



# Production and characterization of astrocyte-derived human apolipoprotein E isoforms from immortalized astrocytes and their interactions with amyloid- $\beta$ <sup>☆</sup>

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The apolipoprotein E (apoE) genotype is an important genetic risk factor for Alzheimer's disease (AD). In the central nervous system (CNS), most apoE is produced by astrocytes and is present in unique high-density lipoprotein (HDL)-like particles that have distinct properties from apoE derived from other sources. To develop an efficient system to produce astrocyte-derived apoE in large quantities, we produced and characterized immortalized cell lines from primary astrocyte cultures derived from human *APOE* knock-in mice. *APOE2*, *APOE3*, and *APOE4* expressing cell lines were established that secrete apoE in HDL-like particles at similar levels, cholesterol composition, and size as those produced by primary astrocytes. In physiological buffers, astrocyte-secreted apoE3 and E4 associated equally well with amyloid- $\beta$ . Under the same conditions, only a small fraction of A $\beta$  formed sodium dodecyl sulfate (SDS)-stable complexes with apoE (E3 > E4). These immortalized astrocytes will be useful for studying mechanisms underlying the isoform-specific effects of apoE in the CNS. © 2004 Elsevier Inc. All rights reserved.

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## Introduction

Epidemiological studies have shown that the  $\epsilon$ 4 allele of the apolipoprotein E gene (*APOE*) is a strong risk factor for both Alzheimer's disease (AD) and cerebral amyloid angiopathy (CAA), whereas the  $\epsilon$ 2 allele is associated with decreased AD risk (Greenberg et al., 1995; Strittmatter and Roses, 1996; Wisniewski et al., 1997). In addition, the  $\epsilon$ 4 allele is associated with increased morbidity following cerebral hemorrhage (Alberts et al., 1995) and head trauma (Chapman et al., 2001b) as well as more rapid progression of disease in patients with multiple sclerosis (Chapman et al., 2001a). A large body of evidence suggests that at least part of the effect of *APOE* isoforms on AD and CAA risk is mediated by interactions of apoE with the amyloid- $\beta$  (A $\beta$ ) peptide, the primary constituent of amyloid plaques that accumulate in the brain of AD patients (reviewed in Holtzman, 2001). ApoE, in an isoform-specific fashion, regulates the time of onset and amount of A $\beta$  deposition via effects on A $\beta$  clearance and fibrillogenesis in APP transgenic mice (Bales et al., 1997; DeMattos et al., 2004; Fagan et al., 2002; Holtzman et al., 2000). In addition, it has been proposed that apoE may also influence AD and other central nervous system (CNS) disorders by influencing additional biological processes such as neural repair and inflammation (Buttini et al., 1999; Lynch et al., 2003; Teter, 2000). Understanding the nature of interactions between apoE and other molecules in the brain is likely to provide important insights into its normal function as well as its role in disease.

ApoE is a 34-kDa glycoprotein that is highly expressed in the liver and in the brain (Elshourbagy et al., 1985; Newman et al.,

<sup>☆</sup> Immortalized astrocyte-derived human ApoE.

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1985). In the plasma, apoE is present in a variety of types of lipoproteins such as large very low-density lipoproteins (VLDL) as well as high-density lipoproteins (HDL). In the cerebrospinal fluid (CSF), apoE is only present in lipoproteins that are HDL-like in size (Borghini et al., 1995; LaDu et al., 1998; Pitas et al., 1987b; Roheim et al., 1979). Interestingly, apoE-containing lipoproteins in the CSF are produced predominantly, if not exclusively, by cells within the CNS (Linton et al., 1991). Unlike apoE in plasma lipoproteins that is present in the same particle along with several other apoproteins, apoE in the CNS is initially secreted predominantly by astrocytes in unique HDL-like particles that contain apoE as the sole apoprotein constituent (Fagan et al., 1999; LaDu et al., 1998). Glia (astrocytes and microglia) are cells in the CNS that produce the majority of apoE (Boyles et al., 1985; LaDu et al., 1998; Nakai et al., 1996; Pitas et al., 1987a; Stone et al., 1997), with astrocytes appearing to produce the preponderance of this protein. It has been demonstrated that astrocytes secrete apoE in nascent HDL-like lipoprotein particles, which contain cholesterol and phospholipid but lack a cholesteryl-ester core (DeMattos et al., 2001b; Fagan et al., 1999; LaDu et al., 1998). Since the physiological activity of apolipoproteins and their interactions with other molecules (e.g., A $\beta$  and receptors) can be markedly influenced by the size, composition, and type of lipoprotein particle with which they are associated, it is important to study the properties and interactions of apoE derived from the CNS or CNS cells. To this end, we recently reported on a method to purify apoE-containing lipoproteins from primary astrocyte cultures to facilitate the characterization of astrocyte-derived human apoE-containing HDL and their interactions with other molecules present in the brain (DeMattos et al., 2001b). Although this method has proved valuable, obtaining large quantities of apoE from primary astrocyte cultures was challenging. To facilitate the preparation and purification of large quantities of human apoE-containing lipoproteins derived from astrocytes, we have established new lines of immortalized astrocytes from human *APOE*-expressing knock-in mice. Herein, we characterize these cells, the apoE-containing lipoproteins they secrete, and the interactions of these lipoproteins with A $\beta$ .

## Materials and methods

### Animals

*APOE* knock-in mice expressing human apoE2, apoE3, or apoE4 (homozygous) under the control of the endogenous mouse apoE promoter were generated by a gene replacement strategy (Knouff et al., 1999; Sullivan et al., 1997, 1998). These mice were used to produce immortalized astrocytes. Wild-type C57Bl/6 mice and apoE<sup>-/-</sup> mice (Jackson labs, Bar Harbor, MN) on a C57Bl/6 background were used as controls. Transgenic mice expressing apoE3 or apoE4 under the control of the glial fibrillary acidic protein (GFAP) promoter (Fagan et al., 1999; Sun et al., 1998) as well as *APOE* knock-in mice were utilized for production of primary astrocyte culture-derived apoE.

### Primary astrocyte cultures

Primary cultures of forebrain astrocytes (>95% pure) were prepared from individual neonatal (1–2 days old) mice in T75 flasks as described (Rose et al., 1993). Growth medium consisted

of Dulbecco's modified Eagle's medium/Ham's F-12 containing 10% fetal bovine serum, 10% horse serum, 1 mM sodium pyruvate, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, 250 ng/ml fungizone, and 10 ng/ml epidermal growth factor.

