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Molecular interactions between apoE and ABCA1: impact on apoE lipidation

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Abstract Apolipoprotein E (apoE)/ABCA1 interactions were investigated in human intact fibroblasts induced with 22(*R*)-hydroxycholesterol and 9-*cis*-retinoic acid (stimulated cells). Here, we show that purified human plasma apoE3 forms a complex with ABCA1 in normal fibroblasts. Lipid-free apoE3 inhibited the binding of ^{125}I-apoA-I to ABCA1 more efficiently than reconstituted HDL particles (IC_{50} = 2.5 ± 0.4 μg/ml vs. 12.3 ± 1.3 μg/ml). ApoE isoforms showed similar binding for ABCA1 and exhibited identical kinetics in their abilities to induce ABCA1-dependent cholesterol efflux. Mutation of ABCA1 associated with Tangier disease (C1477R) abolished both apoE3 binding and apoE3-mediated cholesterol efflux. Analysis of apoE3-containing particles generated during the incubation of lipid-free apoE3 with stimulated normal cells showed nascent apoE3/cholesterol/phospholipid complexes that exhibited preβ-electrophoretic mobility with a particle size ranging from 9 to 15 nm, whereas lipid-free apoE3 incubated with ABCA1 mutant (C1477R) cells was unable to form such particles. These results demonstrate that *1*) apoE association with lipids reduced its ability to interact with ABCA1; *2*) apoE isoforms did not affect apoE binding to ABCA1; *3*) apoE-mediated ABCA1-dependent cholesterol efflux was not affected by apoE isoforms in fibroblasts; and *4*) the lipid translocase activity of ABCA1 generates apoE-containing high density-sized lipoprotein particles. Thus, ABCA1 is essential for the biogenesis of high density-sized lipoprotein containing only apoE particles in vivo.—Krimbou, L., M. Denis, B. Haidar, M. Carrier, M. Marcil, and J. Genest, Jr. **Molecular interactions between apoE and ABCA1: impact on apoE lipidation. *J. Lipid Res.* 2004.** 45: **839–848.**

Supplementary key words ATP binding cassette transporter A1 • lipid efflux • apolipoprotein E isoforms • high density lipoprotein-sized lipoprotein containing only apolipoprotein E particles

Human apolipoprotein E (apoE) is an arginine-rich glycoprotein (34,200 Da) that plays a pivotal role in lipoprotein metabolism and neurobiology through its interactions with heparan sulfate proteoglycans and the LDL receptor family (1). Thus, apoE is believed to play a significant role in the onset and development of coronary artery atherosclerosis (2) and the pathophysiology of Alzheimer's disease (3). The importance of apoE in the pathogenesis of atherosclerosis has been strikingly demonstrated by the presence of spontaneous atherosclerosis in experimental animals made deficient in apoE (4, 5) and conversely by the protection against or regression of atherosclerosis in apoE-deficient animals supplemented with apoE (6, 7). ApoE exists in three isoforms, apoE2, apoE3, and apoE4, each differing by cysteine and arginine at positions 112 and 158. ApoE3, the most common form, contains cysteine and arginine at these positions, respectively, whereas apoE2 contains cysteine and apoE4 contains arginine at both sites (8). These differences have profound effects on the biological functions of apoE. Both apoE3 and apoE4 bind to the LDL receptor with high affinity, whereas apoE2 exhibits defective binding to the LDL receptor and is associated with type III hyperlipoproteinemia (9). ApoE4 is associated with high plasma cholesterol level and increased risk for both coronary heart disease and Alzheimer's disease (2, 10).

Although it is clear that apoE plays an important role in the reverse cholesterol transport (RCT) process, structural determinants of molecular interactions between key proteins involved in RCT and apoE have not yet been elucidated. However, there is ample evidence that apoE can directly affect the ability of HDL particles to mediate cellular lipid efflux. For example, it has been documented that the capacity of apoE-depleted HDL of human or HDL of apoE-deficient mice to promote cholesterol efflux

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Abbreviations: apoE, apolipoprotein E; 2D-PAGGE, two-dimensional polyacrylamide nondenaturing gradient gel electrophoresis; DSP, dithiobis(succinimidylpropionate); FC, free cholesterol; HDL-LpE, high density-sized lipoprotein containing only apoE particles; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; RCT, reverse cholesterol transport; r(LpE3), reconstituted HDL particles; SM, sphingomyelin; TD, Tangier disease.

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from mouse peritoneal macrophages is decreased and can be restored to normal by the addition of apoE (11, 12). A recent study from our laboratory documented that human plasma high density-sized lipoprotein containing only apoE particles (HDL-LpE) is very effective in removing acetyl-LDL-derived [^{3}H]cholesterol from J744 macrophages (13). Phenotype-specific differences have also been shown in the ability of apoE to promote cholesterol efflux from cultured cells. γ-LpE from apoE3/3 individuals stimulated 7- to 13-fold more cholesterol efflux from cultured fibroblasts than the same fraction from apoE2/2 or apoE4/4 individuals (14). Moreover, it was documented that apoE promotes lipid release from astrocytes and neurons in an isoform-dependent manner (15).

The discovery that the low HDL levels associated with Tangier disease (TD) and familial HDL deficiency are attributable to mutations in the ABCA1 gene (16, 17) has revealed that this transporter is crucial for HDL biogenesis, because it mediates the apolipoprotein-dependent transfer of intracellular cholesterol and phospholipids to lipid-free apolipoproteins (18–20).

To better define the role of ABCA1 in the biogenesis of LpE particles, experiments were directed in the present study at defining the mechanism by which apoE is lipidated by ABCA1 and how the interactions of apoE and ABCA1 can be affected by apoE association with lipids, by apoE isoforms, or by naturally occurring mutation of ABCA1.

MATERIALS AND METHODS

Patient selection

For the present study, we selected fibroblasts from three normal control subjects and one patient with TD (compound heterozygous carrying the mutations C1477R and the splice site G→C in exon 24) as previously described (16, 20). The protocol for the study was reviewed and accepted by the Research Ethics Board of the McGill University Health Centre. Separate consent forms for blood sampling, DNA isolation, and skin biopsy were provided.

