Effect of Anti-inflammatory Medications on Neuropathological Findings in Alzheimer Disease

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Background: There has been no analysis of brain tissue from longitudinally observed, cognitively tested patients to validate whether anti-inflammatory medications protect against the pathological changes of Alzheimer disease.

Objective: To investigate the role of anti-inflammatory medications in alleviating the pathological features of Alzheimer disease.

Design and Main Outcome Measures: A 5-year post-mortem tissue collection was performed after a case-control study of Alzheimer disease (approximately 90 [30%] of patients died during follow-up, of whom consent for autopsy was obtained in 44 [50%]). Cases were selected on the basis of (1) adequate clinical histories of nonsteroidal anti-inflammatory drug usage, (2) no neuropathological findings other than Alzheimer disease, and (3) no generalized sepsis at death. Variables analyzed included neuropsychological test scores and amount of tissue inflammation and Alzheimer-type pathological changes. Two-way analysis of variance was used to determine whether drug usage significantly affected these variables.

Setting: The Centre for Education and Research on Ageing and the Prince of Wales Medical Research Institute, Sydney, Australia.

Patients: Twelve patients with Alzheimer disease (5 taking anti-inflammatory drugs) and 10 nondemented controls (3 taking anti-inflammatory drugs) were selected (50% of available sample).

Results: Of the patients with Alzheimer disease, anti-inflammatory drug users performed better on neuropsychological test scores than did nonusers. However, there were no significant differences in the amount of inflammatory glia, plaques, or tangles in either diagnostic group.

Conclusion: Long-term anti-inflammatory medications in patients with Alzheimer disease enhanced cognitive performance but did not alleviate the progression of the pathological changes.

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Brain inflammation is thought to contribute to the pathological features of Alzheimer disease (AD), and it has been postulated that anti-inflammatory drugs protect against this tissue damage.1-3 Numerous cross-sectional and limited longitudinal epidemiological studies have identified a reduced risk of AD in patients who consume anti-inflammatory medications.4,5 An additional benefit of consuming anti-inflammatory medications appears to be better cognitive performance.3,6 The inflammatory response in AD is characterized by the presence of activated microglia (the resident immunocompetent cells of the brain) in close association with neuritic plaques.3,7,8 Current evidence suggests that microglia are primarily involved in phagocytic activity and may be responsible for inducing further neuronal damage by generating oxygen species and proteolytic enzymes.3,10 If anti-inflammatory drugs protect against the neurodegeneration observed in the brains of patients with AD, then patients with a history of anti-inflammatory drug usage should have a reduction in pathological changes in the brain and brain inflammation. The present study aimed to test this hypothesis.

RESULTS

DEMOGRAPHIC DETAILS

There was no significant difference between the mean age of the groups (ANOVA, P for diagnosis, .88, for NSAID usage, .34, for interaction, .18). Control patients had an average (±SEM) age of 77±3 years, AD cases were on average...
SUBJECTS AND METHODS

CASE SELECTION AND CLASSIFICATION

Participants in a case-control study of AD published in 1990 were asked to participate in brain donation for research purposes at the time of their death. The cases selected for the original study were consecutive new referrals to dementia clinics in Sydney, Australia. Participants were recruited into the case-control study to determine risk factors for AD, limiting potential recruitment bias to consent for autopsy for the present study purposes. Details of neurological, neuropsychological, and medical histories, including drug usage, have been previously described in detail. Clinical follow-up consisted of yearly field assessments using the Clinical Dementia Rating Scale and assessment of any changes in medical condition and drug usage. Brain collection was limited to a 5-year follow-up period to maintain temporal relevance for the detailed neuropsychological measures.

Approximately 90 original participants (30% of the original sample) died during the 5-year follow-up period, and for 44 (50%) consent for autopsy was given. All cases and controls were screened for neurodegenerative conditions by means of standardized research protocols, as currently recommended. Strict criteria for selection were used in the present study. Cases were excluded if they took steroid anti-inflammatory drugs, if they had any neuropathological conditions other than that associated with AD (particularly infarction, which increases inflammatory markers), or if they had generalized sepsis at death (which also potentiates cerebral inflammation). Twenty-two cases with matching clinical and neuropathological diagnoses were selected for study, 12 AD cases and 10 controls.