### Immortalization of *APOE* knock-in mice astrocytes

After culturing primary astrocytes in T75 flasks for 7 days, the cultured cells were dislodged by 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA), triturated, and then subcultured on 100-mm petri dishes at a density of  $2\text{--}3 \times 10^5$  cells/dish. After an overnight incubation, the cells were transfected with 10  $\mu$ g of the plasmid pSV3-neo containing the SV40 T antigen early regions and the neomycin resistance gene (Morikawa et al., 2001; Southern and Berg, 1982) using FuGENE 6 (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Three days after transfection, Geneticin (Invitrogen Corporation, Grand Island, NY) was added to select neomycin-resistant colonies. The concentration of Geneticin was gradually increased from 200 to 800  $\mu$ g/ml. After 3 weeks, single colonies of cells were isolated with cloning discs dipped in 0.05% trypsin/0.02% EDTA and placed in a 24-well plate.

Clones were expanded in a 12-well and then a 6-well plate and were then maintained in growth medium with 200  $\mu$ g/ml Geneticin (regular media). The immortalized hypothalamic neuronal cell line GT1-7 (Mellon et al., 1990) was used as a neuronal cell line control for characterization and comparison of cell lines and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Wild-type mouse embryonic fibroblasts (MEF-1 cells), fibroblasts homozygous for disruption of the low density lipoprotein receptor (LDLR)-related protein (LRP) gene (MEF-2 cells), and fibroblasts homozygous for disruption of both the LRP and the LDL receptor genes (MEF-4 cells) were kindly provided by Dr. Guojun Bu (Washington University in St. Louis, School of Medicine) (Narita et al., 2002).

### Media and cell harvesting

Cell clones were plated in 6-well plates and, after reaching confluency, were rinsed two times with phosphate-buffered saline (PBS, pH 7.4) followed by the addition of 800  $\mu$ l of serum-free media, Dulbecco's modified Eagle's medium/Ham's F-12 containing 1% N-2 supplement (Invitrogen Corporation), 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and 250 ng/ml fungizone. Twenty-four hours later, conditioned media were harvested and protease inhibitors (Complete protease inhibitor cocktail tablets; Roche Applied Science and 1 mM phenylmethylsulfonyl fluoride (PMSF)) were added to the media. Cells were washed with PBS and resuspended in 1 ml of cell lysis buffer containing 0.5% Nonidet P-40 and protease inhibitors (Complete protease inhibitor cocktail tablets and 1 mM PMSF). Human CSF, obtained as described (Fagan et al., 2000), and mouse brain lysates were used as controls for the media and cell lysates, respectively.

### ApoE ELISA

Sandwich ELISA for human apoE was performed as described (Mobley et al., 1989), except that the coating antibody was a mouse monoclonal antibody (WU E-4) raised against human apoE (Krul et al., 1988), and the detection antibody was a goat polyclonal antibody (Calbiochem, San Diego, CA) raised against

human apoE. Each antibody recognizes apoE2, apoE3, and apoE4 equally. Recombinant human apoE (Invitrogen Corporation) was used for standards.

#### *Western blot analysis*

Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology Incorporation, Rockford, IL). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed using 7.5–12.5% gels for separating and 5% for stacking with established methods (Laemmli, 1970). Following SDS-PAGE, proteins were transferred to a nitrocellulose membrane. GFAP proteins were detected by incubation with anti-GFAP antibodies (Santa Cruz Biotechnology, Santa Cruz, CA; 1:100 dilution) and the neuronal specific protein NeuN was detected with anti-NeuN antibodies (Chemicon International Incorporation, Temecula, CA; 1:100 dilution). SV40 T antigen was detected with an anti-SV40 T antigen antibody (gift from R. Weiner, University of California, San Francisco; 1:1000 dilution) and human apoE proteins were detected with the antihuman apoE monoclonal antibody (WU E-4; 1:1000 dilution). Mouse LRP and LDLR were detected with the rabbit anti-LRP and LDLR antibody (gift from G. Bu., Washington University in St. Louis, MO; both 1:2000 dilution). Immunoreactivity was visualized with enhanced chemiluminescence (Pierce Biotechnology Incorporation). Images were captured using the Kodak ImageStation 440CF. Densitometry and standard curves were generated using the Kodak 1D Image Analysis software.

#### *Nondenaturing gradient gel electrophoresis (NDGGE)*

Samples were electrophoresed on a nondenaturing 4–25% polyacrylamide gradient gel as described (Holtzman et al., 1999), transferred to nitrocellulose membrane, and probed with a polyclonal antibody to human apoE (Bioscience International, Saco, ME; 1:1000 dilution). Immunoreactivity was visualized with enhanced chemiluminescence (Pierce Biotechnology Incorporation). The size of the apoE-containing lipoproteins was estimated by comparing their location following NDGGE to size markers (Amersham Bioscience, Piscataway, NJ) with known hydrated diameters.

#### *Fractionation*

Serum-free conditioned medium (SFCM) from immortalized astrocyte cell lines expressing apoE2, apoE3, or apoE4 was concentrated 100-fold with a 10-kDa cut-off filter (Millipore Corporation, Bedford, MA) prior to fractionation. One milliliter of concentrated SFCM was fractionated by gel filtration chromatography (Biologic System, Bio-Rad Laboratories, Hercules, CA) with tandem Superose-6 HR 10/30 columns (Amersham Bioscience) in 150 mM NaCl with 1 mM EDTA, and 0.02% sodium azide as described (Fagan et al., 2000). Fractions of 400  $\mu$ l each were collected and analyzed.

#### *Total cholesterol and phospholipid analysis*

Total cholesterol in fractions was quantified with the Red Cholesterol Assay Kit (Molecular Probes, Eugene, OR) and phospholipid was quantified with the phospholipids B kit (Wako, Richmond, VA).

#### *Purification of apoE particles and electron microscopy*

To purify astrocyte-secreted apoE-containing lipoproteins, immunoaffinity columns were prepared as described (DeMattos et al., 1998) using a mouse monoclonal antibody to human apoE (WU E-4). ApoE was then purified from the conditioned media with the anti-apoE columns as described (DeMattos et al., 1998, 2001b). Purified apoE-containing lipoproteins were visualized by electron microscopy as described (Wang et al., 2002). Purified apoE-containing lipoproteins from immortalized astrocytes were adsorbed to glow-discharged carbon-coated copper grids. Grids were washed with two drops of deionized water and stained with two drops of freshly prepared 0.75% uranyl formate. Specimens were inspected with a Philips Tecnai 12 electron microscope operated at 120 kV and images were taken at a nominal magnification of 52,000 $\times$  using low-dose procedures.

