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Domains of apoE4 required for the biogenesis of apoE-containing HDL

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Abstract

Introduction—We have studied the functions of truncated apoE4 forms *in vitro* and *in vivo* in order to identify the domains of apoE4 required for the biogenesis of apoE-containing high density lipoprotein (HDL).

Results—We have found that apoE4-185, -202, -229, or -259 could promote ATP-binding cassette transporter A1 (ABCA1)-dependent cholesterol efflux *in vitro*, although less efficiently than full-length apoE4 and had diminished capacity to activate lecithin cholesterol acyltransferase (LCAT). Formation of HDL *in vivo* was assessed by various methods following gene transfer in apolipoprotein A-I^{-/-} × apoE^{-/-} mice. Fast protein liquid chromatography of plasma showed that the truncated apoE forms, except apoE4-185, generated an apoE-containing HDL peak. Two-dimensional gel electrophoresis of plasma and electron microscopy (EM) showed that truncated apoE forms generated distinct HDL subpopulations and formed discoidal HDL particles which could be converted to spherical by co-administration of truncated apoE4-202 and LCAT.

Conclusion—Overall, the *in vivo* and *in vitro* data are consistent and indicate that apoE4-185 is the shortest truncated form that supports formation of discoidal apoE4 containing HDL particles.

Keywords

Apolipoprotein E; HDL biogenesis; HDL gene transfer; LCAT

Key messages

1. Gene transfer in double deficient apoA-I^{-/-} × apoE^{-/-} mice established that truncated apoE4 forms can generate discrete HDL subpopulations and discoidal HDL particles that can be converted to spherical by excess LCAT.
2. Consistent with the *in vivo* data *in vitro* studies established that truncated apoE4 forms can promote ABCA1-dependent cholesterol efflux, which is the first step in HDL biogenesis, but activate LCAT poorly.
3. The ability of truncated apoE4 variants to form HDL differentiates apoE4 from apoA-I where the C-terminal domain is required for ABCA1 mediated cholesterol efflux and for the biogenesis of HDL.

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Introduction

Apolipoprotein E (apoE) is a very important protein of the lipoprotein transport system, is essential for lipid homeostasis in the circulation, and is protective from atherosclerosis (1-3).

Lipoprotein-bound apoE is the ligand for the LDL receptor as well as other receptors in vitro (4-6). In vivo and in vitro studies have shown that mutations in apoE that diminish binding of apoE-containing lipoproteins to the LDL receptor are associated with high plasma cholesterol levels and cause premature atherosclerosis in humans and experimental animals (1-3). Recently, we have shown that apoE participates in the biogenesis of distinct apoE-containing HDL species with the participation of ATP-binding cassette transporter A1 (ABCA1) and lecithin cholesterol acyltransferase (LCAT) (7). The pathways of biogenesis of apoE- and apoA-I-containing HDL are similar (7;8). The combined properties of apoE to promote formation of apoE-containing HDL and to clear lipoprotein remnants explain, at least partially, the more pronounced contribution of apoE to atheroprotection as compared to apoA-I (1;9;10). ApoE has three common isoforms (apoE2, apoE3 and apoE4) in humans (11). In addition to its role in either the pathogenesis of or protection from cardiovascular disease, the apoE4 isoform is a risk factor for late onset Alzheimer's disease (12;13). ApoE is the only medium-size apolipoprotein expressed in the brain (14) and existing literature suggests that formation of apoE-containing HDL may have beneficial effects in the brain (14-17).

The domains of apoE required for the biogenesis of apoE-containing HDL are not known. We have chosen to work with apoE4 due to the unique involvement of this isoform with both lipid homeostasis and with Alzheimer's disease (12;13). In this manuscript, we show that truncated apoE4 forms can promote ABCA1-dependent cholesterol efflux but have diminished capacity to activate LCAT in vitro. When expressed in double-deficient apoA-I^{-/-} × apoE^{-/-} mice, the truncated apoE forms generate mostly discoidal HDL particles that can be converted to spherical by excess LCAT and form discrete HDL subpopulations. The ability of the truncated apoE4 forms to promote cellular efflux of cholesterol in vitro is consistent with their ability to direct *in vivo* the formation of mostly discoidal HDL particles. This property of the truncated apoE4 variants differentiates apoE from apoA-I, where the C-terminal domain is required for the biogenesis of HDL particles. These newly identified functions of apoE are important for understanding the role of this protein, both in cardiovascular disease and in Alzheimer's disease.

