Quantitation of apoE Domains in Alzheimer Disease Brain Suggests a Role for apoE in Aβ Aggregation

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Abstract. Apolipoprotein E (apoE) and apoE-derived proteolytic fragments are present in amyloid deposits in Alzheimer disease (AD) and cerebral amyloid angiopathy (CAA). In this study, we examined which apoE fragments are most strongly associated with amyloid deposits and whether apoE receptor binding domains were present. We found that both apoE2- and apoE4-specific residues were present on plaques and blood vessels in AD and CAA. We quantified Aβ plaque burden and apoE plaque burdens in 5 AD brains. ApoE N-terminal-specific and C-terminal-specific antibodies covered 50% and 74% of Aβ plaque burden, respectively (p < 0.003). Double-labeling demonstrated that the plaque cores contained the entire apoE protein, but that outer regions contained only a C-terminal fragment, suggesting a cleavage in the random coil region of apoE. Presence of N- and C-terminal apoE cleavage fragments in brain extracts was confirmed by immunoblotting. The numbers of plaques identified by the apoE N-terminal-specific antibodies and the apoE C-terminal-specific antibody were equal, but were only approximately 60% of the total Aβ plaque number (p < 0.0001). Analysis of the size distribution of Aβ and apoE deposits demonstrated that most of the Aβ-positive, apoE-negative deposits were the smallest deposits (less than 150 μm2). These data suggest that C-terminal residues of apoE bind to Aβ and that apoE may help aid in the progression of small Aβ deposits to larger deposits. Furthermore, the presence of the apoE receptor binding domain in the center of amyloid deposits could affect surrounding cells via chronic interactions with cell surface apoE receptors.

Key Words: Amyloid; Lipoprotein; Proteolysis; Receptor.

INTRODUCTION

In Alzheimer disease (AD) and cerebral amyloid angiopathy (CAA), the apolipoprotein E (apoE) protein is associated with the deposits of the Aβ peptide (1, 2). The APOE gene is associated with altered risks of AD and CAA, although the risks follow a surprising pattern: while APOE-ε4 increases the risk for both AD (3) and CAA (4), APOE-ε2 decreases the risk of AD (5) but increases the risk of CAA (6, 7). Neuropathologically, APOE-ε4 is associated with increased amyloid deposition in both AD (8) and CAA (9); APOE-ε2 is associated with decreased parenchymal amyloid deposition (10), but an increased frequency of CAA-associated vascular damage (7, 11). Clinically, APOE-ε4 is associated with an earlier onset in AD patients (12, 13); APOE-ε2 is associated with an increased risk of recurrent hemorrhages in CAA patients (14). From these observations, we have suggested that apoE4 promotes the formation of amyloid deposits and apoE2 promotes the breakdown of amyloid-containing vessels (7).

The molecular mechanisms behind these associations remain undefined. In vitro, studies of apoE’s role in AD and CAA have mainly focused on an altered ability of apoE isoforms to clear Aβ via apoE receptors (15, 16) or to promote Aβ aggregation (17–19). In vivo, these hypotheses may be differentiated by neuropathological analysis of the different apoE domains in AD brain. ApoE contains 3 distinct functional domains (20) (Fig. 1A): the N-terminal receptor binding domain, a random coil region, and the C-terminal lipid-binding domain (reported to bind Aβ in vitro [21, 22]). We examined CAA and AD brains to test whether the apoE receptor binding domain was intact in amyloid deposits and capable of interacting with apoE receptors for clearance. We also compared the distribution of N- and C-terminal domains to analyze apoE metabolism in amyloid deposits. Finally, we examined the Aβ and apoE fragments in the brain parenchyma to test whether apoE domains were present in every type of Aβ deposit. Our findings support a model of apoE involvement in the progression of amyloid deposits (but not in the initial deposition of Aβ), and the selective interaction of apoE with apoE receptors at the core of Aβ deposits.