#### *ApoE particle/A $\beta$ complex formation and assay*

Synthetic human A $\beta$  1–40 peptide (A $\beta$ <sub>40</sub>) (American Peptide Company Incorporation, Vista, CA) was dissolved at 2 mM in 100% dimethyl sulfoxide (DMSO) or in formic acid. For experiments in which A $\beta$  was dissolved in formic acid, vials of lyophilized A $\beta$ <sub>40</sub> (1 mg) were solubilized in 98% formic acid to a final concentration of 10 mg/ml (2 mM). Immediately prior to any mixing experiments with apoE-containing lipoproteins, the pH of the A $\beta$  stock was neutralized by performing a 1:1000 dilution into PBS containing 10 mM Tris pH 7.4. For experiments with DMSO, 1  $\mu$ l of a 2 mM A $\beta$  stock solution was diluted into 15  $\mu$ l of PBS (pH 7.4). Two microliters of this 0.125 mM A $\beta$  solution was gently mixed with 18  $\mu$ l of purified apoE-containing lipoproteins and incubated for 2 h at room temperature with A $\beta$ <sub>40</sub> in PBS (pH 7.4) in a total volume of 20  $\mu$ l. After 2 h, 5 $\times$  nonreducing Laemmli buffer was added to the mixture and complexes were separated on 10–20% polyacrylamide SDS/Tricine gels (Invitrogen Corporation). Alternatively, the gel running buffer (Tris-Glycine Native Running Buffer, Invitrogen Corporation) in the absence of detergent was combined with apoE/A $\beta$  mixtures and run on nondenaturing 3–30% polyacrylamide gradient gels in the absence of 2-mercaptoethanol, transferred to nitrocellulose membranes, and probed with anti-A $\beta$  monoclonal antibodies 2G3 (gift from Eli Lilly; 1:2000 dilution), 3D6 (gift from Eli Lilly; 1:1000 dilution), and 4G8 (Signet Laboratories Incorporation, Dedham, MA; 1:1000 dilution), or with a mouse monoclonal antibody to human apoE (WU E-4; 1:1000 dilution). The amount of A $\beta$  complexed with apoE-containing lipoproteins was estimated with densitometry using the Kodak ImageStation 440CF.

## **Results**

#### *Isolation and characterization of immortalized cells*

We transfected primary astrocyte cultures derived from post-natal day P1 or P2 *APOE2*, *APOE3*, and *APOE4* knock-in mice with a plasmid containing the SV40 T antigen and the selectable marker *neo*. Several weeks after transfection, we were able to obtain multiple cell clones derived from *APOE2* ( $n = 34$ ), *APOE3* ( $n = 22$ ), and *APOE4* ( $n = 22$ ) mice (Table 1). We determined the concentration of human apoE in serum-free conditioned media (SFCM) from each cell line. After 24 h of incubation, apoE levels,

Table 1

Quantitation of the level of apoE present in serum-free conditional media from immortalized astrocytes derived from *APOE2*, *APOE3*, and *APOE4* knock-in mice

Clone No.	apoE (ng/mg protein)	Clone No.	apoE (ng/mg protein)	Clone No.	apoE (ng/mg protein)
apoE2-line 1	894.8	apoE3-line 1	636.6	apoE4-line 1	2720.6
apoE2-line 2	1613.4	apoE3-line 2	678.3	apoE4-line 2	1273.9
apoE2-line 3	96.9	apoE3-line 3	860.7	apoE4-line 3	2066.4
apoE2-line 4	27.1	apoE3-line 4	1133.8	apoE4-line 4	1335.2
apoE2-line 5	2634.5	apoE3-line 5	1423.3	apoE4-line 5	2114.3
apoE2-line 6	2056.2	apoE3-line 6	1394.4	apoE4-line 6	872.7
apoE2-line 7	239.0	apoE3-line 7	3307.8	apoE4-line 7	2330.4
apoE2-line 8	153.7	apoE3-line 8	1695.2	apoE4-line 8	1435.9
apoE2-line 9	400.6	apoE3-line 9	946.9	apoE4-line 9	1220.5
apoE2-line 10	885.3	apoE3-line 10	1094.6	apoE4-line 10	115.5
apoE2-line 11	0.0	apoE3-line 11	1817.0	apoE4-line 11	1573.0
apoE2-line 12	596.1	apoE3-line 12	3674.7	apoE4-line 12	1345.4
apoE2-line 13	362.8	apoE3-line 13	394.0	apoE4-line 13	2376.8
apoE2-line 14	0.0	apoE3-line 14	939.0	apoE4-line 14	943.5
apoE2-line 15	1682.6	apoE3-line 15	1551.9	apoE4-line 15	1504.3
apoE2-line 16	420.2	apoE3-line 16	910.8	apoE4-line 16	899.2
apoE2-line 17	82.5	apoE3-line 17	2462.6	apoE4-line 17	417.6
apoE2-line 18	429.9	apoE3-line 18	736.6	apoE4-line 18	607.6
apoE2-line 19	447.0	apoE3-line 19	3569.7	apoE4-line 19	621.4
apoE2-line 20	342.9	apoE3-line 20	3334.1	apoE4-line 20	540.2
apoE2-line 21	1600.9	apoE3-line 21	1270.1	apoE4-line 21	1547.8
apoE2-line 22	289.5	apoE3-line 22	116.4	apoE4-line 22	1539.5
apoE2-line 23	1398.9				
apoE2-line 24	587.0				
apoE2-line 25	77.9				
apoE2-line 26	328.1				
apoE2-line 27	893.7				
apoE2-line 28	825.4				
apoE2-line 29	919.5				
apoE2-line 30	283.7				
apoE2-line 31	20.7				
apoE2-line 32	15.6				
apoE2-line 33	0.0				
apoE2-line 34	10.7				

Cells were grown in 6-well plates. After reaching confluency, 1 ml of serum-free media was placed on the cells and following 24 h the media were analyzed for apoE concentration by ELISA. ApoE values are expressed as ng per mg of protein in cell lysates. The highlighted cell lines are the highest expressing lines that were further characterized.

as assessed by ELISA, ranged from 1 to 1.7  $\mu\text{g/ml}$  from the highest secreting clones. These apoE levels overlap with levels found in the media derived from primary cultured astrocytes (0.5–2  $\mu\text{g/ml}$ ) prepared in the same fashion (Sun et al., 1998). We then determined the amount of apoE in SFCM from each clone normalized to the amount of cellular protein (Table 1). Cell lines with the highest apoE expression levels (apoE2–5, apoE3–12, and apoE4–7) were characterized in more detail. Cells from all three cell lines exhibited flat, polygonal shapes and formed monolayers that were similar in appearance to primary astrocyte cultures (Fig. 1). Cell lysates and SFCM from these immortalized astrocytes were analyzed by SDS-PAGE followed by Western blotting for a variety of proteins. In SFCM, apoE was detected as a 34- to 36-kDa doublet from *APOE2*-, *APOE3*-, and *APOE4*-expressing cells that comigrated with apoE present in human CSF (Fig. 2A). In nonreducing gels, an ~80-kDa apoE dimer was detected in cells expressing apoE2 and apoE3 due to the presence of one or two cysteine residues, respectively, in each molecule (data not shown). The astrocyte-specific 50-kDa protein GFAP was detected in cell lysates derived from primary astrocytes from wild-type mice as well as from all clones of immortalized astrocytes that were

analyzed (Fig. 2B). GFAP was not detected in cell lysates derived from the neuronal cell line GT1–7 (Fig. 2B). Neu-N, a 46- to 66-kDa neuronal-specific protein, was detected in mouse brain homogenates but not in cell lysates from immortalized astrocytes (Fig. 2C). Two of the main receptors for apoE, the low-density lipoprotein receptor (LDLR) as well as the LDLR-related protein (LRP), were expressed by each *APOE2*, *APOE3*, and *APOE4* expressing cell lines that were assessed (Fig. 2D). The SV40 T antigen was also detected in Western blots of cell lysates from immortalized astrocyte clones (data not shown), demonstrating that the cell lines were derived from cells stably transfected with the SV40 T antigen-containing plasmid.