Materials and Methods

Materials

Materials not mentioned in the *Methods* have been obtained from sources described previously (18;19).

Methods

Recombinant adenoviruses construction—The recombinant adenoviruses were constructed as described using the Ad-Easy-1 system where the adenovirus construct is generated in BJ-5183 bacterial cells (20;21). Following large-scale infection of HEK 293 cell cultures, the recombinant adenoviruses were purified by two consecutive CsCl₂ ultracentrifugation steps, dialyzed and titrated. Usually, titers of approximately 2 to 5×10¹⁰ pfu/ml were obtained.

Animal studies and RNA isolation and apoE mRNA quantitation—Male and female apoE^{-/-} × apoA-I^{-/-} double-deficient mice 8-10 weeks old were used in these studies.

Groups of 4 or more mice were injected intravenously through the tail vein with a dose of $0.5-4 \times 10^9$ pfu of adenoviruses expressing truncated apoE forms. Blood was obtained from the tail vein after a 4h fast preceding adenoviral injection and 1, 2, 3 and 4 days post-infection. The plasma was isolated and stored at 4°C and -20°C. Four or more animals from each group were sacrificed 4 days post-infection, hepatic RNA was isolated by the trizol method (Invitrogen) and the apoE mRNA levels were determined by quantitative PCR using the standard TaqMan thermal cycling program and Applied Biosystems probe sets for human apoE and 18s rRNA (Cat. #HJ00171168-M1 and 4319413E respectively).

Fast protein liquid chromatography analysis of plasma (FPLC), lipid and apoE determinations—Plasma samples, 12 µl of plasma were diluted 1:4 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 of 50 µl volume each were collected for further analysis. Triglycerides and free cholesterol of plasma and the FPLC fractions were determined spectrophotometrically at 540 nm and 490 nm respectively as described previously (20), using the Plasma Triglyceride Kit (Sigma) and Free Cholesterol Kit (Wako), according to the manufacturer's instructions. The total cholesterol in plasma was determined by hydrolysis of cholesterol esters and subsequent determination of the free cholesterol levels using cholesterol E kit (Wako). CE was determined by subtraction of free from total cholesterol levels. Plasma human apoE concentrations were measured using sandwich ELISA (20).

Statistical analysis—Data are presented as the mean $\pm$ standard deviation. Statistically significant differences between two groups of mice were determined using Student's *t* test with unequal variance.

Density gradient ultracentrifugation and electron microscopy (EM) analysis—To assess the ability of WT and truncated apoE forms to associate with different lipoproteins, an aliquot of 0.3 ml of plasma was fractionated by KBr density gradient ultracentrifugation and the fractions were analyzed by SDS-PAGE as described previously (22-24). Aliquots of the 5-8 fractions obtained by density gradient ultracentrifugation that contain most of the apoE were analyzed by EM. The photomicrographs were taken at $\times 75,000$ magnification and enlarged three times as described previously (25).

Non-denaturing two-dimensional electrophoresis—The distribution of HDL subfractions in plasma was analyzed by two dimensional gel electrophoresis as described (26). ApoE was detected by probing the membrane with a goat polyclonal anti-human apoE antibody (Meridian Biosciences K74190G; Saco, ME).

LCAT production, purification and activation by full-length and truncated apoE4 forms—LCAT was purified as described (27-29) from the culture medium of human HTB13 cells infected with an adenovirus expressing the human LCAT cDNA (30). The ^{14}C -cholesterol labeled reconstituted HDL (rHDL) particles used as substrate were prepared by the sodium cholate dialysis method (31;32). The enzymatic reactions were carried out at different concentrations of apoE containing rHDL were determined and the apparent kinetic parameters ($V_{\text{max,app}}$ and $K_{\text{m,app}}$) were obtained by fitting the data to the Michaelis-Menten equation, using the Prism software (GraphPad Software, Inc.) as described (27;31).