MATERIALS AND METHODS

Antibodies

Antibodies to different domains of human apoE were used (Fig. 1A): 6C5 (mouse monoclonal antibody against residues 1–15); 1D7 (mouse monoclonal antibody against residues 140–160); 3H1 (mouse monoclonal antibody against residues 243–272) (each from Ottawa Heart Institute Research Corporation, Ottawa, Ontario); F48.1 (mouse monoclonal antibody against apoE2, American Research Products, Belmont, MA). We also used 15D2, a mouse monoclonal antibody specific for the apoE4 isofrom (Elan Pharmaceuticals, San Francisco, CA). This antibody recognized the 34 kDa apoE4 protein on Western blots.
Fig. 1. Region-specific apoE antibodies recognize amyloid deposits. A: Diagram of apoE, with structural domains and residues recognized by various antibodies: 6C5 recognized the N-terminal residues (aa 1–15); 1D7, the LDL receptor binding area (aa 140–160); and 3H1, the C-terminal residues (aa 243–272). 15D2 recognizes apoE4-specific residues (aa 112) and F48.1 recognizes apoE2-specific residues (aa 158). B: Amyloid deposits in the temporal cortex of an APOE ε4/4 case were recognized by apoE region-specific antibodies. From left to right: 6C5, 15D2, 1D7, and 3H1. The final panel was immunostained with an antibody against Aβ. Scale bar = 200 μm.

and apoE on amyloid deposits in AD brain tissue from APOE-ε4/4 individuals by immunohistochemistry, but did not recognize apoE on amyloid deposits in brain tissue from an APOE-ε2/3 individual with familial AD. Aβ was detected with 10D5 (mouse monoclonal antibody, Elan Pharmaceuticals [23]). For double labeling, 6C5 was directly labeled using the Cy3 Mab labeling kit (Amersham Pharmacia Biotech, Piscataway, NJ). Horseradish peroxidase-linked secondary antibodies and Cy5-linked secondary antibody were purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

Brain Tissue

The Massachusetts Alzheimer Disease Research Center Brain Bank and Harvard Brain Tissue Resource Center provided temporal cortex and occipital cortex from 5 AD cases (each APOE ε4/4) and 5 CAA cases (each APOE ε2/4). For the AD cases, there were 3 women and 2 men, with a mean age of 77 yr (range 67 to 84); each had severe AD, 2 with some evidence of Lewy body disease and 3 with concomitant CAA. For the CAA cases, there were 3 women and 2 men, with a mean age of 75 (range 69 to 83); each also had neuropathological evidence of AD. DNA was isolated from brain tissue and APOE genotypes were determined as described (8). The brain tissues were fixed in paraformaldehyde lysine metaperiodate (Sigma Chemical, St. Louis, MO) for 48 h at 4°C and placed in 15% glycerol in Tris-buffered saline (TBS) (pH 7.4) for 24 h. The blocks were cut into 50-μm sections on a freezing sledge microtome. The free-floating sections were transferred into tubes containing 15% glycerol in TBS (pH 7.4) and stored at −20°C until use.

For immunoblotting, approximately 1 g of brain cortex was homogenized in 50 mM Tris (pH 8.0), 0.5 M NaCl, 0.1% Triton X-100 with 400 μM PMSF, 2 μM pepstatin, 2 μM leupeptin and 1.5 μM aprotinin, and centrifuged briefly to remove debris. Extract proteins (40 μg) were separated under denatured and reduced conditions by Tris-Tricine 10%–20% polyacrylamide gel electrophoresis (Novex, SD, CA). The separated proteins were transferred onto the PVDF membrane at 200 mA for 2 h and blocked with 5% nonfat dry milk. The blot was incubated with antibodies 6C5 (1:2,000), 1D7 (1:2,000), or 3H1 (1:2,000) at 4°C overnight. The horseradish peroxidase-conjugated antimouse Ig secondary antibody was visualized by ECL detection system (Amersham Pharmacia Biotech). Human recombinant apolipoprotein E4 (Biogenesis, Brentwood, NH) was used as apoE standard.

