levels in these mice were very low in the VLDL fraction, and barely detectable in the rest of the lipoprotein fractions (Figure 4C). In contrast, in mice expressing apoE4 5 days post-infection, cholesterol levels were high. Approximately 80% of cholesterol was distributed in VLDL and approximately 20% in HDL (Figure 4B). As expected, triglyceride levels were very high in the VLDL fractions and barely detectable in the rest of the lipoprotein fractions (Figure 4D). As an additional control, infection with 2×10^9 pfu of the control virus AdGFP did not result in any change in the cholesterol and triglyceride profiles of the apoE-deficient mice (data not shown).

Analysis of mouse plasma by sandwich ELISA showed that the average total plasma apoE levels, based on a pool of plasma of three mice, were 60−70 mg/dL for apoE3 and apoE4, and 1−5 mg/dL for apoE4−202 and apoE4−185, respectively. Full-length apoE was distributed mostly in HDL (50%), and VLDL (25%), whereas apoE4−202 was uniformly distributed in all lipoprotein fractions (data not shown). The apparent lower concentration of apoE4−202 in plasma reflects the efficient catabolism of the apoE4−202-containing lipoproteins. SDS−PAGE analysis showed that the VLDL isolated from mice infected with adenoviruses expressing apoE4 had high concentrations of apoE4 and was lacking apoA-IV and apoA-I, whereas VLDL isolated from mice infected with AdGFP-E4−202 had low levels of apoE4−202 and apoA-I and high levels of apoA-IV (Figure 5).

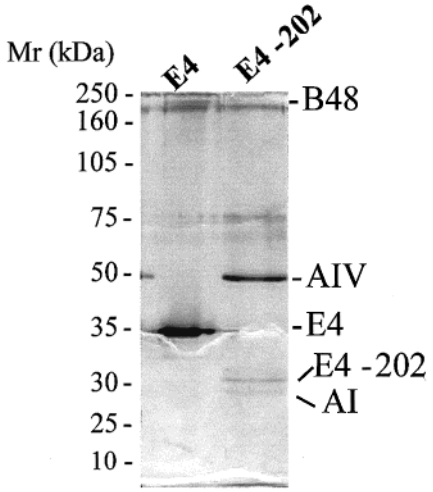

FIGURE 5: SDS−PAGE analysis of VLDL isolated from $E^{-/-}$ mice infected with adenoviruses expressing apoE4 and apoE4−202 forms. Molecular mass markers and the positions of apoE4, apoE4−202, apoA-I, and apoA-IV are indicated. The abnormal mobility of apoE4−202 is the result of O-glycosylation.

The Full-Length ApoE Increases Significantly the Rate of Hepatic VLDL Triglyceride Secretion As Compared to the Truncated ApoE4−185 Forms, and the Control Virus AdGFP. The rate of VLDL triglyceride secretion in the plasma was determined following injection of Triton WR1339 5 days following adenoviral infection. It was found that the rate of triglyceride secretion in mice infected with adenovirus expressing the full-length apoE4 was 8-fold higher, as compared to mice infected with 1×10^{10} pfu of AdGFP-E4−185 (Figure 6). The rate of VLDL triglyceride secretion was 2-fold higher in mice infected with 2×10^9 pfu of AdGFP as opposed to mice infected with AdGFP apoE4−185. The findings suggest that the carboxy-terminal region of apoE influences the rate of VLDL triglyceride secretion.