Cholesterol efflux studies—Experiments were performed as described (18;24). Following 24h of labeling with 0.5 µCi/ml 1,2- ^3H cholesterol in DMEM (high glucose) supplemented with 10 % (v/v) FBS, washing and equilibration in PBS, the cells were

incubated for 1h with DMEM (high glucose) supplemented with 0.2% (w/v) fatty acid free BSA. At the end of this period, cells were incubated with 0.5 ml DMEM (high glucose), supplemented with 0.2% (w/v) fatty acid free BSA with or without 1 to 6 μ M human apoA-I or apoE4 and truncated apoE4 forms at 37°C for 4 h. The media and cell lysates were collected separately, and the radioactivity was determined by scintillation counting. [3 H]Cholesterol efflux was expressed as the percentage of the radioactivity released in the medium relative to the total radioactivity in cells and medium. To calculate the net ABCA1-mediated efflux, the cholesterol efflux of the cells transfected with the control plasmid (mock) was subtracted from the cholesterol efflux of the cells transfected with the ABCA1 plasmid.

LCAT activation by full-length and truncated apoE4 forms—LCAT, apoA-I, full-length and truncated and apoE4 forms were purified from the culture medium of HTB-13 cells infected with adenoviruses expressing the corresponding proteins as described previously (28-31;33). The 14 C-cholesterol labeled reconstituted HDL (rHDL) particles containing the various apoE4 forms used as substrate were prepared by the sodium cholate dialysis method (31;32). The enzymatic reactions carried out at different concentrations of apoE containing rHDL were determined and the apparent kinetic parameters ($V_{max,app}$ and $K_{m,app}$) were obtained by fitting the data to the Michaelis-Menten equation, using the Prism software (GraphPad Software, Inc.) as described (22).

Cholesterol efflux studies—Efflux experiments were performed in HEK293 EBNA-T cells transfected with an ABCA1-expressing plasmid or a control plasmid (mock) as described (18;24), using 1 to 6 μ M WT apoA-I or apoE4 and truncated apoE4 forms as cholesterol acceptors at 37°C for 4 h. Efflux of [3 H]cholesterol from the cells was expressed as the percentage of the radioactivity released in the medium relative to the total radioactivity in cells and medium. The net ABCA1-mediated efflux was determined by subtracting the cholesterol efflux of the cells transfected with the control plasmid (mock) from the cholesterol efflux of the cells transfected with the ABCA1 plasmid.

Results

Ability of apoE forms to promote ABCA1-mediated cholesterol efflux and to activate LCAT

The full-length apoA-I and apoE4 as well as the apoE4 variants were produced by large-scale cultures of HTB-13 cells in roller bottles following infection with adenoviruses expressing the corresponding protein and purified.

ABCA1-mediated cholesterol efflux represents the first step in the biogenesis of HDL. We have compared the ability of full-length apoA-I and apoE, as well as the ability of truncated apoE4 forms, to promote cholesterol efflux from HEK293 EBNA-T cells transfected with an ABCA1-expressing plasmid. It was found that all three natural apoE isoforms, apoE2, apoE3, and apoE4, at either 1 or 3 μ M concentrations, have similar ability to promote ABCA1-dependent cholesterol efflux as compared to wild-type apoA-I. On the other hand, the truncated apoE forms showed a concentration-dependent ability to promote cholesterol efflux. At 1 μ M concentration, the cholesterol efflux for the shorter forms (apoE4-229, apoE4-202, apoE4-185) ranged from 17 to 30% of the full-length apoE4. At 3 μ M concentration, the cholesterol efflux of E4-259 was 73% as compared to the full-length apoE4. At concentration of 6 μ M, the efflux promoted by apoE4-185, -202, and -229 was 70, 69, and 86 percent of the full-length apoE4 respectively (Figure 1A). The ability of truncated apoE forms to promote ABCA1-dependent cholesterol efflux suggested that they may be able to participate in the biogenesis of HDL.

LCAT-mediated esterification of free cholesterol is required for the conversion of discoidal HDL to spherical HDL particles *in vitro* and *in vivo*. We have performed *in vitro* LCAT activation assays using POPC/C/apoE (rHDL) particles containing full-length or truncated apoE4 forms. Using different concentrations of apoE, we obtained the kinetic constants apparent V_{max} ($V_{max_{app}}$) and apparent K_m ($K_{m_{app}}$) for different apoE4 forms. This analysis showed that the catalytic efficiency ($V_{max_{app}}/K_{m_{app}}$) of the enzymatic reaction for truncated apoE4 forms was 14-18% that of full-length apoE (Figure 1B).

