Human Cerebrospinal Fluid Apolipoprotein E Isoforms are Apparently Inefficient at Complexing with Synthetic Alzheimer's Amyloid- β Peptide ($A\beta^{1-40}$) *in vitro*

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Abstract

Background: Variation at the apolipoprotein E locus on chromosome 19 plays a role in more cases of Alzheimer's disease than does any other identified genetic determinant. We have previously reported the isoform-specific interaction of native human apolipoprotein E (*APOE*, gene; apoE, protein) $\epsilon 3$ with the amyloid- β peptide, $A\beta^{1-40}$, the major component of the cerebral amyloid deposits that appear to cause Alzheimer's disease.

Materials and Methods: In order to investigate the apoE: $A\beta$ interaction further, a modified assay was developed based on co-immunoprecipitation of the complex using an anti-apoE antibody (anti-apoE IP assay).

Results: Application of this assay demonstrated that the interaction of $A\beta^{1-40}$ and apoE can be distinguished into two types: sodium dodecyl sulfate (SDS) -resistant and SDS-releasable. The SDS-resistant interaction between $\epsilon 3$ and $A\beta^{1-40}$ is apparently maximal at an $A\beta^{1-40}$ concentration of $\sim 75 \mu M$, and an $A\beta^{1-40}/\epsilon 3$ molar ratio of about

250:1. The major apoE-isoform-specific difference in interaction with $A\beta^{1-40}$ is the ability of $A\beta^{1-40}$ to form SDS-resistant complexes with $\epsilon 3$ but not with $\epsilon 4$. Using the anti-apoE co-IP assay, we found that human cerebrospinal fluid (CSF) $\epsilon 3$ can also form an SDS-resistant complex with $A\beta^{1-40}$ but human CSF $\epsilon 4$ cannot. However, when compared with apoE $\epsilon 3$ collected from the conditioned medium of *APOE* $\epsilon 3$ -transfected cells, the competence of equal concentrations of CSF apoE $\epsilon 3$ to form SDS-resistant complexes with $A\beta^{1-40}$ is apparently diminished. A 1:1 mixture of CSF plus apoE $\epsilon 3$ -containing conditioned medium is associated with diminished $A\beta^{1-40}/\epsilon 3$ complex formation to a greater extent than that observed when an identical volume of phosphate-buffered saline is added to apoE $\epsilon 3$ medium.

Conclusions: These results suggest the presence in CSF of factors that interfere with the formation of complexes between synthetic $A\beta^{1-40}$ and apoE $\epsilon 3$.

Introduction

Alzheimer's disease (AD) is characterized by the invariable deposition of brain amyloid plaques (1). Genetic analysis has revealed that at least one apolipoprotein E (*APOE*) type $\epsilon 4$ allele is present in the genomes of about 70% of late-onset familial AD patients and about 60% of sporadic AD patients (2–6). Three isoforms of apoE exist: $\epsilon 2$ (Cys112, Cys158), $\epsilon 3$ (Cys112, Arg158) and $\epsilon 4$ (Arg112, Arg158), differing from each other by single amino acid substitutions at one of two positions and coded for by the *APOE2*, *APOE3*, and *APOE4* alleles, respectively (7). The mechanism by which the

$\epsilon 4$ isoform of apoE increases the risk for AD is not entirely clear. However, the incorporation of apoE into brain amyloid plaques (8,9), the existence of SDS-resistant apoE/ $A\beta$ complexes in AD brain (10), and the increased amyloid plaque density in AD patients and nondemented elderly individuals carrying an $\epsilon 4$ allele (11–14) all emphasize the likely importance of isoform-specific interactions between apoE and $A\beta$ peptides. The recent demonstration that human *APOE* isoform transgenes control $A\beta$ accumulation in the brains of amyloid plaque-forming mutant *APP*-transgenic/*APOE*-knockout mice provides the most compelling evidence linking *APOE* isoforms and cerebral amyloidosis (15).

