Original Articles

Alzheimer Amyloid-β Peptide Forms Denaturant-Resistant Complex with Type ε3 but Not Type ε4 Isoform of Native Apolipoprotein E

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ABSTRACT

Background: The apolipoprotein E (apoE) type ε4 isoform specifies increased cerebral and cerebrovascular accumulation of amyloid-β protein (Aβ) and contributes to the genetic susceptibility underlying a large proportion (~60%) of typical, sporadic Alzheimer disease. Unfortunately, in vitro biochemical studies of direct apoE isoform–specific interactions with Aβ have been inconsistent, perhaps due to the use by different research groups of apoE isoform preparations in different conformational states (purified denatured versus native).

Materials and Methods: In the current study, we have investigated the possibility that synthetic $A\beta^{1-40}$ preferentially associates with native apoE of either the type ε3 or the type ε4 isoform.

Results: Here, we demonstrate the preferential association of synthetic $A\beta^{1-40}$ with native apoE ε3. The complex between apoE ε3 and $A\beta^{1-40}$ could not be disrupted by sodium dodecyl sulfate. In a parallel assay, no denaturant-resistant association of $A\beta^{1-40}$ with apoE ε4 was detectable.

Conclusions: These results support the notion that the apoE ε4 isoform may foster β-amyloidogenesis because apoE ε4 is inefficient in forming complexes with Aβ.

INTRODUCTION

The apolipoprotein E type ε4 allele contributes to the genetic susceptibility underlying a large proportion (~60%) of typical, sporadic Alzheimer disease (1–5). Correlations between genetic and neuropathological results indicate that the apolipoprotein E (apoE) type ε4 isoform specifies increased cerebral (6–10) and cerebrovascular (11–13) accumulation of amyloid-β protein (Aβ). Conversely, the apoE ε2 isoform can apparently prevent (14,15) the expression of clinical Alzheimer-type dementia, which is otherwise typically associated with amyloidogenic mutations in the amyloid-β protein precursor (AβPP) (16). The importance of apoE in human amyloid-β biology has been emphasized by studies indicating that apoE and Aβ are present as non-dissociable complexes within the cerebral amyloid plaques characteristic of this disease (17,18). Therefore, it is important to search for relevant interaction(s) between Aβ and various isoforms of apoE.

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Currently proposed mechanisms for the proamyloidogenic activity of apoE ε4 include the possibility that apoE ε4 might have a relatively greater propensity (compared with apoE ε3) for modulating Aβ misfolding and fibrillogenesis (19–23), although we recently observed equipotent profibrillogenic activities for native apoE ε3 and ε4 isoforms (Sweeney et al., submitted for publication). Alternatively, apoE ε4 might be inferior to apoE ε3 in some bioactivity related to modulation of Aβ clearance (7,24). Since both Aβ and apoE are secreted proteins, either of these mechanisms might be attributable to direct apoE/Aβ interactions (2,24) in the extracellular space. To date, however, the results of in vitro biochemical studies of direct interactions between Aβ and different apoE isoforms have been inconsistent, perhaps due to the use by different research groups of either purified, denatured (2) or native (24) apoE isoforms. In view of the great importance of clarifying the molecular mechanisms underlying the role of apoE in human amyloid-β biology, we have in the current study compared the ability of native apoE ε3 and ε4 isoforms to associate with synthetic $A\beta^{1-40}$.

MATERIALS AND METHODS

$A\beta^{1-40}$ was prepared at the Keck Foundation Protein Facility at Yale University (New Haven, CT, U.S.A.). RAW264 mouse macrophage cells were obtained from American Type Culture Collection (Rockville, MD, U.S.A.). ApoE serum standards were kindly provided by Dr. Petar Alaupovic of the Oklahoma Medical Research Foundation (Oklahoma City, OK, U.S.A.).

