A Sex Difference and No Effect of ApoE Type on the Amount of Cytoskeletal Alterations in the Nucleus Basalis of Meynert in Alzheimer's Disease

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SALEHI, A., V. GONZALEZ MARTINEZ, AND D. F. SWAAB. A sex difference and no effect of ApoE type on the amount of cytoskeletal alterations in the nucleus basalis of Meynert in Alzheimer's disease. *NEUROBIOL AGING* 19(6) 505–510, 1998.—In the nucleus basalis of Meynert (NBM) we studied the presence of early cytoskeletal alterations as shown by the antibody Alz-50 in ApoE-typed patients. Using an image analysis system, the area covered by Alz-50 staining and the percentage of neurons stained by Alz-50 were determined. There were no significant differences in the area covered by Alz-50 or in the proportion of Alz-50-stained neurons in the nucleus basalis of Meynert of Alzheimer's disease (AD) patients with one or two ApoE ϵ 4 alleles as compared with those without any ApoE ϵ 4 allele. However, there was a significant sex difference in Alz-50 staining: female Alzheimer's disease patients showed more severe early cytoskeletal alterations than males. We also found a significant relationship between the number of Alz-50-stained neurons and the severity of dementia. © 1999 Elsevier Science Inc.

Alzheimer's disease ApoE Alz-50 Neurofibrillary tangles Sex difference Tau

ALZHEIMER'S disease (AD) is characterized by progressive memory loss and other cognitive impairments and by the occurrence of typical neuropathological lesions, i.e., neuritic plaques (NPs), neurofibrillary tangles (NFTs) and neuropil threads (3,5,6). The density of NFTs correlates with the severity of the disease (2). NFTs are composed of paired helical filaments (PHFs), from which the main component is hyperphosphorylated tau, a microtubule-associated protein (MAP) involved in microtubule formation and stabilization (23,24). PHFs also occur in neuropil threads and dystrophic neurites associated with plaques (6). NPs mainly contain a deposit of 40–42/43 amino acid peptide derived through the processing of amyloid precursor protein (17).

Epidemiological studies have revealed that the genetic variation in apolipoprotein E (ApoE) is an important risk factor for AD (36–38,45). ApoE is a 37 kDa protein encoded by a four-exon gene of 3.6 kb located on the long arm of chromosome 21. The inheritance of one or two ApoE ϵ 4 alleles increases the risk of AD and decreases the age of onset of the disease (36,37), whereas ApoE ϵ 2 appears to reduce the risk of AD and to increase the age of onset (11). It has been reported that carriers with ApoE ϵ 4 have a higher amyloid load (46), a higher number of plaques (13,15,30),

an increased number of silver stained NFTs (30), more severe cholinergic dysfunctioning (35) and reduced neuronal activity (44). The exact mechanism by which ApoE influences the course of AD is not known. It has been suggested that ApoE participates in the pathogenesis of AD, e.g., by: 1) modulating the process of amyloid fibrillogenesis (40,54); 2) influencing the function and integrity of the cholinergic system (35); 3) influencing neuronal metabolic rate (44); or 4) affecting abnormal phosphorylation of tau and thus NFT formation (47). There are several studies in the literature supporting the last possibility. For instance, it has been reported that in vitro ApoE ϵ 3 interacts with tau and MAP2c, whereas ApoE ϵ 4 does not (25,47). Furthermore, none of the ApoE isoforms binds to hyperphosphorylated tau. These observations have led to the hypothesis that ApoE ϵ 3 protects against the development of AD by preventing hyperphosphorylation of tau and by increasing tau, leading to microtubule stabilization and reduction of NFT formation (47). Regarding the interaction between the ApoE genotype and other risk factors, it has been shown that the negative effect of ApoE ϵ 4 is influenced by sex. Unlike men, ϵ 4 heterozygote women are more at risk than those without ϵ 4 alleles (33). Furthermore, having one ϵ 4 allele is

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TABLE 1.
CLINICO-PATHOLOGICAL INFORMATION ON ALZHEIMER PATIENTS WITH DIFFERENT APOE GENOTYPES