We have previously reported the preferential interaction of $A\beta^{1-40}$ with native human $\epsilon 3$ derived from mammalian cell lines (16,17), confirming the results of an earlier study by LaDu, Frail, and

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colleagues (18). In order to investigate further the isoform-specific interaction of apoE and A β ¹⁻⁴⁰, we have developed a new *in vitro* assay utilizing an anti-apoE antibody to co-immunoprecipitate both apoE and any associated A β , including the apoE/A β complex. The complex is subsequently visualized following SDS-PAGE and immunoblotting. Using this assay, we found that there exist two distinct types of binding between A β ¹⁻⁴⁰ and apoE isoforms: one that is SDS-resistant and another that is SDS-releasable. The major difference between ϵ 3 and ϵ 4 isoforms in their interactions with A β ¹⁻⁴⁰ *in vitro* is the preferential ability of ϵ 3 to form SDS-resistant complexes with A β . Using human cerebrospinal fluid (CSF) as a source of apoE, we found that ϵ 3 in human CSF can form an SDS-resistant complex with A β ¹⁻⁴⁰, but ϵ 3 cannot. However, the A β ¹⁻⁴⁰-binding activity of CSF ϵ 3 is markedly reduced when compared with that of an equal concentration of apoE ϵ 3 prepared from the conditioned media of transfected cells. A 1:1 mixture of human CSF and native ϵ 3 from conditioned media led to diminution of the level of apoE ϵ 3/A β ¹⁻⁴⁰ complex formation to a level below that expected from dilution alone. This suggests the existence of a factor(s) in CSF that regulates apoE-isoform-specific interactions with A β ¹⁻⁴⁰.

Materials and Methods

Materials

A β ¹⁻⁴⁰ was obtained from the Keck Foundation Protein Facility at Yale University. The goat anti-human apoE antibody was from INCSTAR (Stillwater, MN, USA). Monoclonal anti-human apoE antibody 9-H8 was purchased from PerImmune (Rockville, MD, USA). Anti-A β antibody 6E10 was purchased from SENETEK (Maryland Heights, MO, USA). Human apoE-containing serum standards were kindly provided by Dr. Petar Alaupovic of the Oklahoma Medical Research Foundation (Oklahoma City, OK, USA).

Cell Culture and Conditioned Medium Preparation

Mouse macrophage RAW264 cells stably transfected with human *APOE* gene constructs were used to generate conditioned media from human ϵ 3- or ϵ 4-transfected, or vector-transfected control, conditioned media as described (16). ApoE concentrations in conditioned media were determined by quantitative Western blotting using human serum apoE standards. The apoE concentration in conditioned media was between 12 and 25 μ g/ml.

Human CSF and APOE Genotyping

One cognitively normal volunteer and two patients with probable AD contributed cerebrospinal fluid and venous blood. *APOE* genotyping was carried out on DNA extracted from blood leucocytes and was

performed using a modification of a standard PCR protocol (19).

The cognitively normal subject, a 47-year old female with a history of multiple lumbar schwannomas and mild communicating hydrocephalus, possessed an *APOE* ϵ 3/ ϵ 3 genotype. Of the other two subjects, one was a female diagnosed with probable AD at age 56 (*APOE* ϵ 3/ ϵ 4), and the other was a male diagnosed with AD at age 61 (*APOE* ϵ 4/ ϵ 4).