#### Purification and size analysis of apoE-containing lipoproteins secreted by immortalized astrocytes

Primary astrocytes secrete nascent murine and human apoE into apoE-containing HDLs that are disc-like in appearance since they contain cholesterol and phospholipid but no cholesteryl-ester core (DeMattos et al., 2001b; Fagan et al., 1999; LaDu et al., 1998). To determine if the apoE2-, apoE3-, and apoE4-secreting



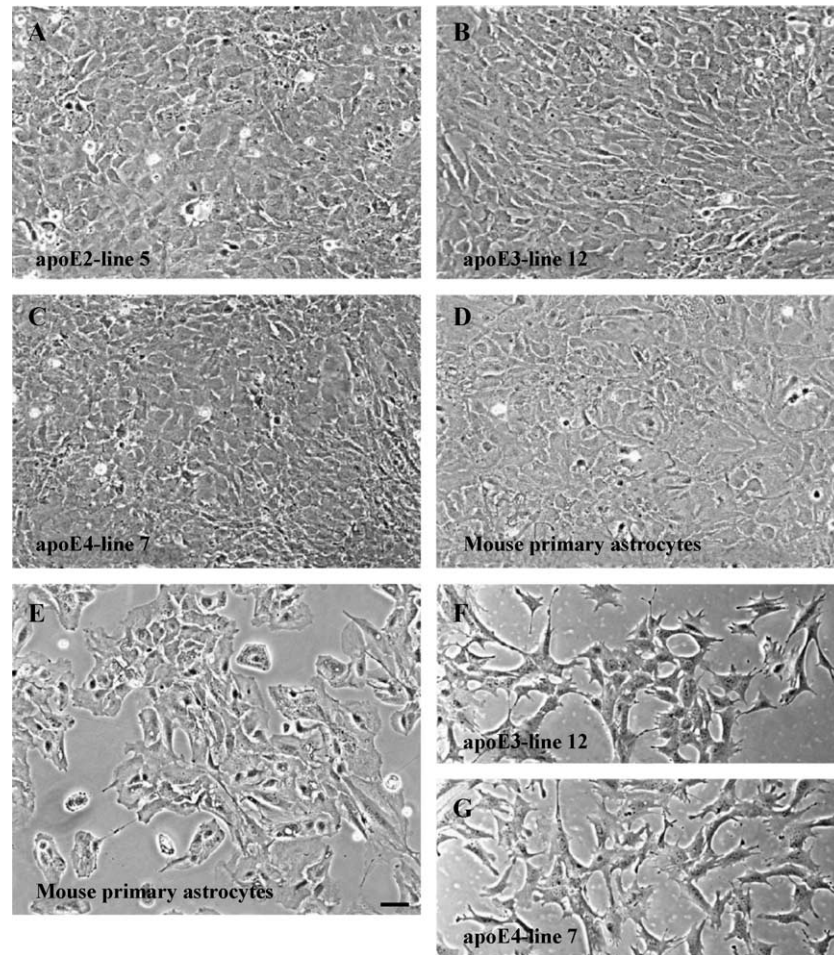


Fig. 1. The morphology of apoE-secreting immortalized astrocytes is similar to that of primary astrocytes. Phase contrast images of confluent (A–C) and subconfluent cultures (F–G) of immortalized astrocytes that secrete (A) apoE2, (B and F) apoE3, and (C and G) apoE4 revealed that the cell lines form monolayers with a “cobblestone” appearance that is of similar morphology to that seen from primary cultured murine astrocytes derived from P1 mice (D and E). Scale bar in E equals 90  $\mu$ m.

cell lines secrete apoE in similar particles, we examined concentrated SFCM as well as immunoaffinity purified apoE particles from these cells and assessed them using nondenaturing gradient gel electrophoresis (NDGGE) followed by Western blotting as well as electron microscopy. Analysis of ApoE present in SFCM and after immunoaffinity purification by these methods reveals particles ranging in diameter from  $\sim$ 7 to 17 nm (Figs. 3A and B). In some experiments, there were no always discreet bands of particles in the larger size range (12–17 nm) as assessed by NDGGE; however, there was no consistent difference between the apoE isoforms or cell lines in this regard. The size of these particles is very similar to that seen with particles derived from primary astrocytes, 8–17 nm (Fagan et al., 1999; DeMattos et al., 2001a,b and see Fig. 7). The apparent larger diameter of apoE in human CSF is consistent with the fact that apoE in CSF acquires a cholesteryl-ester core (Fig. 3A). No apoE immunoreactivity was detected in SFCM derived from apoE<sup>-/-</sup> astrocytes (Fig. 3A). Analysis of apoE particles purified by immunoaffinity chromatography by SDS-PAGE followed by Western blot revealed that apoE was still present as a 34- to 36-kDa doublet (Fig. 3C) as was seen with SFCM from these cells. In addition, we analyzed the appearance of purified apoE particles from these cells by electron microscopy (Fig. 4). Electron microscopy

images of immunoaffinity purified ApoE particles from these cells revealed predominantly disc-like particles ranging in diameter from 8 to 19 nm. The size and morphology distribution of ApoE2-, E3-, and E4-containing particles here are virtually identical to those observed for ApoE particles secreted by primary astrocytes (DeMattos et al., 2001b).

#### *Immortalized astrocytes secrete apoE in HDL-like lipoproteins*

Primary astrocytes secrete apoE in nascent HDL containing similar amounts of apoE, cholesterol, and phospholipid (DeMattos et al., 2001b; Fagan et al., 1999; LaDu et al., 1998). To determine if apoE particles secreted by immortalized astrocytes were similar in composition to particles secreted by primary astrocytes, we collected SFCM from an immortalized astrocyte cell line secreting apoE3, concentrated it, and subjected it to size exclusion chromatography. ApoE and cholesterol were at highest concentrations in fractions 30–48, which corresponds in size to HDL-like lipoproteins seen in plasma, CSF, and from primary astrocyte cultures (Fig. 5) (Fagan et al., 1999, 2000). Superimposition of the same data from primary astrocytes revealed similar levels of apoE and cholesterol in overlapping fractions (Fig. 5; Fagan et al., 1999; Sun et al., 1998). The mass of apoE to cholesterol was in the range

