Apolipoprotein E does not cross the blood-cerebrospinal fluid barrier, as revealed by an improved technique for sampling CSF from mice

Min Liu, David G. Kuhel, Ling Shen, David Y. Hui, and Stephen C. Woods

1Department of Pathology and Laboratory Medicine, University of Cincinnati College of Medicine, Cincinnati, Ohio; and 2Department of Psychiatry and Behavioral Neuroscience, University of Cincinnati College of Medicine, Cincinnati, Ohio

Submitted 11 May 2012; accepted in final form 29 August 2012

Liu M, Kuhel DG, Shen L, Hui DY, Woods SC. Apolipoprotein E does not cross the blood-cerebrospinal fluid barrier, as revealed by an improved technique for sampling CSF from mice. Am J Physiol Regul Integr Comp Physiol 303: R903–R908, 2012. First published August 29, 2012; doi:10.1152/ajpregu.00219.2012.—Apolipoprotein E (apoE) is a 34-kDa glycoprotein that is important in lipoprotein metabolism both peripherally and centrally. Because it is primarily produced in the liver, apoE observed in the brain or cerebrospinal fluid (CSF) could have originated in the periphery; i.e., circulating apoE may cross the blood-brain barrier (BBB) and/or enter CSF and be taken up by brain cells. To determine whether this occurs, a second-generation adenovirus encoding human apoE3 was administered intravenously (iv) to C57BL/6J mice, and the detection of human apoE3 in the CSF was used as a surrogate measure of central availability of this protein utilizing an improved method for sampling CSF from mice. This improved technique collects mouse CSF samples with a 92% success rate and consistently yields relatively large volumes of CSF with a very low rate of blood contamination, as determined by molecular assessment of apolipoprotein B, a plasma-derived protein that is absent in the central nervous system. Through this improved method, we demonstrated that in mice receiving the administered apoE3 adenovirus, human apoE3 was expressed at high levels in the liver, leading to high levels of human apoE3 in mouse plasma. In contrast, human apoE3 levels in the CSF, as assessed by a sensitive ELISA, were essentially undetectable in human apoE3 adenovirus-treated mice, and comparable to levels in LacZ adenovirus-treated control mice. These data indicate that apoE in the CSF cannot be derived from the plasma pool and, therefore, must be synthesized locally in the brain.

Cerebrospinal fluid collection; cisternum magnum; adenovirus; apolipoprotein E

APOLIPOPROTEIN E (APOE) IS A 299-AMINO-ACID PROTEIN WITH A 34-KDA MOLECULAR WEIGHT (33). Although it is synthesized in several areas of the body, the liver accounts for most circulating apoE (19). ApoE plays a key role in lipid transport and lipoprotein metabolism (18). It mediates the uptake and degradation of chylomicron and very low-density lipoprotein remnants by acting as a ligand for the low-density lipoprotein (LDL) receptor and the LDL receptor-related protein 1 (LRP1) (1, 18). Because apoE is involved directly in the uptake and distribution of plasma lipids, it is not surprising that it has been implicated in cardiovascular disease, and apoE deficiency is associated with high serum cholesterol and triacylglycerol levels and leads to premature atherosclerosis (23).

ApoE is also present in the brain. Central apoE is the most abundant component of HDL-like lipoproteins in the cerebrospinal fluid (CSF) (22), and it has a pivotal role in brain lipid transport and the maintenance of cell membranes, myelin, and neural networks under normal conditions (3, 11). Recently, we demonstrated that central apoE is important in the control of food intake and body weight (27, 28), and these effects might be mediated by the LRP1 receptor (16). Because apoE is present in both blood and CSF, any apoE found in the brain could have originated in the periphery; i.e., circulating apoE may cross the blood-brain barrier (BBB) and/or enter CSF and be taken up by brain cells. One of main goals of the current studies was to determine whether apoE enters the brain from the blood as assessed by changes in CSF levels when its plasma levels are increased.