Conditioned Media ApoE/A β Interaction Assay

Conditioned media prepared from RAW264 mouse macrophage cells transfected with human *APOE* ϵ 3 or ϵ 4 constructs were used for standard assays. Synthetic A β ¹⁻⁴⁰ was first dissolved in distilled water at a concentration of 5.0 mg/ml and then added to 100 or 200 μ l of conditioned media that contained standard concentrations (12 μ g/ml) of either ϵ 3 or ϵ 4, yielding a final A β ¹⁻⁴⁰ concentration of 0.5 mg/ml (100 μ M). This apoE/A β mixture was incubated at room temperature for 1–2 hr. Then, 1 ml of Dulbecco's Modified Eagle (DME) medium containing 0.1% bovine serum albumin (BSA) was added to the mixture. After preclearing with 15 μ l of protein A CL-4B sepharose beads (prepared as a 50% suspension in phosphate buffered saline [PBS]), free apoE and apoE/A β complexes were recovered by co-immunoprecipitation using 2 μ l of polyclonal anti-apoE antibody (INCSTAR) and 40 μ l of protein A sepharose beads. Immunoprecipitates were washed with Tris buffered saline supplemented with 0.1% Tween 20 (TBST), and free apoE and apoE/A β complexes were eluted by boiling the beads with 1X Laemmli buffer for 3 minutes and then analyzed by electrophoresis in 10–20% Tricine SDS-polyacrylamide gels (Novex) under nonreducing conditions. After electrotransfer to nitrocellulose paper (Schleicher & Schuell), A β ¹⁻⁴⁰ (recovered by virtue of its association to the immunocollected apoE) was detected by immunoblotting with monoclonal anti-A β antibody 6E10. The apoE content of each precipitate was monitored by immunoblotting with a monoclonal anti-human apoE antibody 9-H8 (PerImmune). A Molecular Analyst phosphorimager (Bio-Rad) was used for all analyses.

Human CSF ApoE/A β Interaction Assay

A 70 μ l aliquot of human CSF was used for each assay. A β ¹⁻⁴⁰ was prepared immediately before each experiment at a concentration of 5 mg/ml in distilled water, and CSF was added to yield a final concentration of 0.5 mg/ml. After a 1 hr incubation at room temperature, 1.0 ml of DME containing 0.1% BSA and an appropriate amount of A β ¹⁻⁴⁰ were added to yield an A β ¹⁻⁴⁰ concentration identical to that used in assays of apoE-containing conditioned media. The CSF apoE-associated A β was detected using the anti-apoE IP protocol described above. For each experiment, some untreated CSF samples were processed in parallel (ie., no synthetic A β ¹⁻⁴⁰ was added). To

test the possibility that CSF might inhibit apoE/A β binding, an equal volume of CSF and human ϵ 3-containing conditioned medium were preincubated. In some experiments, PBS was substituted for CSF. The apoE/A β interaction assay was then performed according to the standard protocol.

Results

Two Types of ApoE/A β Interactions

We have previously reported the preferential association of A β ¹⁻⁴⁰ with the ϵ 3 isoform of cell-secreted apoE (16). However, that technique focused on the detection of SDS-resistant apoE/A β complexes. In order to perform a more comprehensive study of

apoE/A β interactions, we developed a modified protocol utilizing a polyclonal anti-apoE antibody to immunoprecipitate all apoE molecules following a preincubation reaction with A β ¹⁻⁴⁰. The apoE-associated A β ¹⁻⁴⁰ was therefore co-precipitated by the anti-apoE antibody and then detected by immunoblotting with antibody 6E10, allowing us to detect apoE-associated (but SDS-releasable) A β ¹⁻⁴⁰ near the dye front (Fig. 1a). The apoE-associated A β ¹⁻⁴⁰ can be distinguished into two types: one, SDS-releasable, was dissociable from apoE in the presence of SDS and migrated at the bottom of the gel. Most of this A β ¹⁻⁴⁰ was monomeric (*), but some dimeric and oligomeric (**) A β could also be detected (Fig. 1a). The other type of association was