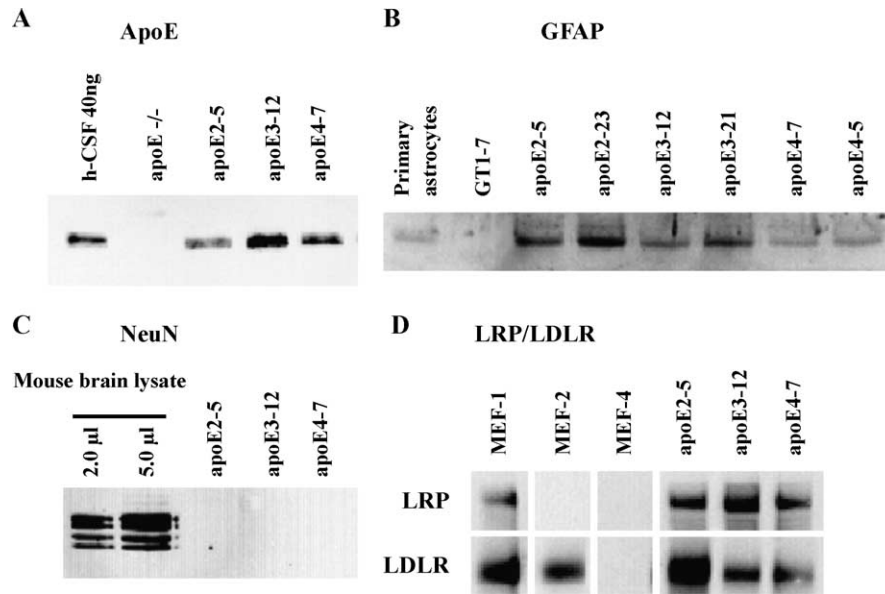


Fig. 2. Immortalized astrocytes from *APOE* knock-in mice express glial and not neuronal markers. (A) ApoE (40 ng) from serum-free conditioned media or (B and C) cell lysates (40 μl, 10–20 μg protein/lane) were subjected to 12% SDS-PAGE followed by Western blot. (A) ApoE is present in media from E2, E3, and E4 secreting cells but not in media from apoE<sup>-/-</sup> astrocytes. H-CSF = human CSF containing 40 ng apoE was loaded as a positive control. (B) Western blot for the astrocyte-specific protein GFAP reveals that several lines of immortalized astrocytes express GFAP. No GFAP is detected in cell lysates (10 μg protein/lane) from the hypothalamic neuronal cell line GT1–7. (C) Western blot for the neuronal-specific protein Neu-N reveals the presence of Neu-N in mouse brain lysates but not in the immortalized astrocyte cell lines. (D) Cell lysates (10 μg protein/lane) from mouse embryonic fibroblasts (MEF)-1 (expressing LRP and LDLR), MEF-2 (lacking LRP expression), MEF-4 (lacking LRP and LDLR expression), and apoE2, E3, and E4 expressing astrocyte cell lines, were subjected to 3–8% SDS-PAGE followed by Western blot for LRP and LDLR. Three representative cell lines express the apoE receptors LRP and LDLR.

of 3–5:1 which is just slightly higher than that found in particles derived from primary astrocytes (2–3:1) (DeMattos et al., 2001b). Analysis of apoE and cholesterol in apoE2 and apoE4 particles revealed similar particle composition.

*Interaction of astrocyte-secreted apoE with Aβ*

Prior experiments have shown that cell line-derived apoE can form an SDS-stable complex with Aβ (apoE3 > apoE4) (LaDu et

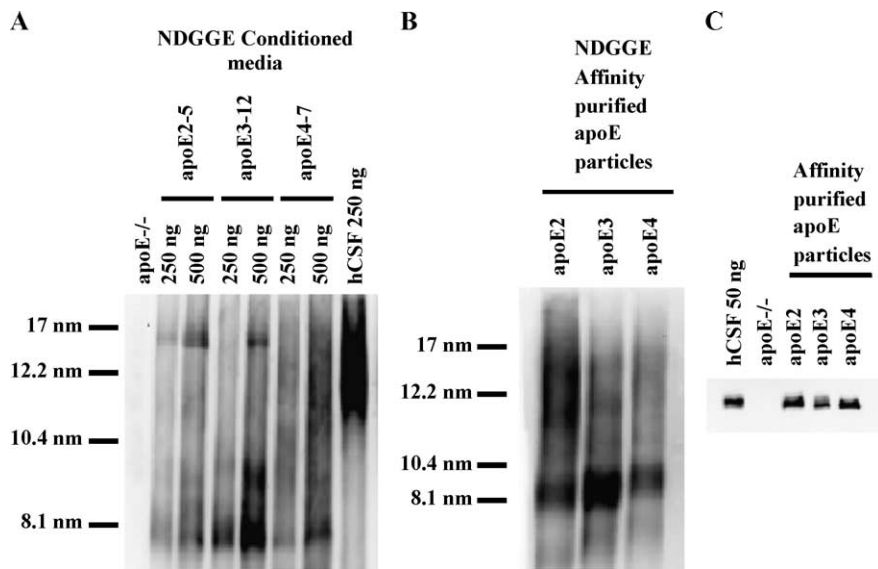


Fig. 3. Human apoE secreted by immortalized astrocytes is present in HDL-like particles. (A) Five milliliters of serum-free conditioned media was concentrated to 1 ml. Following apoE ELISA, the amount of conditioned media containing the indicated amount of apoE was loaded into each lane. Samples were analyzed by nondenaturing gradient gel electrophoresis (NDGGE 4–25%) followed by Western blot for apoE. The same analysis was done in (B) except that the apoE (600 ng) derived from immortalized astrocytes was first immunoaffinity purified on an anti-apoE column. ApoE secreted by the astrocyte cell lines is present in 7- to 17-nm particles. In (A), hCSF = human CSF, apoE<sup>-/-</sup> is conditioned media from apoE<sup>-/-</sup> astrocytes. Size markers in (A) and (B) represent the hydrated diameter, in nanometers, of known proteins run as standards. (C) SDS-PAGE of immunoaffinity purified apoE-containing HDL from immortalized astrocytes followed by Western blot for apoE. Fifty nanograms of apoE was loaded per lane. ApoE is seen as a doublet at ~34–35 kDa.

