Inflammation, Aβ Deposition, and Neurofibrillary Tangle Formation as Correlates of Alzheimer’s Disease Neurodegeneration

LIH-FEN LUE, PhD, LIBUSE BRACHOVA, PhD, W. HAROLD CIVIN, MD, AND JOSEPH ROGERS, PhD

Abstract. We evaluated entorhinal cortex and superior frontal gyrus for hallmarks of Alzheimer’s disease (AD) pathology, including inflammation, in three patient sets: AD patients, nondemented elderly patients with few or no neurofibrillary tangles (NFTs) and amyloid β peptide (Aβ) deposits, i.e. normal controls (NC), and nondemented elderly patients with profuse entorhinal cortex NFTs and neocortical Aβ deposits, i.e. high pathology controls (HPC). Membrane attack complex (C5b-9) immunoreactivity and immune activation of microglia (MHCII expression) were used as general markers for inflammation. Compared to NC patients, AD patients exhibited significant cortical synapse loss, Aβ deposition, NFT formation, and inflammation. HPC patients also had significantly elevated Aβ deposition and NFT formation, but there was no evidence of synapse loss and little or no evidence of inflammation. Across patients and brain regions the measures of inflammation each accounted for significant percentages of the variance in synaptophysin immunoreactivity and each was more highly correlated with synapse estimates than NFT formation or Aβ deposition.

Key Words: Alzheimer’s disease; Amyloid β peptide; Inflammation; Membrane attack complex; Microglia; Neurofibrillary tangle; Synapse.

INTRODUCTION

We (1) and others (2–6) have noted subsets of patients who come to autopsy without clinical history of dementia but who nonetheless exhibit sufficient neocortex amyloid β peptide (Aβ) deposition and entorhinal cortex neurofibrillary tangle (NFT) formation to otherwise qualify for the diagnosis of Alzheimer’s disease (AD) (7). We have termed this subset “high pathology controls” (HPC) to distinguish them from the more usual nondemented elderly control patients with little or no AD pathology (normal controls) (NC).

HPC patients may provide opportunities to address several pervasive controversies in AD research—in particular, whether various aspects of AD pathology are both necessary and sufficient to account for AD dementia and the neurodegeneration that underlies AD dementia (c.f., 3). Accordingly, we have evaluated HPC, NC, and AD patients using markers for neurodegeneration, Aβ deposition, NFT formation, and inflammation.

MATERIALS AND METHODS

Patients

From among 84 routine brain autopsies of control patients presenting without prior medical history of dementia, 6 were obtained that exhibited sufficient neocortex Aβ plaques and entorhinal cortex NFTs to otherwise qualify for the diagnosis of AD (7). These HPC patients were contrasted with 6 randomly selected NC patients who had limited AD pathology and no prior medical history of dementia and 6 randomly selected AD patients who had previously received a clinical diagnosis of probable AD that was confirmed neuropathologically at autopsy (Table 1). Absence of material symptoms of dementia in HPC and NC patients was confirmed by reference to medical records and interviews with relatives, attending physicians, and attending nurses. Nonetheless, in the absence of more definitive premortem cognitive status data, the present research focuses on a correlate of dementia that is quantifiable postmortem, synapse loss (3), and not on dementia itself.

Brain Samples and Processing

Brains were removed within 5 hours of death (X ± SEM = 2.8 ± 0.3 hours), weighed, and immersed in ice cold 0.1 M phosphate buffer (pH 7.4). They were then sectioned coronally at 1 cm intervals and blocks of the entorhinal cortex (including the transentorhinal area) at the level of the anterior hippocampus and superior frontal gyrus at the level of the genu of the corpus callosum were dissected. These samples were postfixed for 24 to 36 hours in ice cold 4% buffered paraformaldehyde (pH 7.4), cut at 40 μm on a freezing microtome, and subjected to histochemical and immunohistochemical procedures. Contralateral entorhinal cortex and superior frontal gyrus samples were snap frozen and stored at −80°C until assay by Western blot analysis.