Fig. 1. ApoE-associated A β ¹⁻⁴⁰ can be distinguished into two types: SDS-resistant and SDS-releasable. **a)** Immunoblot illustrating two types of interactions involving apoE and A β ¹⁻⁴⁰. Conditioned media were incubated with synthetic A β ¹⁻⁴⁰ (100 μ M) at room temperature for 1 hr and then subjected to immunoprecipitation using goat anti-human apoE antibody. The co-immunoprecipitated products were analyzed following electrophoresis in 10–20% SDS-Tricine gels. After electrotransfer to nitrocellulose, transferred proteins were blotted with either monoclonal anti-human apoE antibody 9-H8 (lanes 1 to 5) or anti-A β antibody 6E10 (lanes 6 to 8). Lanes 1 and 8 are from vector-transfected control conditioned medium. Lanes 3, 5 and 7 are from ϵ 3-containing conditioned medium. Lanes 2, 4 and 6 are from ϵ 4-containing conditioned medium. Samples applied to lanes 1–3 were assayed in parallel but A β ¹⁻⁴⁰ was omitted from the reaction mixture. CM = conditioned media. **b)** Quantitative analysis of the fold-differences between SDS-resistant and SDS-releasable A β ¹⁻⁴⁰ associated with CM- ϵ 3 versus CM- ϵ 4. Data are representative of results from each of five experiments; $p < 0.05$. For each experiment, the amount of SDS-resistant A β ¹⁻⁴⁰ associated with CM- ϵ 4, or the amount of monomeric, SDS-releasable A β ¹⁻⁴⁰ associated with CM- ϵ 4, respectively, were arbitrarily assigned a value of 1.0.

SDS-resistant. Under denaturing but nonreducing conditions, this SDS-resistant apoE-associated A β ¹⁻⁴⁰ migrated slightly more slowly than did apoE, appearing as a shifted band at about 40 kDa (Fig. 1a). Quantitative analyses revealed a ~9-fold increase in the SDS-resistant association of A β ¹⁻⁴⁰ with ϵ 3 over that with ϵ 4, and a ~2-fold increase in the SDS-releasable association with ϵ 3 over ϵ 4 (Fig. 1b; $p < 0.05$). The majority of ϵ 3-associated A β ¹⁻⁴⁰ was of the SDS-resistant type.

A mouse monoclonal anti-human apoE antibody, 9-H8, was used to monitor the apoE levels in the immunoprecipitates (Fig. 1a). This antibody recognizes both ϵ 3 and ϵ 4 isoforms, and both monomeric and dimerized apoE (Fig. 1a). Using this antibody, we found that about 30% of the apoE ϵ 3 in conditioned media existed as a dimer. However, ϵ 4 existed exclusively as a monomer (Fig. 1a). A β ¹⁻⁴⁰ bound to apoE ϵ 3 monomers to form SDS-resistant complexes, but no SDS-resistant association of A β ¹⁻⁴⁰ with apoE ϵ 3 dimers was detected (Fig. 1a).

Interactions Between Human CSF ApoE and A β ¹⁻⁴⁰

As a step toward understanding the interactions of apoE isoforms and A β peptides in the brain, we used human CSF as a source of apoE to test apoE binding to synthetic A β ¹⁻⁴⁰ (Fig. 2). CSF from individuals with genotypes of ϵ 3/ ϵ 3, ϵ 3/ ϵ 4 and ϵ 4/ ϵ 4 (one sample from each) were tested. When analyzed by SDS-Tricine gel electrophoresis under nonreducing conditions, most of the CSF ϵ 3 existed as the monomeric form migrating as a 34 kDa band (Fig. 2). About 30% of CSF ϵ 3 appeared as a dimer, migrating at 60 to 70 kDa (Fig. 2). As previously observed in ϵ 4-containing conditioned media, ϵ 4 in human CSF existed only in the monomeric form (Fig. 2).

SDS-resistant apoE/A β complexes could also be visualized when human CSF was used as the source of apoE (Fig. 2). Using the trace amount of SDS-resistant ϵ 4/A β ¹⁻⁴⁰ complex detectable in conditioned media as a standard, the ability of CSF apoE from the ϵ 3/ ϵ 3 donor to form SDS-resistant complexes with A β was about 3-fold higher than was the observed capacity for complex formation when either ϵ 3/ ϵ 4 or ϵ 4/ ϵ 4 CSF samples were used (Fig. 2). However, when the CSF ϵ 3 complex capacity was compared with that of a similar amount of ϵ 3 from conditioned media, the ability of CSF ϵ 3 to form a complex with A β ¹⁻⁴⁰ was dramatically lower (Fig. 2), suggesting that CSF might contain factors that inhibit complex formation.