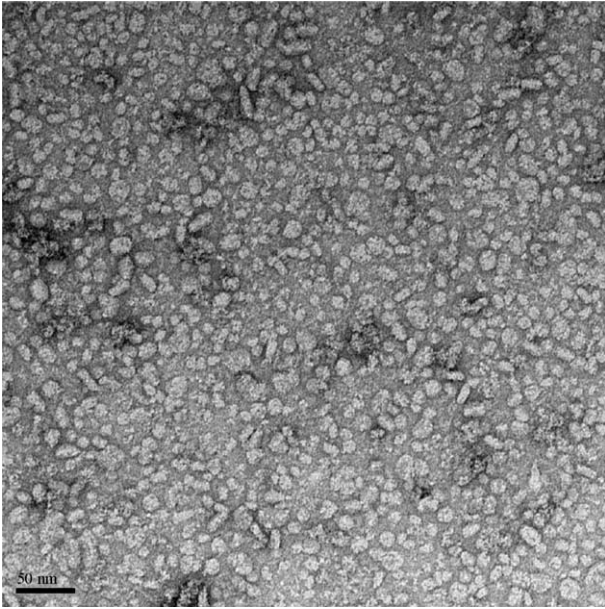


Fig. 4. Electron microscopic image of negatively stained immunoaffinity purified apoE3-containing lipoproteins from immortalized astrocytes. Scale bar is equal to 50 nm.

al., 1994, 1995). To determine if this was the case with astrocyte-derived apoE, we incubated immunoaffinity purified apoE3 and apoE4 (2  $\mu$ g) with A $\beta$ <sub>40</sub> (1  $\mu$ g) for 2 h (molar ratio apoE/A $\beta$  1:4.5) and looked for evidence of complex formation by denaturing as well as nondenaturing gel electrophoresis. Under denaturing conditions, >99% of the A $\beta$  was present as monomers, dimers, and trimers as visualized after a 30-s exposure of the Western blot (Fig. 6A). When A $\beta$  is first dissolved in formic acid instead of DMSO and then diluted, almost all the A $\beta$  is monomeric with almost no A $\beta$  dimers and trimers seen (data not shown). After a longer exposure time (5 min), an apparent apoE3-A $\beta$  complex running at ~40 kDa could be detected, likely representing an SDS-stable apoE-A $\beta$  complex (Fig. 6B) that has previously been described (Aleshkov et al., 1997; LaDu et al., 1994, 1995; Yang et al., 1997). A faint band likely representing an apoE4/A $\beta$  complex could be seen at even longer exposure times (data not shown). We have seen very similar results utilizing apoE3 and apoE4 derived from primary astrocytes (data not shown). After stripping the same membrane followed by apoE Western blot, a 34-kDa apoE was detected (Fig. 6C). To mimic more physiological interactions that might occur between apoE (secreted by glia) and soluble A $\beta$  (predominantly secreted by neurons) in the brain interstitial fluid (ISF) space, we also incubated immunoaffinity isolated apoE particles (2  $\mu$ g) with A $\beta$  (1–5  $\mu$ g) (molar ratio apoE/A $\beta$  range 1:4.5–1:22.5) and looked for the presence of complex formation under nondenaturing conditions. To investigate the possibility that apoE interaction with A $\beta$  results in the formation of complexes that could be SDS sensitive, the apoE and A $\beta$  mixtures were loaded and analyzed by NDGGE (in the absence of detergent) followed by Western blotting. Interestingly, a much larger percentage of A $\beta$  interacted with apoE-containing HDL-like particles under these nondenaturing (5–20%) as compared to denaturing conditions (<1%) (Figs. 7C and B) as determined by densitometry. A $\beta$  comigrated with the larger apoE-containing HDLs that were ~10–17 nm in size, suggesting greater interaction with larger particles containing more lipid (Fig. 7B). There was no clear difference

between the amount of A $\beta$  that associated with apoE3- or E4-containing HDL under these conditions. In experiments with apoE2 and A $\beta$ , we found qualitatively similar results as with apoE3 and E4 (data not shown). In addition to comparing the interaction between A $\beta$  and astrocyte cell line-secreted apoE, we also examined the interaction between A $\beta$  and apoE particles

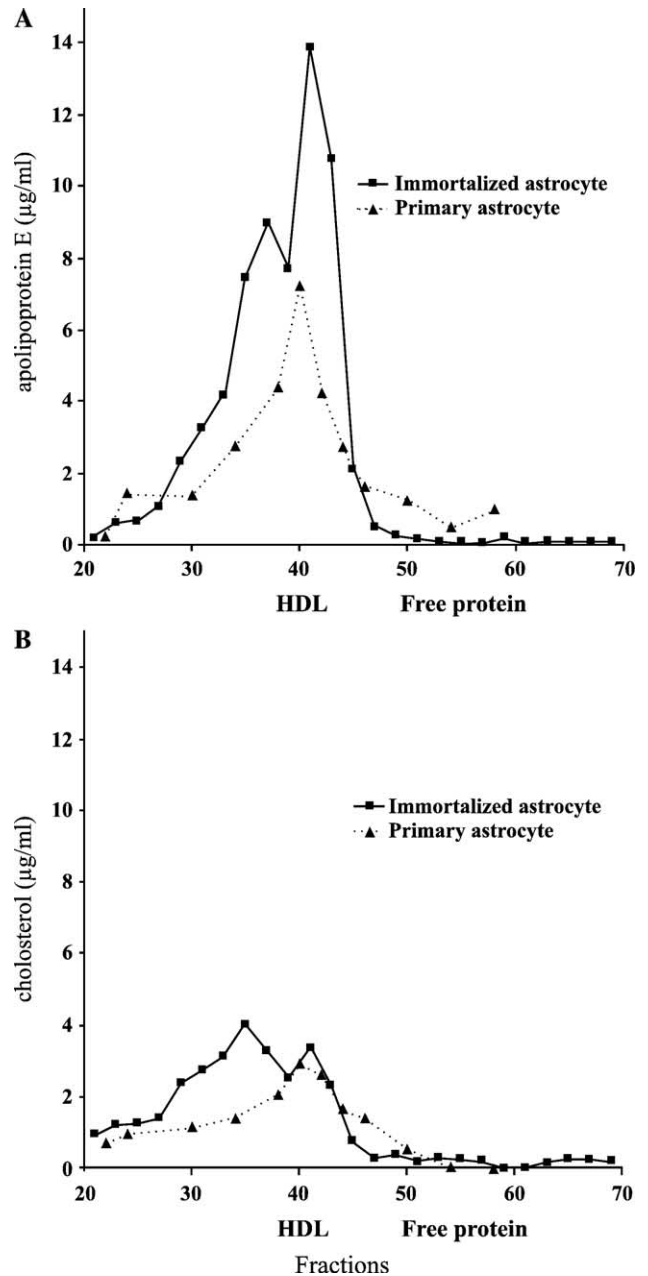


Fig. 5. Size exclusion chromatography of serum-free conditioned media from a human apoE3-secreting immortalized astrocyte cell line (squares) and primary astrocyte cultures from an apoE4-transgenic mouse (Fagan et al., 1999) (triangles) reveal that apoE and cholesterol are colocalized. One hundred milliliters of serum-free conditioned media derived from apoE3-line 12 and 50 ml from primary cultures from apoE4-transgenic mice were concentrated to 1 ml and then fractionated via size exclusion chromatography. Four hundred-microliter fractions were analyzed for (A) apoE protein by ELISA and (B) cholesterol. ApoE and cholesterol are colocalized in the plasma HDL size range indicating that apoE is present in HDL-like particles.



