

Research report

Apolipoprotein E genotype, Alzheimer's pathologies and related gene expression in the aged population

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Abstract

We have investigated the effect of genotypes of apolipoprotein E (ApoE) on the pathologies found in Alzheimer's disease (AD) and its related gene expression in 38 aged human brains obtained from consecutive autopsied cases. ApoE2/3, -3/3, -3/4, and -4/4 were typed in those aged brains, with ApoE3/3 being most prevalent. The AD pathologies were undetectable in ApoE2/3 brains, but were frequently observed in the other ApoE groups. In ApoE3/3 brains, 55%, 34%, and 24% of the cortical sections examined showed senile plaques (SPs), neurofibrillary tangles (NFTs), and cerebral amyloid angiopathy (CAA), respectively. In ApoE4/4 brains, the SP formation was significantly higher. The ApoE genotype neither affected ApoE, APP, or tau mRNA level, nor the differential expression of the latter two. These results suggest that ApoE4/4 accelerates and ApoE2/3 decelerates the development of the AD pathologies in the aged brain, but this is not through alterations of the APP and tau gene expression.

Keywords: Apolipoprotein E; Genotype; Senile plaque; Neurofibrillary tangle; Cerebral amyloid angiopathy; β -Amyloid protein precursor; Tau; Gene expression

1. Introduction

Alzheimer's disease (AD) is characterized by innumerable senile plaques (SPs) and neurofibrillary tangles (NFTs) throughout the cortex. Also AD brain is often complicated with cerebral amyloid angiopathy (CAA), β -amyloid deposition in meningeal vessels, arterioles and capillaries (for review see ref. [44]). The immunocytochemical analysis of Down's syndrome brains of various ages revealed the temporal relationship of the above three AD pathologies; SP, NFT, and CAA appear in this order chronologically [21].

The biochemical studies in the past several years showed that β -protein [5,22] and tau [17,19,47] are the major constituents of β -amyloid and paired helical filaments (PHF), a unit fibril of NFT, respectively. β -Protein is a small protein with $M_r \sim 4000$ which is proteolytically derived from β -amyloid protein precursor (APP) with features of glycosylated cell surface

receptor [14]. There are three major forms of APP, APP-695, -751, and -770 in the brain, which are generated by alternative splicing from a single pre-mRNA [14,16,33,41]. On the other hand, tau is a microtubule-associated phosphoprotein and is considered to stabilize microtubules in vivo [13]. Tau also has molecular diversity which results in part from alternative splicing at both amino- and carboxyl-terminal regions [7,8]. The insertion at the carboxyl-terminal portion produces two types of tau, three- and four-repeat tau.

It has recently been demonstrated that ApoE allele $\epsilon 4$ is a risk factor in sporadic, and both early-onset and late-onset familial AD (FAD); the $\epsilon 4$ allele frequency is significantly high in those forms of AD as compared to that in the general population [1,34,35,37,43]. Three major ApoE isoforms, E2, E3, and E4, are products of three alleles, $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$, on chromosome 19, respectively. From the expression of any two of the three alleles, three homozygous phenotypes, ApoE2/2, -3/3, and -4/4, and three heterozygous phenotypes, ApoE2/3, -2/4, and -3/4, arise.

The role of ApoE as a risk factor for AD raises

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questions as to whether the ApoE genotype or its mRNA level has an effect on the AD pathologies in a large series of aged brains and whether it is related to the expression of β -amyloid protein precursor (APP) and tau mRNAs. This is because in Down's syndrome brains, which are known to develop β -amyloid prematurely [21], both APP and tau mRNAs are upregulated [31] and in aged subjects both mRNA levels are strictly proportional to each other [29]; higher APP mRNA levels accompany higher tau mRNA levels. We report here that the ApoE genotype is related to the degree of the AD pathologies, but not to the expression of APP and tau genes in the aged brain.

