The Cholinergic Deficit Coincides with Aβ Deposition at the Earliest Histopathologic Stages of Alzheimer Disease

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Abstract. Effective therapeutic intervention in Alzheimer disease (AD) will be most effective if it is directed at early events in the pathogenic sequence. The cholinergic deficit may be such an early event. In the present study, the brains of 26 subjects who had no history of cognitive loss and who were in early histopathologic stages of AD (average Braak stage less than II) were examined at autopsy to determine whether a cortical cholinergic decrement was associated with Aβ concentration or deposition. In the superior frontal and inferior temporal gyri, the choline acetyltransferase (ChAT) activity of plaque-containing cases was significantly decreased (p < 0.05, unpaired, two-tailed t-tests), measuring 70.9% and 79.5%, respectively, relative to plaque-free cases. In the inferior temporal gyrus, Spearman’s rank correlation analysis showed that ChAT activity had a significant inverse correlation with Aβ concentration (p = 0.075; r = −0.3552). The results indicate that the cholinergic deficit is established at an early histopathologic stage of AD, before the onset of clinical symptoms.

Key Words: Acetylcholine; Aging; Alzheimer disease; Amyloid β-peptide; Cerebral cortex.

INTRODUCTION

Alzheimer disease (AD) is a family of diseases in which there are multiple etiologies. Mutations in 3 different genes can all cause an early-onset form of AD, which is inherited as an autosomal dominant condition (1). The majority of AD is not inherited, however, at least not in a single gene, Mendelian fashion. It is likely that this common form of the disease, “sporadic AD,” results from the interaction of multiple genes with environmental influences and aging changes. The development of effective treatments for sporadic AD depends on an understanding of the initiation and development of the pathogenic cascade. Potentially, treatment strategies could be directed at any of the steps within the cascade, but the most useful treatments will be those which are directed at early events. If AD can be detected and treated preclinically, it may be possible to halt the neurodegenerative process before it has a significant impact on quality of life.

Cortical cholinergic denervation may be an early step in AD pathogenesis. The cholinergic deficit has been noted to be the earliest and most consistent neurochemical change in AD (2, 3). Our finding that cholinergic fiber loss is associated with the presence of plaques in the nondemented elderly (4) suggests that the loss of cholinergic neuronal elements is quite likely a preclinical event, and is at least coincidental with the first appearance of Aβ deposition. We have also recently reported that neurofibrillary tangles are extremely common and perhaps universal in the nucleus basalis of Meynert in nondemented older people (5), further supporting an early involvement of this cholinergic cell group. Other work (6) has also indicated a preclinical onset for the cholinergic deficit in AD.

In this study, we have sought further evidence of the place of the cholinergic deficit in the AD pathogenetic sequence. Specifically, we wished to determine whether the cholinergic deficit is linked to Aβ deposition at the earliest histopathologic stages of AD, before the onset of clinical disease.

MATERIALS AND METHODS

Brains were obtained postmortem from 26 nondemented elderly persons. The ages of the subjects ranged between 63 and 94 years. All were volunteer donors to the brain autopsy program at Sun Health Research Institute in Sun City, Arizona. The presence of dementia was excluded by specific questioning as to whether the donor had experienced any cognitive deterioration, or had ever had a diagnosis of dementia or other neuropsychological condition. This was done with telephone or in-person interviews, first with the donor at the time of enrollment into the program, and again with family members and/or caregivers at the time of death. In addition, all medical records from the 2 years preceding death were obtained and reviewed. Cases with clinically recognized deficits in memory or cognition, or with a history of neurological disease were excluded. Furthermore, the Washington University Clinical Dementia Rating (7) was administered postmortem for 18 of the 26 cases, using the closest living family member as the informant. Sixteen cases received a score of 0 (nondemented) while 2 were scored at 0.5 (questionable dementia). Cases that possessed the e4 allele of the apolipoprotein E gene were excluded because they were scarce in this population, and because both choline acetyltransferase (ChAT) activity and cortical Aβ levels have been reported to be independently affected by this allele (8–11).
The left half of each brain was sliced coronally into 0.8-cm slabs and fixed for 2 days in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, at 4°C. After cryoprotection in 30% ethylene glycol/30% glycerol/PB, 40-μm sections were taken on a freezing microtome. Coronal slices from the right half of each brain were frozen rapidly between slabs of dry ice.