Cell culture

Human skin fibroblasts were obtained from 3.0 mm punch biopsies of the forearm of the patient and healthy control subjects and cultured in DMEM supplemented with 0.1% nonessential amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FBS.

Human plasma apoE

Purified human plasma apoE isoforms were a gift from Dr. Karl H. Weisgraber (Gladstone Institutes of Cardiovascular Disease, San Francisco, CA). Before use, either lyophilized apoE or apoA-I (Biodesign) was resolubilized in 4 M guanidine HCl and dialyzed extensively against Tris buffer (10 mM Tris, 150 mM NaCl, and 1.0 mM EDTA, pH 8.2) as described previously (21).

Cellular cholesterol efflux and lipid labeling

Cholesterol efflux was determined as previously described (20) with minor modifications. Briefly, 50,000 cells were seeded in 12-well plates. At midconfluence, the cells were labeled with 0.2 μCi/ml [^{3}H]cholesterol (Perkin Elmer) for 48 h. At confluence, cells were cholesterol-loaded (20 μg/ml) for 24 h. During a 24 h equilibration period, cells were stimulated or not with 2.5 μg/ml 22(*R*)-hydroxycholesterol and 5 μM 9-*cis*-retinoic acid for 20 h. Cholesterol efflux was determined for the indicated concentration and time as follows: ^{3}H cpm in medium/(^{3}H cpm in medium + ^{3}H cpm in cells); the results were expressed as percentage of total radiolabeled cholesterol. Cells from a normal and a TD subject were grown to confluence in 100 mm diameter dishes, labeled with 15 μCi/ml [^{14}C]cholesterol (Perkin Elmer) for 48 h, and then cholesterol-loaded, equilibrated, and stimulated as described above. Cellular [^{14}C]cholesterol labeling was used for the characterization of apoE-containing particles generated during the incubation of lipid-free apoE3 with stimulated cells. Cell phospholipids were labeled with [^{32}P]orthophosphate as follows: fibroblasts from a control and a TD subject were grown to confluence in 100 mm diameter dishes and incubated for 72 h with 300 μCi of [^{32}P]orthophosphate mixed with DMEM. After the first 24 h of incubation with [^{32}P]orthophosphate, cells were cholesterol-loaded (20 μg/ml) for 24 h, and then during a 24 h equilibration period, the cells were stimulated as described above before incubation with apoE3.

Preparation of reconstituted HDL particles

Complexes comprising apoE3, POPC, and cholesterol were prepared using the sodium cholate dialysis method described by Jonas, Steinmetz, and Churgay (22). An apoE3/POPC/cholesterol molar ratio of 1:100:5 was used in this experiment. Reconstituted HDL particles [r(LpE3)] were further concentrated by ultrafiltration (spiral ultrafiltration cartridge, MWCO 50,000; Amicon) to discard any lipid-free apoE3 or proteolytic peptides. ApoE3/lipid complex formation was verified by analysis with native polyacrylamide gradient (8–25%) gel electrophoresis.

Competitive binding of apoE3 and r(LpE3)

The competitive binding assay was performed as previously described (23). Briefly, apoA-I isolated from human plasma (Biodesign) was iodinated with ^{125}I by IODO-GEN® (Pierce) to a specific activity of 800 cpm/ng apoA-I. Cells were grown on 24-well plates and stimulated with 2.5 μg/ml 22(*R*)-hydroxycholesterol and 5 μM 9-*cis*-retinoic acid for 20 h. Cells were then incubated for 2 h at 37°C with 1 μg/ml ^{125}I-apoA-I in DMEM/BSA (1 mg/ml) in the presence or absence of increasing amounts of the unlabeled competitor [apoA-I, apoE3, and r(LpE3)]. The cells were then washed rapidly two times with ice-cold PBS/BSA and two times with cold PBS and lysed with 0.1 N NaOH. The amount of bound iodinated ligand was determined by γ counting, and the protein content was measured using a modified Lowry method as described previously (24).

Chemical cross-linking and immunoprecipitation analysis

Chemical cross-linking was performed as described by Wang et al. (25) with a minor modification. Fibroblasts were grown to confluence in 100 mm diameter dishes and then stimulated or not with 2.5 μg/ml 22(*R*)-hydroxycholesterol and 5 μM 9-*cis*-retinoic acid for 20 h in DMEM/BSA. Cells were incubated in the presence or absence of 3 μg/ml apoE2, apoE3, apoE4, or apoA-I in DMEM/BSA for 1 h at 37°C. Cells were then placed on ice for 15 min and washed three times with PBS. Dithiobis(succinimidylpropionate) (DSP; cross-linker agent) was dissolved immediately before use in DMSO and diluted to 250 μM with PBS. Eight milliliters of DSP solution was added in each well. Cells were then incubated at room temperature for 1 h; the medium was removed, and the cells were washed twice with PBS. Cells were lysed at 4°C with immunoprecipitation buffer containing 20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, and 1% Triton X-100 (Invitrogen), and the suspension was allowed to stand for 30 min

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at 4°C in the presence of a protease inhibitor cocktail (Roche Diagnostics). ApoE/ABCA1 and apoA-I/ABCA1 complexes were immunoprecipitated with an affinity-purified polyclonal anti-ABCA1 antibody (Novus Biologicals) as described (26) or used for solid-phase binding assay. Immunoprecipitated complexes were separated on SDS gels as previously described (26). ApoE or apoA-I associated with ABCA1 was detected by affinity-purified human anti-apoE or anti-apoA-I antibodies (12171-21E and 12171-21A, respectively; Genzyme Corp.).

Preparation of total microsomal fraction

Fibroblasts were grown to confluence in 100 mm diameter dishes and then stimulated or not for 20 h. Cells were incubated with either apoE or apoA-I (3 μg/ml), and then chemical cross-linking was performed as described above. The cells were swollen on ice for 10 min and then homogenized with 20 strokes in a tight-fitting Dounce homogenizer. After centrifugation at 1,000 *g* and 4°C for 3 min to remove unbroken cells and nuclei, the supernatant was recentrifuged at 100,000 *g* and 4°C for 60 min. The resulting supernatant was discarded, and the final microsomal fraction pellet was resuspended in 250 μl of immunoprecipitation buffer. Total microsomal fraction containing apoE/ABCA1 and apoA-I/ABCA1 complexes were used for solid-phase binding assays.