Pathologic Variables

Synaptophysin immunoreactivity (Boehringer-Manheim, Indianapolis) provided a measure of synapse loss (3), which has been suggested to be the most consistent marker of neurodegeneration in AD brain and the best correlate of AD dementia severity (3). Aβ 10D5 monoclonal antibody (Athena Neuroscience, San Francisco) was used to reveal both diffuse and compacted Aβ deposits (8). Thioflavin histofluorescence and morphologic criteria were employed to identify compacted, cross-β-pleated Aβ deposits (9). C5b-9 (membrane attack complex)
TABLE 1
Patient Demographics

<table>
<thead>
<tr>
<th>Group</th>
<th>Age ± 4</th>
<th>Sex</th>
<th>Postmortem interval (hours)</th>
<th>Brain weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>81 ± 4</td>
<td>3M/3F</td>
<td>3.2 ± 0.4</td>
<td>1,103 ± 30</td>
</tr>
<tr>
<td>HPC</td>
<td>78 ± 4</td>
<td>5M/1F</td>
<td>3.2 ± 0.5</td>
<td>1,290 ± 51</td>
</tr>
<tr>
<td>NC</td>
<td>77 ± 3</td>
<td>2M/4F</td>
<td>1.9 ± 0.3</td>
<td>1,133 ± 43</td>
</tr>
</tbody>
</table>

(MAC) immunoreactivity (Calbiochem, La Jolla, CA) was chosen as a marker for inflammation because the MAC is the lytic, culminating step in full complement activation; its presence presupposes other components of complement activation, including anaphylotoxins, opsonins, and cytokine stimulators (10–12). Association of C5b-9 immunostaining with Aβ deposits and NFTs was measured using a thioflavin counterstain (c.f., 12). Activation of microglia, as measured by expression of major histocompatibility complex type II cell surface glycoprotein (LN3 monoclonal antibody) (ICN, Irvine), has also been widely used as a marker for heightened immune activity in AD (13–16) and was employed here. The density and close apposition of activated microglia, particularly in AD brain, make counts of individual cells extremely difficult. For this reason, spatially distinct clusters of two or more LN3+ microglia were recorded.

Western Blot Analysis

Equivalent amounts of protein were electrophoresed for 24 hours (h) on 3.5 mm thick SDS-polyacrylamide gels (7.5%) and electrotransferred overnight (0.4A, 4°C). Membranes were blocked with 5% nonfat dry milk for 2 h, then incubated with 1:1000 synaptophysin primary monoclonal antibody (Boehringer-Manheim) for 24 h (4°C). For immunodetection, ECL Western blot analysis kits (Amersham Life Science) were employed, with horseradish peroxidase-conjugated ECL mouse IgG (1:1000) and ECL detection reagents. Optical densities of immunoreactive bands were recorded by densitometry. A representative blot is shown in Figure 1.

Histochemistry and Immunohistochemistry

Entorhinal cortex and superior frontal gyrus sections from each patient were processed in triplicate for thioflavine S histochemistry and Aβ, LN3, and C5b-9 immunohistochemistry as previously described (12).

Quantification of Pathology

Sections were imaged at 100× using bright field or fluorescence optics. A total of 20 fields per slide from superior frontal gyrus lamina I–VI and entorhinal cortex pyramidal, polymorphic, and molecular layers were digitized and displayed. Stained elements within fields (e.g. Aβ immunoreactive plaques, clusters of activated microglia) were counted by an observer blind to patient condition.

Statistics

The data were analyzed by 2-way repeated measures ANOVA, with disease state (AD, NC, HPC) as the first factor and brain region (entorhinal cortex, superior frontal gyrus) as the second. Where dictated by significant interaction terms and a priori hypotheses, 1-way ANOVAs, t-tests, and within-subject correlations (from stepwise regression) were pursued.

RESULTS

Summary demographic data and means (± SEM) for the pathologic variables are given in Tables 1 and 2, respectively. Alzheimer’s disease, HPC, and NC groups did not differ significantly with respect to age, sex, or postmortem interval, indicating that the patients were well matched. By 2-way ANOVA, AD, HPC, and NC groups differed significantly on all the pathologic variables. Representative micrographs illustrating these changes are provided in Figure 2.