Group	Age	Sex	Bw (g)	Pmd (h)	Fix (d)	GDS
ApoE ϵ 3/3	70.7 $\pm$ 3.6*	8f/7m	1073.6 $\pm$ 41.0	4.1 $\pm$ 0.2	33.0 $\pm$ 2.9	6.3 $\pm$ 0.2
ApoE ϵ 3/4	74.4 $\pm$ 4.4	7f/4m	1198.0 $\pm$ 79.2	4.2 $\pm$ 0.2	35.1 $\pm$ 3.5	6.5 $\pm$ 0.2
ApoE ϵ 4/4	72.7 $\pm$ 2.8	6f/4m	1206.8 $\pm$ 51.0	4.5 $\pm$ 0.3	30.1 $\pm$ 1.9	6.1 $\pm$ 0.4

Abbreviations: m, male; f, female; Pmd, postmortem delay (in hours); Fix, fixation time (in days); Bw, brain weight (in grams); GDS, Global Deterioration Scale (39).

*Data are expressed as mean $\pm$ SEM.

enough to shift the onset of AD to a younger age in women, but not in men (34).

The present study was carried out in order to determine the relationship between the presence of early cytoskeletal alterations, the number of ApoE ϵ 4 alleles and sex. As an indicator of early cytoskeletal alterations, the antibody Alz-50 was used. Alz-50 is highly selective for neurofibrillary pathology partly because it binds to a conformational epitope of tau, which is stabilized and prevalent in PHFs. Therefore, Alz-50 is one of the most robust antibodies compared with other anti-tau antibodies which staining the fibrillary pathology (10,50). We studied the relationship between Alz-50 staining intensity and the number of ApoE ϵ 4 alleles in the nucleus basalis of Meynert (NBM) of AD patients in relation to their sex.

MATERIALS AND METHODS

Tissue Preparation

Brains of 36 AD patients ranging in age from 49 to 96 years were obtained at autopsy (see Table 1 for details and clinicopathological information). The patients were clinically assessed and diagnosed as "probable AD" by excluding other possible causes of dementia by history, physical examination and laboratory tests according to the diagnostic criteria as described by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA (26)). All patients had a global deterioration scale (GDS) of 4–7 for severity of dementia (39). The clinical diagnosis AD was neuropathologically confirmed. Neuropathological examination showed extensive neocortical and hippocampal NPs, NFTs and dystrophic neurites (43). Tissue blocks that contain hypothalamus and the NBM, were dissected and fixed in 4% formaldehyde in phosphate-buffered saline (PBS), pH 7.4, at room temperature, for about 1 month. The fixed tissue blocks were dehydrated in graded ethanols, embedded in paraffin and cut serially in 6- μ m coronal sections. For anatomical orientation, every 50th section was mounted, deparaffinized, hydrated and stained with thionine (0.5%). Based on the number of ApoE ϵ 4 alleles, the AD patients were divided into three groups: 1) AD patients without any ApoE ϵ 4 alleles (70.7 $\pm$ 3.6 years of age), 2) AD patients with one ApoE ϵ 4 allele (74.4 $\pm$ 4.4 years); and 3) those with two ApoE ϵ 4 alleles (72.7 $\pm$ 2.8 years). The Kruskal-Wallis test showed no significant differences in age ($p = 0.556$), brain weight ($p = 0.170$), fixation time ($p = 0.474$), postmortem delay ($p = 0.655$) or GDS ($p = 0.801$) among the three groups.

Immunocytochemistry

As primary antibody the monoclonal Alz-50 was used. This is a monoclonal IgG antibody (clone #p-42) directed against the N-terminus of tau (14,20). Epitopes have been demonstrated in amino acid sequences 2–10 of tau protein, which are apparently

not phosphorylation-dependent (14). Alz-50 recognizes pathological PHF-containing structures before the cell contains silver staining NFTs (19) and is therefore considered to be a marker of early cytoskeletal changes in AD (55,56). The development of Alz-50 immunoreactivity during the course of AD parallels that of hyperphosphorylated tau as determined by the antibody AT8 in AD (4). On immunoblots, Alz-50 recognizes all human tau isoforms in normal adult and fetal brains (22), and also A68 (19,20). Unlike the human adult brain, in which tau is phosphorylated to a very limited extent, in AD A68 is abundantly phosphorylated at multiple sites, including serine 202 and serine 396, which are not phosphorylated in normal adult autopsy tissue (7,20). More recent data have shown that individual tau monomers adopt a specific conformation preceding or during filament formation, which is selectively recognized by Alz-50 (10).