While murine apoE is monomorphic, apoE occurs in three major common isoforms in humans. ApoE3 (112 Cys, 158 Arg) is the most common isoform and is considered the wild type. ApoE4 (112 Arg, 158 Arg) and apoE2 (112 Cys, 158 Cys) differ from apoE3 by single amino acid substitutions. The homology of mouse and human apoE is ~70%, but human and mouse apoE have similar molecular weights (34 vs. 33 kDa) (35). We used a second-generation adenovirus to express human apoE3 uniquely in the liver of C57BL/6J mice. When human apoE3 is induced in mouse liver, it is secreted into the blood, and its levels can be assessed differentially from native mouse apoE. If this human apoE3 appears in the CSF, it is, therefore, likely that it came from the circulation, either directly via passage across the choroid plexus or indirectly by passage through the BBB followed by diffusion/convection transport from the brain interstitial fluid to the CSF (26). We reasoned that assessing the CSF for human apoE3 in apoE3 adenovirus-treated mice would enable us to ascertain possible transport. If both the plasma and CSF have human apoE3 protein, and synthesis is restricted to the liver, it would imply that the apoE enters the brain from the blood. If, on the other hand, human apoE3 is only present in blood but not present in the CSF, it would imply that apoE does not cross the BBB and that native mouse apoE that is found in the CSF is likely made within the brain.

Key to such experiments is the ability to obtain CSF samples from mice that have absolutely no blood contamination. Because mice are small, making it difficult to obtain blood-free CSF samples in sufficient quantities for assessing its constituents, a reliable approach had to be devised. Therefore, we modified a technique for obtaining relatively large samples and developed a novel method to screen CSF samples for blood contamination. Using this improved technique, we demonstrated that the human apoE3 induced by adenovirus does not exist in mouse CSF, indicating that apoE is not able to cross the BBB, and the central apoE is produced within the brain.
MATERIALS AND METHODS

Animals. Adult male C57BL/6J mice (22–28 g, Jackson Laboratory, Bar Harbor, ME) were housed in a temperature- and humidity-controlled vivarium on a 12:12-h light-dark cycle (lights on at 0600) with ad libitum access to pelleted laboratory chow (Teklad 7912; Madison, WI) and water. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

Materials. A second-generation recombinant adenovirus encoding human apoE3 (AdhapoE3) was constructed by Tsukamoto et al. (31). Previous studies have demonstrated that this adenovirus sustains expression of human apoE3 uniquely in the liver of apoE-deficient mice (31, 32). An electrode holder with a 90° bend at its distal end (model 1769, Kopf, Tujunga, CA) (Fig. 1A) was attached to a rat stereotaxic instrument (Stoelting, Wood Dale, IL). A glass capillary tube (cat. no. 53508–466; VWR, Radnor, PA) was heated and pulled manually. The tapered end of the glass capillary had an approximate diameter of 0.5 mm and was sharpened with a fine whetstone to provide a sharp tip (Fig. 1B). The rabbit polyclonal antibody against rodent anti-apoB, reported previously (17), was obtained from Dr. Patrick Tso’s laboratory. The chemicals were purchased from Sigma (St. Louis, MO).

Procedures. The CSF sampling procedure is a modification and improvement of a previously reported technique (14), and we additionally included a sensitive molecular method for identifying and thus discarding any blood-contaminated samples. To collect CSF, a straight, pulled, and sharpened glass capillary tube was mounted onto the electrode holder and adjusted, so that it was perpendicular to the ear bars and thus parallel to the long axis of the stereotaxic apparatus (Fig. 1A). Each mouse was anesthetized with inhaled isoflurane (2% ketamine (87 mg/kg body wt) plus xylazine (13 mg/kg), and the area between the shoulders and below the skull was shaved. The mouse was then placed prone on the stereotaxic instrument and secured in the incisor bar. The incisor bar was adjusted to the lowest possible position (model 1769, Kopf, Tujunga, CA) (Fig. 1A) was attached to a rat stereotaxic instrument (Stoelting, Wood Dale, IL). A glass capillary tube (cat. no. 53508–466; VWR, Radnor, PA) was heated and pulled manually. The tapered end of the glass capillary had an approximate diameter of 0.5 mm and was sharpened with a fine whetstone to provide a sharp tip (Fig. 1B). The rabbit polyclonal antibody against rodent anti-apoB, reported previously (17), was obtained from Dr. Patrick Tso’s laboratory. The chemicals were purchased from Sigma (St. Louis, MO).