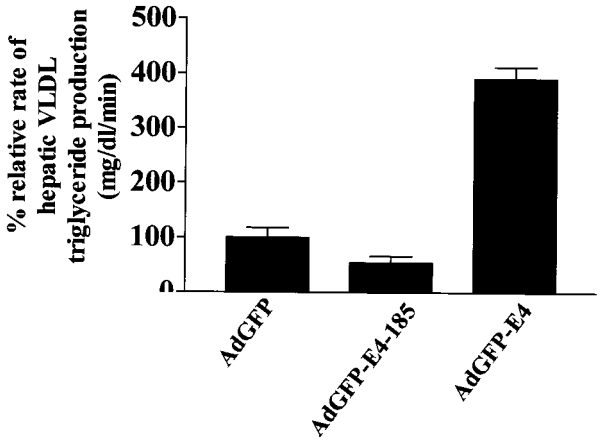

FIGURE 6: Hepatic VLDL triglyceride production analysis in mice infected with AdGFP, AdGFP-E4, or AdGFP-E4−185. Triton WR1339 (500 mg/kg body weight) was injected into three fasted mice per virus group. Serum samples were collected at 20, 40, and 60 min after the injection with the detergent. As control, serum samples were isolated 1 min immediately after the injection with the detergent. Serum triglyceride levels were determined, and a linear graph of serum triglyceride concentration vs time was generated. The rate of VLDL triglyceride secretion expressed in milligrams per deciliter per minute was calculated from the slope of the linear graph for each individual mouse. The bar-graph represents the mean ± standard deviation of the individual rates of VLDL triglyceride production per virus group.

DISCUSSION

Background. ApoE is a very important protein of the cholesterol transport system. Initial studies centered on the role of apoE as a ligand for apoE-recognizing receptors (*1*−*5*). It was found that mutation in apoE within the vicinity of amino acids 130−160 affected the recognition of this protein by the LDL receptor and resulted in dominant or recessive forms of type III hyperlipoproteinemia (*6*−*13*, *46*−*48*). Two additional properties of apoE pertinent to the secretion and lipolysis of VLDL triglycerides were suggested by the analysis of transgenic animals expressing different levels of apoE. The first property involves the contribution of apoE in VLDL triglyceride secretion, perhaps through the mobilization of membrane triglycerides (*34*, *39*, *40*, *49*, *50*). The second property of apoE is its ability to increase the plasma triglyceride levels when apoE is overexpressed (*25*, *26*, *34*−*38*, *51*). This apoE function may account for the correlation of plasma apoE levels with plasma triglyceride levels in the human population (*25*).

The Amino-Terminal Segment 1−185 of ApoE Suffices for the in Vivo Catabolism of Lipoprotein Remnants. Receptor-mediated catabolism of apoE implies the association of apoE with lipoprotein particles and their subsequent recognition by cell receptors (*1*−*6*). Heparan sulfate proteoglycans may also be involved in this process (*52*−*55*). Several domains of apoE have been described which are presumably involved in receptor binding (*46*−*48*, *56*), heparin binding (*57*, *58*), and lipid and lipoprotein binding (*59*, *60*). The receptor binding domain is found between residues 136 and 152 while neighboring residues may also indirectly affect receptor binding (*47*, *48*).

It has been proposed that apoE contains three heparin binding domains. One of them overlaps with the receptor binding domain between residues 142 and 147, while two other heparin binding domains were localized between residues 211−218 and 243−272 (*57*, *58*). Previous studies indicated that the 142−147 domain is directly involved in the binding of apoE-containing lipoproteins to heparan sulfate proteoglycans, and their subsequent internalization with or without the participation of LRP and the LDL receptors (*2*, *6*, *55*). The prevailing concept regarding lipid and lipoprotein binding was that the region of apoE between residues 244 and 299 contributes to the binding of apoE to lipids and lipoproteins, whereas the amino-terminal region of apoE lacks the determinants required for association with lipoproteins (*60*). It was also reported that residues 267−299 are crucial for the tetramerization of apoE (*60*). The present study refines the domains of apoE required for clearance of lipoprotein remnant by the liver. One major finding is that the amino-terminal residues 1−185 contain the domains necessary for the clearance of cholesteryl ester-rich lipoprotein remnants in vivo. It has been proposed that in addition to the numerous lipoprotein receptors, heparan sulfate proteoglycans participate in the clearance of apoE-containing lipoproteins by the liver (*57*). Although apoE4−185 contains only the 142−147 heparin binding domain, it is cleared efficiently by the liver. Since the 142−147 region also contains the receptor binding domain, binding of apoE to the heparan sulfate proteoglycans should mask the receptor binding domain and prevent recognition by cell receptors. Thus, any contribution of heparan sulfate proteoglycans in the uptake of apoE4−185-containing lipoproteins may either involve apoB−48 or direct endocytosis of heparan sulfate proteoglycan-bound remnants.