2. Materials and methods

2.1. Tissue sources and immunocytochemistry

The present study was based on tissue blocks from three areas, frontal, temporal, and occipital lobes of 37 brains obtained at autopsy at Tokyo Metropolitan Geriatric Hospital and one brain obtained at a local psychiatric hospital [28,30]. The ages of patients ranged from 50 to 101 years, but the majority were more than 75 years old. The data on mental status were not available in this series except four AD cases.

Semiquantitative grading of SPs and NFTs in the tissue section were previously described [28,30]. The numbers of SP, NFT, and CAA were rated as follows: (–), none; (+), some; and (++) , moderate; (+++) , many.

2.2. Chemicals

Nuclease S1 was purchased from Boehringer Mannheim, Germany. All DNA-modifying enzymes and pUC118 were from Takara Shuzo, Kyoto, Japan. [α -³²P]dCTP was from Amersham, England.

All oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer (Foster City, CA, USA) by the phosphoramidite method.

2.3. Preparation of total RNA

Total RNA was prepared from 0.5–1.5 g of frozen brains as described previously [28].

2.4. ApoE genotyping

ApoE genotyping was carried out by DNA amplification by the polymerase chain reaction (PCR) and *Hha*I digestion, essentially as described [10], except for using cDNA instead of genomic DNA. ApoE cDNA was amplified in two steps, using two pairs of oligonucleotide primers. The forward and reverse primers for the 1st PCR were 5'-GACCCCGGTGGCGGAGGAGA-3', which corresponded to bases 246–265, and 5'-GCCCGCACGCGGCCCTGTTC-3', which was complementary to bases 556–575 [24], respectively. First stranded cDNA synthesis and 1st PCR reaction were performed essentially as described previously [28] except for using the above primer DNAs. The amplified cDNA was electrophoresed on a 6% polyacrylamide gel containing 7 M urea. The target cDNA with expected length (330 bases) was recovered from the gel and an aliquot (1 μ l from 100-fold diluted sample) was used as a template for the second PCR. The second PCR in the presence of [α -³²P]dCTP was carried out essen-

tially as described previously [35] using two primers [10]. The forward and reverse primers were 5'-TAAGCTTGGCACGGCTGTC-CAAGGA-3' and 5'-ACAGAATTCGCCCCGGCCTGGTACAC-3', respectively. The amplified 244 base pair DNA was digested with *Hha*I and the DNA fragments were separated on a 6% polyacrylamide gel containing 7 M urea. After electrophoresis, the gel was dried and analyzed by a Fujix Bioimage Analyzer BAS 2000 (Fuji Photo Film, Kanagawa, Japan).

2.5. Construction of probe DNA

A probe DNA for quantifying ApoE mRNA was prepared from the cDNA amplified by PCR using synthetic oligonucleotides and Taq DNA polymerase. The forward primer was 5'-GGAGATGGG-CAGCCGGACCC-3', which corresponded to bases 657–676, and the reverse primer was 5'-CCAGGGGCTCGAACCAGCTC-3' which was complementary to bases 786–805 of the ApoE sequence [24]. The amplified cDNA was electrophoresed on a 6% polyacrylamide gel and the target cDNA with expected length was recovered from the gel. The 5'-end of each purified DNA was phosphorylated by T4 polynucleotide kinase. The cDNA fragment encoding ApoE (149 bases) was subcloned to pUC118 linearized by digestion with *Hinc*II. The resulting plasmid was digested with *Ava*II and *Hind*III to yield the 154 base fragment, of which 3'-20 bases was derived from the polylinker region of pUC118. A probe DNA for β -actin was prepared as described previously [28,29].

The probe DNAs were labeled at the 3'-ends by the end-filling reaction with Klenow fragment of DNA polymerase I in the presence of [α -³²P]dCTP and other dNTPs and subjected to strand separation to recover the ³²P-labeled antisense probe DNAs.

2.6. Nuclease S1 protection analysis

The mRNA levels and differential expression of both APP and tau for each case were previously reported [28,29]. The levels of ApoE mRNAs were quantified by the nuclease S1 protection method essentially as described previously except for using ApoE probe DNA [29,30].