Solid-phase binding assay of apoE isoforms to ABCA1

To quantitate apoE isoform binding to ABCA1, we used solid-phase binding assays. Ninety-six-well microtiter plates (Nunc Immunosorb modules) were coated overnight at 4°C with either 5 μg/ml anti-ABCA1 antibody or albumin in PBS. Unbound proteins were washed from the wells, and nonspecific binding sites were blocked by incubation with 5% BSA and 0.05% Tween 20 in PBS for 1 h at room temperature. Direct binding assay was performed by adding either apoE isoforms or apoA-I associated with ABCA1 from both stimulated and unstimulated cells prepared as described above. Isolated microsomal fraction (120 ng) was added to immobilized anti-ABCA1 antibody or BSA-coated plates in Tris-buffered saline, pH 7.5, containing 1% BSA, 0.01% Tween 20, and 1 mM $CaCl_2$. Then, the plates were incubated for 16 h at 4°C. Unbound proteins were aspirated, and the wells were washed three times with PBS. To detect bound apoE or apoA-I, the plates were incubated with ^{125}I-labeled affinity-purified polyclonal anti-human apoE or apoA-I antibody for 1 h at 37°C. The wells were then washed three times with PBS, and bound radioactivity was removed with 10% SDS and counted. To detect nonspecific binding, all assays were done simultaneously on plates coated with BSA alone as described above. The background binding to BSA was subtracted from all samples and represented less than 5% of total binding.

Separation of lipoproteins by two-dimensional polyacrylamide nondenaturing gradient gel electrophoresis

ApoE3-containing particles were separated by two-dimensional polyacrylamide nondenaturing gradient gel electrophoresis (2D-PAGGE) as previously described (27, 28). Briefly, samples (30–100 μl) were separated in the first dimension (according to their charge) by 0.75% agarose gel electrophoresis (100 V, 3 h, 4°C) and in the second dimension (according to their size) by 5–23% polyacrylamide concave gradient gel electrophoresis (125 V, 24 h, 4°C). An iodinated high molecular weight protein mixture (7.1–17.0 nm; Amersham) was run as a standard on each gel. Electrophoretically separated samples were electrotransferred (30 V, 24 h, 4°C) onto nitrocellulose membranes (Hybond ECL; Amersham). ApoE3-containing particles were detected by incubating the membranes with immunopurified polyclonal anti-apoE antibody labeled with ^{125}I. The presence of either [^{14}C]cholesterol or [^{32}P]phospholipids was detected directly by autoradiography using Kodak XAR-2 film.

RESULTS

In agreement with previous studies (29, 30), pretreatment of fibroblasts with 22(*R*)-hydroxycholesterol and 9-*cis*-retinoic acid (stimulated cells), as described in Materials and Methods, increased the level of ABCA1 protein by ∼4-fold (data not shown). This induction of ABCA1 increased apoE3-mediated cholesterol efflux by ∼2.5-fold and apoE binding to ABCA1 by 3-fold (see data below). It is well established that oxysterols, including 22(*S*)-hydroxycholesterol, are high-affinity endogenous ligands for liver X receptor, the nuclear receptor that upon dimerization with retinoid X receptor induces ABCA1 gene transcription in macrophages and other cells (31, 32). In the present study, we have examined the binding of apoE3 to ABCA1 in normal cultured stimulated cells. As shown in **Fig. 1A** (left panels), a marked and consistent association of apoE3 with ABCA1 was detected using chemical cross-linking, as described in Materials and Methods. ApoA-I was used as a control for this experiment (Fig. 1A, right panels).

It is well established that the conformation of apolipoproteins within HDL particles is affected by their association with lipid molecules. Therefore, it was of interest to determine whether apoE conformation/organization within particles would affect its interaction with ABCA1. Competition assays were performed to determine the ability of lipid-free apoE3, as well as discoidal r(LpE3) with a molecular diameter of 13 nm, to compete for the binding of ^{125}I-apoA-I to ABCA1 in stimulated cells. As shown in Fig. 1B, both lipid-free apoE3 and apoA-I have similar capacities to compete for binding to ABCA1. In contrast, lipid-free apoE3 inhibited the binding of ^{125}I-apoA-I to ABCA1 more efficiently than did r(LpE3) (IC_{50} = 2.5 ± 0.4 μg/ml vs. 12.3 ± 1.3 μg/ml). Control experiments were conducted to examine whether the apparent decrease in cell binding of the labeled apoA-I may be attributable to ^{125}I-apoA-I binding to different competitor particles instead of the cells. An experiment was carried out in which apoE3, r(LpE3), and r(LpA-I) particles were incubated with ^{125}I-apoA-I under conditions similar to those used for the apoA-I binding assay, and the samples were separated by fast protein liquid chromatography. No significant amount of ^{125}I-apoA-I was associated with apoE3, r(LpE3), or r(LpA-I) (data not shown), supporting our results shown in Fig. 1B.