Apolipoprotein E (ApoE) genotyping was conducted by polymerase chain reaction (PCR) from DNA extracted from formalin-fixed, paraffin-embedded tissue. Details of the DNA extraction have not previously been published. Four 10-µm tissue sections from each case were dewaxed, rehydrated, and scraped into sterile 1.5-mL standard micro–test tubes (Eppendorf; Hamburg, Germany) by means of a sterile scalpel. Tissue was homogenized in 50 µL of 1× PCR buffer (Gibco BRL, Melbourne, Australia) by means of a cordless handheld homogenizer (Kontes Glass Co, Vineland, NJ). Fifty microliters of 20-mg/mL proteinase K (Sigma-Aldrich Corp, St Louis, Mo) was added and samples were incubated at 55°C overnight. After digestion, samples were heated to 90°C for 10 minutes to inactivate any remaining enzyme, and the DNA stock was stored at −20°C until used. Amplification of the ApoE gene was performed with a mastercycler gradient (Eppendorf). DNA samples (200-400 ng) were amplified in the presence of 2.5 µL of 10× PCR buffer, 1.5 µL of magnesium chloride (50 mmol/L), 2.5 µL of dimethylsulfoxide, 0.2 µL of Taq DNA polymerase, and 2.5 µL of 20-nmol/µL ApoE primer stocks 5′-TCC AAG GAG CTG CAG GCG GCG CA-3′ and 5′-ACA GAA TTC GCC CCG GCC TGG TAC ACT GCC A-3′ (35 cycles at 94°C for 45 seconds, 63°C for 1 minute, and 72°C for 1 minute). Reactions were all made up to a final concentration of 25 µL with ultrapure water (all reagents were from Gibco BRL). To ensure that amplification of the correct product size had occurred (227 base pairs), 6 µL of each sample was run out on a 1% agarose gel (Ultrapure; Gibco BRL). Remaining aliquots of successful PCR samples were then digested with Hhal according to methods previously described. Samples were run out on an 8% polyacrylamide gel to determine the ApoE genotype. Each genotype possessed a unique combination of fragments generated by cleavage with Hhal (91, 81, 72, and 45 base pairs).

Pharmacological exposures and medical treatments were included as a major category in the risk factor interview for the case-control study. Daily medication use for at least 6 months was used as the criterion for significant drug usage. For the present study, cases taking nonsteroidal anti-inflammatory medications (NSAIDs) had arthritis and were monitored for continuation of their NSAID use during the follow-up period. Five cases with AD and 3 controls had consumed NSAIDs for arthritis for a considerable time before death. The remaining cases studied had no documented use of any anti-inflammatory drugs.

79±3 years old, NSAID users had a mean age of 80±3 years, and nonusers were 77±3 years old. Similarly, there was no significant difference in postmortem delay between groups (<45 hours in all cases; mean delay, 19±3 hours; ANOVA, P = .05). Because of the recruitment process (newly diagnosed dementia cases with a 5-year maximum follow-up), there was no significant difference between groups in the time elapsed between the first symptom of dementia and death (average of 4±1 years for NSAID users and 6±2 years for nonusers; P = .21). All AD cases examined died with a Clinical Dementia Rating of 3, indicating severe dementia. Most cases and controls had ApoE ε3/3 alleles, with only 1 AD and 1 control case having the ε3/4 genotype. These variables were therefore not considered further.

NEUROPSYCHOLOGICAL FUNCTION

In all cognitive domains tested, the AD cases taking anti-inflammatory medications performed better than their nonmedicated counterparts (Table). In particular, the tests for attention and speed of information processing, language, apraxia, and visual agnosia reached statistical significance between these groups (Table). The data are consistent with previous findings from the larger case-control study of a negative association between arthritis and AD, and a negative association between analgesic use and late-onset AD.