Immunohistochemistry

Temporal lobe of AD cases (including the superior temporal sulcus) and available sections of the occipital lobe of CAA cases were immunostained for Aβ and apoE. Free floating sections were pretreated with 10 mM citrate buffer pH 6.0 at 95°C for 10 min and then 70% formic acid at room temperature for 10 min. 0.5% Triton X-100 in 3% H2O2 was applied to the sections for 20 min followed by 1 h of blocking in 3% nonfat dry milk in TBS (pH 7.4) at room temperature. Sections were incubated overnight at 4°C with primary antibody diluted in 1.5% normal goat serum: 6C5 (1:300), 1D7 (1:200), 3H1 (1:300), F48.1 (1:200), 15D2 (1:100), 10D5 (1:350). Immunoreactivity was visualized with horseradish peroxidase linked secondary antibody and 3,3′-diaminobenzidine (DAB) as chromogen. For sequential double labeling, the first primary antibody was detected with Cy5-labeled anti-mouse secondary antibody. After thorough washing, the section was probed with second primary antibody directly linked with Cy3. Fluorescence was observed using the BioRad MRC-1024 confocal microscope (Cy3: excitation at 568 nm, emission at 605 nm, Cy5:
excitation at 647 nm, emission at 680 nm). No plaque or vessel staining was observed when primary apoE and Aβ primary antibodies were not included in the immunohistochemical analyses.

Quantitative Analysis

A strip of cortex, approximately 1,500 μm wide by the depth from the white matter to the pia, in the inferior bank of the superior temporal sulcus was examined as we have previously described (13). Video images were captured using a DAGE MTI CCD72 camera. Data were recorded using a Bioquant Microquant Image Analysis System (Knoxville, TN) on a motorized stage which moved to non-overlapping fields (24). Areas of staining above background within this field were identified and circumscribed manually by a single examiner; plaques (and rare instances of neurofibrillary tangles) were included but blood vessels and artifacts were eliminated. Every plaque within the field was measured and the areas of plaques were recorded. The plaque burden (%) was defined as a percentage of the area of cortex covered by immunostaining. The plaque number was defined as the number of plaques in 1 mm² of gray matter immunostained. The relative apoE plaque burden (%) and plaque number (%) for each case were recorded as percentages of Aβ plaque burden and plaque number in order to control for the variation of amyloid deposition in the different cases. The relative plaque burdens and the relative plaque numbers were statistically compared by an ANOVA test.

RESULTS

ApoE2 and apoE4 Are Present on Plaques and CAA

Genetic studies have implicated APOE ε2 and APOE ε4 in the pathogenic processes of AD and CAA. ApoE protein or protein fragments are known to be present on amyloid deposits in these diseases. We tested whether the isoform-specific domains of apoE2 and apoE4 were present in both types of amyloid deposits. Various regions of the occipital cortex from 5 APOE ε2/4 brains were examined with apoE2- and apoE4-specific antibodies. The apoE2-specific antibody immunostained plaques and blood vessels (Fig. 2A, C), as did the apoE4-specific antibody (Fig. 2B, D). Amyloid in plaques and blood vessels were equally well stained for apoE2 and apoE4. As controls, we found no cross-reactivity of apoE2 or apoE4 antibodies with other apoE isoforms: the apoE2-specific antibody did not immunostain APOE ε3/4 AD brain and apoE4-specific antibody did not immunostain APOE ε2/3 AD brain.

All Domains of apoE Are Present on Amyloid Deposits

Figure 1A shows apoE amino acid residues recognized by 4 apoE antibodies, 1 specific to apoE4, and the other 3 isoform-independent. We immunostained temporal cortex of AD brains from 5 APOE ε4/4 individuals and found that each antibody identified numerous parenchymal amyloid deposits (Fig. 1B); most apoE antibodies also identified a few neurofibrillary tangles in some cases.