Because phenotype-specific differences have been shown in the ability of apoE to interact with many proteins (9, 33–35), we posed the question of whether the interaction of apoE with ABCA1 could be affected by apoE isoforms. Cross-linking experiments were carried out with purified plasma apoE2, apoE3, or apoE4. As seen in **Fig. 2** (upper panel), all three naturally occurring isoforms of apoE can cross-link to ABCA1 in stimulated cells. To quantitate the relative binding of apoE isoforms to ABCA1, we

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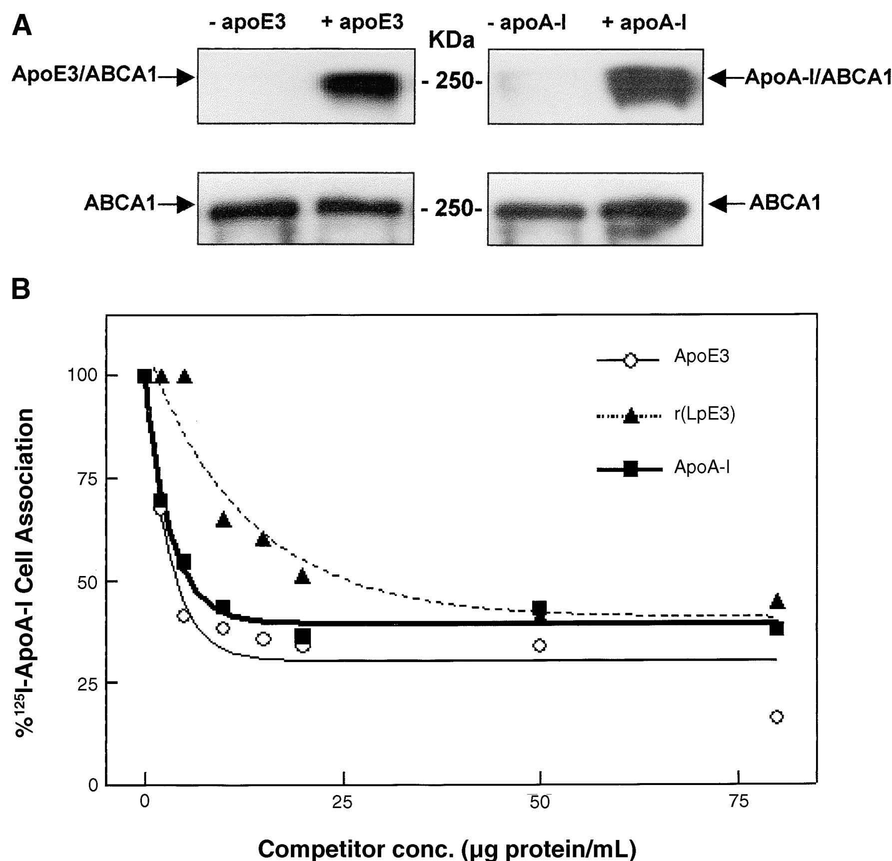

Fig. 1. Cross-linking of apolipoprotein E3 (apoE3) and apoA-I to ABCA1 and the ability of lipid-free apoE3 and reconstituted HDL particles [r(LpE3)] to interact with ABCA1. A: Normal fibroblasts were stimulated with 2.5 μg/ml 22(*R*)-hydroxycholesterol and 5 μM 9-*cis*-retinoic acid for 20 h and incubated with 3 μg/ml apoE3 or apoA-I at 37°C for 1 h. Cells were washed three times with cold PBS and exposed to the dithiobis(succinimidylpropionate) cross-linker for 1 h at room temperature. Either apoE3 (left panels) or apoA-I (right panels) associated with ABCA1 was detected as described in Materials and Methods. B: Normal cells were plated in 24-well plates and stimulated for 20 h. Cells were then incubated with 1 μg/ml ^{125}I-apoA-I (1,200 cpm/ng) for 2 h at 37°C with increasing amounts of lipid-free apoE3, r(LpE3), or unlabeled lipid-free apoA-I (0, 2, 5, 10, 15, 20, 50, and 80 μg protein/ml). Cells were then washed rapidly three times with ice-cold PBS/BSA and then PBS alone. ^{125}I-apoA-I associated with cells was determined as described in Materials and Methods. Values shown represent means from duplicate wells. Representative curves from two independent experiments are shown. IC_{50} values shown were determined using GraphPad Prism 4.00 software. Conc., concentration.

used the solid-phase binding assay described in Materials and Methods. First, apoE2, apoE3, apoE4, or apoA-I was cross-linked to ABCA1 in stimulated and unstimulated cells, and total microsomal fractions were isolated. ApoE isoforms or apoA-I associated with ABCA1-containing microsomal fraction (120 ng of protein) was immunoprecipitated with an affinity-purified anti-ABCA1 antibody, which had been immobilized by passive adsorption to microtiter plates. Either apoE or apoA-I associated with ABCA1 was quantitated as described in Materials and Methods. In separate experiments, the saturation of anti-ABCA1 antibody was determined by incubating increasing amounts of total microsomal fraction containing apoE/ABCA1 complex with immobilized anti-ABCA1 antibody, and apoE associated with ABCA1 was detected by anti-apoE antibody. In the present binding assay, saturation was reached at 300 ng of protein. Figure 2 (lower panel) shows that under the same conditions, the binding of apoE2, apoE3, and apoE4 to ABCA1 is similar. As expected, both apoE isoforms and apoA-I bound to ABCA1 approximately three times more in stimulated than in unstimulated cells. ApoA-I binding to ABCA1 was used as a control in this assay.

Phenotype-specific differences have been shown in the ability of apoE to promote cholesterol efflux from cultured cells (14, 15, 36). We examined the effect of apoE isoform on the ability of apoE to mediate ABCA1-dependent cellular cholesterol efflux. As shown in **Fig. 3** (upper panel), the three apoE isoforms mediate cellular cholesterol efflux approximately 2.5 times more in stimulated than in unstimulated cells in a dose-dependent manner. At the same time, apoE2, apoE3, and apoE4 showed similar abilities to induce cellular cholesterol efflux in a time-dependent manner. Furthermore, in our stimulated cell culture system, apoE-mediated cholesterol efflux reached saturation after a 16 h incubation (Fig. 3, lower panel).