ANALYSIS OF PATHOLOGICAL FINDINGS

As expected, cases with AD had significantly more plaques and tangles, as well as significantly more inflammatory microglia than controls had (ANOVA, P < .05; Table; Figure). No significant differences were found in the amount of GFAP-positive astrocytes between any groups (Table). In contrast to the differences observed in neuropsychological test scores, there was no significant decrease in any of the neuropathological markers analyzed between cases taking NSAIDs and those not taking these drugs (ANOVA, P > .05, Table). Power analysis was conducted to determine whether the number of AD cases...
VISUALIZATION AND QUANTITATION OF PATHOLOGICAL FINDINGS

Apart from the classic Alzheimer-type pathological features (neuritic plaques and neurofibrillary tangles), inflammatory microglia were detected by means of immunohistochemistry for the expression of major histocompatibility complex II cell surface glycoprotein HLA-DR. In addition, the involvement of other glial components, glial fibrillary acidic protein (GFAP)-positive astrocytes, was analyzed. Formalin-fixed cortical tissue was sampled from the superior frontal, anterior cingulate, inferior temporal, hippocampal, and parahippocampal regions. Two tissue blocks were sampled from each region. One was embedded in paraffin and sectioned at 10 µm, and the other underwent antigen retrieval with 4% sodium citrate buffer, as previously described, before cryoprotection and sectioning at 50 µm on a cryostat. Paraffin-embedded, serial 10-µm sections were stained with the modified Bielschowsky silver stain and peroxidase immunohistochemical reaction for GFAP (Dako, Glostrup, Denmark; Z334 polyclonal antibody diluted 1:750) to visualize neuritic plaques, neurofibrillary tangles, and GFAP-positive astrocytes. Frozen 50-µm sections were stained to visualize activated microglia by means of peroxidase immunohistochemical reaction for HLA-DR (Dako M775 monoclonal antibody diluted 1:1000), as previously described. The specificity of the immunohistochemical reactions was tested by omitting the primary antiseraum samples. No peroxidase reaction was observed in these test sections.

Areal fraction counts obtained with an 11 × 11 eyepiece graticule were used on sections of the superior frontal, inferior temporal, and parahippocampal cortices to quantify pathological changes. Each slide for each case was assessed and the counts were expressed as a percentage of the total number of grid points. This method is the simplest way of reporting how much of a structure there is in the sections. The area of cortex occupied by diffuse and neuritic plaques (Figure, A-C) was assessed in 10 random strips of cortex perpendicular to the pial surface and spanning the entire cortical width magnified at ×200. The area of cortex occupied by neurofibrillary tangles (Figure, D) was assessed in 10 random strips of cortex perpendicular to the pial surface and spanning the entire cortical width magnified at ×400. The area of cortex occupied by inflammatory microglia (Figure, E-G) and reactive astrocytes was assessed in 1 field per cortical region containing the greatest density of immunoreactive structures magnified at ×400. Five repeated measures on the same cortical strip by 2 investigators gave 10% or less difference for all measures with 5% or less difference between the mean score for 5 cortical strips.

ANALYSIS

For the autopsy sample, analysis of clinical test scores gathered at the time of the case-control analysis was performed to see whether the AD cases taking NSAIDs performed better than those who did not. Examination of the neuropsychological tests scores for the participants has been previously published. The cognitive tests were given either according to their standardized method of administration as published in the test manual or by protocols developed for the study. Scores from multiple tests were grouped into the following cognitive domains: attention, language, memory, visuospatial, frontal-executive, apraxia, and agnosia. For the present study, test z scores for each domain were calculated from control values, and differences between AD cases either consuming or not consuming NSAIDs were examined by means of t tests (Levene’s test for homogeneity at P<.05).