Thus, we used these 4 apoE antibodies directed against different apoE domains to examine the interaction of Aβ with apoE domains in vivo.

ApoE N-terminus is not Present on the Periphery of Plaques

To examine in detail the distribution of apoE domains in amyloid deposits, we double-labeled AD temporal cortex with the N-terminal-specific antibody and the C-terminal-specific antibody (Fig. 3). The N-terminal-specific antibody, 6C5-Cy3 (in red), and the C-terminal-specific antibody, 3H1 detected with Cy5 (in blue), greatly co-localized (in pink). However, C-terminal apoE was present in the outer portions of the plaques without any N-terminal apoE (although it is technically possible that the N-terminus of apoE is present but masked in these regions). To control for differences due to detection of the fluorophores, we reversed the labeling and observed the same disparity in amyloid deposits immunostained with N-terminal and C-terminal apoE antibodies.

Quantification of Aβ and apoE Domains in Amyloid Deposits

In order to quantitatively compare the deposition of the different domains of apoE to the deposition of Aβ in the
Fig. 3. Double-labeling of apoE with an N-terminal-specific antibody and a C-terminal-specific antibody. Temporal cortex from APOE ε4/4 case was immunostained with 6C5 for N-terminal apoE residues (panel A, in red) and with 3H1 for C-terminal apoE residues (panel B, in blue). Co-localization of N- and C-terminal apoE residues (panel C, in pink) appears at the centers of deposits (see arrowheads). Arrows denote areas where apoE N-terminal residues are not present with apoE C-terminal residues. Scale bar = 100 μm.

AD brain, we measured plaque burden in the superior temporal sulcus after immunostaining with Aβ or apoE antibodies, imaging with DAB, and counting by the Bioquant Analysis System. Plaque burden was defined as a percentage of the area of cortex covered by immunostaining. Five APOE ε4/4 cases were examined.

The mean of Aβ plaque burden in these 5 cases was 12% (SD ± 2%). ApoE plaque burdens were significantly lower for each of the 4 apoE antibodies, covering 47%–74% of the Aβ plaque burden: 6C5, 47% (SD ± 16%, p < 0.0001); 15D2, 58% (SD ± 17%, p < 0.0001); 1D7, 47% (SD ± 12%, p < 0.0001); 3H1, 74% (SD ± 10%, p < 0.003) (Fig. 4). These data were supported by qualitative analysis with Aβ/apoE double-labeling, showing frequent Aβ-positive, apoE-negative plaques but only very rare Aβ-negative, apoE-positive plaques (data not shown). Figure 4 also demonstrated that the 3 N-terminal apoE antibodies stained 22%–36% less area than the C-terminal apoE antibody (6C5 (p < 0.003), 15D2 (p < 0.06), 1D7 (p < 0.003)). ApoE burdens between 6C5, 15D2, and 1D7 were not significantly different. From these data, we determined that a significant fraction of amyloid-associated apoE was present as C-terminal fragment without N-terminal residues, consistent with the greater C-terminal apoE immunostaining observed in Figure 3.

We tested for an in vivo cleavage between the N- and C-termini of apoE in brain by immunoblotting AD brain extract with N- and C-terminal-specific antibodies (Fig. 5). While each apoE antibody identified primarily full-length 36 kDa apoE, the 2 N-terminal apoE antibodies identified significant breakdown products of 23–25 kDa (Fig. 5, upper arrow). While more fragments were identified with the C-terminal apoE antibody, a major breakdown product of 10 kDa was observed (Fig. 5, lower arrow). N- and C-terminal apoE fragments were observed in extracts from AD as well as control brains.