Transfected Cells

RAW264 cells were stably transfected with apoE isoforms using the SV40 early promoter to drive expression of genomic apoE DNA fragments which extended from the *Aat*II site in exon 1 to the *Eco*RI site 626 bp 3′ to the end of the apoE gene (25). The initial clone contained the apoE ε4 allele, and the apoE ε3 clone was created by exchange of two 2-kb genomic *Eco*RI cassettes. Stably expressing lines were prepared by cotransfection of apoE with pUCneo. Geneticin (Life Technologies, Inc. Gaithersburg, MD, U.S.A.) resistant colonies were expanded and screened for apoE production by in situ immunocytochemistry of samples of colonies, using a commercial apoE antibody (INCSTAR Stillwater, MN, U.S.A.).

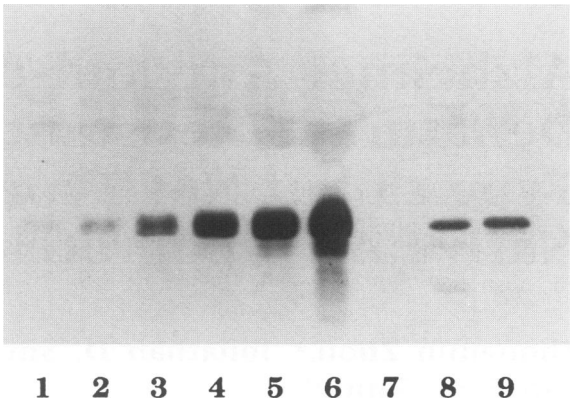

FIG. 1. Quantitative immunoblotting for apoE content of conditioned media

Anti-apoE antibody was used to probe electroblotted conditioned medium proteins following SDS-PAGE in a 10% gel. Lanes 1–6, human plasma apoE standards of 10 ng (Lane 1), 20 ng (Lane 2), 50 ng (Lane 3), 100 ng (Lane 4), 200 ng (Lane 5), and 400 ng (Lane 6); Lane 7, conditioned medium from untransfected RAW264 cells; Lane 8, conditioned medium from apoE ε3–transfected RAW264 cells; Lane 9, conditioned medium from apoE ε4–transfected RAW264 cells.

Preparation of Conditioned Media

ApoE isoform–transfected RAW264 cells were used to generate conditioned media containing native apoE isoforms. Conditioned media containing apoE were prepared using Dulbecco's minimal essential medium, which in some cases was supplemented with 0.2% (w/v) bovine serum albumin. Media were harvested after a 24-hr conditioning period, and apoE isoform levels were determined by quantitative immunoblotting of conditioned media and serum standards containing apoE (Fig. 1). ApoE concentrations were adjusted to 20 μg/ml using medium conditioned by untransfected RAW264 cells as diluent. These apoE concentrations are within the range reported for human cerebrospinal fluid (26). Aliquots were stored at −120°C until use.

Detection of ApoE/$A\beta^{1-40}$ Complexes by Immunoblotting

ApoE/$A\beta^{1-40}$ complexes were studied using a modification of the procedure of LaDu et al. (24). Synthetic $A\beta^{1-40}$ was freshly prepared as a 5.0 mg/ml stock solution in distilled water. Aliquots of this stock solution were added to conditioned medium samples containing various amounts of either apoE ε3 or apoE ε4, yielding a final $A\beta^{1-40}$

concentration of 0.5 mg/ml (~100 μM). The reaction mixtures were incubated at room temperature for 2 hr, and then aliquots were removed and analyzed by SDS-PAGE in a 10–20% tricine gel under nonreducing conditions. ApoE/Aβ^{1-40} complexes were detected by immunoblotting with either antibody 6E10 against Aβ^{1-17} (Senetek, Maryland Heights, MO, U.S.A.) or anti-apoE antibody (INCSTAR).

RESULTS

Preferential association of Aβ^{1-40} with native apoE ε3 was demonstrated using an experimental protocol based on immunoblotting of denaturant-resistant apoE/Aβ complexes, as previously described by LaDu et al. (24). In the current study, conditioned media containing native apoE isoforms were derived from stably transfected RAW264 macrophage cells. Human kidney 293 cells were used in the study by LaDu et al. (24).