Complete neuropathological examinations were performed on all brains using paraffin sections stained with hematoxylin and eosin (H&E). Alzheimer-type histopathology was staged according to the CERAD protocol for neuritic plaques (12) and the Braak protocol for neurofibrillary changes (13) using sections stained with the Campbell-Switzer, Gallyas (14), and Thioflavine-S methods from a minimum of 5 tissue blocks, including all cerebral lobes as well as amygdala, hippocampal formation, and parahippocampal gyrus.

Frozen samples of superior frontal gyrus (level of genu of corpus callosum) and inferior temporal gyrus (level of lateral geniculate nucleus) were assayed for ChAT activity and Aβ peptide content. The ChAT activity was determined according to the method of Fonnun (15). Levels of Aβ (N-40) and Aβ (N-42) were assayed as described previously (16). The tissue was initially homogenized in 4 volumes of extraction buffer (20 mM Tris–HCl, pH 7.4, 3 mM EDTA, 500 ng/ml leupeptin, 700 ng/ml Pepstatin, 30 μg/ml PMSF). Samples were centrifuged at 100,000 g for 30 minutes and the supernatant and pellet separated. The supernatant, containing “soluble” Aβ, was assayed directly, while the pellet was first solubilized in formic acid as described (17). To detect Aβ (N-40) and (N-42), the C-terminal-specific antibodies R163 and R165 were used. These were raised in rabbits against the peptide sequences acids 34–40 and 36–42, respectively (18). The monoclonal antibody 4G8 (19, 20) (Senetek PLC, St. Louis, MO) was used as the reporter antibody. Microtiter plates (NUNC-ImmunoPlate, Maxisorp, 96 well; NALGE-NUNC Int. Corp., Naperville, IL) were coated with primary antibodies at a concentration of 4 μg/ml. Wells were blocked with 1% bovine serum albumin for 2 hours at room temperature, rinsed, and then filled with 100 μl of the specimen or of Aβ standards (synthetic Aβ1–40 or 1–42, obtained from California Peptides, Napa, CA). Following a 1-hour incubation, wells were rinsed and incubated with Europium-labelled 4G8 (Delfia kit obtained from Wallac, Inc., Gaithersburg, MD). After a final rinse, Europium enhancement solution (Wallac) was added and the plates were read in a fluorometer using excitation and emission wavelengths of 330 nm and 615 nm, respectively. Values for each sample were the average of triplicate wells, adjusted to standard curves generated on each plate.

Values for ChAT activity in plaque-free and plaque-containing cases were compared in the 2 brain regions using unpaired, two-tailed t-tests. The relationship between cortical Aβ levels and ChAT activity was examined using Spearman’s rank correlation.

RESULTS

Neuropathology

Of the 26 cases, 8 were plaque-free, containing neither diffuse nor neuritic plaques. The 18 cases with plaques had neuritic plaque densities varying from 0 (diffuse plaques only) to frequent, using the diagrammatic guidelines provided by CERAD (12). Conversion of the CERAD qualitative ratings (none, rare, sparse, moderate, frequent) to numerical scores (0, 1, 2, 3, 4; the category of “rare” is not provided by CERAD, but is defined here as cases showing only widely-scattered plaques not reaching the “sparse” density) enabled an average neuritic plaque density score to be obtained, which, for the plaque-containing group was 1.64. Braak staging for neurofibrillary tangles established that the cases ranged between stages I and IV. The average Braak stage of the plaque-containing cases was 1.83, while the plaque-free cases had an average score of 1.25. Eight cases had small, old cerebral infarctions totalling less than 5 cc. The frequency of infarctions in plaque-free cases was 2 out of 8, while in plaque-containing cases it was 6 out of 18. The mean age of plaque-containing and plaque-free cases was 81.3 years, and 75.8 years, respectively. The difference did not meet the significance level of 0.05 on an unpaired, two-tailed t-test and age did not significantly correlate with ChAT activity in these subjects.

ChAT Activity

In both the superior frontal gyrus and inferior temporal gyrus, the plaque-containing group had significantly lower ChAT activity, with p values of 0.015 for both comparisons. The ChAT activity in the plaque-containing cases was 70.9% and 79.5% of that in the plaque-free cases in superior frontal gyrus and inferior temporal gyrus, respectively (Fig. 1).

Aβ Concentration and Correlation with ChAT Activity

Plaque-containing cases had significantly more insoluble Aβ (N-42) than plaque-free cases, but other forms of Aβ were present in roughly equivalent amounts in the 2 groups (Table). The amount of insoluble Aβ (N-42) in plaque-containing cases was increased 9.1-fold and 6.9-fold in superior frontal gyrus and inferior temporal gyrus, respectively, relative to plaque-free cases.

