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Apolipoprotein E: High-avidity binding to β-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease

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ABSTRACT **Apolipoprotein E is immunochemically localized to the senile plaques, vascular amyloid, and neurofibrillary tangles of Alzheimer disease. *In vitro*, apolipoprotein E in cerebrospinal fluid binds to synthetic βA4 peptide (the primary constituent of the senile plaque) with high avidity. Amino acids 12–28 of the βA4 peptide are required. The gene for apolipoprotein E is located on chromosome 19q13.2, within the region previously associated with linkage of late-onset familial Alzheimer disease. Analysis of apolipoprotein E alleles in Alzheimer disease and controls demonstrated that there was a highly significant association of apolipoprotein E type 4 allele (*APOE*-ε4) and late-onset familial Alzheimer disease. The allele frequency of the *APOE*-ε4 in 30 random affected patients, each from a different Alzheimer disease family, was 0.50 ± 0.06; the allele frequency of *APOE*-ε4 in 91 age-matched unrelated controls was 0.16 ± 0.03 ($Z = 2.44$, $P = 0.014$). A functional role of the apolipoprotein E-E4 isoform in the pathogenesis of late-onset familial Alzheimer disease is suggested.**

Apolipoprotein E (*APOE*, gene; ApoE, protein) is a plasma protein involved in cholesterol transport (1). ApoE is produced and secreted in the central nervous system by astrocytes (2–4). Brain contains large quantities of *APOE* mRNA, second only to the liver (5). ApoE synthesis is increased following injury and is implicated in the growth and repair of the nervous system during development or after injury. In the peripheral nervous system, the synthesis of ApoE increases 250- to 350-fold within 3 weeks of sciatic nerve crush, and ApoE constitutes 5% of the total soluble extracellular protein (2, 6). After crush injury to the central nervous system optic nerve, ApoE is bound to the degenerating optic tracts and to the retino-recipient layers of the lateral geniculate nucleus and the superior colliculus (7). ApoE is also increased in several chronic neurodegenerative diseases. In Alzheimer disease, ApoE is bound to extracellular senile plaques, to intracellular neurofibrillary tangles, and at sites of cerebral vessel congophilic angiopathy (8). ApoE is also bound in the amyloid plaques of another dementing degenerative disease, Creutzfeld–Jakob disease (8). The amyloid deposits in Creutzfeld–Jakob disease contain the prion protein, not the βA4 peptide in Alzheimer disease (9). *APOE* mRNA is increased severalfold in the brain of patients with Alzheimer disease and in the brain of animals with scrapie (10), a transmissible disease in animals that is similar to Creutzfeld–Jakob disease in humans and also exhibits prion protein plaques (9).

Extracellular senile plaques contain an amyloid core formed by the βA4 peptide (11), a peptide of at most 43 amino acids formed by proteolytic processing of the amyloid precursor protein (APP). Several additional proteins are associated with βA4, including ApoE, APP, α_1-antichymotrypsin, complement factors, and immunoglobulins (12). These proteins, as well as the βA4 amyloid peptide, are also found surrounding cerebral vessels in Alzheimer disease, producing the "congophilic angiopathy" (12). The mechanism of assembly and the putative role in pathogenesis of the senile plaque and congophilic angiopathy are not known.

The avidity and specificity of protein binding to βA4 peptide immobilized on a membrane support can be examined *in vitro*. We used this assay to screen partially purified serine-protease inhibitors ("serpins") for their ability to bind βA4 peptide and found that ApoE was a minor protein contaminant that remained tightly bound to βA4 peptide. We show here that (*i*) ApoE in cerebrospinal fluid (CSF) binds with high avidity to immobilized βA4 peptide and to small peptide domains of βA4 peptide; (*ii*) senile plaques, vascular amyloid, and neurofibrillary tangles *in vivo* contain ApoE; and (*iii*) *APOE* allele frequency analyses in patients with familial Alzheimer disease reveal an unexpected overrepresentation of the *APOE*-ε4 allele in late-onset familial Alzheimer disease compared with age-matched, unrelated controls.