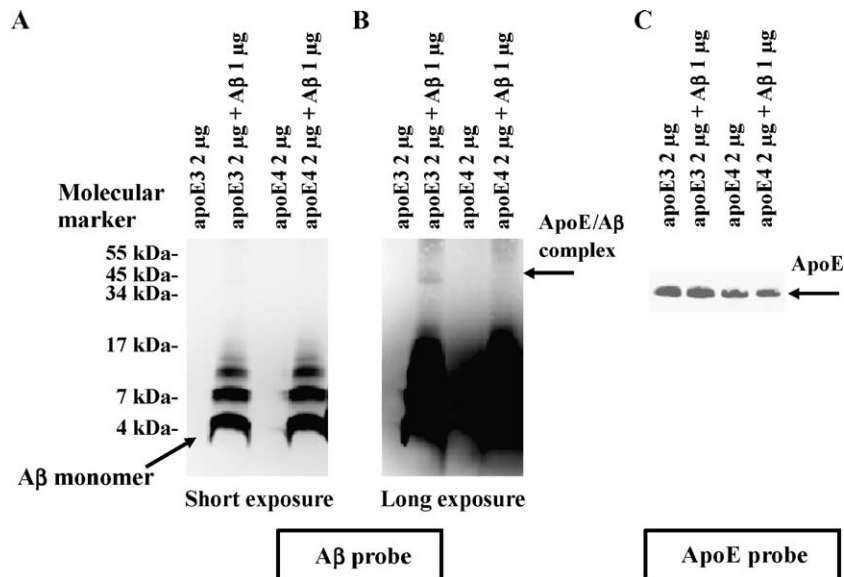


Fig. 6. Interaction of immortalized astrocyte-derived apoE isoforms with  $A\beta_{40}$  under denaturing conditions by SDS-PAGE. Immunoaffinity purified apoE3 and apoE4 (2  $\mu$ g, respectively) was incubated with synthetic human  $A\beta_{40}$  (1  $\mu$ g). Following their incubation for 2 h at room temperature, apoE- $A\beta$  complex formation was assessed under denaturing conditions by 10–20% SDS-Tricine-PAGE. Gels were then analyzed by Western blot for  $A\beta$  (A and B) and apoE (C). Short exposure (30 s) of the blot shown in (A) and long exposure of the blot (5 min) is shown in (B).  $A\beta$  monomers, dimers, and trimers were detected after short exposure (A). After long exposure, an apoE3/ $A\beta$  complex was detected at  $\sim$ 40 kDa. The  $A\beta$  in this complex is  $<$ 1% of the  $A\beta$  loaded in the lane as detected by densitometry. After stripping the same membrane followed by Western blot for apoE, apoE was detected with a molecular weight of  $\sim$ 34 kDa (C). Size markers are in kilodaltons (kDa).

purified from primary astrocyte cultures. In this experiment, molar ratios of apoE/ $A\beta$  of 1:1 or 1:0.5 were utilized (600 ng apoE, 70 or 37.5 ng  $A\beta$ ). As with our cell line-secreted apoE, apoE3 and apoE4 were in particles that ranged in size from  $\sim$ 8 to 17 nm (Fig. 7C). After mixing  $A\beta_{40}$  with apoE3 and apoE4,  $\sim$ 20% of the  $A\beta$  comigrated with the larger 10- to 17-nm particles (Fig. 7D), as was seen with the astrocyte cell line-derived particles (Fig. 7B). Qualitatively, there was no clear isoform-specific difference in the interaction.

## Discussion

ApoE plays an important role in plasma lipoprotein metabolism. In the CNS, its function is less clear; however, *APOE* allelic differences are associated with differential risk for developing AD, CAA, as well as for altered clinical progression following the onset of certain CNS diseases such as multiple sclerosis, brain hemorrhage, and trauma (for a review, see Holtzman, 2001; Teter, 2000). In order to better understand the normal biological role of apoE and its role in the pathogenesis of AD and other CNS diseases, it would be useful to study apoE using preparations of apoE-containing lipoproteins that are similar to that found in the parenchyma of the CNS. While we have previously developed a method to purify apoE-containing HDL particles from primary astrocytes, obtaining large quantities of apoE from primary cultures for detailed biochemical studies remains a challenging task due to the large number of animals required and the relatively small amount of apoE that can be purified from each set of cultures. To facilitate studies of astrocyte-derived human apoE, we have established new lines of immortalized astrocytes from human *APOE*-expressing knock-in mice. We confirmed that these cell lines express astrocytic but not neuronal proteins. In addition, we

found that several cell lines secrete apoE2, E3, and E4 in HDL-like lipoproteins that have similar biochemical and structural properties to primary astrocyte-derived apoE particles, including lipid content, morphology, and molecular size. We also examined the interaction of apoE-containing lipoproteins derived from these cells with  $A\beta_{40}$ . While we found differential interactions between apoE3 and apoE4 with  $A\beta_{40}$  in regard to sodium dodecyl sulfate (SDS)-stable complex formation (Fig. 6B), a very small percentage of  $A\beta$  ( $<$ 1%) formed an SDS-stable complex. In contrast, a relatively large percentage of  $A\beta_{40}$  formed a complex between apoE3 and apoE4 particles in the presence of physiological buffers and there was a similar degree of association between the isoforms (Figs. 7B and D). This suggests that under physiological conditions such complexes may be important to modulate the ability of apoE to influence soluble  $A\beta$  clearance and diffusion in the CNS parenchyma. These cell lines should be useful tools for studying cell biological, biochemical, and other properties of astrocyte-derived apoE-containing lipoproteins and their role in health and disease.

The apoE particles secreted by the cell lines appear to have properties very similar to lipoprotein particles secreted by primary astrocytes. We and others have previously shown that primary astrocytes secrete apoE as nascent HDL-like lipoproteins that (1) range in size from 7 to 17 nm; (2) are disc-like in appearance; (3) contain apoE, phospholipids, and cholesterol but no cholesteryl ester core; and (4) contain apoE as their sole apoprotein constituent (DeMattos et al., 2001b; Fagan et al., 1999; LaDu et al., 1998; Pitas et al., 1987a). Virtually identical properties were seen from the astrocyte cell line secreted apoE. Importantly, compared to apoE particles that are secreted by transfected cell lines, the nascent astrocyte-secreted particles contain a much greater amount of cholesterol. For example, the mass ratio of apoE/cholesterol in apoE particles secreted by a neuronal cell line was 73–88:1