Probe DNAs for β -actin and ApoE mRNAs were hybridized with 5–10 μ g of RNA in 30 μ l solution containing 40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) (pH 6.4), 1 mM EDTA, 0.4 M NaCl, and 80% formamide, at 46°C for 18 h. The RNA–DNA hybrid was mixed with 300 μ l of an ice-cold solution (50 mM sodium acetate (pH 4.6), 0.28 M NaCl, 4.5 mM ZnSO₄, and 800 U of nuclease S1), which was incubated at 37°C for 30 min. After terminating the digestion by adding 50 μ l of 6 M ammonium acetate, 0.1 M EDTA, the nuclease-resistant DNA was precipitated with 2-propanol and then analyzed on a 6% polyacrylamide gel containing 7 M urea. The relative radioactivities of the nuclease-resistant fragments were determined with the Bioimage Analyzer. The expected size of the protected fragments was 134 bases for ApoE.

2.7. Statistical analysis

The data were subjected to statistical analysis using χ^2 -test, *F*-test and Student's *t*-test.

3. Results

3.1. ApoE genotype and AD pathologies in aged human brains

The majority of patients in this study were 70–90 years old (see ref. [28]) and there was no relationship

between ApoE genotype frequency and age (data not shown). Of 38 aged brains, ApoE2/3, -3/3, -3/4, and -4/4 were 3 (8%), 27 (73%), 4 (11%), and 3 (8%) cases, respectively (one brain was not determined). ApoE2/2 and -2/4 genotypes were absent in our series, which reflects the lowest frequency of both phenotypes among Japanese ($0 \sim 0.9\%$) [4,42]. Of four typical AD brains based on the established criteria [15,23], two were of ApoE4/4 (cases 20 and 25 in ref. [28]) and the others were of ApoE3/3 (cases 26 and 27). It is of note that the frequency of ApoE3/3 in Japanese is 72.1% in the

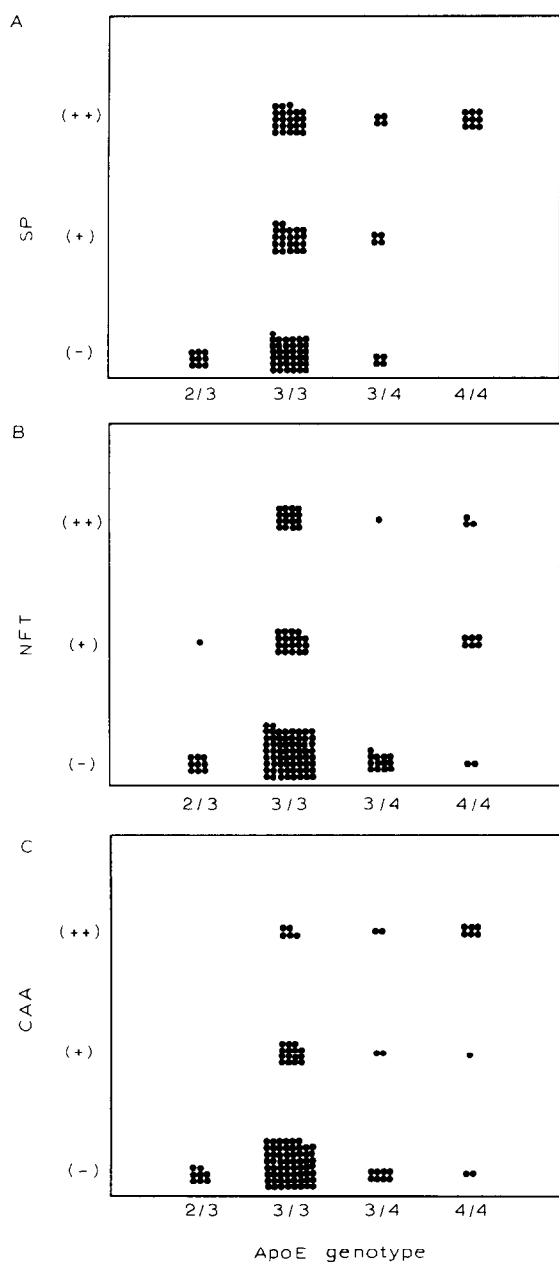


Fig. 1. ApoE genotype vs abundance of SP (A), NFT (B), and CAA (C). Each closed circle represents the degree of each lesion in a given tissue section.