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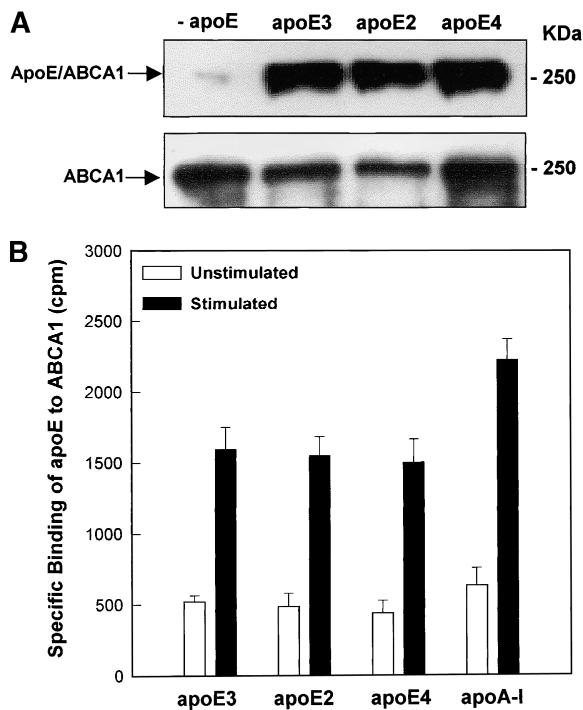

Fig. 2. Effect of apoE isoforms on apoE/ABCA1 association. A: Stimulated cells were incubated with 3 μg/ml apoE2, apoE3, or apoE4 for 1 h at 37°C, and then cross-linking was performed. Immunoprecipitation and apoE detection were performed as described for Fig. 1A. B: Cells were stimulated or not and incubated with 3 μg/ml apoE2, apoE3, apoE4, or apoA-I for 1 h at 37°C, and then cross-linking was performed. ApoE isoforms and apoA-I associated with ABCA1 in both unstimulated and stimulated cells were quantified by the solid-phase binding assay as described in Materials and Methods. Values represent means ± SD from triplicate wells. Similar results were obtained from three other independent experiments.

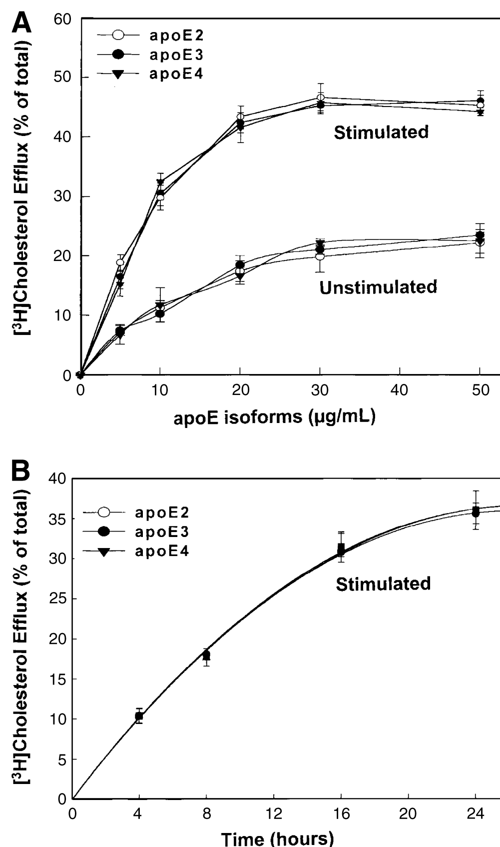

Fig. 3. Concentration and time dependence of apoE isoform-mediated cellular cholesterol efflux. A: Normal fibroblasts were labeled with [^{3}H]cholesterol and cholesterol-loaded, then stimulated for 20 h or not. Stimulated and unstimulated cells were incubated with increasing amounts of apoE isoforms (0, 5, 10, 20, 30, and 50 μg/ml) for 24 h at 37°C. Cholesterol efflux was determined as described in Materials and Methods. Values represent means ± SD from triplicate wells. B: Fibroblasts were cholesterol-loaded and labeled with [^{3}H]cholesterol, then stimulated. Cells were incubated with 10 μg/ml apoE3 for the indicated times, and cholesterol efflux was determined. Values represent means ± SD from triplicate wells.

To determine whether naturally occurring mutants of ABCA1 might affect apoE3 binding, cross-linking of apoE3 to mutant ABCA1 (C1477R) was examined. As shown in **Fig. 4**, C1477R mutant abolished both apoE3 cross-linking and apoE3-mediated cholesterol efflux. On the other hand, C1477R mutant was found to be present at the plasma membrane, as determined by cell surface biotinylation (data not shown).

To investigate the nature of apoE3-containing particles generated by ABCA1, stimulated cells from either normal control or TD (C1477R) subjects in 100 mm diameter dishes were incubated with 5 μg/ml lipid-free apoE3 in DMEM for 24 h at 37°C. The medium was concentrated, and apoE3-containing particles were electrophoretically separated and detected by immunopurified polyclonal anti-apoE antibody labeled with ^{125}I. As shown in **Fig. 5B**, apoE3-containing particles generated by stimulated normal cells exhibited preβ electrophoretic mobility with a particle diameter ranging from 9 to 15 nm, which are designated preβ-LpE3-like particles. In contrast, lipid-free apoE3 incubated with stimulated TD cells was unable to form such particles (Fig. 5E), which had a particle diameter similar to that of lipid-free apoE3 incubated in the same conditions without cells (Fig. 5A). To demonstrate that preβ-LpE3-like particles contained cholesterol, we incubated lipid-free apoE3 with stimulated fibroblasts from normal and TD subjects that were labeled with radioactive cholesterol (^{14}C) for 24 h at 37°C. Labeled cholesterol was found in all preβ-LpE3-like subspecies generated by normal cells (Fig. 5C). Importantly, we have not been able to detect any [^{14}C]cholesterol associated with apoE3 during incubation with TD cells (Fig. 5F). We next determined the phospholipid content of preβ-LpE3-like particles; cells were first labeled with [^{32}P]orthophosphate and then stimulated and incubated with 5 μg/ml lipid-free apoE3 for 24 h at 37°C, as described in Materials and Methods. The medium was concentrated and dialyzed, and then [^{32}P]apoE3-containing particles were analyzed by 2D-PAGGE. As shown in Fig. 5D, ^{32}P-phospholipidated apoE3 from stimulated normal cells colocalized with apoE3-containing particles revealed with anti-apoE antibody and [^{14}C]cholesterol-lipidated LpE3 particles (Fig. 5B, C, re-