It is also quite possible that the major route of clearance of the lipoproteins containing apoE4−185 is via the LDL receptor and LRP pathways as opposed to the heparan sulfate proteoglycan pathway. The potential participation of the LRP and the LDL receptor in the clearance of apoE4−185-containing remnants may make the uptake of VLDL and the subsequent cholesterol clearance more efficient and thus account for the observed properties of apoE4−185 in lipoprotein clearance.

Work by others has shown that injection of two ^{125}I truncated apoE forms from residues 1−191 and 1−255 respectively in rabbits resulted in their fast and very efficient removal from plasma (*60*). These observations are consistent with the finding of this study, which shows that the efficient removal of lipoprotein remnants also removes concomitantly apoE molecules, thus leading to lower levels of steady-state plasma apoE levels. The steady-state plasma apoE levels of the full-length apoE observed in this study are in the range of 60−70 mg/dL, and those of the truncated apoE forms are in the range of 1−5 mg/dL.

Full-Length ApoE Induces High Cholesterol and Triglyceride Levels in C57BL6 Mice. Induction of Combined Hyperlipidemia Requires the Carboxy-Terminal Segment of ApoE. The present study established that overexpression of full-length apoE3 or apoE4 in the liver induces combined hyperlipidemia in C57BL6 mice characterized by high plasma cholesterol and triglyceride levels, whereas overexpression of apoE4−202 has no effect. This finding provides two important clues: (a) The induction of combined hyperlipidemia is the result of overexpression of apoE and is independent of the apoE phenotype. (b) Induction of combined hyperlipidemia requires the carboxy-terminal

region between amino acids 203 and 299. Similar conclusions can be reached by overexpression of the wild-type and truncated apoE forms in $E^{-/-}$ mice. As explained above, apoE4−185 clears the lipoprotein remnants of $E^{-/-}$ mice without induction of hypertriglyceridemia, whereas full-length apoE4 does not clear the lipoprotein remnants, and in addition induces hypertriglyceridemia. Since apoE−185 does not contain the O-glycosylation site which is on Thr-194, our findings also confirm that apoE glycosylation has no effect on the clearance of apoE-containing lipoproteins. A previous study by Tsukanoto and co-workers (*61*) suggested that infection of apoE-deficient mice with (4.5−5) $\times 10^9$ pfu of second-generation adenoviruses expressing apoE resulted in the clearance of the lipoprotein remnants from the plasma of these mice, and only apoE2 induced hypertriglyceridemia (*61*). A subsequent study concluded that hypertriglyceridemia was independent of the apoE phenotype (*62*). These later findings are consistent with the results of Figure 2, which show that doses as low as 1×10^9 pfu of adenovirus expressing apoE4 or apoE3 can induce combined hypertriglyceridemia in C57BL6 mice.

Potential Causes of Induction of Hyperlipidemia by Overexpression of Full-Length ApoE. The de novo induction of combined hyperlipidemia by the full-length apoE but not by the truncated forms could reflect differences in the levels of apoE expression or a specific effect of apoE on hepatic VLDL secretion and catabolism. The levels of apoE expression were assessed by Northern blot analysis of total RNA isolated from the livers of infected mice. This analysis has established unequivocally that under conditions of comparable steady-state apoE mRNA levels, mice expressing the full-length apoE develop hyperlipidemia whereas those expressing truncated apoE forms do not (Figure 3A−C). This analysis indicates that it is unlikely that decreased synthesis of the truncated apoE forms accounts for the absence of hyperlipidemia. In fact, the levels of apoE mRNA in livers of mice infected with 1×10^{10} pfu of adenoviruses expressing the apoE4−185 form are much higher than the levels in mice infected with the adenoviruses expressing apoE3 and apoE4. This finding is consistent with tissue culture experiments showing that C127 cell lines (mouse mammary tumor cells) stably transfected and expressing the full-length and truncated apoE forms, or HTB-13 cell cultures infected with apoE-expressing recombinant adenovirus, secrete similar amounts of wild-type and truncated forms. These observations indicate that the truncated apoE forms are stable and are secreted as efficiently as their wild-type full-length apoE counterparts (Figure 1).