Neurodegeneration

Synaptophysin immunoreactivity was similar in entorhinal cortex and superior frontal gyrus when viewed across all patients. Indeed, the densitometric estimates of synapse density for these two brain regions were highly correlated within subjects (R = 0.819, P < 0.001). When synapse measures were compared among AD, HPC, and NC patients, however, overall (F2,15 = 6.91, P = 0.007) and individual differences emerged. Consistent with their putatively nondemented status, HPC patients showed no evidence of synapse loss, with levels of synaptophysin immunoreactivity that were, if anything, higher than those of NC patients. By contrast, AD patients exhibited significantly lower synaptophysin levels compared to both HPC patients (t10 = 3.36, P = 0.007, entorhinal cortex, and t10 = 2.59, P = 0.027, superior frontal gyrus) and NC patients (t10 = 3.22, P = 0.009, entorhinal cortex, and t10 = 2.2, P = 0.050, superior frontal gyrus). Brain weights also differed significantly (F2,15 = 5.70, P = 0.014), with the HPC group yielding significantly higher mean values than the AD group (t10 = 3.17, P = 0.010) or the NC group (t10 = 2.37, P = 0.039), replicating a previous finding (2).

Aβ Deposition

Counts of Aβ immunoreactive plaques (F2,15 = 5.79, P = 0.014) and compacted, thioflavine histofluorescent plaques (F2,15 = 6.30, P = 0.010) differed significantly among the groups. HPC and AD patients did not differ
**TABLE 2**

*Alzheimer’s Pathology (per mm²)*

<table>
<thead>
<tr>
<th>Group</th>
<th>Aβ⁻ plaques</th>
<th>Thioflavine⁺ plaques</th>
<th>C5b-9⁻ plaques</th>
<th>Thioflavine⁺ NFTs</th>
<th>C5b-9⁻ NFTs</th>
<th>LN3⁺ microglia clusters</th>
<th>Synaptophysin⁺ (OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entorhinal cortex:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AD</td>
<td>71.8 ± 24.2</td>
<td>21.6 ± 2.6</td>
<td>4.9 ± 2.5</td>
<td>98.2 ± 22.0</td>
<td>32.0 ± 2.3</td>
<td>10.8 ± 4.6</td>
<td>2.6 ± 0.5</td>
</tr>
<tr>
<td>HPC</td>
<td>51.3 ± 5.8</td>
<td>14.5 ± 3.3</td>
<td>0.5 ± 0.1</td>
<td>28.1 ± 11.5</td>
<td>1.0 ± 0.4</td>
<td>3.1 ± 1.2</td>
<td>5.4 ± 0.7</td>
</tr>
<tr>
<td>NC</td>
<td>1.1 ± 1.1</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.2 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>0.6 ± 0.6</td>
<td>4.4 ± 0.3</td>
</tr>
<tr>
<td>Superior frontal gyrus:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AD</td>
<td>214.0 ± 83.8</td>
<td>51.8 ± 14.7</td>
<td>11.1 ± 3.3</td>
<td>21.6 ± 10.2</td>
<td>0.0 ± 0.0</td>
<td>9.8 ± 2.0</td>
<td>2.8 ± 0.7</td>
</tr>
<tr>
<td>HPC</td>
<td>237.0 ± 62.9</td>
<td>53.3 ± 21.2</td>
<td>3.6 ± 1.1</td>
<td>0.1 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>2.3 ± 0.6</td>
<td>5.0 ± 0.5</td>
</tr>
<tr>
<td>NC</td>
<td>1.4 ± 1.1</td>
<td>0.2 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>1.5 ± 1.5</td>
<td>4.6 ± 0.4</td>
</tr>
</tbody>
</table>

significantly on these measures. However, both groups had significantly more Aβ immunoreactive and thioflavine histofluorescent plaques than NC patients, who had few, if any, of these pathologic markers. With respect to the two brain regions, Aβ⁺ plaques (F₁,₁₅ = 11.68, P = 0.004) and thioflavine⁺ plaques (F₁,₁₅ = 9.47, P = 0.008) were significantly elevated in superior frontal gyrus compared to entorhinal cortex. Neither measure of Aβ deposition correlated well with synapse loss (R = -0.105, P = 0.543 for Aβ⁻ plaques and R = -0.129, P = 0.454 for thioflavine⁺ plaques).

**NFT Formation**

NFT counts differed significantly among the groups (F₁,₁₅ = 12.76, P = 0.001). In entorhinal cortex, HPC patients exhibited substantial NFT formation, with levels that were nearly 100-fold greater than NC patients, though still less than AD patients. In superior frontal gyrus, NFTs were rare in HPC patients and not detected in NC patients, both groups having dramatically fewer NFTs than AD patients. With respect to the two brain regions, NFT formation predominated in the entorhinal cortex compared to the superior frontal gyrus (F₁,₁₅ = 21.11, P < 0.001) and was a marginal (R = -0.374, P = 0.025) correlate of synapse loss.