MATERIALS AND METHODS

Preparation of Immobilized Peptides. βA4 peptides (Bachem) and other peptides were covalently immobilized on Immobilon AV affinity membrane (Millipore). βA4 peptides, or other peptides, were dissolved at 10 μg/μl in distilled water. Samples (10 μl, containing 100 μg of peptide) were applied to 13-mm-diameter Immobilon discs and allowed to dry overnight at room temperature. Peptide was in large excess to the number of functional binding groups on the membrane. Control membranes were prepared by incubation with 2.0 M ethanolamine in 1.0 M $NaHCO_3$ (pH 9.5) to block the reactive groups on the membranes. Membranes were stored at −20°C in a desiccator. Prior to use the membranes were washed with phosphate-buffered saline. The hydropathic mimic and even-hydropathy analogues of the βA4-(12–28) peptide (see Fig. 3) were custom synthesized in the Howard Hughes Medical Institute Biopolymer Resource Facility at Duke Medical Center by using standard fluoren-9-ylmethoxycarbonyl synthesis on an Applied Biosystems 430A peptide synthesizer. Purity of the peptides was verified by reverse-phase high-performance liquid chromatography.

Binding of CSF Proteins to Immobilized Peptides. Immobilon AV membranes derivatized with βA4 peptides, with ethanolamine, or with the hydropathic mimic or even-hydropathy peptide were incubated with 100 μl of CSF (previously filtered through a 0.22-μm filter) and 50 μl of phosphate-buffered saline (pH 7.3) for 30 min at room temperature. After incubation the membranes were placed in a Millipore Swinnex filter holder and washed with 3 ml of phosphate-buffered saline followed by 700 μl of 5% (wt/vol)

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Abbreviations: ApoE, apolipoprotein E; CSF, cerebrospinal fluid.
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sodium dodecyl sulfate (SDS). The membranes were removed from the filter holder, cut in half, and placed in 150 μl of Laemmli buffer [2% SDS/5% (vol/vol) 2-mercaptoethanol/10% (vol/vol) glycerol/62.5 mM Tris·HCl, pH 6.8] and boiled for 5 min to solubilize retained proteins. Forty-five microliters of Laemmli buffer with solubilized protein was loaded in each of 10 lanes of a Bio-Rad minigel apparatus, with a stacking gel of 4% (wt/vol) polyacrylamide, and a separating gel of 12% polyacrylamide with 2% SDS. Electrophoresed proteins were then either visualized by silver stain (Bio-Rad) or transferred to Immobilon P membrane.

Immunoblot Detection of ApoE. Electrophoresed proteins were transferred to Immobilon P by standard Western transfer techniques. After transfer the membrane was incubated in blotto [5% dried milk in Tris-buffered saline (pH 7.6) with 0.10% Tween 20 (Pierce)] at room temperature for 1 hr. The membrane was next incubated with goat anti-rabbit ApoE antibody at 1:1000 dilution (kindly supplied by Joel Morrisett, Department of Medicine, Baylor College of Medicine, Houston) in blotto overnight at 4°C, then washed five times in blotto. The membrane was exposed to rabbit anti-goat secondary antibody conjugated with horseradish peroxidase (1:10,000 dilution) for 1 hr at room temperature, then washed seven times in blotto. Horseradish peroxidase was visualized with an enhanced chemiluminesce detection kit (Amersham) and exposed to Hyperfilm ECL (Amersham).

Amplification and Restriction Isotyping of *APOE*. Genomic DNA was amplified by polymerase chain reaction (PCR) in either a Stratagene SCS-96 or a Techne MW-2 thermocycler using Techne Hi-Temp 96-well plates and the primers described by Wenham *et al.* (13). The PCR protocol was derived from those described by Wenham *et al.* (13) and Hixson and Vernier (14). Each reaction mixture contained 20 ng of genomic DNA, 15 pmol of each primer, 10% (vol/vol) dimethyl sulfoxide (Sigma), 200 μM each dNTP (Pharmacia), 2.0 μCi of [α-^{32}P]dCTP (800 Ci/mol in 10 mM Tricine, NEN; 1 Ci = 37 GBq); 0.75 unit of *Taq* DNA polymerase, and 1× incubation buffer (Boehringer Mannheim) in a final volume of 15 μl. After electrophoresis, the products were transferred to Whatman 3MM chromatography paper, dried, and autoradiographed for 1 hr with Kodak XAR-5 film.