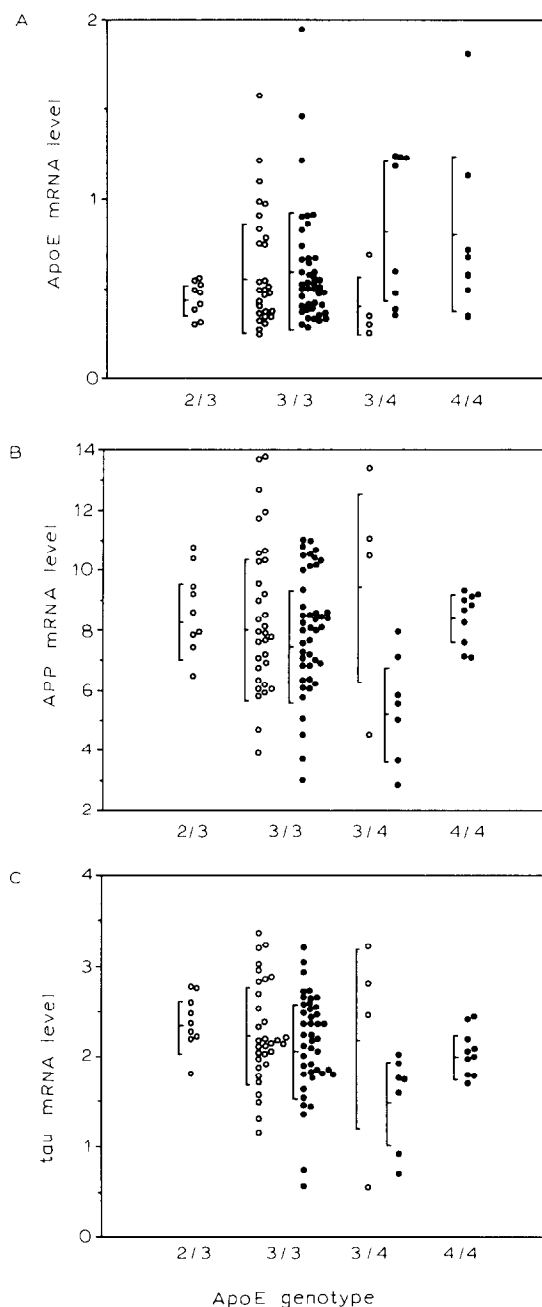


Fig. 2. ApoE genotype vs ApoE (A), APP (B), or tau mRNA level (C). Open and closed circles represent SP-free and SP-bearing areas, respectively. Bars indicate mean and standard deviation. A: ApoE levels in ApoE2/3 are significantly lower as compared to those in ApoE3/4 SP(+) or ApoE4/4 ($P < 0.05$). B,C: APP and tau mRNA levels in ApoE3/4 SP(+) are significantly lower than those in other groups ($P < 0.05$).

two other studies [4,42], which indicates higher allele frequency of $\epsilon 3$ among Japanese.

We first compared the AD pathologies in four ApoE groups. As shown in Fig. 1A–C, ApoE2/3 brains were distinct from other brains, in particular ApoE3/3 brains; SP, NFT, and CAA were hardly detected in ApoE2/3 brains (statistically significant ($P < 0.05$) for

SP, but not for NFT or CAA), while varying degrees of the three lesions were frequently observed among other brains. In ApoE3/3 brains, 55%, 34%, and 24% of the cortical sections exhibited SP, NFT, and CAA, respectively. In ApoE3/4 brains, those values were 67%, 7%, and 33%, respectively. In ApoE4/4 brains, there were higher degrees of the three pathologies (statistically significant ($P < 0.05$) for SP but not for NFT or CAA). Interestingly, SP and CAA often coexisted in ApoE4/4, while SP did not necessarily accompany CAA in other genotypes. These results indicate that ApoE genotype affects the three AD pathologies in general aged population.