Figure 2 illustrates typical results obtained from one of three independent experiments. Aβ^{1-40} was incubated with either conditioned medium from control untransfected RAW264 cells (Fig. 2, Lanes 1 and 4), apoE ε3–transfected RAW264 cells (Fig. 2, Lanes 2 and 5), or apoE ε4–transfected RAW264 cells (Fig. 2, Lanes 3 and 6). Following incubation, aliquots were removed, mixed with an equal volume of nonreducing Laemmli buffer, and boiled for 3 min prior to loading and separation by SDS-PAGE in a 10–20% tricine gel. Separated proteins were then electroblotted to nitrocellulose and probed with either antibody 6E10 (Fig. 2, left panel) against Aβ^{1-17} or anti-apoE antibody (Fig. 2, right panel).

Free Aβ^{1-40} was visible as a diffusely migrating, 6E10-immunoreactive species at the bottom of the gel, near the bromphenol blue tracking dye indicator (Fig. 2, left panel). Using antibody 6E10, an apoE/Aβ^{1-40} complex (Fig. 2, arrowhead) was detectable following co-incubation of Aβ^{1-40} with apoE ε3 (Fig. 2, Lane 2), but not following co-incubation of Aβ^{1-40} with apoE ε4 (Fig. 2, Lane 3). As expected, apoE ε3 and ε4 were detected only in media from transfected RAW264 cells (Fig. 2, Lanes 5 and 6). An apoE ε3/Aβ^{1-40} complex was also visible as the slowly migrating component of the apoE ε3 band, as detected with the anti-apoE antibody (Fig. 2, Lane 5). ApoE ε4/Aβ^{1-40} complexes were not detectable in any of three identical independent experiments.

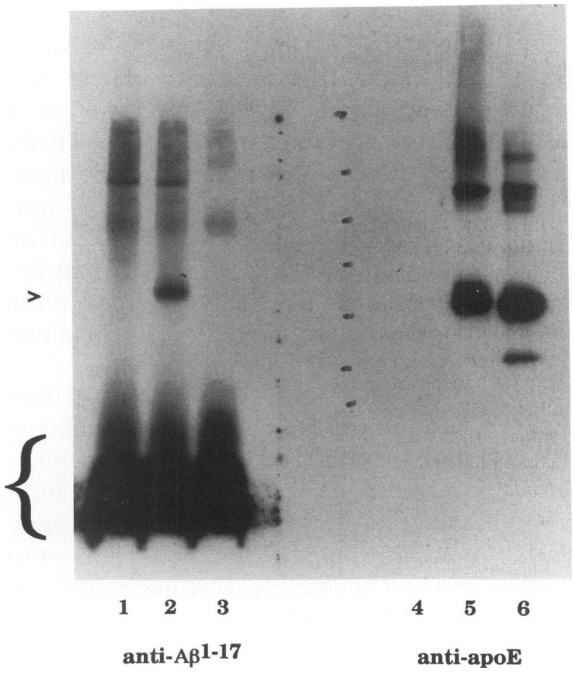

FIG. 2. Comparison of ability of native apoE ε3 and ε4 to form a denaturant-resistant complex with synthetic Aβ^{1-40}

Aβ^{1-40} (0.5 mg/ml) was added to each of the three types of conditioned media. For media containing apoE isoforms, apoE concentration was adjusted to 20 μg apoE/ml as described in Materials and Methods. Incubations were carried out for 2 hr at room temperature. Aliquots of the reaction mixtures were separated by SDS-PAGE in a 10–20% tricine gel, electroblotted to nitrocellulose and probed with either anti-Aβ^{1-17} antibody 6E10 (left panel) or anti-apoE antibody (right panel). Lanes 1 and 4, conditioned medium from untransfected RAW264 cells; Lanes 2 and 5, conditioned medium from apoE ε3–transfected RAW264 cells; Lanes 3 and 6, conditioned medium from apoE ε4–transfected RAW264 cells. Arrowhead indicates the location of apoE ε3/Aβ complex which migrates as a discrete band in Lane 2 and as the slowly migrating component of the apoE ε3 band in Lane 5. Bracket indicates position of free Aβ^{1-40}, visible in Lanes 1–3. Markings in center of figure represent positions of molecular weight standards of 200, 97.4, 69, 46, 30, 21.5, and 14.3 kD, respectively, from top to bottom.