Immunocytochemistry of ApoE. Blocks of brain tissue freshly dissected from autopsy specimens (Joseph and Kathleen Bryan Alzheimer's Disease Research Center Rapid Autopsy Program) were immersed in buffered 10% formalin for 12–18 hr and then placed in phosphate-buffered saline at 4°C for storage. Vibratome sections (30–40 μm thick) were cut from these blocks and treated briefly with 10% methanol/3% hydrogen peroxide to block endogenous peroxidase. In some cases, sections were also exposed to 10% formic acid for 10 min. Immunocytochemical localization of ApoE was carried out by standard avidin–biotin detection using the same antiserum utilized in Western blotting, at dilutions from 1:5000 to 1:50,000. A primary incubation of 1 hr at room temperature was followed by an overnight incubation at 4°C. Visualization was performed with diaminobenzidine as a chromogen. Similar dilutions of normal goat serum served as control for nonspecific binding of immunoglobulins. Sections of control human brain and Alzheimer disease brain were examined, as well as sections from a very old *Lemur fulvus collaris* (wild-caught; estimated age, 38 years) euthanized by the Duke Primate Center because of untreatable illness and extreme age. Presence of amyloid plaques was verified in all material by use of a monoclonal antibody to βA4 peptide (4D12, a gift from David Allsop and George Glenner, University of California, San Diego).

CSF and Blood Samples. CSF was obtained from clinical diagnostic lumbar punctures performed following informed consent and stored at −80°C. Both control CSF and Alzheimer patient CSF were obtained from the Joseph and Kathleen Bryan Alzheimer's Disease Research Center Brain Bank. The diagnosis of Alzheimer disease was confirmed by neuropathologic examination.

Association Studies. Affected individuals used in the allele association study were randomly selected subjects from each of 30 families with familial Alzheimer disease. The diagnostic criteria used for these families have been described (15). In 28 of the families the disease was of the late-onset type (mean age of onset > 60 years of age), and in 2 of the families, early onset (mean age of onset < 60 years of age). Controls used in the study were 91 unrelated grandparents from the Centre d'Etude du Polymorphisme Humain (16). To compare the allele frequencies between the various groups we applied the *Z* statistic as outlined by Schellenberg *et al.* (17).

RESULTS

Potter *et al.* (18) reported that βA4 peptide bound α_1-antichymotrypsin *in vitro*. Using the *in vitro* assay described here, we found that antithrombin, a related protein, did not bind the βA4 peptide but a contaminant protein did. This contaminant protein was identified by protein microsequencing to be ApoE. We then characterized the *in vitro* binding of ApoE in CSF to immobilized βA4 peptide. CSF contains many proteins that are retained on both the immobilized βA4-(12–28) peptide and the even-hydropathy control peptide (see Fig. 3) following a wash with phosphate-buffered saline (Fig. 1 *Upper*, lanes 1). Among these retained proteins is ApoE (Fig. 1 *Lower*, lanes 1). CSF contains two species of ApoE that are resolved by gel electrophoresis. The slower migrating, upper band is sialylated ApoE; the faster migrating ApoE does not contain sialic acid (19). Many of the CSF