Plasma lipid and apoE levels of apoA-I^{-/-} × apoE^{-/-} mice expressing WT and truncated apoE forms

To map the domains of apoE4 required for the biogenesis of HDL, we have used adenovirus-mediated gene transfer of full-length and truncated apoE4 forms into apoA-I^{-/-} × apoE^{-/-} mice. These mice were chosen in order to rule out formation of apoE-containing HDL particles by the endogenous mouse apoE.

This analysis showed that when mice were infected with high doses of recombinant adenovirus (1.5×10^9 to 4×10^9 pfu), all truncated apoE forms cleared cholesterol without induction of hypertriglyceridemia (Figure 2A,B). Similar clearance of cholesterol with induction of mild hypertriglyceridemia was observed when the mice were infected with low doses (5×10^8 pfu) of an adenovirus expressing wild-type apoE4 (Figure 2A,B).

The dose of adenoviruses used was selected so that mice expressing the truncated apoE4 forms had comparable or higher hepatic apoE4 mRNA levels to the mRNA levels of mice expressing low doses of full-length apoE4 (Figure 2C). The plasma apoE4 levels four days post-infection were higher in mice expressing full-length apoE4, were comparable in mice expressing E4-229 and -259, and were reduced in mice expressing E4-202 and E4-185 as compared to mice expressing E4-229 and E4-259 (Figure 2D).

Assessment of the formation of HDL particles by FPLC, density gradient ultracentrifugation, electron microscopy, and two-dimensional gel electrophoresis

FPLC analysis of plasma showed that expression of all the truncated apoE4 forms tested, with the exception of apoE4-185, resulted in the formation of an HDL peak that was enriched in free cholesterol (Figure 3A,B; Supplementary Figure 1A-D). Similarly enriched in free cholesterol were the peaks of VLDL, IDL, and LDL. Analysis of mice infected with low doses of adenoviruses expressing full-length apoE4 showed that the HDL peak consisted mostly of esterified cholesterol (CE/TC=0.61) (Figure 3B). FPLC analysis of plasma of uninfected apoA-I^{-/-} × apoE^{-/-} mice showed the presence of a large VLDL peak with CE/TC ratio of 0.52 (Supplementary Figure 1D).

Density gradient ultracentrifugation of plasma of uninfected and infected apoA-I^{-/-} × apoE^{-/-} mice showed that apoE4-185 floats in the VLDL, IDL, LDL, and HDL2, and exists in low concentrations in plasma, whereas apoE4-202, -229, -259, and full-length apoE float predominantly in the HDL3 region, to a much lesser extent in the VLDL, IDL, LDL, and HDL2 region, and their concentration is much greater than that of apoE4-185 (Figure 3C,D; Supplementary Figure 2A-D).

Fractions 6 to 8, which are enriched in apoE4 and float in the HDL-density region, were analyzed further by electron microscopy. HDL obtained from mice expressing apoE4-185 contained a small amount of discoidal particles (Figure 4A). HDL obtained from mice expressing E4-202 and -229 contained a large amount of discoidal particles (Figure 4B,C). HDL obtained from mice expressing E4-259 contained a mixture of discoidal and spherical particles (Figure 4D). HDL obtained from mice infected with low doses (5×10^8 pfu) of adenoviruses expressing full-length apoE4 contained only spherical particles (Figure 4E),

whereas HDL obtained from mice infected with high doses (1×10^9 pfu) of adenoviruses expressing full-length apoE4 contained a mixture of spherical and discoidal particles (Figure 4F). HDL fractions 6 to 8 obtained from plasma of uninfected apoA-I^{-/-} × apoE^{-/-} or ABCA1^{-/-} mice contained a sparse population of particles (Figure 4G).