3.2. ApoE genotype and ApoE mRNA level

Recent observations suggest that ApoE is involved in the regeneration of PNS [11] and CNS [20]; axonal

injuries in CNS are reported to induce high levels of ApoE mRNA in reactive astrocytes [32]. These suggest the possibility that a higher mRNA level may represent higher regenerative activities, which could provide more resistance to the destructive activities of AD (see also ref. [27]). Thus, ApoE mRNA levels were determined in those brains. The levels varied considerably among individuals with SP(–) and SP(+) ~ (++) (Fig. 2A). The mean value of ApoE mRNA levels in ApoE2/3 was slightly lower than those in other ApoE groups (statistically significant ($P < 0.05$) between ApoE2/3 and -3/4 SP(+) or -4/4). Some brains in ApoE3/3, -3/4, and -4/4 showed increased ApoE expression, but there were no statistically significant differences among the levels of those ApoE groups ($P < 0.05$; Fig. 2A). In addition, there were no statistical differences in its levels between SP(–) and SP(+) ~ (++) groups in each of ApoE3/3 and -3/4 brains ($P < 0.05$).

3.3. ApoE genotype and APP and tau gene expression

We also examined whether the ApoE genotype is related to APP or tau mRNA level, or their differential expression. There were no statistically significant differences among APP and tau mRNA levels in the four ApoE groups, except for significantly lower levels of APP and tau mRNAs in ApoE3/4 SP(+) ($P < 0.05$; Fig. 2B,C). In addition, there were no correlations between ApoE genotypes and relative abundance of APP-751 and four-repeat tau mRNAs, except for higher APP-751 ratio in ApoE3/4 SP(–) and lower four-repeat tau ratio in ApoE3/4 SP(+) ($P < 0.05$; Fig. 3A,B).

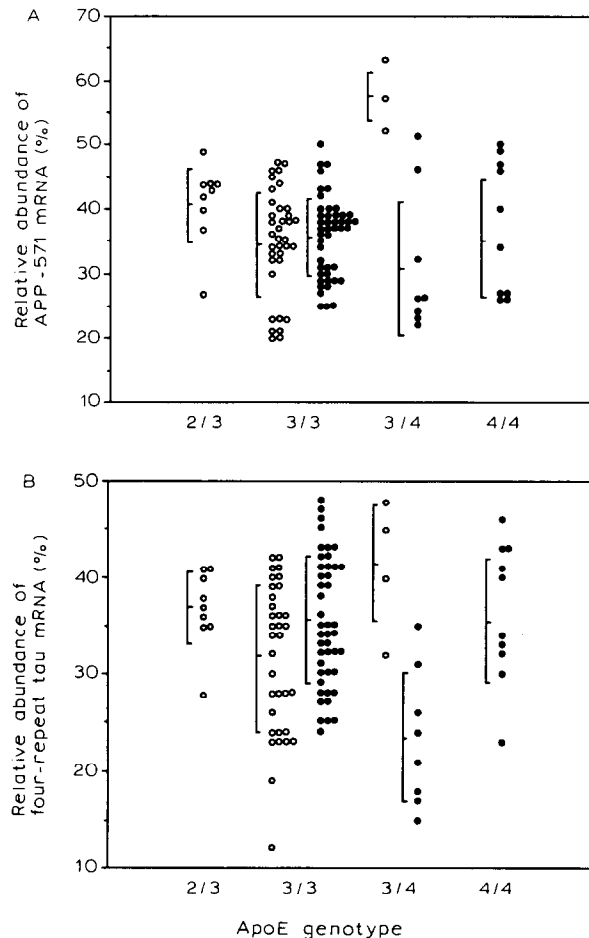


Fig. 3. ApoE genotype vs relative abundance of APP-751 (A) or four-repeat tau mRNA (B). Open and closed circles represent SP-free and SP-bearing areas, respectively. Bars indicate mean and standard deviation. A: APP-751 ratio in ApoE3/4 SP(–) is significantly higher than those in other groups ($P < 0.05$). B: four-repeat tau ratio in ApoE3/4 SP(+) is significantly lower than those in other groups ($P < 0.05$).