Discussion

Strittmatter et al. (3) first reported that apoE ϵ 4 bound amyloid peptide more avidly than ϵ 3. However, our previous two reports (16,17) on the isoform-specific interaction between A β ¹⁻⁴⁰ and native human apoE secreted from transfected RAW264 and CHO cells

Fig. 2. At equivalent apoE concentrations, human CSF apoE binds less synthetic A β ¹⁻⁴⁰ than does apoE from the conditioned media of transfected cells. Human CSF from individuals bearing *APOE* genotypes of ϵ 3/ ϵ 3 (upper panel), ϵ 3/ ϵ 4 (middle panel) or ϵ 4/ ϵ 4 (lower panel) were incubated with synthetic A β ¹⁻⁴⁰ (100 μ M) at room temperature for 1 hr and then immunoprecipitated using a polyclonal anti-human apoE antibody (INCSTAR). The apoE-bound A β ¹⁻⁴⁰ was detected by immunoblotting the separated immunoprecipitates using antibody 6E10 (lanes 5–9). The amounts of monomeric and dimeric apoE in immunoprecipitates were monitored by immunoblotting the separated immunoprecipitates using a monoclonal anti-human apoE antibody, 9-H8 (lanes 1 to 4). Conditioned media prepared from human *APOE* ϵ 3- or ϵ 4-transfected cell lines as well as from vector-transfected control cell lines were studied in parallel. Lanes 1 and 5, human CSF incubated with A β ¹⁻⁴⁰; lanes 2 and 6, CSF without exogenous A β ¹⁻⁴⁰; lanes 3 and 7, ϵ 4-containing conditioned medium incubated with A β ; lanes 4 and 8, ϵ 3-containing conditioned medium incubated with A β ; lane 9, control conditioned medium prepared from vector-transfected cell line. CM = conditioned media.

differ from Strittmatter et al. (3) but are in agreement with the report of LaDu et al. (18), indicating that native human ϵ 3 binds more A β ¹⁻⁴⁰ than does ϵ 4. Here we reconfirm this isoform-specific difference using a novel protocol. Furthermore, we demonstrate that in addition to the SDS-resistant interaction of A β ¹⁻⁴⁰ and ϵ 3, there is also SDS-dissociable binding of A β ¹⁻⁴⁰ to both ϵ 3 and ϵ 4. This SDS-dissociable interaction also preferentially involves ϵ 3. LaDu et al. (20) have shown that the interaction of A β ¹⁻⁴⁰ with ϵ 3 is attenuated by apoE purification employing organic solvents, suggesting that changes in the conformation of apoE during denaturation and purification might alter its interaction with A β ¹⁻⁴⁰. We would conclude that purification/denaturation of apoE probably underlies the apparently artefactual preference of A β for apoE ϵ 4 reported by Strittmatter et al. (3).

It should be kept in mind that supraphysiological concentrations of A β are used in all these *in vitro* protocols, and the physiological importance and exact mechanisms underlying apoE-isoform-specific interactions with A β are not well understood. Structurally, apoE consists of two distinct domains (21). While the amino-terminal four-helix bundle domain (residues 1–191) contains the lipoprotein receptor binding region, the carboxyl-terminal part (residues 216–299) contains most of the lipid binding activity. Human ϵ 3 differs from ϵ 4 by only one residue at position 112, which is a cysteine in ϵ 3 and an arginine in ϵ 4. There are no other cysteines in ϵ 3, and ϵ 4 has no cysteines at all. These cysteines allow ϵ 3, but not ϵ 4, to form homodimers. Our observations: (1) that ϵ 3 monomer can form an SDS-resistant complex with A β ^{1–40} but that ϵ 4 cannot; (2) that ϵ 3 dimers cannot complex with A β ^{1–40}; and (3) that the presence of dithiothreitol abolishes the interaction of ϵ 3 and A β (17); suggest that Cys112 may play an important role in formation of the SDS-resistant complexes. The SDS-resistant complex between ϵ 3 and A β ^{1–40} is stable even with 2% SDS and boiling, implying a strong intermolecular interaction. However, since there is no cysteine residue within A β ^{1–40}, apoE ϵ 3 and A β cannot participate in disulfide bonding. By using antibodies that recognize different regions of apoE, Naslund et al. (10) found that the apoE/A β complexes in AD brain are composed of the C-terminal part of apoE. Thus, it is possible that free Cys112 is crucial for an intramolecular interaction within monomeric ϵ 3, specifying a conformation of apoE ϵ 3 which, in turn, determines the ability of ϵ 3 (and ϵ 2; 17) to form SDS-resistant and isoform-specific complexes with A β ^{1–40}.