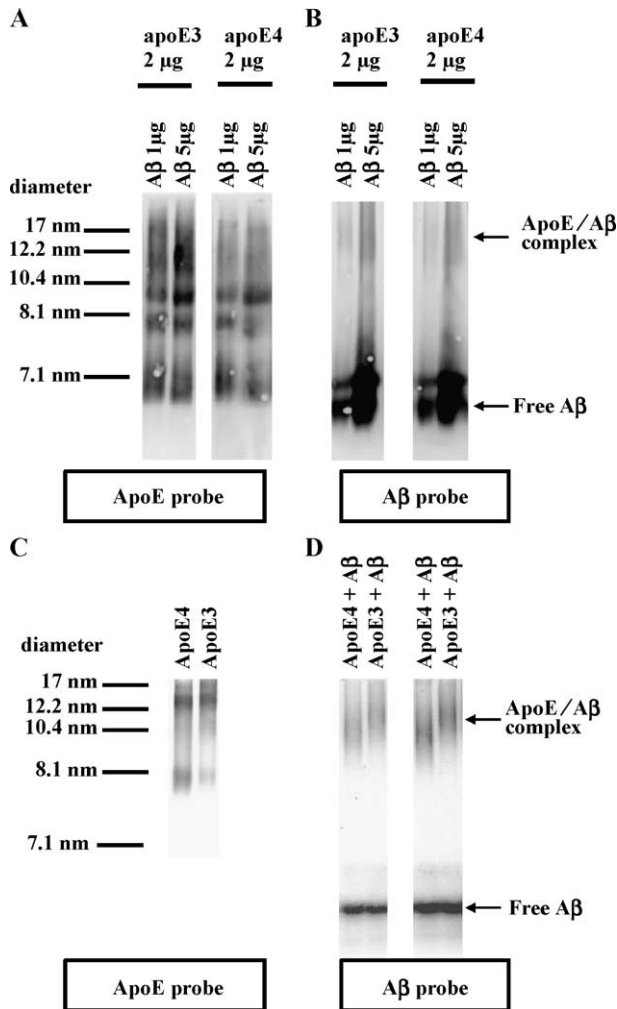


Fig. 7. Interaction of immortalized astrocyte-derived and primary astrocyte-derived apoE isoforms with A $\beta_{40}$  under nondenaturing conditions as assessed by NDGGE. (A and B) Immunoaffinity purified apoE3 and apoE4 from immortalized astrocytes were incubated with synthetic A $\beta_{40}$ . Following their incubation for 2 h at room temperature, apoE–A $\beta$  complex formation was assessed under nondenaturing conditions by NDGGE. Gels were then analyzed by Western blot for apoE (A) and A $\beta$  (B). ApoE is present in HDL-like particles ranging in size from 7 to 17 nm. The amount of A $\beta$  that associates with the purified apoE3- and apoE4-containing HDL when analyzed under nondenaturing conditions is similar. A $\beta$  associates with the larger 10- to 17-nm HDL-like particles. (C and D) Immunoaffinity-purified apoE3 and apoE4 (C) from primary astrocyte cultures were mixed with A $\beta_{40}$  that had previously been dissolved in formic acid. After resolving the complexes by NDGGE, the relative amount of A $\beta$  associated with apoE particles was determined by  $^{125}\text{I}$ -linked to streptavidin followed by phosphorimager quantitation (D). Purified apoE3 and apoE4 were seen in particles that ranged in size from ~8 to 17 nm (C). After mixing A $\beta_{40}$  with apoE3 and apoE4 (molar ratio: apoE vs. A $\beta$  = 1:0.5 (left column) and 1:1 (right column), respectively), A $\beta$  comigrated with the larger 10- to 17-nm particles (D) as was seen in (B). Phosphorimager analysis revealed that ~20% of the A $\beta$  had associated with the apoE particles. Size markers represent the hydrated diameter, in nanometers, of proteins run as standards.

(DeMattos et al., 2001a), whereas the ratio of apoE/cholesterol with the immortalized astrocytes is ~3–5:1, similar to that seen with primary astrocytes. The apoprotein/lipid ratio of lipoprotein particles can markedly influence properties such as receptor binding (Narita et al., 2002) and interactions with other molecules

such as A $\beta$  (LaDu et al., 1994, 1995; Tokuda et al., 2000). Importantly, two recent studies demonstrate that if apoE is poorly lipidated in the CNS due to the absence of the protein ATP-binding cassette transporter A-1 (ABCA1), apoE particles are rapidly metabolized in the brain resulting in levels that are 2% of normal in the CSF and <20% of normal in the cortex (Hirsch-Reinshagen et al., 2004; Wahrle et al., 2004). Thus, studying apoE derived from astrocyte cell lines that are lipidated by the cellular machinery possessed by these cells is likely to be very useful to better model apoE's effects and interactions in the CNS.

ApoE has been demonstrated to modulate A $\beta$  clearance and fibrillogenesis both in vitro (Beffert et al., 1999; Castano et al., 1995; Evans et al., 1994; Koistinaho et al., 2004; Ma et al., 1994; Yang et al., 1997, 1999) and in vivo (Bales et al., 1997; DeMattos et al., 2004; Holtzman et al., 2000; Shibata et al., 2000). In vivo, human apoE suppresses the onset of A $\beta$  deposition and subsequently results in an isoform-specific effect (E4 > E3 > E2) on the amount of A $\beta$  deposition, fibril formation, and neuritic plaque formation (Fagan et al., 2002; Holtzman et al., 2000). To date, while the effects of apoE on A $\beta$  clearance have been seen in in vitro and in vivo studies, an isoform-specific effect of apoE has been difficult to identify (DeMattos et al., 2004; Koistinaho et al., 2004). Some in vitro studies do show differential effects of apoE isoforms on A $\beta$  fibrillogenesis (E4 > E3) with some studies demonstrating that apoE inhibits and others showing apoE enhances A $\beta$  fibril formation (Castano et al., 1995; Evans et al., 1994; Ma et al., 1994). Importantly, while lipidated apoE has been used in some A $\beta$  binding and association studies (LaDu et al., 1994, 1995; Tokuda et al., 2000), lipidated apoE has not been extensively utilized in studies examining the effect of apoE on A $\beta$  clearance and fibrillogenesis. The apoE particles derived from astrocytes should provide a novel tool to assist in studying the effects of apoE on A $\beta$  fibrillogenesis and clearance using more physiological reagents. This should allow for a better determination of the extent to which apoE is influencing A $\beta$  conformation, clearance, or both, and provide new insights into the effects of apoE on A $\beta$  in the interstitial fluid of the brain. Such effects are likely to be relevant to mechanisms underlying A $\beta$  deposition and toxicity in AD and CAA.

In conclusion, apoE influences risk for AD, CAA, and outcome after certain CNS diseases. Studies of apoE that is in unique HDL-like lipoproteins secreted by astrocytes may allow for better modeling of both normal and disease-related functions of apoE in the brain. Our studies suggest that A $\beta_{40}$  can directly interact with astrocyte-derived apoE3- and apoE4-containing lipoproteins to a similar degree in physiological buffers. The immortalized astrocyte cell lines that express human apoE isoforms will be a useful tool to study astrocyte-derived apoE and its functions.

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