Innovative Methodology

A second-generation recombinant adenovirus encoding human apoE3 (AdhapoE3) was constructed by Tsukamoto et al. (31). Previous studies have demonstrated that this adenovirus sustains expression of human apoE3 uniquely in the liver of apoE-deficient mice (31, 32). An electrode holder with a 90° bend at its distal end (model 1769, Kopf, Tujunga, CA) (Fig. 1A) was attached to a rat stereotaxic instrument (Stoelting, Wood Dale, IL). A glass capillary tube (cat. no. 53508–466; VWR, Radnor, PA) was heated and pulled manually. The tapered end of the glass capillary had an approximate diameter of 0.5 mm and was sharpened with a fine whetstone to provide a sharp tip (Fig. 1B). The rabbit polyclonal antibody against rodent anti-apoB, reported previously (17), was obtained from Dr. Patrick Tso’s laboratory. The chemicals were purchased from Sigma (St. Louis, MO).

Method to assess circulating human apoE3 in CSF. Mice were divided into two groups (n = 10 mice/group) with equal body weight. Via intravenous (iv) administration at the retroorbital sinus (4), group 1 received 100 μl of AdhapoE3 (1 × 1010 particles/ml), and group 2 received 100 μl of control adenovirus encoding LacZ cDNA (AdLacZ, 1 × 1010 particles/ml). Three days after intravenous injection, CSF was withdrawn. The still-anesthetized mice were then killed, and their blood and a piece of liver were collected. The CSF, plasma, and liver were stored at −80°C for subsequent molecular studies.

Measurements of human apoE3 in plasma and CSF by ELISA. Human apoE3 levels in both plasma and CSF were determined by a sandwich ELISA from Mabtech (Cincinnati, OH). Briefly, plasma and CSF samples were diluted 1:1,000 and 1:50, respectively. Maxisorp 96-well plates (Nunc, Rochester, NY) were coated with mAB-E276 mouse anti-human apoE3 (rabbit mAb-E887-biotin (1:500) for 1 h. Streptavidin-ALP was then added and detected by p-nitrophenyl phosphate, disodium salt (PNPP) (Thermo Fisher Scientific, Rockford, IL), and the absorbance was measured at 405 nm.

Western blot of human apoE3 in plasma. Eighty micrograms total protein extracted from mouse liver was heat-denatured, and separated by 4 to 15% SDS-PAGE gradient gels and then electro-transferred to PVDF membranes (Millipore, Bedford, MA). The membranes were then incubated with a polyclonal rabbit anti-apoB antibody (1:5,000 dilution) (17) overnight at 4°C with gentle shaking. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000, Dako, Carpinteria, CA). The amount of immune complexes was quantified using an enhanced chemiluminescence detection system (Millipore, Billerica, MA). The reacted membranes were exposed to X-ray film (Kodak Scientific, Rochester, NY).

Western blot of mouse apoB. Western blot of mouse apoB. Two-fold serial dilutions of plasma, corresponding to 2.5–0.078% plasma in water (vol/vol), were prepared as standards using mouse plasma. Twenty microliters of the standards and individual mouse CSF samples (5 μl, 1:4 dilution) were heat-denatured, and separated by 4 to 15% SDS-PAGE gradient gels (Bio-Rad Life Sciences, Hercules, CA) and then electro-transferred to PVDF membranes (Millipore, Bedford, MA). The membranes were then incubated with a polyclonal rabbit anti-apoB antibody (1:5,000 dilution) (17) overnight at 4°C with gentle shaking. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000, Dako, Carpinteria, CA). The amount of immune complexes was quantified using an enhanced chemiluminescence detection system (Millipore, Billerica, MA). The reacted membranes were exposed to X-ray film (Kodak Scientific, Rochester, NY).