For immunocytochemistry (43,51), mounted sections were hydrated and stained according to following procedure: 1) pretreatment of the sections in 3% hydrogen peroxide (Merck) and 0.2% Triton X-100 (Sigma) in TBS for 30 min; 2) incubation of sections in 5% nonfat dried milk; 3) incubation with Alz-50 (1:100) in a 5% milk solution.

The slides were used to determine by image analysis the area covered by Alz-50 staining. This was followed by taking the coverslip off and counterstaining the slides with Hematoxylin Eosin in order to counterstain all neurons. Then, using a different image analysis program, the proportion of Alz-50-stained neurons was determined.

Since the NBM is a very extensive system, the measurements were performed in a standardized part of the NBM, i.e., the medial and lateral subdivisions of Ch4a, according to Mesulam's nomenclature (28). NBM-containing sections were selected on the basis of a standardized location of the fornix, anterior commissure, optic tract and supraoptic nucleus (42).

Alz-50 Load

Alz-50 stains intracellular NFTs, neuropil threads and dystrophic neurites (5,6,52,55,56). Because the NBM does not show many neuritic plaques (1), most of the staining by Alz-50 is due to intracellular NFTs and neuropil threads (1,48).

The Alz-50 load of the NBM, i.e., the area stained by Alz-50, was determined using an IBAS-KAT image analysis system (Kontron-AT based system). The image analysis system was connected to a Bosch TYK9B TV camera equipped with a chalnycan tube mounted on a Zeiss microscope. The microscope was equipped with Planapo objectives. All measurements were performed using a 560-nm small band DEPAL filter (Schott, Germany), which coincides with the maximum absorption of the diaminobenzidine/nickel sulfate precipitate in the sections.

Random sampling consisted of the following two steps: 1) area selection and 2) determination of the total area covered by Alz-50 staining per field. Sampling and area selection were performed as

follows: in each section to be analyzed, an area widely covering the NBM was loaded into the IBAS, using the 2.5 $\times$ objective of the microscope, and displayed on the image analysis monitor. The position of the section under the microscope was stored using the x-y coordinates of the scanning stage. In this image, the contour of the NBM was outlined manually. In order to select a number of fields in the NBM, a grid which consisted of areas corresponding to the image size at a 500 $\times$ magnification (40 $\times$ objective) was superimposed over the NBM area. From this grid, 50 fields (out of an average of 100 fields) were randomly selected. The position of each microscopic field belonging to this sample was again expressed in the x-y coordinates of the scanning stage. On the basis of these coordinates, the selected fields were retrieved for measurement.

In order to determine the total area covered by Alz-50 staining, the 40 $\times$ objective was positioned in the microscope and the scanning stage moved to the previously defined positions of the high-magnification measuring areas. At each position, a 768 $\times$ 512 pixel large image was loaded. Subsequently, a mask of the Alz-50 area in all stored images was calculated. The Alz-50 masks were stored together with the images. Finally, the total area of the mask was calculated.

Alz-50-Stained Neuronal Counting

A different image analysis system (Kontron) mounted with a CCD video camera (Sony XC-77CE) connected to an Axioskop microscope (Zeiss) was used for cell counting. The microscope was equipped with an X and Y scanning stage (Märzhäuser) and a Z-motor (Zeiss).

In the same sections as used for the previous measurements (two to three sections per subject) the proportion of neurons stained with Alz-50 was determined. In each section the analysis consisted of seven steps: 1) outlining the NBM area in an image loaded with a 2.5 $\times$ objective; 2) superimposing a grid of rectangular areas "seen" through a 40 $\times$ objective by the camera; 3) storage of the coordinates of all those fields of the grid covered by the NBM outline; 4) systematic random selection of 50 fields by the computer; 5) automatic loading of the images of randomly selected fields with a 40 $\times$ objective into the image analyzer; 6) manual labeling (either positive or negative) of each neuronal profile containing a nucleolus by the observer (in case of doubt, the section was scanned in the Z-direction to identify the nucleolus); and 7) calculation of the total number of stained and unstained profiles.