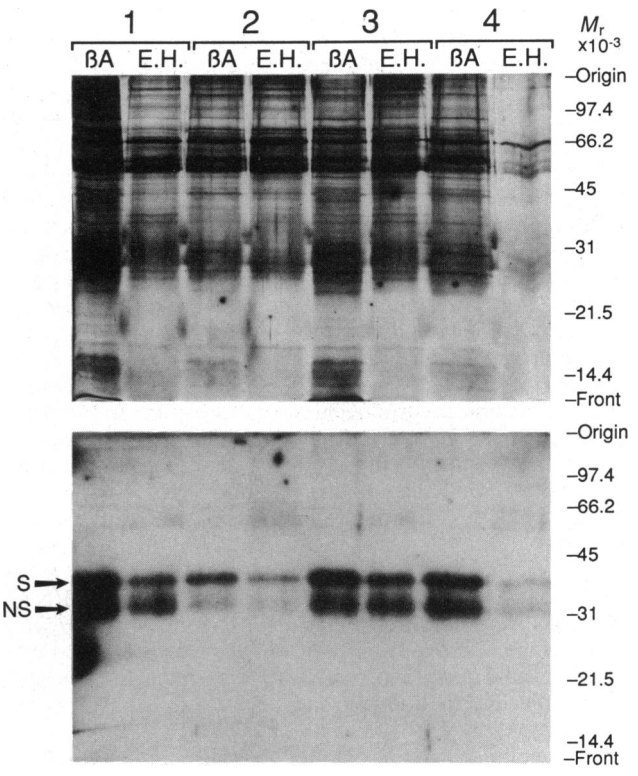

FIG. 1. Binding of CSF proteins to immobilized peptides following elution. CSF proteins were visualized by silver-staining (*Upper*), or CSF ApoE was visualized by immunoreactivity (*Lower*), after incubation with immobilized βA4-(12–28) or the even-hydropathy peptide (E.H.; see Fig. 3) and elution with phosphate-buffered saline alone (lanes 1) or containing 5% SDS (lanes 2), 4 M urea (lanes 3), or 6 M guanidine hydrochloride (lanes 4). Arrows: S, sialylated ApoE; NS, nonsialylated ApoE.

proteins are eluted from both immobilized peptides with 5% SDS (Fig. 1, lanes 2); by 4 M urea (lanes 3); or by 6 M guanidine hydrochloride (lanes 4). Guanidine hydrochloride did not elute ApoE from the βA4-(12–28) peptide but did elute virtually all the ApoE bound to the even-hydropathy control peptide (Fig. 1 *Lower*, lanes 4).

Binding of ApoE to various immobilized peptides is shown in Fig. 2. ApoE in CSF remained bound to immobilized βA4-(1–40), βA4-(1–28), and βA4-(12–28) after incubation and washing with phosphate-buffer saline plus 5% SDS. The even-hydropathy peptide contains the same amino acids as βA4-(12–28) but has a hydropathic profile different from βA4-(12–28) (Fig. 3). ApoE did not bind to this immobilized peptide (Fig. 2 *Lower*). The 12–28 hydropathic mimic peptide contains different amino acids than βA4-(12–28) but has a hydropathy profile very similar to βA4-(12–28) (Fig. 3). This peptide also bound ApoE after washing with phosphate-buffered saline plus 5% SDS (Fig. 2).

Antiserum to ApoE stained amyloid plaques in tissues from Alzheimer disease patients and an aged lemur (Fig. 4 *B* and *E*). This immunolocalization was observed even at the highest dilutions of antiserum and was very intense compared with control sections (Fig. 4*A*). Plaques were the last structures to lose immunopositivity. Formic acid pretreatment increased ApoE immunoreactivity of amyloid plaques, similar to the effect of formic acid on βA4 immunoreactivity. ApoE immunoreactivity (Fig. 4*B*) closely imitated the distribution of βA4 amyloid (Fig. 4*C*), even being observed near glial processes (Fig. 4 *B* and *C*, arrowheads). In addition, intraneuronal staining was observed, which may represent staining of neurofibrillary tangles (8) or early degeneration of neurons (Fig. 4 *D–F*). Immunohistochemical identification of ApoE surrounding blood vessels was similar in appearance to