Method to assess circulating human apoE3 in CSF. Mice were divided into two groups (n = 10 mice/group) with equal body weight. Via intravenous (iv) administration at the retroorbital sinus (4), group 1 received 100 μl of AdhapoE3 (1 × 1010 particles/ml), and group 2 received 100 μl of control adenovirus encoding LacZ cDNA (AdLacZ, 1 × 1010 particles/ml). Three days after intravenous injection, CSF was withdrawn. The still-anesthetized mice were then killed, and their blood and a piece of liver were collected. The CSF, plasma, and liver were stored at −80°C for subsequent molecular studies.

Measurements of human apoE3 in plasma and CSF by ELISA. Human apoE3 levels in both plasma and CSF were determined by a sandwich ELISA from Mabtech (Cincinnati, OH). Briefly, plasma and CSF samples were diluted 1:1,000 and 1:50, respectively. Maxisorp 96-well plates (Nunc, Rochester, NY) were coated with mAB-E276 mouse anti-human apoE3 (rabbit mAb-E887-biotin (1:500) for 1 h. Streptavidin-ALP was then added and detected by p-nitrophenyl phosphate, disodium salt (PNPP) (Thermo Fisher Scientific, Rockford, IL), and the absorbance was measured at 405 nm.

Western blot of human apoE3 in plasma. Eighty micrograms total protein extracted from mouse liver was heat-denatured, and separated by 4 to 15% SDS-PAGE gradient gels and then electro-transferred to PVDF membranes (Millipore). After incubating in blocking buffer, membranes were incubated with a polyclonal rabbit anti-human apoE antibody (1:1,000, Dako) overnight at 4°C with gentle shaking. After washing with PBS, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000;

Fig. 1. A: photo of the stereotaxic instrument with the electrode holder bent at 90° at its distal end. B: an amplified view of the tapered and sharpened tip of the capillary tube. C: position of the mouse during the procedure. The incisor bar in the stereotaxic device is positioned so that the mouse’s neck is maximally ventroflexed. D and E: Close-up views of the membrane of exposed cisternum magnum before and after penetration of the capillary tube.
The amount of immune complexes was quantified using an enhanced chemiluminescence detection system (Millipore). The reacted membranes were exposed to X-ray film (Kodak Scientific).

Statistics. All data are presented as means ± SE. Differences between sets of data were determined using Student’s t-test for comparison of treatments. P values less than 0.05 were considered statistically significant.

RESULTS

CSF sampling procedure parameters. As depicted in Fig. 1, a straight and sharpened glass capillary tube was mounted onto the electrode holder and adjusted by the worm-gear-driven drives of stereotaxic instrument to penetrate the cisternum magnum. This method typically allowed collection of 10–15 μl of CSF within 6 min per mouse, including 3 min from the time of insertion of the tip of capillary tube to completion of CSF sampling. The temporal limitation of 3 min was imposed to minimize the possibility that continued drainage of the CSF might affect its composition.

Determination of blood contamination in CSF samples. CSF was collected from 86 mice. By visual inspection, more than 97% of the CSF samples were clear; i.e., appeared to have no blood contamination. This was further evaluated using Western blotting for apoB, a protein found in high concentration in plasma but absent in the central nervous system (CNS) (29). We conducted Western blot analysis using plasma diluted in water, spanning a range of concentrations corresponding to 2.5–0.078%, as standards. As depicted in Fig. 2, this immunoblot was able to detect apoB levels as low as 0.078% in aqueous solutions. The lack of apoB-immunoreactive bands in the two depicted CSF samples (Fig. 2) indicates the absence of detectable plasma contamination. Any CSF samples with detectable apoB were not used (around 5% of samples).

Liver is the primary organ expressing the human apoE3. To confirm the expression of the human apoE3 in vivo, total protein was extracted from the livers, and the contents of human apoE3 were determined by Western blot analysis. As depicted in Fig. 3, the administration of AdhapoE3 induced significant expression of human apoE3 in mouse liver. The human apoE3 bands were observed only in the liver of AdhapoE3-treated mice, and almost none was detected in that of AdLacZ-treated controls. These data are consistent with a previous report (31, 32) and confirm in vivo expression of the expected human apoE3 protein in mouse liver.