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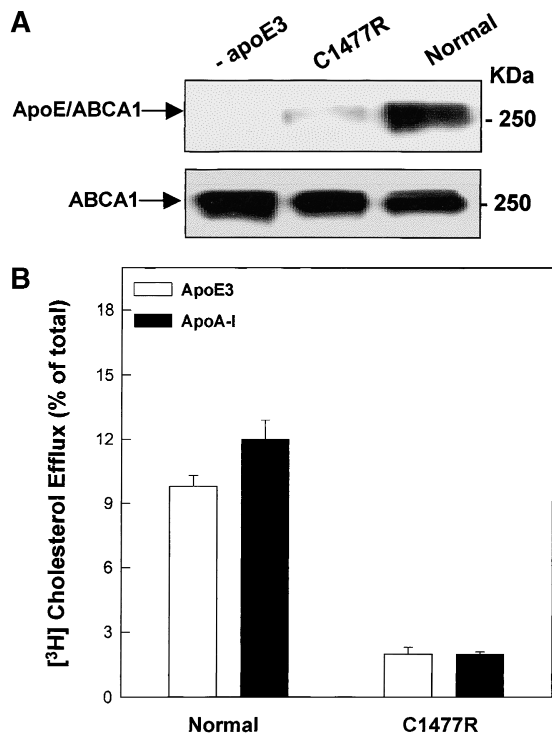

Fig. 4. Effect of naturally occurring mutations of ABCA1 on apoE3 binding and cholesterol efflux. A: Fibroblasts from a normal control and a Tangier disease (TD) (C1477R) subject were stimulated and incubated with 3 μg/ml apoE3. Cross-linking, immunoprecipitation, and detection of apoE3 associated with ABCA1 were performed as described in Materials and Methods. B: Fibroblasts from a normal control and a mutant (C1477R) subject were labeled with [^{3}H]cholesterol and cholesterol-loaded, then stimulated for 20 h. Cells were incubated with 10 μg/ml apoE3 or apoA-I for 24 h, and cholesterol efflux was determined. Values represent means ± SD from triplicate wells.

spectively). In contrast, apoE3 was unable to recruit phospholipids from stimulated TD cells (Fig. 5G). To determine the relative phospholipid composition of preβ-LpE3-like particles, after incubation of apoE3 with stimulated cells for 24 h, the medium was concentrated and dialyzed, and apoE3-containing particles were immunoprecipitated with anti-apoE antibody. The ^{32}P-labeled phospholipids sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylethanolamine (PE), lysophosphatidylcholine (LPC), and phosphatidylinositol (PI) were extracted from immunoprecipitated medium and then separated in triplicate by TLC and quantitated by densitometric scanning. The results in **Fig. 6** show the relative percentage phospholipid composition of preβ-LpE3-like particles and the whole-cell phospholipid species distribution. PC and SM constituted 89% of the total phospholipids in preβ-LpE3-like particles, whereas PE, LPC, and PI contributed the additional 11%. Despite the fact that PC and PE appeared to be the most abundant phospholipid species in whole cells, the PC/PE ratio was 9-fold lower in the preβ-LpE3-like particles than in the whole cell.

DISCUSSION

It is well accepted that the RCT is a dynamic process in which multiple protein components are involved (37, 38). In this context, there has been increasing interest in understanding the molecular and physiological mechanisms of apoE-mediated cellular lipid efflux. Here, we investigated apoE/ABCA1 interactions and their impact on the lipidation of apoE. Consistent with an earlier study (39), we show that apoE is able to form a complex with ABCA1 in stimulated cells as well as apoA-I (Fig. 1A). This result suggests that the specific amino acid sequence of either apoE or apoA-I is not critical for binding to ABCA1; rather, some structural motifs common to apoE and apoA-I are necessary, such as the amphipathic α-helical domains that are the major element of secondary structure (39–42). On the other hand, we observed that plasma membrane PC or SM hydrolysis did not prevent apoE3/ABCA1 complex formation (data not shown), consistent with our recent finding that treatment of intact cells with phospholipases (PC-specific phospholipase C or sphingomyelinase) affected neither the specific binding of apoA-I to ABCA1 nor apoA-I/ABCA1 cross-linking (23). This suggests that both apoE and apoA-I interactions with ABCA1 are attributable to direct protein-protein contact.

ApoE is a polymorphic protein, and its three isoforms, apoE2, apoE3, and apoE4, differ by only a single amino acid substitution, yet these changes have an important effect on their interactions with many proteins, including the microtubule-associated protein tau (33), amyloid protein (Aβ) (34), and α_2-macroglobulin (35), among others. Here, we demonstrate that all three naturally occurring isoforms of apoE have similar binding to ABCA1 (Fig. 2). This indicates that specific interactions of amino- and carboxyl-terminal domains of apoE molecules, suggested to be responsible for the apoE4 preference for VLDL and the apoE2 or apoE3 preference for HDL (43), are not implicated in the apoE isoform/ABCA1 interaction. It is likely that the amphipathic α-helix common to the three apoE isoforms interacts with ABCA1, rather than a specific domain, as has been established for both apoA-I/ABCA1 and apoE isoform/scavenger receptor class B type I interactions (41, 42, 44). This conclusion is consistent with the finding that the three apoE isoforms showed identical kinetics in their abilities to induce ABCA1-dependent cellular cholesterol efflux (Fig. 3). In contrast to the present study, it was documented that exogenous apoE promotes lipid release from astrocytes and neurons in an isoform-dependent manner (15). The same group also reported that the amount of cholesterol released into the culture medium from the apoE3-expressing astrocytes was 2.5-fold greater than that from apoE4-expressing astrocytes (36). Furthermore, allele-specific differences have been demonstrated by Cullen et al. (45) in the ability of macrophages to take up, secrete, and accumulate cholesterol. However, the cells used in the aforementioned studies were apoE-secreting cells, indicating that the cholesterol efflux in their system could have been mediated by endogenous apoE, which may be distinct from that me-