ApoE Genotyping

ApoE genotyping was performed on frozen tissue from the cerebellum of the AD patients. The genotype of each extracted DNA sample was determined by polymerase chain reaction amplification. Then the polymerase-chain-reaction product was digested and subsequently separated by electrophoresis in a 5% agarose gel (53).

Statistical Methods

Differences among the groups were tested using the Kruskal-Wallis test. The Spearman's correlation coefficient was used to study the associations among various parameters. The critical level for statistical significance was 5%.

RESULTS

Immunocytochemistry

In all AD patients immunocytochemical staining with Alz-50 revealed a very intense staining of NBM neuronal perikarya,

FIG. 1. Immunocytochemical staining of Alz-50 in the NBM of an Alzheimer patient with ApoE ϵ 3/4. Note the clear staining of cells bodies, dystrophic neurites and neuropil threads. Scale bar = 28 μ m

neuropil threads and some dystrophic neurites (Fig. 1). Qualitative microscopic analysis showed that both the area occupied by Alz-50 staining (Alz-50 load) and the proportion of Alz-50-stained neurons in AD patients with the ApoE genotype ϵ 3/3 showed much interindividual variation.

Morphometry

Alz-50 Load or the Total Area Covered by Alz-50 Staining. A total of 5,264 images (each image 37,456 μ m²) were analyzed. In order to determine the variability of the measurements of the area covered by Alz-50 staining by the image analysis system, the same section from an AD patient was measured 10 times. Using an ANOVA test, no significant difference was found between the measurements ($p = 0.928$, $F = 0.410$). Furthermore, the standard error of the mean was less than 5% of the mean value of all measurements (mean $\pm$ SEM; 0.922 ± 0.037).

There was a significant negative correlation between the Alz-50 load and brain weight ($p = 0.003$). No significant correlation was found between the Alz-50 load and 1) age ($p = 0.490$), 2) GDS ($p = 0.114$) or 3) postmortem delay ($p = 0.632$). The mean percentage of area occupied by Alz-50 staining was (5.18 ± 7.40) in ApoE ϵ 3/3, (3.97 ± 5.2) in patients with ApoE ϵ 3/4 and (4.78 ± 6.43) in AD patients with two ApoE ϵ 4 alleles. The Kruskal-Wallis test did not show any significant ($p = 0.719$) differences in Alz-50 load among the three groups.

The Proportion of NBM Neurons Stained by Alz-50

A total of 13,639 neurons were analyzed. The Kruskal-Wallis test only showed a trend in the proportion of Alz-50-stained neurons among the three groups of AD with different amounts of ApoE ϵ 4 alleles ($p = 0.062$) neither was there any significant difference in the number of Alz-50-stained neurons between AD patients with one and those with two ApoE ϵ 4 alleles. However, Spearman's correlation test showed a significant positive correlation ($p = 0.030$) between the percentage of Alz-50-stained neurons and the severity of dementia as indicated by GDS. Moreover, no significant correlation between the percentage of Alz-50 stained neurons and i) age ($p = 0.341$) or ii) postmortem delay ($p = 0.217$) of the patients was found.

Effect of Gender

Alz-50 Load. The Alz-50 load in females ($3.5\% \pm 0.5$) was not significantly different from that of males ($2.8\% \pm 0.5$). In order to

FIG. 2. Immunocytochemical staining of Alz-50 in the NBM of (A) a male and (B) a female AD patient. Note the increase in the number of Alz-50-stained cells in the female AD patient. Scale bar = 80 μ m.

exclude the effect of ApoE on the analysis of sex effects, all patients were matched for the ApoE ϵ 4 allele, after which the difference between males ($n = 15$) and females ($n = 21$) for the area covered by Alz-50 remained nonsignificant (Fig. 2)

Alz-50-Stained Neurons. The percentage of neurons stained by Alz-50 in the NBM of female patients (49.5 ± 6) was significantly ($p = 0.039$) higher than that of male patients (32.0 ± 5 ; Fig. 3).