Figure 4 showed that 26% of Aβ plaque area was not associated with any apoE. This reduction could have been due to a subset of Aβ deposits that had no apoE, or to 26% of each deposit that was apoE-negative, or to some combination of these possibilities. Thus, in addition to measuring the percentage of area immunostained by Aβ and apoE (plaque burden), we counted the numbers of plaques immunostained (plaque number). The mean number of Aβ plaques was 209/mm² (SD ± 56) from the 5 APOE ε4/4 cases. The numbers of plaques immunostained with the different apoE antibodies were significantly lower than the number of Aβ plaques for each of.
Fig. 5. Western blot of apoE and apoE fragments from AD brain. Homogenized AD brain tissue was immunoblotted for apoE protein fragments using the N-terminal apoE antibody (6C5, lane 1), an antibody to the receptor binding domain (1D7, lane 2), and the C-terminal apoE antibody (lane 3). Lane 4 contains recombinant human apoE4. The upper arrow indicates apoE fragments containing the N-terminus, and the lower arrow indicates a fragment specific to the apoE C-terminus. These blots indicate that in brain there is a major cleavage site of apoE in its random coil domain.

Fig. 6. Relative plaque numbers. Plaque numbers in the gray matter of temporal cortex were counted with an antibody that recognizes Aβ and 4 different apoE antibodies that recognize different residues of apoE. Five APOE ε4/4 AD brains were examined. The mean of Aβ plaque number per mm² of gray matter was 209 (SD ± 56) and compared with other apoE plaque numbers. Plaque numbers with apoE antibodies were lower than with the Aβ antibody: 6C5, 51% (SD ± 8%, p < 0.0001); 15D2, 61% (SD ± 22%, p < 0.0001); 1D7, 54% (SD ± 9%, p < 0.0001); 3H1, 60% (SD ± 10%, p < 0.0001) (Fig. 6). These data suggest that approximately 40% of Aβ plaques are not associated with apoE. Interestingly, the numbers of plaques immunostained with each of the apoE antibodies were very similar (51% to 61%), despite the differences in plaque burden.

To determine which types of Aβ deposits were apoE-negative, we plotted the distribution of Aβ plaques and apoE plaques according to their sizes. Figure 7 shows the distribution from 1 brain, which is representative of all 5 brains examined. In all 5 cases, there were many more small Aβ deposits than small apoE deposits. Examining only the smallest category (<150 μm²), we found there was only 42%–59% as many apoE-positive plaques as Aβ-positive plaques in these 5 AD brains. Thus, many of the smallest Aβ deposits lack any fragment of apoE.

**DISCUSSION**

We performed detailed analyses of apoE domains in AD brain using diverse techniques, including measurement of plaque size and number, double immunolabeling, and immunoblotting. Observations based on these approaches allowed us several interesting conclusions: 1) ApoE in the brain undergoes cleavage in the random coil domain; 2) the C-terminus of apoE is most strongly associated with Aβ deposits; 3) a significant fraction of Aβ deposits have no apoE, and these are generally the smallest deposits; and 4) the apoE receptor binding domain is present at the core of amyloid deposits and could have chronic effects on apoE receptors. Each of these observations and conclusions is considered in more detail below.

Our quantitation of apoE deposits determined that there was 22%–36% less N-terminal apoE than C-terminal apoE (Fig. 4). The apoE N-terminus co-localizes with the C-terminus at the core of the plaque, showing that the core contains holo-apoE or both N- and C-terminal apoE fragments. The outer portions of the deposit contain only C-terminal apoE immunostaining (Fig. 3), suggesting that the N-terminus of apoE is removed from the outer portions of the plaque while the C-terminus remains bound to the Aβ. With another apoE N-terminal antibody (against apoE 82–97), Aizawa et al also found that apoE N-terminal immunoreactivity was qualitatively weak in AD brain (25). The 3 N-terminal apoE antibodies used in this study, spanning amino acids 1 to 160, show similar stainings, suggesting cleavage of apoE to remove the N-terminus occurs after residue 160. Immunoblot data
of AD brain extracts (Fig. 5) support this localization, with cleavage of the 36 kDa apoE producing an N-terminal fragment of approximately 25 kDa and a C-terminal fragment of 10 kDa. Cleavage of apoE at a thrombin cleavage site within the random coil domain of apoE (amino acids 180–200) had previously been demonstrated in human brain (26). Our immunostaining of amyloid deposits suggests that this apoE cleavage may be occurring in the outer regions of plaques and thus, that the periphery of amyloid deposits may be more dynamic than the core.