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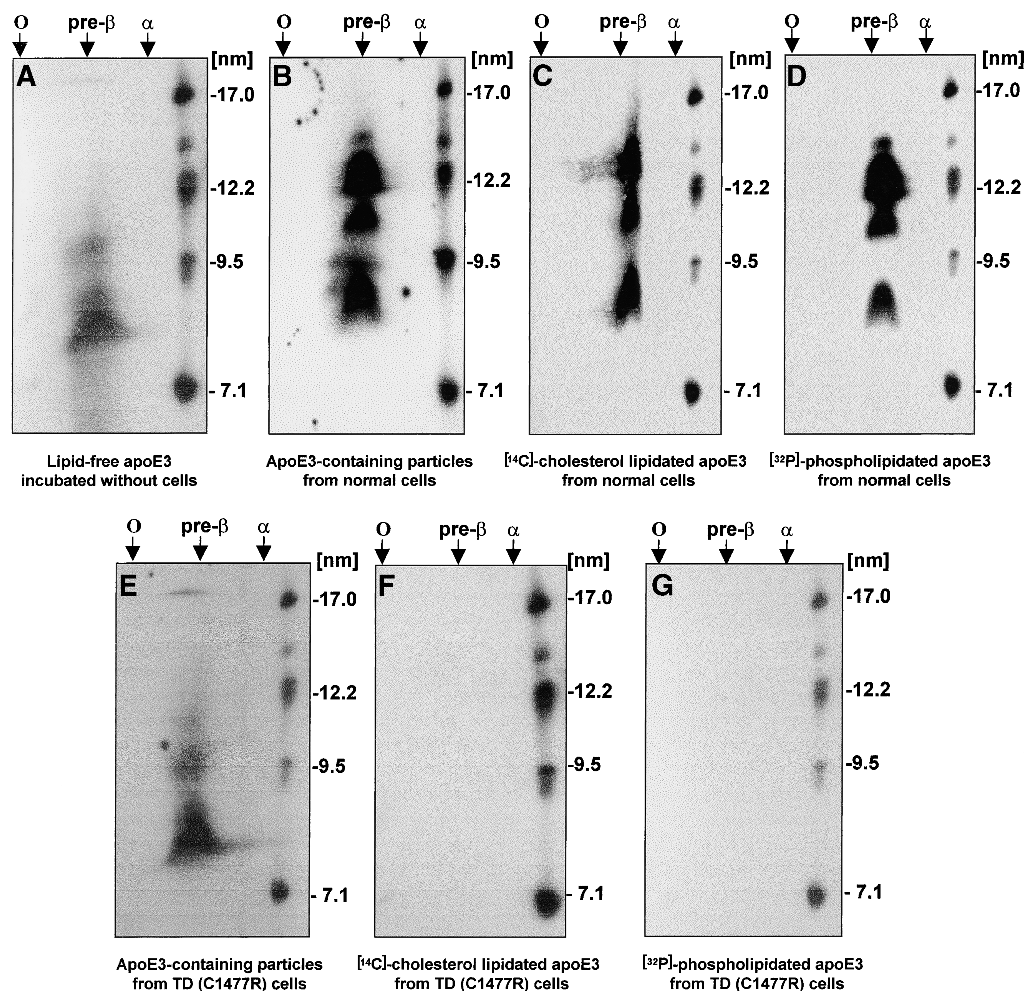

Fig. 5. Characterization of lipidated apoE3-containing particles generated during apoE3 incubation with stimulated fibroblasts from a normal and a TD (C1477R) subject. A, B, and E: Lipid-free apoE3 (5 μg/ml) was incubated in DMEM/BSA for 24 h at 37°C without cells or with stimulated normal and TD cells. Samples were separated by two-dimensional polyacrylamide nondenaturing gradient gel electrophoresis (2D-PAGGE), and apoE was detected with immunopurified polyclonal anti-apoE antibody labeled with ^{125}I. C and F: [^{14}C]cholesterol-labeled normal and TD cells were stimulated and then incubated with 5 μg/ml apoE3 for 24 h at 37°C. The medium was recovered, concentrated, and separated by 2D-PAGGE. D and G: [^{32}P]orthophosphate-labeled normal and TD cells were stimulated and then incubated with 5 μg/ml apoE3 for 24 h at 37°C. The medium was recovered, concentrated, and dialyzed. Samples were separated by 2D-PAGGE. The presence of either [^{14}C]cholesterol or ^{32}P-labeled phospholipids associated with apoE3-containing particles from normal or TD cells was directly detected by autoradiography using Kodak XAR-2 film. Molecular size markers are indicated on the right side of each gel.

diated by exogenous added apoE. On the other hand, in our cell culture system, ABCA1 was induced with 22(*R*)-hydroxycholesterol and 9-*cis*-retinoic acid. Our results demonstrate that apoE isoforms did not affect either apoE binding or apoE-mediated ABCA1-dependent cellular cholesterol efflux. This is consistent with the finding of Smith and coworkers (46) that in the presence of a cyclic AMP analog, which also stimulates ABCA1 expression, apoE2, apoE3, and apoE4 were found to be equally efficient at promoting lipid efflux from macrophages.

The structural requirements of apoE lipidation by ABCA1 have not yet been determined. However, in an attempt to understand this process in fibroblasts, we characterized apoE3-containing particles generated during the incubation of apoE3 with either stimulated normal or TD fibroblasts. We show that a heterogeneous species of lipid particle is formed. There are at least three types of apoE3-containing particles exhibiting preβ electrophoretic mobility with a particle size ranging from 9 to 15 nm. In contrast, apoE3 incubated with TD cells (C1477R) was unable to form such particles (Fig. 5); because this is the plasma HDL-LpE3 size range having preβ electrophoretic mobility (13), these particles are designated preβ-LpE3-like particles. Furthermore, all preβ-LpE3-like particle subspecies generated by normal cells contained both cholesterol and phospholipids (Fig. 5). In contrast, apoE3 was unable to recruit cholesterol and phospholipids from TD cells (Fig. 5F, G, respectively). These results are consistent with our