Spearman’s rank correlation analysis of Aβ (N-42) levels against ChAT activity showed that there was an inverse relationship between the 2 parameters (Fig. 2). The rank correlation coefficients were −0.2053 for superior frontal gyrus and −0.3552 for inferior temporal gyrus. The correlation was statistically significant only in the inferior temporal gyrus (two-tailed p = 0.075).

DISCUSSION

The cholinergic hypothesis of AD arose from the findings of decreased cortical ChAT activity in patients with the disease (21–23). The hypothesis was advanced both as an explanation of the clinical symptoms and as a pathogenetic event. When cholinergic therapy proved
Fig. 1. A: ChAT activity in superior frontal gyrus. Plaque-containing cases have a mean ChAT activity, which is 70.9% of that of plaque-free cases. The difference is significant ($p < 0.05$). Horizontal bar represents the mean for each category. B: ChAT activity in inferior temporal gyrus. The ChAT activity in plaque-containing cases is 79.5% of that in plaque-free cases. The difference is significant ($p < 0.05$).

Fig. 2. A: Linear regression of ChAT activity vs Aβ concentration in superior frontal gyrus. There is a trend for Aβ to increase with decreasing ChAT. The Spearman's rank correlation coefficient is $-0.2053$ (ns). Triangles represent cases without plaques, while squares are cases with plaques. B: Linear regression of ChAT activity vs Aβ concentration in inferior temporal gyrus. The Aβ concentration increases with decreasing ChAT. Spearman’s rank correlation coefficient is $-0.3552$ with a two-tailed $p$ value of 0.075. Triangles represent cases without plaques; squares are cases with plaques.

This is somewhat surprising since biopsy studies have indicated that the cholinergic deficit is present in early AD (2), and a well-known study by Katzman et al (6) found that putatively preclinical cases of AD (nondemented but with high plaque densities) already showed decreased cortical ChAT activity. We have recently shown that nondemented plaque-containing elderly persons have decreased cholinergic fiber densities relative to plaque-free persons (4), and that neurofibrillary change is extremely common, if not universal, in the nucleus largely disappointing, and subsequent research found evidence of other neurotransmitter deficits in AD, the cholinergic hypothesis was virtually abandoned, and in fact has come to be regarded as a secondary change, a retrograde degenerative response to cortical pathology (24).

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Plaque status</th>
<th>sAβ (n-40)(ng/g)</th>
<th>sAβ (n-42)(ng/g)</th>
<th>isAβ (n-40)(μg/g)</th>
<th>isAβ (n-42)(μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFG</td>
<td>Plaque-negative</td>
<td>1.34 (0.422)</td>
<td>0.66 (0.28)</td>
<td>0.83 (0.50)</td>
<td>0.51 (0.31)*</td>
</tr>
<tr>
<td></td>
<td>Plaque-positive</td>
<td>3.02 (0.71)</td>
<td>0.83 (0.15)</td>
<td>1.01 (0.69)</td>
<td>8.59</td>
</tr>
<tr>
<td>ITG</td>
<td>Plaque-negative</td>
<td>6.69 (2.86)</td>
<td>2.85 (0.74)</td>
<td>&lt;0.04*</td>
<td>0.53 (0.29)*</td>
</tr>
<tr>
<td></td>
<td>Plaque-positive</td>
<td>12.12 (2.19)</td>
<td>2.84 (0.25)</td>
<td>&lt;0.04*</td>
<td>3.63 (0.81)*</td>
</tr>
</tbody>
</table>

* Detection limit of assay was 0.04 μg/g. Data represent means and standard errors of the means (the latter in parentheses). Statistical significance was determined using unpaired, two-tailed $t$-tests; $p = 0.0048$ for $^*$ vs $^*$; $p = 0.0017$ for $^*$ vs $^*$. 

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basalis of Meynert of elderly nondemented persons (5). This evidence, and the results presented in this communication, strongly favor a preclinical occurrence of the cholinergic deficit in AD.

In both superior frontal gyrus and inferior temporal gyrus, plaque-free cases had significantly higher ChAT activity than plaque-containing cases. This indicates that the cortical cholinergic deficit becomes evident at the first appearance of histologic Aβ deposition, before clinical dementia is apparent. The result confirms our earlier finding, in which we used cholinergic fiber densities as a measure of cortical cholinergic innervation (4).