The greater immunostaining with the C-terminal apoE antibody suggested that the C-terminus of apoE is the domain that forms the most stable interaction with Aβ. This region is the lipid-binding domain of apoE (20), and seems a logical domain for interaction with the hydrophobic Aβ molecule. Strittmatter et al showed that amino acids 244–272 of apoE were responsible for the binding of apoE to Aβ in vitro (21), a finding supported by Pillot et al (22). This region was also found to be important for binding to non-Aβ amyloids, amyloid A and amyloid L (27). However, some studies have found that both N- and C-terminal regions of apoE are important in the formation of apoE-Aβ complexes in vitro (28, 29). Our findings suggest that whichever residues are initially responsible for the binding of apoE to Aβ, it is the C-terminal domain that forms the most stable complex in vivo. This hypothesis is consistent with the finding that a C-terminal fragment of apoE (amino acids 216–299) co-purified with Aβ from AD brain (30).

Approximately 40% of Aβ deposits have no apoE (Fig. 6), consistent with earlier, more qualitative studies showing some apoE-negative Aβ deposits (31–33). Together these studies demonstrate that apoE is not necessary for Aβ deposition. Analysis of plaque size distributions (Fig. 7) showed many more small Aβ deposits than apoE deposits, but almost equal numbers of larger deposits. There are several models that could explain this preferential distribution of apoE on larger Aβ deposits. One model that we favor is that apoE binding aids the growth or stabilization of Aβ deposits. This model is consistent with studies of transgenic APP mice showing that apoE is necessary for the accumulation of Aβ deposits (34, 35). This model is also supported by our previous study of human CAA that found that APOE genotype affected the progression of small Aβ deposits to larger deposits in the cerebrovasculature (9). In this study, we controlled for APOE genotype by analyzing only APOE e4/4 cases, and the data again supported a role for apoE in the growth of Aβ deposits. It will be interesting to determine if the same pattern is found in APOE-e3/3 individuals who demonstrate lesser Aβ burdens (8, 36) and fewer Aβ deposits (37).

The effect of the presence of the apoE receptor binding domain in amyloid deposits in CAA and AD (Fig. 2) is unknown. ApoE in the brain is induced in response to acute damage (38) and acts to clear debris from the neuropil (39, 40). Normally apoE would be quickly cleared and degraded through interactions with members of the LDL receptor family. In response to ligand binding, these receptors can, in addition to mediating endocytosis, transmit intracellular signals (41, 42), including changes in calcium homeostasis (43–45). These findings provide support for studies showing neuronal toxicity of apoE4 via interactions with 1 member of this receptor family, LRP (26, 43, 46, 47). In the AD brain, the binding of apoE to amyloid deposits can stabilize its presence in the brain and potentially alter these signaling mechanisms for as long as the N-terminus of apoE remains associated with the amyloid. There are some indications that apoE is found more often on plaques associated with dystrophic neurites (32, 33), raising the possibility that apoE could be enhancing the toxic characteristics of Aβ deposits.

In summary, our data suggest that Aβ can form deposits without binding to apoE. ApoE is found preferentially with the larger Aβ deposits, perhaps enabling them to accumulate greater levels of Aβ or perhaps stabilizing their presence in the brain. The apoE found on
these deposits is dynamic, undergoing cleavage in the outer regions of the deposit, releasing the N-terminus of apoE. However, many Aβ deposits in the cerebrovascularature and the brain parenchyma contain the ligand-bind-
ing domain of apoE, which could alter signaling mech-
nisms in the surrounding cells, and, over time, be a source of toxicity. How apoE isoforms differ in these stages will continue to be an active area of research.

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