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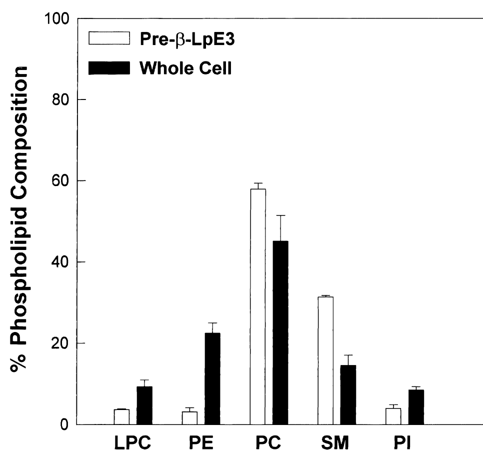

Fig. 6. Phospholipid composition of apoE3-containing particles generated during apoE3 incubation with stimulated normal fibroblasts. Normal fibroblasts were labeled with [^{32}P]orthophosphate and stimulated as described in Materials and Methods. Stimulated cells were incubated with 5 μg/ml apoE3 for 24 h at 37°C, the medium was concentrated and dialyzed, and apoE3-containing particles were immunoprecipitated with anti-apoE antibody. ^{32}P-labeled phospholipids in the medium and the whole cells were extracted and separated by TLC. The ^{32}P-labeled phospholipids sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylethanolamine (PE), lysophosphatidylcholine (LPC), and phosphatidylinositol (PI) were quantified by densitometry scanning. Percentage phospholipid compositions of preβ-LpE3 particles and whole cells are presented. Values represent means ± SD from triplicate analyses. Similar results were obtained from three other independent experiments.

observations that ABCA1 mutation in the second large extracellular loop (C1477R) abolished both apoE3 binding and apoE3-mediated cholesterol efflux (Fig. 4). One important observation reported here is that the newly formed preβ-LpE3-like particles had distinctly different sizes, suggesting that the heterogeneity of those particles may be attributable to the phospholipid-free cholesterol ratio and/or the number of apoE3 molecules within each subspecies. Furthermore, the size of preβ-LpE3-like particles is consistent with that of typical discoidal apoE3/phospholipid complexes containing two or three apoE3 molecules (22). Interestingly, the phospholipid composition of preβ-LpE3-like particles did not reflect the distribution of the phospholipid species in whole cells (Fig. 6), which supports the idea that ABCA1 may have specificity for PC and SM or, alternatively, that the phospholipid that may be a substrate for ABCA1 comes from distinct microdomains of the plasma membranes or from endosomal compartments (47).

It has been suggested that apoE, as well as other apolipoproteins, induces cellular lipid efflux by membrane microsolubilization (48). It is possible that the interactions of apoE with ABCA1 lead to the lipidation of apoE through a membrane microsolubilization process whereby there is simultaneous release of phospholipids and free cholesterol to apoE, as was recently suggested by the same group for the lipidation of apoA-I (49). This is in agreement with the idea that the transfer of phospholipids and cholesterol from the active site of the ABCA1 transporter to the apoE molecule weakens the interaction of apoE and ABCA1 and causes dissociation of the lipidated apoE product. This concept is supported by recent work in our laboratory (23) demonstrating that *1*) specific apoA-I dissociation from ABCA1 is rapid (t 1/2 = 1.4 ± 0.66 h) and *2*) the association of apoA-I or apoE3 (Fig. 1B) with lipids reduces their abilities to interact with ABCA1. On the other hand, in our stimulated cell culture system, apoE-mediated cholesterol efflux reached saturation after 16 h of incubation (Fig. 3B). This is in agreement with the idea that each ABCA1 molecule at the cell surface may have multiple lipidation cycles, which may result in the lipidation of many apoE molecules by the same ABCA1 molecule. This concept is supported by a recent study by Tall and coworkers (50) demonstrating that apoA-I/ABCA1 interactions result in the dephosphorylation of the ABCA1 PEST sequence and thereby inhibit calpain degradation, leading to an increase of both ABCA1 cell surface expression and activity.

γ-LpE, a SM-rich lipoprotein that contains apoE as its sole protein component (51, 52) and that is present in HDL-LpE subpopulations (13), has been proposed to play a role in cellular cholesterol efflux by acting, like preβ_1-LpA-I, as an initial acceptor of cell-derived cholesterol in normal and HDL-deficient plasma subjects (14, 53). In the present study, we observed that apoE3 incubated with stimulated normal fibroblasts did not cause γ-LpE formation (Fig. 5B–D). Furthermore, neither human macrophages nor hepatoma cell lines (HepG2) were found to secrete γ-LpE-like particles (13), consistent with the concept that in vivo lipolysis of triglyceride-rich lipoproteins may generate these particles (13, 52). However, preβ-LpE3-like particles were similar in size to plasma HDL-LpE3, which are present in the plasma of ABCA1-deficient subjects (13). This suggests that HDL-LpE3 found in the plasma of ABCA1-deficient subjects may have hepatic, macrophage, or lipolytic origins (13). Based on previous studies and our result showing that r(LpE3) had a 5-fold lesser efficiency in interacting with ABCA1 relative to lipid-free apoE3 (Fig. 1B), it is more likely that r(LpE3), γ-LpE, or HDL-LpE3 removed cellular lipid by an aqueous diffusion process than by an ABCA1-dependent pathway (54, 55).

Evidence has been presented here demonstrating that only lipid-free apoE is able to interact efficiently with ABCA1 in vitro (Fig. 1B). However, lipid-free apoE molecules are not normally present in significant quantities in plasma. It is possible that lipid-free apoE generated during the lipolysis of apoE-containing particles is rapidly lipidated by ABCA1 and forms plasma HDL-LpE. Our cell culture system represents a relatively simple model. However, in peripheral tissues and interstitial fluid, many other lipid-free apolipoproteins (e.g., apoA-I, apoJ, apoA-IV) might compete with apoE for ABCA1 binding and consequently cellular lipid release. Although the physiological significance of apoE/ABCA1 interactions remain to be determined, both apoE and ABCA1 have been implicated in the efflux, transport, and redistribution of cholesterol in the central nervous system, in which apoE is a

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major lipid transport protein (3, 10). Thus, the interactions of apoE and ABCA1 as documented in the present report may be of pathophysiological significance, which deserves further investigation.

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