LCAT can promote the maturation of the discoidal HDL generated by the truncated form apoE4-202

To assess the ability of LCAT to convert discoidal HDL particles containing apoE4-202 into spherical particles, mice were co-infected with 1.5×10^9 pfu of adenovirus expressing apoE4-202 and 5×10^8 pfu adenovirus expressing human LCAT, or 1.5×10^9 pfu adenovirus expressing apoE4-202 and 5×10^8 pfu of adenovirus expressing GFP. Both treatments cleared cholesterol without induction of hypertriglyceridemia (Supplementary Figure 3A,B). Plasma apoE levels were reduced significantly in mice co-infected with adenoviruses expressing apoE4-202 and LCAT as compared to mice co-infected with adenoviruses expressing apoE4-202 and GFP (Supplementary Figure 3C,D), whereas the hepatic mRNA levels of the two groups were comparable (Supplementary Figure 3G).

FPLC analysis of plasma showed that the HDL cholesterol of mice treated with apoE4-202 and GFP was not esterified (CE/TC ratio = 0.04) whereas HDL cholesterol of mice treated with apoE4-202 and LCAT was esterified (CE/TC ratio = 0.65) (Supplementary Figure 3E,F).

Electron microscopy of the HDL fractions 6 and 7, corresponding to the fractions shown in Supplementary Figure 3C,D showed that apoA-I^{-/-} × apoE^{-/-} mice infected with adenovirus expressing apoE4-202 and GFP form discoidal particles (Figure 4H), whereas the mice infected with apoE4-202 and LCAT formed mostly spherical particles (Figure 4I). These findings indicate that the discoidal apoE4-202-containing HDL particles can be converted to spherical by excess LCAT.

The formation of apoE4-containing HDL was also assessed by two-dimensional gel electrophoresis (2D-GE) that employs agarose gel electrophoresis in the first dimension and native PAGE in the second dimension. This analysis showed the formation of a small diffuse population of particles that were barely detectable in mice infected with high doses (4×10^9 pfu) of an adenovirus expressing apoE4-185 (Figure 5A). Infection of mice with adenoviruses expressing apoE4-202 and apoE4-229 promoted the formation of two distinct subpopulations of apoE-containing particles (Figure 5B,C).

Infection of mice with adenoviruses expressing apoE4-259 produced a diffused population of apoE-containing particles (Figure 5D). A similar two-dimensional pattern was observed when mice were infected with low doses (5×10^8 pfu) of an apoE4-expressing adenovirus, which cleared plasma cholesterol and induced only mild hypertriglyceridemia (Figure 5E). However, when mice were infected with higher doses of an apoE4-expressing adenovirus (1×10^9 pfu), which increased plasma cholesterol and induced severe hypertriglyceridemia, an additional slower migrating population of lipoprotein particles was detected (Figure 5F). The slower migrating population most likely represents apoE-containing VLDL/IDL/LDL particles.

Discussion

Background

A series of recent studies have established that biogenesis of apoA-I-containing HDL is a continuous pathway where apoA-I and various participating proteins interact successively to form initially a lipidated intermediate and subsequently discoidal and spherical HDL

particles (34). Previous studies showed that C-terminal truncations that remove the 220 to 231 residues of apoA-I diminish the ability of the protein to promote cholesterol efflux and prevent formation of discoidal or spherical HDL particles (18;34;35).

ApoE and apoA-I have striking structural and functional similarities as well as differences (1;18;27;35-40). ApoE contains N- and C-terminal domains which unfold independently (36;37), whereas apoA-I gives a single unfolding curve following chemical or thermal denaturation (38). Both lipid-free apoA-I or apoE promote ABCA1-dependent cholesterol efflux which represents the first step in the biogenesis of HDL (Figure 1A) (18). In addition, similarly to apoA-I lipid-bound apoE activates LCAT (Figure 1B) (41;42) and promotes SR-BI-dependent cholesterol efflux (23). Both apoA-I and apoE participate in the biogenesis of distinct HDL particles that contain the corresponding protein (7;34). The objective of this study was to assess the importance of the C-terminal region of apoE4 in the biogenesis of apoE-containing HDL.

A property of apoE that is not shared by apoA-I is its ability to bind to various cell receptors (1-3) and promote in vivo clearance of lipoprotein remnants and other apoE-containing lipoproteins via the LDL receptor (1;43). This may explain why apoE deficiency leads to severe atherosclerosis (1), whereas apoA-I deficiency only exacerbates the atherosclerosis of susceptible mouse strains (9;10).