Fig. 2. Determination of apolipoprotein B (apoB) levels by Western blot analysis. Twofold serial dilutions of plasma, corresponding to 2.5–0.078% plasma in water (vol/vol), were prepared as standards using the plasma from one mouse. These standards were run in parallel. A level as low as 0.078% blood contamination (lane 6) was observed as clear bands. Two cerebrospinal fluid (CSF) samples (lanes 7 and 8, 1:4 dilution, 20 μl/lane) from two individual mice were used as examples to determine potential blood contamination. No apoB-immunoreactive bands (apoB100 and apoB48) were observed, indicating no blood contamination in either CSF sample.

Human apoE3 levels in plasma and CSF following intravenous adenovirus administration. As depicted in Fig. 4, high levels of human apoE3 in plasma were found in AdhapoE3-treated mice, compared with AdLacZ-treated controls. In contrast, human apoE3 levels in CSF were low, and there was no significant difference in CSF apoE3 levels between AdhapoE3- and AdLacZ-treated mice (4.8 ± 0.61 vs. 3.31 ± 0.29 ng/ml). The box within Fig. 3 depicts our ELISA standard curve. Such extremely low content of apoE3 in the CSF could be considered as background level because normal native mouse apoE in CSF was reported more than 1,100 ng/ml (34).

DISCUSSION

CSF is frequently analyzed in clinical populations for assessing biomarkers of drug efficacy or disease progression in
Innovative Methodology

CSF is derived in part from the extracellular fluid in the CNS, and it is segregated from plasma by the blood-CSF barrier. It is estimated that 80% of CSF proteins come from the plasma, but the protein concentration of whole blood is 200- to 400-fold higher than that in CSF (8, 30). Consequently, even a small proportion of blood contamination during CSF collection can dramatically alter the content profile of some constituents, leading to potential misinterpretation of the experimental data. Conventionally, blood contamination in CSF has been examined by visual observation, and some investigators simply used blood-contaminated CSF after centrifugation of the samples (6). However, those measurements are not sufficiently sensitive to assess a low level of blood contamination in CSF. Therefore, we used a highly sensitive molecular method to screen for potential blood contamination of the CSF by measuring apoB, a plasma-derived protein that is absent in the CNS (29). As depicted in Fig. 2, our detection limit reached as low as 0.0781% (vol/vol) blood contamination in CSF by Western blot analysis. When no apoB is detected, there is a high degree of confidence that the CSF sample is uncontaminated by blood. Although not tested in the current study, a few blood-specific, highly abundant proteins, e.g., hemoglobin, catalase, peroxiredoxin, and carbonic anhydrase I, could be similarly used as blood contamination markers, according to previous reports (21, 37).

Using these improved techniques, we asked whether circulating apoE crosses the BBB and enters CSF. ApoE is primarily produced in the liver of both humans and rodents (19). Roheim and colleagues (24) first reported that apoE is present in the CSF (24), and they suggested that it was either synthesized locally in the brain and then diffused into the CSF, or else that it crossed through the BBB from the plasma. We previously observed apoE mRNA in the hypothalamus (27), implying that at least a portion of the apoE protein present in the brain was actually synthesized in the brain (27), but those findings did not address the possibility that circulating apoE crosses the BBB.

Zlokovic and colleagues (20, 38) administered \(^{125}\text{I}\)-labeled human apoE systemically to guinea pig and then measured radioactive counts of brain tissue. They reported that the apoE did not cross the BBB based on low radioactive counts in the brain. However, such a statement seems inconclusive because of the facts that (1) apoE is quickly metabolized in liver (9) and (2) the authors did not measure the \(^{125}\text{I}\)-labeled apoE levels in the blood at the time when the animals were killed for determining \(^{125}\text{I}\)-labeled radioactivity in homogenized brain tissue. Without knowing the concentration of \(^{125}\text{I}\)-labeled apoE in blood, potential problems for their negative result could occur because of an insufficient amount of \(^{125}\text{I}\)-labeled apoE3 was infused and/or the infused \(^{125}\text{I}\)-labeled apoE3 was rapidly metabolized in the liver, leading to low level of \(^{125}\text{I}\)-labeled apoE3 in the blood and undetectable levels in the brain. Therefore, those studies alone could not rule out the possibility that apoE crosses the BBB.