The observation that both hypertriglyceridemia and hypercholesterolemia are induced de novo in C57BL6 mice, which normally have low cholesterol triglyceride levels, strongly points to the direction of an effect of intracellular apoE forms in lipoprotein secretion. This possibility was investigated by determining the rate of hepatic VLDL triglyceride secretion elicited by the truncated apoE4−185 and the full-length apoE4 forms following injection of Triton WR1339. This analysis showed that the rate of hepatic VLDL triglyceride secretion in $E^{-/-}$ mice infected with the adenoviruses expressing the full-length apoE4, 5 days post-infection, was 8-fold higher as compared to the rate of hepatic VLDL triglyceride secretion in mice infected with the adenovirus expressing the apoE4−185 form. It is interesting that the rate of VLDL triglyceride secretion in mice infected with the truncated apoE4−185 form is even lower, by 50%, than that of mice infected with the control AdGFP adenovirus. A rigorous proof of the role of the carboxy-terminal segment of apoE in the development of hyperlipidemia will require the induction of hyperlipidemia by the carboxy-terminal 203−299 region of apoE alone, or abolishment of hypertriglyceridemia by infection of $E^{-/-}$ mice with adenoviruses expressing point mutants in the carboxy-terminal region. This question is the subject of ongoing research.

Although the de novo induction of combined hyperlipidemia could be accounted to a large extent by the apoE-mediated increase in lipoprotein secretion, the lack of clearance of the lipoprotein remnants by the full-length apoE may, in addition, be affected by further diminished lipoprotein clearance. This, for instance, could be due to compositional changes in the plasma lipoproteins. Analysis of the apoprotein composition of VLDL particles of mice expressing E4, and E4−202, showed that other proteins present in triglyceride-rich VLDL, such as apoA-IV and apoA-I, are also displaced by full-length apoE4, but not by apoE−202 (Figure 5). Previous studies also established that overexpression of apoE displaced apoCII from VLDL and reduced lipolysis (*34, 36*). X-ray crystallography and computer modeling showed that the amino-terminal domains 23−185 of apoE contain antiparallel helices (*63, 64*). Based on our findings, it is reasonable to assume that in the absence of the carboxy-terminal region, the predicted amino-terminal helices may have a favorable conformation on the lipoprotein particles. Such a conformation may promote strong interactions with cell receptors and possibly heparan sulfate proteoglycans. One may then assume that when the full-length apoE is overexpressed it inserts itself into a newly synthesized lipoprotein particle and assumes a conformation that hinders receptor recognition. Thus, despite the excess of full-length apoE, the triglycerides of the resulting lipoprotein particle cannot be further hydrolyzed and cannot be removed efficiently through catabolism by cell receptors or other mechanisms. As pointed out by others, lipoprotein lipase and apoCII may also be affected by the excess apoE (*34, 36*).

Overall, the current study indicates that the amino-terminal 1−185 residues of apoE are sufficient for efficient clearance of lipoprotein remnants by the liver. In addition, overexpression of full-length apoE increases lipoprotein secretion and causes combined hyperlipidemia. One major parameter in successful gene therapy applications is gene dosage and expression levels. The induction of hyperlipidemia by overexpression of apoE diminishes significantly its therapeutic potential. The inability of the truncated apoE forms that lack the carboxy-terminal region to induce hyperlipidemia, coupled with its intact ability to clear lipoprotein remants, makes it an attractive candidate in future gene therapy applications to correct remnant removal disorders.

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