**Inflammation**

Inflammation, as measured by C5b-9 immunoreactivity, was profusely present in AD entorhinal cortex plaques, NFTs, and neuropil threads, and in AD superior frontal gyrus plaques. C5b-9 staining of Aβ deposits differed significantly with respect to patient group (F₂,₁₅ = 6.83, P = 0.008) and brain region (F₁,₁₅ = 12.91, P = 0.003), with HPC patients exhibiting 3- to 10-fold fewer C5b-9⁺ plaques than AD patients. These low levels were more comparable to the absence of C5b-9 staining in NC patients. Entorhinal cortex C5b-9-positive NFTs also differed significantly among the three groups (F₂,₁₅ = 9.34, P = 0.002). In AD patients, approximately 1 out of 3 NFTs were associated with C5b-9, whereas only 1 in 28 NFTs was associated with C5b-9 in HPC patients. NC patients had no C5b-9 immunostained NFTs, nor was C5b-9 immunoreactivity observed in superior frontal gyrus NFTs of any patient. Across all patients and brain regions, C5b-9 immunoreactivity was the most sensitive correlate of synapse loss (R = -0.429, P = 0.009 for C5b-9⁺ plaques and R = -0.399, P = 0.016 for C5b-9⁺ NFTs) compared to the other measures of AD pathology.

Activated, LN3⁺ microglia, the second measure of inflammation, also differed significantly among the patient groups (F₂,₁₅ = 6.17, P = 0.011), with AD patients having the highest values, NC patients having negligible values, and HPC patients having intermediate values more comparable to NC than AD. There was no significant difference in LN3 staining of entorhinal cortex and superior frontal gyrus. As a correlate of synapse measures, LN3 immunoreactivity was second only to C5b-9 immunoreactivity (R = -0.390, P = 0.019).

**Relationships among the Pathologic Variables**

No significant correlations of NFTs with Aβ⁺ or thioflavine⁺ plaques were observed within or across the two brain regions examined. However, each of the pathologic variables in entorhinal cortex was correlated with its counterpart in superior frontal gyrus: Aβ⁺ plaques (R = 0.652, P = 0.003), thioflavine⁺ plaques (R = 0.470, P = 0.049), C5b-9⁺ plaques (R = 0.790, P < 0.001), thioflavine⁺ NFTs (R = 0.655, P = 0.003), and LN3⁺ microglia clusters (R = 0.818, P < 0.001). Because activated microglia may provide a brain endogenous source of complement proteins (17), we also sought and observed a significant within-subject correlation of C5b-9 and LN3 immunoreactivities (for C5b-9 plaques R = 0.737, P < 0.001, for C5b-9 NFTs R = 0.547, P = 0.001).

**DISCUSSION**

Consistent with their absence of overt symptoms of dementia, HPC patients show no evidence of synaptic
Fig. 2. Entorhinal cortex pathology in AD patients (left panels), HPC patients (middle panels), and NC patients (right panels). The top three micrographs are stained for Aβ immunoreactivity and show substantial numbers of Aβ⁺ plaques in AD and HPC patients, but not NC patients. The panels immediately below use thioflavine histofluorescence to illustrate plaques and tangles, which are numerous in AD and HPC patients, but negligible in NC patients. Note the profuse staining of entorhinal cortex island cells. The three micrographs next to the bottom show C5b-9 immunoreactivity of plaques and tangles. Like NC patients, HPC
loss, and in fact have slightly higher synapse measures than NC patients in both superior frontal gyrus and entorhinal cortex. Interestingly, HPC patients also have significantly higher brain weights than NC patients, a finding consistent with previous studies (2). These results suggest that increased brain reserves prior to the neurodegenerative onslaught of AD may raise the threshold for the expression of overt dementia.