DISCUSSION

The present results confirm the observations of LaDu et al. (24), who also used native apoE isoforms. The results obtained by LaDu et al.

(24), using kidney 293 cells, and in the present study using RAW264 macrophage cells, differ from those obtained by Strittmatter et al. (2), who described preferential association of apoE ε4 with Aβ. The latter results are most likely attributable to the use of purified, delipidated, denatured apoE isoforms (27). Our RAW264-produced apoE isoforms are lipid–poor (6.0 ± 2.6 μg phospholipid/100 μg apoE), suggesting that denaturation of apoE during purification (27) is the most likely explanation for the results obtained by Strittmatter et al. (2).

Since apolipoprotein E ε4–related Alzheimer disease is the most prevalent molecular subtype of the disease identified to date (4,5), an elucidation of the molecular pathology resulting from the apolipoprotein E ε4 genotype is particularly crucial. Along this line, the ability of apoE ε4 to promote cerebral and cerebrovascular accumulation of Aβ (6–13) would appear to be an important clue, especially considering that genetically heterogeneous forms of Alzheimer disease foster Aβ accumulation via at least three independent amyloidogenic pathways: excess Aβ precursor expression (28), excess Aβ generation (29), or generation of aberrant, excessively aggregable Aβ (16,30).

Studies of Aβ levels and plaque accumulation in affected individuals support the notion that the role of apoE ε4 in β-amyloidogenesis might involve a deficiency of Aβ clearance. Soluble Aβ levels in human cerebrospinal fluid are apparently unaffected by the apoE ε4 allele (31). Parenchymal amyloid plaque *density* (plaques/mm^2) is increased by apoE ε4, while plaque *size* is increased in Down syndrome (32). These data are consistent with a proposed model (32) in which elevations in soluble Aβ concentration (as would be predicted to occur in Down syndrome) lead to enlarged plaque size whereas the apoE ε4 "effect" leads to an increased frequency of plaque initiation. The current data demonstrating differences in the association of specific apoE isoforms with Aβ indicate the importance of assessing the role of the apoE ε3/Aβ complex in the fate of Aβ. It will be important to determine the fate of both extracellular and cell-associated Aβ. The use of primary brain cultures as well as in vivo approaches should be of value in determining the importance of apoE in Aβ clearance.

Alternatively, with regard to the molecular basis of cognitive decline in Alzheimer disease, apoE ε4 may well be less efficient than apoE ε3 in exerting important neurobiologic effects via an as yet unclarified role in neuronal or synaptic plasticity (33). This possibility is supported by recent evidence that apoE-deficient mice exhibit learning and memory deficits (34,35), even in the absence of consistent obvious structural neuropathology (35). It is encouraging to note that many of the proposed actions for apoE isoforms are experimentally testable. Data from such experiments may provide valuable opportunities for slowing or preventing the clinical course of Alzheimer disease.