When all cases were combined, Spearman's rank correlation analysis showed that ChAT activity in the inferior temporal gyrus was significantly correlated with Aβ concentration, with a Spearman's correlation coefficient of −0.3552. The correlation did not reach the significance level in the superior frontal gyrus; the reason for this is unclear, and may simply be due to greater variability in the data for this area. In both frontal and temporal cortex, decreased ChAT activity was significantly associated with histologic Aβ deposition, as discussed above. Frontal and temporal cortex receive similar densities of cholinergic innervation, but the temporal lobe shows earlier and more pronounced evidence of cholinergic denervation in AD (25).

These results strongly suggest that the cholinergic deficit coincides with Aβ deposition at a very early stage of AD. Supporting the assertion that these cases are early-stage AD are the Braak scores; as the average Braak score for both the plaque-free and plaque-containing groups was less than stage II. The fact that most of the Aβ present was of species ending at amino acid 42 also supports that these cases represent an early stage of Aβ deposition (26–28). Although these cases had not received extensive neuropsychological testing, of the 18 cases in which the Clinical Dementia Rating was performed retrospectively, 16 received a score of 0 (non-demented), while 2 were rated as 0.5 (questionable dementia). Also, in each case, the next-of-kin and/or caregivers stated that they were cognitively unimpaired until the time of death and review of each patient’s medical records confirmed that none had ever presented with, or been found to have, symptoms of cognitive impairment. The possibility that mild dementia may have been present in 1 or more cases cannot be ruled out, but this would not prevent their classification as early stage AD.

Some evidence has been presented recently which suggests, counter to that presented already, that the cholinergic deficit is a late change in AD. Davis et al (29) reported that individuals testing as mildly demented, with a CDR score of 0.5, did not have decreased ChAT activity relative to nondemented cases with a CDR of 0, whereas more severely demented cases did show a decrement in ChAT activity. Cognitive measures, however, are not a specific measure of AD stage, since a plethora of processes can contribute to cognitive dysfunction in aging individuals. Cognitive measures are also relatively insensitive at detecting the earliest histopathologic stages of AD. Cases with a CDR of 0.5, who are at the earliest detectable stage of dementia, often contain abundant plaques and tangles (30). We suggest, as have others (13, 30), that histopathologic changes are the most sensitive and specific means with which to classify the earliest stages of AD.

Cortical cholinergic denervation may be not only an early step, but a critical one in the development of AD as it may lead directly to Aβ deposition, through altered metabolism of the β-amyloid precursor protein (β-APP). A substantial body of recent work has indicated that cholinergic neurotransmission affects β-APP processing. Activation of protein kinase C through muscarinic m1 and m3 receptor binding stimulates the non-amyloidogenic pathway, resulting in increased release of sAPPα and reduced Aβ production (31–37). Cholinergic neurotransmission therefore might be considered to have a protective effect against Aβ formation. Conversely, the loss of cortical cholinergic innervation could lead to increased production of Aβ and amyloid deposition.

Cortical deposition of Aβ as senile plaques is not restricted to AD but is in fact an extremely common event in normal aging. Cortical cholinergic denervation is also part of normal aging, beginning around age 50 (38). An age-related elevation in biochemically-detectable cortical Aβ also begins at about this time (39) and is followed, within about 10 years by age-related Aβ deposition (40). This is consistent with the hypothesis that cholinergic denervation may in fact cause Aβ deposition.

In this study, we have provided additional evidence that cortical cholinergic denervation begins at the earliest stages of AD, before the disease is clinically apparent. The cholinergic deficit appears to coincide with the first accumulation of Aβ deposits. It is unlikely that cortical neurofibrillary pathology is responsible for the relative cholinergic deficit in the plaque-containing cases, since the mean Braak stage of both the plaque-containing and plaque-free groups was less than stage II. At this stage, tangles are confined to the parahippocampal gyrus and hippocampal formation, so it is difficult to imagine how this localized pathology could result in widespread neocortical loss of cholinergic afferents. Neurofibrillary change in neurons of the nbM might, however, be an important mechanism leading to cell death and subsequent neocortical deafferentation (5).

Human autopsy studies can indicate whether 2 pathologic phenomena are present and statistically associated with each other, but cannot definitively establish the temporal sequence of their occurrence, or whether one may
cause the other. It is possible, for example, that cholinergic denervation occurs early in AD only because cholinergic neurons are extraordinarily vulnerable to the putatively toxic effects of Aβ. Animal experimentation can most effectively answer this question, and some recent work with transgenic mouse models of AD has already shed light on the issue. Two lines of transgenic mice (41, 42) showing very heavy cortical deposition of Aβ (43) could be the other. It is possible, for example, that cholinergic replacement could be a predictor of cholinergic deficits and treatment outcome in Alzheimer disease. Neurology 1991;41:479–86 


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