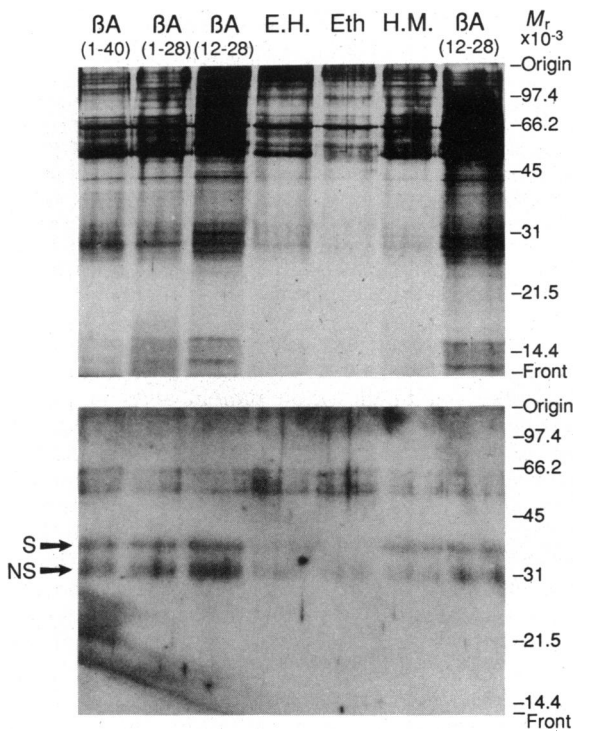

FIG. 2. Binding of CSF proteins to immobilized peptides. CSF proteins were visualized by silver staining (*Upper*), or CSF ApoE was visualized by immunoreactivity (*Lower*), after incubation with immobilized βA4-(1–40), βA4-(1–28), even-hydropathy peptide (E.H.), ethanolamine (Eth), or the hydropathic mimic (H.M.; see Fig. 3) and elution with phosphate-buffered saline containing 5% SDS. Arrows: S, sialylated ApoE; NS, nonsialylated ApoE.

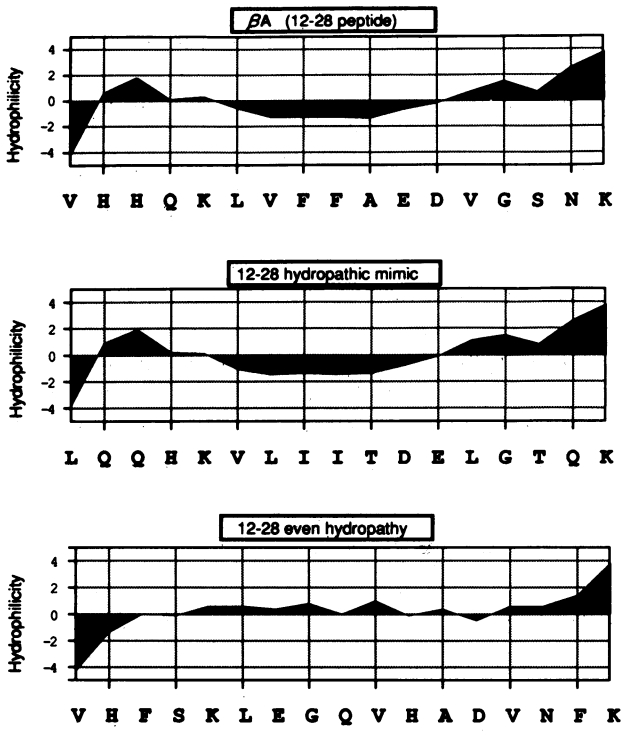

FIG. 3. Amino acid sequences (one-letter symbols) and hydropathy plots of βA4-(12–28) (*Top*), 12–28 hydropathic mimic (*Middle*), and 12–28 even-hydropathy peptide (*Bottom*). Hydropathic profiles were generated by Kyte–Doolittle analysis with a window of seven amino acids, using the MACVECTOR program.

βA4 amyloid immunostaining of vascular amyloid deposits (Fig. 4*F*).

There are three major isoforms of ApoE identified by amino acid sequencing, designated ApoE-E3, ApoE-E4, and ApoE-E2. The two uncommon isoforms, E2 and E4, vary from the common E3 isoform by the substitution of one amino acid (1). ApoE-E4 contains an arginine at residue 112, whereas E3 has a cysteine. ApoE-E2 contains a cysteine at residue 158, whereas E3 has an arginine. There are other rare isoforms (1). PCR-based restriction isotyping of *APOE* alleles has been developed for use in population studies (13, 14). Association studies of *APOE* alleles using DNA samples from one randomly selected familial Alzheimer disease patient from each of 30 families and from 91 age-matched unrelated controls showed that the frequency of the *APOE-ε4* allele in the 30 patients (0.50 ± 0.06) differed significantly from the frequency found in the 91 controls (0.16 ± 0.03; $Z = 2.44$, $P = 0.014$). The allele frequency of *APOE-ε4* found in our control population was similar to that found in a larger population study (20). *APOE-ε4* frequency was similar for all 83 tested Alzheimer disease patients in the 30 families, indicating that the random sample used in the association analysis accurately represented the ε4 allele frequency in the families (Table 1).