Truncated apoE4 forms promote the formation of discoidal HDL which can be converted to spherical by excess LCAT

The ability of truncated apoE forms to promote ABCA1-dependent cholesterol efflux at 3 to 6 μ M concentration suggested that the product of ABCA1-apoE interactions might proceed further to form HDL in vivo. To address this question we employed adenovirus-mediated gene transfer of truncated apoE forms in apoA-I^{-/-} $\times$ apoE^{-/-} double-deficient mice.

The in vivo experiment provided novel information on the ability of truncated apoE4 variants to form apoE4-containing HDL as well as the types of the HDL particles generated. Following infection with the recombinant adenoviruses, apoE4-185 was distributed in the VLDL/IDL/LDL region. However all other truncated apoE4 forms were distributed predominantly in the HDL region. The buildup of apoE4 in the HDL or, in the case of apoE4-185, in the VLDL/IDL/LDL region inhibited the esterification of the cholesterol of HDL and VLDL/IDL/LDL. Based on this finding the expectation was that if HDL particles were formed they should have discoidal shape. Indeed, EM analysis established for the first time that apoE4-185 and -202 formed exclusively discoidal particles and apoE4-229 and apoE4-259 formed a mixture of discoidal and spherical particles. The formation of discoidal HDL particles is consistent with the diminished capacity of the truncated apoE4 forms to activate LCAT in vitro. A mixture of discoidal and spherical particles was also produced by high levels of expression of full-length apoE4 that induces dyslipidemia, whereas low levels of expression of apoE4 or apoE3 that clears the cholesterol of the double-deficient mice generated only spherical particles (Figure 4E and Supplementary Figure 4). The association between induction of dyslipidemia and formation of discoidal HDL particles was also observed in experiments with apoE^{-/-} mice (44).

The property of truncated apoE4 forms to promote formation of discoidal apoE-containing HDL differentiates apoE from apoA-I where deletion of the carboxy-terminal domain prevented the formation of discoidal or spherical HDL particles (35). The esterification of the HDL cholesterol and the conversion of the discoidal to spherical HDL by excess LCAT suggest that the truncated apoE4 forms are poor activators of LCAT in vivo. It is also possible that mice that express the truncated apoE4 forms have LCAT insufficiency. This insufficiency may have resulted from a fast removal of complexes of LCAT with the

minimally lipidated apoE forms or the discoidal HDL species containing truncated apoE forms by the kidney, as it happens for the discoidal apoA-I-containing HDL species (45).

The distinct HDL subpopulations generated by apoE4-202 and apoE4-229 with similar mobility and size are very interesting. These HDL particles that have been observed for the first time are associated with the formation of discoidal only HDL. In contrast, the formation of a more complex set of HDL species by apoE4-259 and low doses of full-length apoE4 may be indicative of formation of at least some mature spherical HDL particles.

Equally important is the observation that under conditions of dyslipidemia in addition to the apoE4-containing HDL particles we detect by two dimensional gel electrophoresis larger size lipoproteins which may represent apoE4-containing VLDL/IDL/LDL particles.

Overall the current study demonstrates that truncated apoE forms can generate apoE-containing discoidal HDL particles that can be converted to spherical by excess LCAT and that apoE4-185 is the shortest truncated apoE form that supports formation of discoidal apoE4 containing HDL particles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

EM	electron microscopy
apoE4	apolipoprotein E4
K_m_{app}	apparent K _m
V_{max}_{app}	apparent V _{max}
ABCA1	ATP-binding cassette transporter A1
HDL	high density lipoprotein
LCAT	lecithin cholesterol acyltransferase
2D-GE	two-dimensional gel electrophoresis

Figure 1. A,B: Functional properties of full-length and truncated apoE or apoA-I forms
A: Cholesterol efflux by full-length apoE and apoA-I and truncated apoE4 forms from HEK293 EBNA-T cells. The concentration of the acceptor apoE or apoA-I in the culture medium was either 1 or 3 or 6 μM, as indicated. Experiments were performed as described in the *Methods*. Values are the mean ± standard deviation from two independent experiments performed in duplicate. **B: Activation of LCAT by rHDL containing full-length and truncated apoE4 forms.** Experiments were performed as described in *Methods*. A range of apoE concentrations were used in order to obtain the apparent kinetic parameters. $K_{m_{app}}$ and $V_{max_{app}}$ and the catalytic efficiency $V_{max_{app}}/K_{m_{app}}$ of the enzymatic reaction for each apoE form incorporated into POPC/cholesterol/apoE proteoliposomes. Values are the means ± the standard deviation from three independent experiments performed in duplicate.