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REFERENCES

1. Strittmatter WJ, Saunders AM, Schmechel D, et al. (1993) Apolipoprotein E: High-avidity binding to β amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc. Natl. Acad. Sci. U.S.A.* **90:** 1977–1981.
2. Strittmatter WJ, Weisgraber KH, Huang DY, et al. (1993) Binding of human apolipoprotein E to synthetic amyloid β peptide: Isoform-specific effects and implications for late-onset Alzheimer disease. *Proc. Natl. Acad. Sci. U.S.A.* **90:** 8098–8102.
3. Corder EH, Saunders AM, Strittmatter WJ, et al. (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* **261:** 921–923.
4. Mayeux R, Stern Y, Ottman R, et al. (1993) The apolipoprotein epsilon 4 allele in patients with Alzheimer's disease *Ann. Neurol.* **34:** 752–754.
5. Saunders AM, Strittmatter WJ, Schmechel D, et al. (1993) Association of apolipoprotein E allele epsilon 4 with late-onset familial and sporadic Alzheimer's disease. *Neurology* **43:** 1467–1472.
6. Schmechel DE, Saunders AM, Strittmatter WJ, et al. (1993) Increased amyloid β-peptide deposition in cerebral cortex as a consequence of apolipoprotein E genotype in late-onset Alzheimer disease. *Proc. Natl. Acad. Sci. U.S.A.* **90:** 9649–9653.
7. Rebeck GW, Reiter JS, Strickland DK, Hyman BT. (1993) Apolipoprotein E in sporadic

Alzheimer's disease: Allelic variation and receptor interactions. *Neuron* **11:** 575–580.
8. Berr C, Hauw JJ, Delaere P, Duyckaerts C, Amouyel P. (1994) Apolipoprotein E allele ε4 is linked to increased deposition of the amyloid β-peptide (A-β) in cases with or without Alzheimer's disease. *Neurosci. Lett.* **178:** 221–224.
9. Polvikoski T, Sulkava R, Haltia M, et al. (1995) Apolipoprotein E, dementia, and cortical deposition of β-amyloid protein. *N. Engl. J. Med.* **333:** 1242–1247.
10. Hyman BT, West HL, Rebeck GW, Lai F, Mann DMA. (1995) Neuropathological changes in Down's syndrome hippocampal formation: Effect of age and apolipoprotein E genotype. *Arch. Neurol.* **52:** 373–378.
11. Greenberg SM, Rebeck GW, Vonsattel JPG, Gomez-Isla T, Hyman BT. (1995) Apolipoprotein E ε4 and cerebral hemorrhage associated with amyloid angiopathy. *Ann. Neurol.* **38:** 254–259.
12. Jordan B, Kanik A, Horwich M, et al. (1995) Apolipoprotein E ε4 and fatal cerebral amyloid angiopathy associated with dementia pugilistica. *Ann. Neurol.* **38:** 698–699.
13. Kalaria RN, Premkumar DRD. (1995) Apolipoprotein E genotype and cerebral amyloid angiopathy. *Lancet* **346:** 1424.
14. St George-Hyslop P, Crapper McLachlan D, Tuda T, et al. (1994) Alzheimer's disease and possible gene interaction. *Science* **263:** 537.
15. Sorbi S, Nacmias B, Forleo P, Piacentini S, Latorraca S, Amaducci L. (1995) Epistatic effect of APP717 mutation and apolipoprotein E genotype in familial Alzheimer's disease. *Ann. Neurol.* **38:** 124–127.
16. Suzuki N, Cheung T, Cai X-D, et al. (1994) An increased percentage of long amyloid β protein secreted by familial amyloid β protein precursor (βAPP717) mutants. *Science* **264:** 1336–1340.
17. Naslund J, Thyberg J, Tjernberg L, et al. (1995) Characterization of stable complexes involving apolipoprotein E and the amyloid β-peptide in Alzheimer disease brain. *Neuron* **15:** 219–228.
18. Wisniewski T, Lalowski M, Golabek A, Vogel T, Frangione B. (1995) Is Alzheimer's disease an apolipoprotein E amyloidosis? *Lancet* **345:** 956–958.
19. Sanan D, Weisgraber K, Russell S, et al. (1994) Apolipoprotein E associates with β amyloid peptide of Alzheimer's disease to form novel monofibrils: Isoform apoE4 associates more efficiently than ApoE3. *J. Clin. Invest.* **94:** 860–869.
20. Ma J, Yee A, Brewer HB, Das S, Potter H. (1994) Amyloid-associated proteins α_1-antichymotrypsin and apolipoprotein E promote assembly of Alzheimer β-protein into filaments. *Nature* **372:** 92–94.
21. Wisniewski T, Castano EM, Golabek A, Vogel T, Frangione B. (1994) Acceleration of Alzheimer's fibril formation by apolipoprotein E in vitro. *Am. J. Pathol.* **145:** 1030–1035.
22. Castano EM, Prelli F, Wisniewski T, et al. (1995) Fibrillogenesis in Alzheimer's disease of amyloid β peptides and apolipoprotein E. *Biochem. J.* **306:** 599–604.
23. Evans KC, Berger EP, Cho C-G, Weisgraber KH, Lansbury PT. (1995) Apolipoprotein E is a kinetic but not a thermodynamic inhibitor of amyloid formation: Implications for the pathogenesis and treatment of Alzheimer's disease. *Proc. Natl. Acad. Sci. U.S.A.* **92:** 763–767.
24. LaDu M, Falduto M, Manelli A, Reardon C, Getz G, Frail D. (1994) Isoform-specific binding of apolipoprotein E to β-amyloid. *J. Biol. Chem.* **269:** 23403–23406.
25. Smith JD, Melian A, Leff T, Breslow J. (1988) Expression of the human apolipoprotein E gene is regulated by multiple positive and negative elements. *J. Biol. Chem.* **263:** 8300–8308.
26. Lehtimaki T, Pirttila T, Mehta PD, Wisniewski HM, Frey H, Nikkari, T. (1995) Apolipoprotein E (apoE) polymorphism and its influence on apoE concentrations in the cerebrospinal fluid in Finnish patients with Alzheimer's disease. *Hum. Genet.* **95:** 39–42.
27. Rall SC, Weisgraber KH, Mahley RW. (1986) Isolation and characterization of apolipoprotein E. *Methods Enzymol.* **128:** 273–287.
28. Goldgaber D, Lerman MI, McBride OW, Saffioti U, Gajdusek DC. (1987) Characterization and chromosomal localization of a cDNA encoding brain amyloid of Alzheimer's disease. *Science* **235:** 877–880.
29. Citron M, Oltersdorf T, Haass C, et al. (1992) Mutation of the β-amyloid precursor protein in familial Alzheimer's disease causes increased β-protein production. *Nature* **360:** 672–674.
30. Wisniewski T, Ghiso J, Frangione B. (1991) Peptides homologous to the amyloid protein of Alzheimer's disease containing a glutamine for glutamic acid substitution have