DISCUSSION

Earlier studies had shown that the NBM is not only heavily stained by Alz-50, but also by other antibodies showing cytoskeletal alterations. In AD, NBM neurons are, e.g., intensely stained by the 60e (against NFTs), tau-1 (against tau) and 3-39 (against ubiquitin) antibody. However, Alz-50 showed the most intense staining in the NBM of AD patients (48).

Alz-50 not only stains AD changes, but also cell bodies, e.g., in the periventricular nucleus of the hypothalamus of nondemented young controls (41). Some of the healthy neurons stained by Alz-50 in nondemented controls are also stained by antibody directed toward somatostatin 15-30 (52). One may question, therefore, whether the staining by Alz-50 in the present study is really due to cytoskeletal alterations. Such crossreactivity of Alz-50 with non-diseased neurons does, however, not take place in the NBM because: 1) in young and even many old controls NBM neurons are not stained with Alz-50 (48,51); and 2) in AD patients not only Alz-50 but also other antibodies show cytoskeletal changes in this nucleus, e.g., the antibodies against paired helical filaments, tau (i.e., 60e and tau-1) and anti-ubiquitin (3-39,48). The prominent staining of the NBM by Alz-50 in AD is thus considered to show indeed, cytoskeletal alterations.

Our study shows that there is no effect of ApoE ϵ 4 on early cytoskeletal alterations in the NBM of AD patients. This is in agreement with other studies showing that the presence of one or two ApoE ϵ 4 alleles is not associated with increased numbers of neurofibrillary tangles, either intra- or extracellular (15,29), and that there is no significant change in the level of normal tau protein in relation to ApoE subtype (29). Part of the variance in the amount of Alz-50 staining in the AD patients may be related to the

FIG. 3. Graph depicting the mean of Alz-50 load (a) and mean percentage of Alz-50 stained neurons (b) in males and females. Note that female subjects showed a significant increase in the mean percentage of Alz-50 stained neurons compared with males. * $p = 0.039$.

presence of polymorphism in subregions of this gene. Recently, Bullido et al. (9) showed that the presence of polymorphism in the regulatory region of the ApoE gene is associated with a higher frequency of AD, independent of the ApoE- ϵ 4 status.

Payami et al. (34) suggested that gender may influence the increased risk of ApoE ϵ 4 in AD patients. However, dividing our AD patients into a male and female group did not reveal any effects of ApoE ϵ 4 on early cytoskeletal alterations in either males or females, suggesting that such a sex difference is probably not accompanied by a difference in cytoskeletal alterations.

Our results also show that there is a significant positive relationship between the number of Alz-50-stained neurons and the severity of dementia as scored by GDS (39). This is in accordance with other studies which reported that NFTs correlate better with the severity of dementia than plaques do (4,15). Gomez-Isla et al. (15) found that NFT numbers are closely correlated with the duration of illness and with the Blessed dementia scale score, while there was no significant interaction of ApoE genotype with NFTs. This again indicates that the effects of ApoE ϵ 4 does not take place via tangle formation. An alternative explanation for the lack of relationship between the number of ApoE ϵ 4 alleles and Alz-50 staining is that several reports suggest that ApoE does not affect the course (16,21) or mortality of AD (15).

The fact that the severity of early cytoskeletal alterations as indicated by the number of Alz-50-stained neurons is more pronounced in female than male AD patients (Fig. 3) is in agreement with recent epidemiological data showing the higher prevalence of AD in women compared with men (8,12). In a longitudinal study, Fratiglioni et al. (12) reported a clearly higher

risk of AD for women compared with men. Interestingly, McMillan et al. (27) showed that ovariectomy in adult female Sprague-Dawley rats causes a significant reduction in ChAT and trkA receptor expression, which were both reversed by short-term estrogen therapy. This is in accordance with previous data showing the colocalization of low-affinity neurotrophin receptors with estrogen receptors in the basal forebrain of rats (49). Recently a number of studies have been published data indicating the beneficial effect of estrogen therapy on female AD patients (31,32,49). Improvements have been reported in attention, memory, calculation, orientation and social interaction following estrogen administration (18). The question that arises from our work is whether estrogen therapy will be able to reverse the occurrence of early cytoskeletal alterations, e.g., in the NBM, or stop the process of neurodegeneration.

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