Figure 2. A-D: Measurements following adenovirus mediated gene transfer of wild-type and truncated apoE forms in apoA-I^{-/-} × apoE^{-/-} mice

A: Plasma cholesterol, **B:** plasma triglycerides, **C:** hepatic apoE mRNA levels of apoA-I^{-/-} × apoE^{-/-} mice infected with recombinant adenoviruses expressing truncated and full-length apoE4 forms and **D:** plasma apoE. Doses of adenovirus used were 4×10^9 pfu apoE4-185, 2×10^9 pfu apoE4-202, 1.5×10^9 pfu apoE4-229, 3×10^9 pfu apoE4-259, and 5×10^8 pfu full-length apoE4. Plasma lipids were determined on days 0 through 4 and hepatic apoE mRNA and plasma apoE levels are determined on day 4.

Figure 3. A-D: Fractionation of plasma by FPLC and density gradient ultracentrifugation
A,B: FPLC profiles of plasma from apoA-I^{-/-} × apoE^{-/-} mice infected with (A) 1.5×10^9 apoE4-229 (B) 5×10^8 full-length apoE4. The position of VLDL/IDL and HDL fractions as well as the cholesterol ester to total cholesterol (CE/TC) ratio are indicated. The distribution of apoE in different lipoprotein fractions, as determined by Western blotting, is indicated below panels A & B **C,D: Distribution of apoE in different lipoprotein fractions following density gradient ultracentrifugation of plasma.** Samples obtained from apoA-I^{-/-} × apoE^{-/-} mice four days post-infection with adenovirus expressing full-length apoE4-229 or apoE4 were fractionated by density gradient ultracentrifugation and analyzed by SDS-PAGE as described in the *Methods*. The density of each fraction and the position of apoE in the VLDL/IDL, LDL, and HDL fractions is indicated in each panel. **Panel C** shows analysis of plasma of mice infected with 1.5×10^9 apoE4-229. Similar distribution was obtained by analysis of plasma of mice infected with 2×10^9 pfu of apoE4-202, 3×10^9 apoE4-259 (see Supplementary Figure 2A-D). **Panel D** shows analysis of plasma of mice infected with 5×10^8 full-length apoE4.

Figure 4. A-I: Electron micrographs of HDL fractions separated by density gradient ultracentrifugation

Fractions of Figure 3C,D and Supplementary Figure 2A,D were pooled and analyzed by EM. The samples analyzed in panels A through I were obtained from mice expressing **A**: apoE4-185; **B**: apoE4-202; **C**: apoE4-229; **D**: apoE4-259; **E**: a low dose of apoE4 wild-type (5×10^8 pfu); **F**: high dose (2×10^9 pfu) apoE4 wild-type; **G**: uninfected apoA-I^{-/-} × apoE^{-/-} mice. A similar pattern was obtained in ABCA1^{-/-} mice. **H**: mice infected with 1.5×10^9 apoE4-202 pfu and 5×10^8 GFP; **I**: HDL obtained from mice infected with 1.5×10^9 pfu apoE4-202 and 5×10^8 LCAT.

Figure 5. A-F: Two-dimensional gel electrophoresis of plasma of apoA-I^{-/-} × apoE^{-/-} mice infected with adenoviruses expressing full-length and truncated apoE4 forms

Plasma was obtained from mice infected with the following doses of adenovirus: **A:** 4×10^9 pfu apoE4-185, **B:** 2×10^9 apoE4-202, **C:** 1.5×10^9 apoE4-229, **D:** 4×10^9 apoE4-259, **E:** 5×10^8 pfu full-length apoE4, **F:** 1×10^9 pfu full-length apoE4.