DISCUSSION

Binding of proteins to the βA4 peptide can be studied *in vitro* by covalently binding βA4 peptide, or fragments of βA4 peptide, to a membrane support. Using this technique, we have demonstrated high-avidity binding of ApoE in CSF to βA4 peptide. Binding of ApoE to βA4, to the hydropathic mimic peptide, and to the prion protein plaque may require the hydrophobic domains of each of these peptides. Namba *et al.* (8) and Wisniewski and Frangione (21) have recently shown ApoE immunoreactivity in several types of cerebral

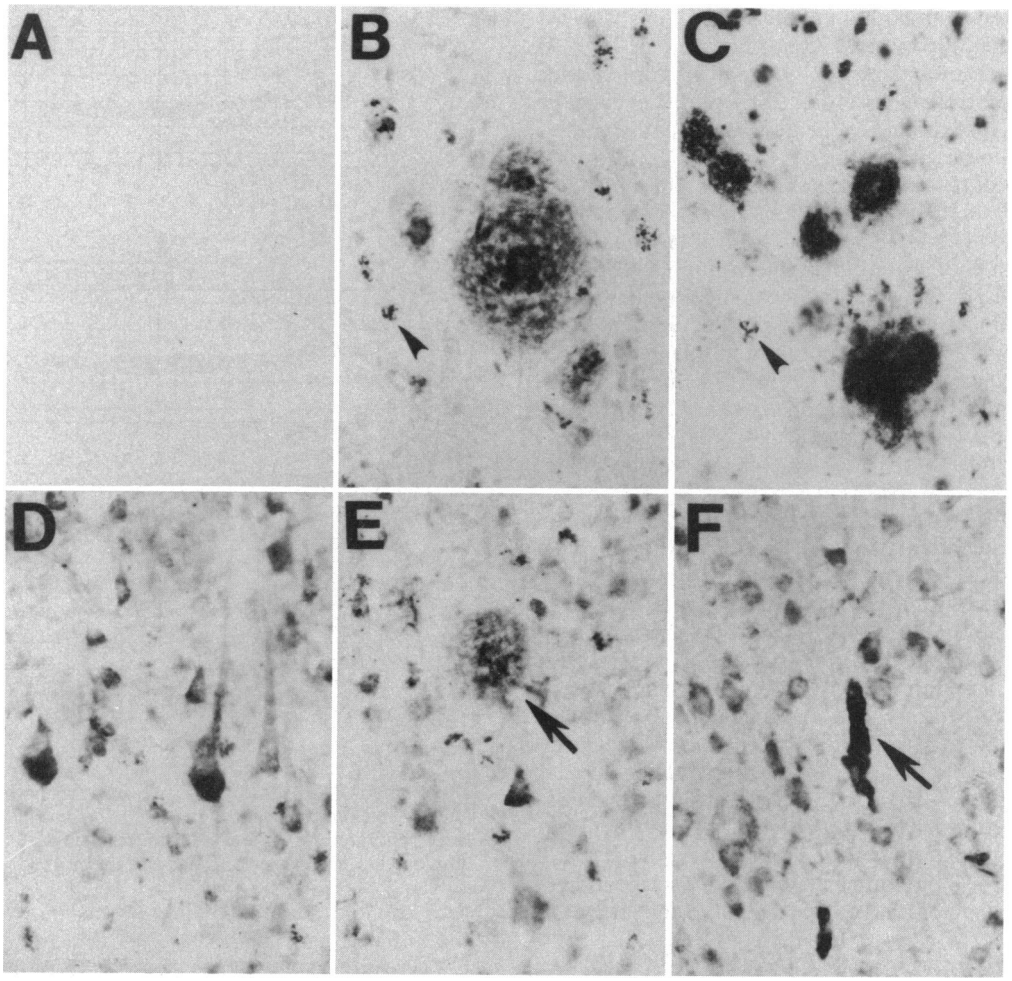

FIG. 4. Photomicrographs of sections of human and lemur brain stained for immunocytochemical localization of ApoE or for βA4 amyloid deposits. All anti-ApoE antibody dilutions were 1:10,000. (*A*) Control section from section of hippocampus from brain of patient with Alzheimer disease stained with normal goat serum (1:10,000 dilution). (*B*) Adjacent section to *A* stained with anti-apoE in parallel and showing staining of plaque and also of glial profiles (arrowhead). (*C*) Adjacent section to *A* stained with monoclonal antibody to βA4 amyloid peptide (4D12, 1:1000 dilution) showing staining of amyloid plaques and also small glial profiles (arrowhead) similar to *B*. (*D*) Section from entorhinal cortex of same case showing staining of neurons by anti-apoE. Neuronal staining varied in intensity and was generally strongest for neurons and neuronal classes with neurofibrillary tangles. (*E*) Section from neocortex of elderly, 38-year-old *Lemur fulvus collaris* showing small anti-apoE-immunoreactive amyloid plaque (arrow) and extensive neuronal staining. (*F*) Another region of the lemur's neocortex showing very intense apoE immunoreactivity around cerebral vessel (arrow). This animal had extensive vascular amyloid deposits, and some amyloid plaques (*E*). Both plaque and vascular amyloid were immunoreactive with monoclonal antibody 4D12, which reacts with βA4 amyloid protein deposits in human and primate material. (×260.)

and systemic amyloid. The latter authors suggested that ApoE may act as a molecular chaperone, defined as "unrelated proteins that mediate β-pleated amyloid formation of polypeptide fragments." Our combined peptide binding data, immunochemistry, and allele association analyses suggest a more integrated role of ApoE in βA4 metabolism and targeting in Alzheimer disease.

ApoE may be a common denominator in either the pathogenesis or the cellular response of several dementing diseases. ApoE is found in senile plaques, in neurofibrillary tangles, and at the sites of congophilic angiopathy of Alzheimer disease and in the prion protein amyloid plaque of Creutzfeld–Jakob disease and scrapie. The localization of ApoE to the defining pathological lesions of Alzheimer disease suggests a functional pathogenetic role for ApoE. Binding of ApoE to βA4 peptide, or to other hydrophobic peptides, may be a requisite step in targeting these