Pathological markers were analyzed to see whether cases taking NSAIDs had fewer pathological changes in the brain than those who did not take NSAIDs. Two-way analysis of variance (ANOVA) with diagnosis and NSAID usage as factors was used to evaluate differences in these variables. Variables tested included the areal fraction of diffuse plaques, neuritic plaques, neurofibrillary tangles, inflammatory microglia, and GFAP-positive astrocytes. Fisher protected least-square difference post hoc tests were used to identify the specific group or groups affected. P<.05 was accepted as the level of significance for all tests.

Comment

The findings of the present study cannot support the thesis that long-term usage of NSAIDs protects against or alleviates the brain changes of AD. While better cognitive performance occurred in the AD cases taking NSAIDs, these cases had as much tissue abnormality as their nonmedicated counterparts. In particular, the number of activated inflammatory microglia was not reduced in patients taking NSAIDs, suggesting that these medications may be ineffective in combating inflammation in the brain in AD. Although the power calculation was weak for this variable (52%), the study design controlled for variables that may affect inflammatory microglia, enhancing the opportunity to detect such pathological change. If anything, there were more inflammatory microglia and more neuropathological changes in the AD cases who used NSAIDs. This may seem surprising considering the recent report of a small but significant decrease in the number of activated microglia in nondemented controls who
Photomicrographs showing some of the neuropathological findings in different cases. A and G are from the same control case; B through E are from the same patient with Alzheimer disease. A, Control tissue showing little or no plaque formation or neurofibrillary tangles. B through D, Cortical neuritic pathological changes in a case of Alzheimer disease (A-D, modified Bielschowsky silver stain; A and B, original magnification ×300; C, original magnification ×400; D, original magnification ×200). E through G, Activated microglia visualized with peroxidase immunohistochemistry with the use of HLA-DR antibodies. Significantly more activated microglia were seen in the cases of Alzheimer disease (E) than in controls either taking nonsteroidal anti-inflammatory drugs (F) or not (G) (E through G, original magnification ×200).
used NSAIDs. However, in these controls neither the level of plaques nor that of tangles within the brain was reduced. Thus, the data suggest that the age-related increase in glial activation can be alleviated by treatment with NSAIDs, but give no indication that the age-related AD neuropathological changes can be stopped. The results of the present study provide direct evidence against the hypothesis that NSAIDs protect against the tissue changes of AD.

The patients in the present study were recruited for an alternative purpose and hence sample bias is minimized to that associated with consent for autopsy. As with all long-term prospective studies, the number of cases consenting to autopsy and therefore available for postmortem analysis is relatively small. Our power calculations suggest that at least some pathological measures should have been reduced if the NSAIDs had a direct effect on the brain. Similar case numbers have been studied previously to show increased HLA-DR glia in AD, as seen in the present study. Thus, we believe the data are representative and provide compelling evidence that considerably more work is required to prove current assumptions associated with the underlying mechanisms of drug action in improving cognition in elderly patients.

The majority of data implicating NSAIDs as a viable treatment strategy for the prevention of AD has been gathered from cross-sectional epidemiological studies. However, there are now data from 3 longitudinal studies analyzing the question of drug protection in AD. The Baltimore Longitudinal Study of Aging examined 1686 people during a 5-year period for type and duration of anti-inflammatory medication use. Recruited volunteers were relatively young (younger than 65 years), with education levels higher than a college degree. Both of these factors decrease the risk of AD. This study found a reduced risk of AD among those who consumed NSAIDs. Furthermore, it was observed that there was an increased protective effect, as indicated by a lower relative risk of 0.40 for >2 years compared with 0.65 for <2 years, the longer NSAIDs were used. A similar lowering of risk (0.74) was observed in subjects who consumed aspirin, but not for those who consumed another analgesic without anti-inflammatory action. The Medical Research Council Treatment Trial of Hypertension in Older Adults examined NSAID usage and cognitive decline in 2651 subjects during a 4- to 5-year period. These volunteers averaged 70±3 (SD) years of age and had moderate hypertension and also relatively high premorbid intelligence. The results showed (1) a relatively small but significant protection against cognitive decline in those consuming NSAIDs and (2) that this effect was absent in people older than 74 years. The latter finding may explain the negative results obtained by Henderson and colleagues in a study of 1045 Australians aged 70 years or older (mean age, 80 years). They found that neither NSAIDs nor aspirin provided protection against cognitive decline or incidence of dementia during a 3- to 4-year period. These longitudinal studies suggest that NSAIDs provide a modest protective effect against cognitive decline in some people younger than 75 years. The patients in the present study had a mean age of 78 years, and certain cognitive domains were improved by NSAID treatment compared with their nontreated counterparts. The similarity in underlying tissue damage in the brains of these patients suggests alternative mechanisms for any cognitive improvement.