4. Discussion

Despite recently performed extensive studies, it has not been clear whether the three AD pathologies, SP, NFT, and CAA, found in general aged population [29] are related to ApoE genotype or its mRNA levels. In addition, it remains unclear whether ApoE genotype is related to its mRNA levels or APP and tau gene expression. The experiments described here were initiated to explore possible effects of ApoE genotypes and ApoE mRNA levels on the appearance of three major AD pathologies and the AD-related gene expression in aged human brains obtained from consecutive autopsy rather than comparing AD with non-AD brains. The present data showed that (i) the ApoE genotype is indeed related to the AD pathologies among aged human brains (Fig. 1A–C); ApoE2/3 brains have the least degrees of three AD pathologies, while ApoE4/4 brains have higher degrees of SP; and (ii) the ApoE genotype has no correlation with ApoE, APP and tau mRNA levels, or differential expression of the latter two genes (Figs. 2A–C and 3A,B).

The AD pathologies were undetectable in ApoE2/3 brains (only the absence of SP is statistically significant), but were frequently observed in the aged brains with other ApoE types. This is consistent with the recent observation that ApoE2 provides a protective effect on the age of onset of AD [2]. Some ApoE3/3 brains showed high degrees of the AD pathologies. They are associated with neither APP mutation [6,26] (data not shown) nor altered gene expressions of APP and tau (Figs. 2B,C and 3A,B). This suggests that there may be another genetic factor working in ApoE3/3 brains. ApoE4/4 brains appeared to have higher prevalences and higher degrees of the three lesions, though only the SP formation was statistically significant (Fig. 1A). Regarding NFT, a recent observation has shown that NFT density is independent of the ApoE genotype in late onset FAD [36]. Although statistically not significant, the prevalence of NFT seemed to be higher in ApoE4/4 brains in the present series. To address the issue, a much larger series should be investigated.

It is known that CAA does not necessarily complicate AD brain [40,44]. In Down's syndrome brains, CAA is the last to appear, just following NFT [21]. In a whole series of ApoE3/3 brains, the frequency of the lesions is SP, NFT, and CAA in this order (data not shown). This should reflect the temporal relationship of the three lesions, originally observed in Down's syndrome brain [21]. In ApoE4/4 brains almost all areas were accompanied by CAA, the last lesion to appear. This is also consistent with the view that ApoE4 accelerates the AD pathologies (see also ref. [36]).

It is reasonable to postulate that the ApoE genotype enhances the AD pathologies by affecting the pathologic cascade of AD [9,21]. A possible target of ApoE would be β -protein because ApoE is associated with β -amyloid in situ [25]. In fact, ApoE in cerebrospinal fluid binds to synthetic β -protein in vitro [37,46]. Furthermore, ApoE4 forms an SDS-resistant aggregate with β -protein more rapidly than ApoE3 [38]. In addition, there are marked differences in the β -amyloid deposition between AD brains with ApoE4 and those without ApoE4 [36]. On the other hand, since ApoE is found to be associated with several kinds of amyloids [25] (see also ref. [3]), its association with β -protein may not be so significant. It has recently been reported that tau binds to ApoE3, but not to ApoE4 and a causal relationship of ApoE genotype to PHF formation has been hypothesized [39]. Although it is an intriguing hypothesis, its validity remains to be confirmed.

We looked into another possibility: a potential relationship to the expression of APP and tau genes. According to our previous data, the differential expression and mRNA levels of both APP and tau do not differ between lesion-free and bearing areas [28,30],

which agrees with the two published works [12,18]. Compatible with these, we found no significant correlation of ApoE genotype with the above variables, suggesting that it is not through the expression of APP and tau that the ApoE genotype affects the cascade.

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