To address this, we used an animal model with sustained high levels of human apoE3 by adenovirus in the circulating system. This recombinant second-generation adenovirus encoding human apoE3 cDNA has previously been used for somatic, liver-directed gene transfer in mice (31, 32). When injected into mice, the viruses induce a high level of expression of the human apoE3 isoform in the liver within 1 day, and it is
still expressed 3 mo after the injection (31). In the present study, we selected day 3 to collect the plasma and CSF samples because previous studies demonstrated that it was the time of peak expression and plasma levels (31).

Consistent with the previous report, there was a high level of human apoE3 in the plasma of AdhapoE3-treated mice relative to that in AdLacZ-treated controls. The liver samples from AdhapoE3-treated mice contained human apoE3 bands, whereas almost none was observed in the liver of AdLacZ-treated controls, confirming the in vivo expression of the human apoE3 protein (Fig. 3). There was a weak “background” in the LacZ control mice when human apoE3 in plasma was determined by ELISA (Fig. 4). This could be caused by 1) a minor cross-binding of the anti-human apoE antibody to native mouse apoE protein; and/or 2) an unknown protein induced by the adenovirus, which could nonspecifically interact with human apoE3 antibody at a very low level. Fortunately, this influence was trivial (<2.5%) in terms of the ratio of the background of LacZ control mice to apoE3 levels in the mice treated with human apoE adenovirus. Importantly, this was not an issue because human apoE3 level in the CSF was very low, as assessed by ELISA, and the human apoE3 levels in human apoE3 adenovirus-treated mice were comparable to that in LacZ adenovirus-treated mice (Fig. 4).

Human apoE induced by adenovirus has been broadly used to study endogenous apoE functions in mice (13, 31, 32). Besides having similar molecular size to mouse apoE, the human apoE3 induced by adenovirus in mice has been demonstrated functionally to interact with mouse apoE receptors, e.g., LDL receptor (LDLR) and LDL receptor-related protein 1 (LRP1) (13). Because its molecular weight is 34 kDa, apoE should theoretically be excluded from the CNS by the BBB. To carry the protein into the brain, a transporter would be required. In this case, the apoE receptor(s) are most likely to serve as transporters, just like leptin receptor.

In this case, the apoE receptor(s) are most likely to serve as transporters, just like leptin receptor. A transporter would be required. The apoE could interact with mouse apoE receptors, it seems most likely that the apoE could be transported into mouse brain through the same or similar mechanisms if native mouse apoE could cross the BBB. Because there is no obvious relationship between human apoE3 levels in the plasma and the CSF, the pools of plasma human apoE3 and central apoE3 appear to be distinct, we, therefore, conclude that apoE identified in the brain and/or CSF is produced in the brain and is not derived from peripheral tissues.

Perspectives and Significance

An improved procedure for CSF sampling from the cisterna magna in mice has been described. Compared with existing techniques, this procedure provides the advantages of consistently yielding large volumes of blood-free CSF and sensitive detection of blood contamination using an assay for apoB. This method could be valuable in studies determining the changes in the levels of specific constituents in the CSF of mice to reveal biological and functional changes in brain activity under various physiological and pathological states. In the present study, through this improved technique, we demonstrated that the human apoE3 induced by adenovirus does not exist in mouse CSF, indicating that peripheral apoE is not able to cross the BBB. Since homozygosity of the ApoE4 allele on chromosome 19 has been associated with an increased risk of sporadic Alzheimer’s disease (7), determining whether the plasma-derived apoE4 contributes its level in the CSF due to blood-brain barrier leakage in Alzheimer’s disease (2) and understanding the related mechanisms might yield new insights into the prevention and treatment of Alzheimer’s disease.

ACKNOWLEDGMENTS

We thank Dr. Philip Shaul and the Core facility at the University of Texas Southwestern Medical Center, Dallas, Texas for amplifying the adenoviruses used in the present study, and Colleen Goodin for their technical assistance.

REFERENCES


apoE DOES NOT CROSS BBB