Cognitive improvement with NSAID usage may occur via cerebrovascular mechanisms. Platelet fluidity increases the risk of AD, with the degree of cognitive impairment correlating with peripheral indexes of platelet activation. Platelet activation releases β-amyloid, explaining some of the increase in plasma concentrations of β-amyloid in AD. Several laboratories have shown that an increase in the concentration of luminal β-amyloid affects the blood-brain barrier and vascular reactivity. Both Aβ1-40 and Aβ1-42 vasoconstrict vascular smooth muscle cells. In vivo infusions of Aβ1-40 decrease blood flow and increase vascular resistance specifically in the brain. These data suggest that increased platelet activation leads to a cascade of events that may contribute to the dementing process via cerebrovascular mechanisms.

Overall, these studies suggest that alternative hypotheses exist for mechanisms of cognitive improvement with NSAIDs, particularly if cerebrovascular processes play a more significant role. Morphological and biochemical alterations to the cerebral vasculature could affect brain perfusion and blood-brain barrier permeability and thereby affect cognition. These factors appear to underlie the clinical effects observed with NSAID usage, rather than a direct action against pathological changes in the brain. Further studies are now required to test all possible hypotheses for drug action and further our understanding of any protective mechanisms against the disease process.

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**Table: Antemortem Neuropsychological Test Scores and Postmortem Quantitation of Neuropathological Findings**

<table>
<thead>
<tr>
<th></th>
<th>Alzheimer Disease (n = 12)</th>
<th>Control (n = 10)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>User (n = 5)</td>
<td>Nonuser (n = 7)</td>
</tr>
<tr>
<td>Antemortem z scores</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Attention/speed</td>
<td>−1.10*</td>
<td>−3.89</td>
</tr>
<tr>
<td>Memory</td>
<td>−1.97</td>
<td>−2.11</td>
</tr>
<tr>
<td>Language</td>
<td>−1.45*</td>
<td>−4.08</td>
</tr>
<tr>
<td>Executive function</td>
<td>−1.87</td>
<td>−2.73</td>
</tr>
<tr>
<td>Visuospatial ability</td>
<td>−1.29</td>
<td>−2.01</td>
</tr>
<tr>
<td>Apraxia</td>
<td>−2.67*</td>
<td>−6.83</td>
</tr>
<tr>
<td>Visual agnosia</td>
<td>−2.72*</td>
<td>−5.46</td>
</tr>
<tr>
<td>Postmortem tissue area</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diffuse plaque</td>
<td>10 (4)*</td>
<td>6 (1)*</td>
</tr>
<tr>
<td>Neuritic plaque</td>
<td>1.2 (0.3)*</td>
<td>1.0 (0.5)*</td>
</tr>
<tr>
<td>Microglia</td>
<td>1.7 (0.4)*</td>
<td>1.1 (0.2)*</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>2.2 (0.4)*</td>
<td>2.0 (0.5)*</td>
</tr>
<tr>
<td>Perivascular space</td>
<td>2.5 (0.6)</td>
<td>1.9 (0.2)</td>
</tr>
</tbody>
</table>

*Statistically better performance or more pathological features (P < .05).
†Test scores of the control cases were used as part of a larger cohort to transform test scores of the patients into z scores. Differences between the groups of patients presented are highlighted.
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