peptides for their final intra- or extracellular metabolism. The possibility of differences in the binding of βA4 peptide to each of the ApoE isoforms may provide insight into the functional role of ApoE-E4 in Alzheimer disease (22). ApoE is involved in the regenerative response of injured nerve and is found in the pathologic lesions of these age-dependent degenerative diseases. Pharmacologic interventions that alter the binding of βA4 peptide to ApoE isoforms and interdict molecular pathogenesis may be important therapeutic areas to explore.

Table 1. *APOE* alleles of 83 patients in 30 families with familial Alzheimer disease (FAD)

Allele	FAD	Controls	
		This work*	Ref. 20
ε2	0.04	0.10	0.08
ε3	0.44	0.73	0.78
ε4	0.52	0.16	0.14
	(n = 166)	(n = 182)	(n = 2000)

n, No. of chromosomes.
*Ninety-one unrelated grandparents from the Centre d'Etude du Polymorphisme Humain.

APOE is on the proximal long arm of chromosome 19, at 19q13.2 (1). With nonparametric linkage analysis methods (15), genetic markers from chromosome 19q13.1–q13.3 had previously suggested linkage to late-onset familial Alzheimer disease. However, it could not be determined at that time

whether the significant results indicated association rather than true linkage (15). The association of the *APOE-ε4* allele with familial Alzheimer disease was significantly increased compared to the population controls and is consistent with prior analyses.

The finding that the *APOE-ε4* allele is associated with familial Alzheimer disease patients has two major implications. First, there may be allele-specific functions that contribute to the molecular mechanism of the disease expression. Second, there could also be another intragenic polymorphism on an *APOE-ε4* background that confers susceptibility to Alzheimer disease. A similar mechanism has been described for fatal familial insomnia (23) and one form of Creutzfeld–Jakob disease (24, 25). Two distinct phenotypes share the same prion protein codon-178 mutation, with disease expression determined by the inheritance of another intragenic prion polymorphism (25).

Another report (22) will describe the status of *APOE* alleles in sporadic Alzheimer disease. With the recent appreciation that βA4 amyloid is a normal extracellular product in many cell types (26), the discovery of specific interactions between ApoE and βA4 could define additional mechanisms of Alzheimer disease pathogenesis. It is intriguing that antibodies to ApoE stain both intracellular and extracellular structures in Alzheimer disease, Creutzfeld–Jakob disease, and a primate model of βA4 amyloidosis. ApoE functions to transport lipids into the cell. A function involving the binding, transport, and targeting of βA4 (or other peptides) may link the diagnostic lesions and genetic susceptibility, suggesting a functional role for ApoE isoforms in the mechanism of Alzheimer disease pathogenesis.

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