accelerated amyloid fibril formation. *Biochem. Biophys. Res. Commun.* **179:** 1247–1254.
31. Motter R, Vigo-Pelfrey C, Kholodenko D, et al. (1995) Reduction of β-amyloid peptide$_{42}$ in the cerebrospinal fluid of patient's with Alzheimer's disease. *Ann. Neurol.* **38:** 643–648.
32. Hyman BT, West HL, Rebeck GW, et al. (1995) Quantitative analysis of senile plaques in Alzheimer disease: Observation of log-normal size distribution and molecular epidemiology of differences associated with apolipoprotein E genotype and trisomy 21 (Down syndrome). *Proc. Natl. Acad. Sci. U.S.A.* **92:** 3586–3590.
33. Poirier J. (1994) Apolipoprotein E in animal models of CNS injury and in Alzheimer's disease. *Trends Neurosci.* **17:** 525–530.
34. Gordon I, Grauer E, Genis I, Sehayek E, Michaelson DM. (1995) Memory deficits and cholinergic impairments in apolipoprotein E-deficient mice. *Neurosci. Lett.* **199:** 1–4.
35. Gandy S, Stafstrom C, Turner B, et al. (1995) Aged apoE deficient mice: Abnormal learning & glial markers but no obvious neuropathology. *Soc. Neurosci. Abstr.* **21:** 5.

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