For all the pathologic features measured there are significant differences among the AD, HPC, and NC groups across the two cortical regions, entorhinal cortex and superior frontal gyrus. Of the variables, Aβ deposition accounts least well for the differing synaptophysin levels observed in the patients. Indeed, HPC patients exhibit similar or higher numbers of Aβ+ and thioflavine-I plaques than AD patients, yet they show no apparent loss of synapses. Although thioflavine-I NFTs do differ significantly among AD and HPC patients, HPC patients still have sufficient numbers of entorhinal cortex NFTs to otherwise meet criteria for AD (7). By contrast, both measures of inflammation, C5b-9 and LN3 immunoreactivity, are significantly reduced in HPC patients and are typically comparable to values for NC patients. Moreover, of all the pathologic variables examined, the best predictors of synaptic changes (i.e., the variables that accounted for the highest proportions of synapse variance) are those related to inflammation, C5b-9 immunoreactivity and activated LN3+ microglia. Taken together, these data suggest that elderly patients may present at autopsy with profuse cortical plaques and entorhinal cortex NFTs, but may not evidence synaptic loss unless these changes are accompanied by inflammatory reactions. Inflammation may therefore be one of the final common pathways through which Aβ deposits and NFTs manifest their neurodegenerative effects, an hypothesis further supported by the consistent association of inflammatory markers with Aβ deposits and NFTs (reviewed in 11, 14, 18) and the fact that both Aβ (12) and NFTs (Brachova et al, in preparation) stimulate inflammatory processes in vitro. Alternatively, the significant but low R2 values for the inflammation variables should make clear that other mechanisms must also be involved in AD pathogenesis.

Our data may also be informative with respect to relationships among the classic hallmarks of AD, plaques and NFTs, Aβ+ and thioflavine-I plaques are the predominant form of AD pathology in superior frontal gyrus, and appear to precede the formation of NFTs there. That is, numerous patients with superior frontal gyrus plaques are observed who have not developed superior frontal gyrus NFTs, whereas no patients are observed who have superior frontal gyrus NFTs but no plaques. This pattern could not occur if NFTs preceded plaques or if both arose concurrently in the superior frontal gyrus.

There are also significantly more (by a factor of three) Aβ+ and thioflavine-I plaques in superior frontal gyrus than entorhinal cortex. The converse is true with respect to NFTs: entorhinal cortex NFTs significantly outnumber (by a factor of three) superior frontal gyrus NFTs. Each of these hallmark pathologies is highly correlated with its counterpart across structures: entorhinal cortex Aβ+ deposits with superior frontal gyrus Aβ+ deposits, entorhinal cortex thioflavine-I compacted plaques with superior frontal gyrus thioflavine-I compacted plaques, and entorhinal cortex thioflavine-I NFTs with superior frontal gyrus thioflavine-I NFTs. Taken together, these results suggest that the driving mechanisms for plaque and tangle formation operate globally in the cortex, but individual cortical structures differ in their response to these global forces. For example, despite the significant correlation of entorhinal cortex and superior frontal gyrus NFTs, the island neurons of the entorhinal cortex (19) must have some special vulnerability to NFT formation compared to other cortical neurons. They are always heavily impacted in any patient exhibiting NFTs both in our study and those of others (20, 21), and indeed they appear to be the first cortical neurons to develop NFTs, as originally suggested by Hyman and colleagues (20). That is, we observe numerous patients who have NFTs only in entorhinal cortex island cells and nowhere else, as well as numerous patients who have NFTs in both entorhinal cortex island cells and other cortical regions; but we never observe patients who have no NFTs in island cells and who do exhibit NFTs in other cortical regions. As before, this pattern logically requires that NFTs develop first in entorhinal cortex island cells, or else in this relatively broad sample we should have observed at least one patient without island cell NFTs but with NFTs elsewhere.

Despite previous hypotheses that have sought to link Aβ deposition with NFT formation (c.f., 22, 23), no relationship between plaques and NFTs is observed within or across brain structures in the present study.

We believe the present results are consistent with the hypothesis that HPC patients are in an early, preclinical stage of AD (2–6). Antecedents of neurodegeneration such as Aβ deposition and NFT formation are in place, but they lack some other factor or factors critical for their
transformation into overt synapse loss and, ultimately, dementia. Although many such factors could be listed, inflammation is a highly tenable candidate. It is an inherently destructive process. It is typically a reactive process, and so should begin to be observed only after more fundamental antecedents are established. It is interactive with classical AD pathology, including in situ colocalization of inflammatory elements with Aβ deposits and NFTs (11, 14, 18), in vitro activation of complement (12), and in vitro stimulation of cytokine expression (17, 24). We conclude, therefore, that Aβ deposition and NFT formation are important early markers of AD pathogenesis. Although both of these AD hallmarks are demonstrably neurotoxic, other reactive, neurotoxic phenomena, particularly inflammation, appear to be necessary to tip the scales to overt dementia.

REFERENCES


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