How might this isoform-specific interaction lead to enhanced accumulation of amyloid plaques in *APOE* ϵ 4 allele-bearing individuals? Amyloid fibril formation has been proposed to be a nucleation-dependent process, and the formation of the nucleus is believed to be the rate-limiting step (22). Evans et al. (22) have reported that low concentrations of apoE ϵ 3 delay the onset of amyloid fibril formation. Another report (23) indicates that the purified apoE/A β complex is a poor nucleus for A β fibril formation. Our finding of a preferential interaction between ϵ 3 and A β ^{1–40} peptide is consistent with these models: in other words, the preferential interaction of ϵ 3 with A β peptide may directly inhibit or compete with the interaction between one A β peptide and another, therefore delaying A β fibrillogenesis by preventing A β monomers from forming the initial A β nucleus.

Alternatively, the apoE/A β interaction may play a role in clearing A β peptide from the extracellular space in human brain. In plasma, apoE functions in lipid and cholesterol metabolism by binding to, and subsequently being internalized through, low density lipoprotein (LDL) receptors or the LDL receptor-related protein (LRP) (24–26). Both LDL receptors and LRP are expressed in human brain (11,27). Rebeck et al. (11) have proposed that apoE-bound

A β could be internalized through such receptors, leading to a difference in A β clearance in ϵ 3-versus ϵ 4-carrying individuals. Since ϵ 3 and ϵ 4 do not differ in their ability to bind to LDL receptors (28,29), such decreased clearance could result from altered capacity of apoE isoforms to bind A β . The preferential interaction of A β ^{1–40} and ϵ 3 would also be consistent with this model.

In summary, our data indicate that apoE in CSF from ϵ 3/ ϵ 3 individuals can form SDS-stable complexes. However, when compared with an equivalent concentration of ϵ 3 from the conditioned medium of non-astrocytic cells, CSF ϵ 3 has an apparently diminished capacity for binding A β ^{1–40}. Furthermore, preincubation of a mixture of CSF (ϵ 3/ ϵ 3) and ϵ 3-containing conditioned media decreases complex formation beyond that expected from dilution alone (not shown). While it is theoretically possible that the A β -binding sites on CSF apoE might have been already occupied by endogenous A β , no obvious endogenous apoE/A β complexes were detectable in our assay. Alternatively, CSF apoE might have a conformation or lipidation state that is different from that of conditioned media and has lower affinity for A β . It is also possible that factors other than apoE and A β are present in human CSF and are involved in regulating the apoE/A β reaction. Such factors might include reducing agents or compounds that interact with cysteine residues. Apolipoprotein J, serum albumin and transthyretin are all known to bind CSF A β (30,31), and it is possible that one or all of these A β -binding proteins modulates apoE/A β complex formation. Indeed, apoJ appears to be the major CSF A β -binding protein (30). Identification of important A β -binding proteins might illuminate novel strategies for the therapeutic modulation of amyloidogenesis, and it will be important to determine whether CSF A β -binding proteins accurately reflect the A β -binding proteins of the brain interstitium, where apoE is initially secreted by astrocytes (32). Further investigation will be required to identify the modulators of apoE/A β complex formation and to establish the role of apoE/A β complexes in the sequence of events by which apoE ϵ 4 fosters cerebral A β accumulation.

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