Absence of Abnormal Hyperphosphorylation of Tau in Intracellular Tangles in Alzheimer's Disease

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Abstract. Adult human nerve cells contain tau protein, a phosphorylated microtubule-associated protein, that is hyperphosphorylated in the fetus and in patients with Alzheimer's disease. Hyperphosphorylation, which diminishes the microtubule-binding capacity of tau, destabilizes microtubules and may enhance the formation of paired helical filaments that constitute neurofibrillary tangles in Alzheimer's disease. Here, we use phosphorylation-dependent anti-tau antibodies to detect specific epitopes that characterize hyperphosphorylated tau. Our demonstration of intracellular tangles containing full-length tau that are not immunolabeled by these antibodies suggests that hyperphosphorylation of tau is not obligatory in the formation of neurofibrillary tangles in Alzheimer's disease.

Key Words: Alzheimer's disease; Hyperphosphorylation; Neurofibrillary tangles; Tau.

INTRODUCTION

A close relationship between neurofibrillary tangles (NFT) and dementia in Alzheimer's disease (AD) has been established (1–5). Neurofibrillary tangles, which are well developed in pyramidal neurons of hippocampus, are composed of densely packed bundles of microscopic-size filaments, made up largely of paired helical filaments (PHF) (6, 7). Paired helical filaments are known to contain tau protein as an integral constituent (8, 9). In addition to NFT, PHF occur in neuropil threads and dystrophic neurites associated with plaques (10).

A monoclonal antibody, Alz50, recognizes pathological PHF-containing structures and certain tangle-free neurons, presumably before the formation of NFT (11, 12). On immunoblots, it recognizes all human tau isoforms in normal adult and fetal brains (13, 14) and A68, a mixture of sarkosyl-insoluble tau proteins with apparent molecular mass of 60–68 kDa prepared from brains of AD patients (15). Whereas normal adult tau protein is phosphorylated to a limited extent (2–3 mols phosphate per mol tau) in postmortem brain, A68 is abundantly phosphorylated at multiple sites, including serine 202 and serine 396, which are not phosphorylated in normal adult, autopsy-derived tau (15, 16). Phosphorylation of these two sites also occurs in fetal tau (16, 17).

Phosphorylated residues that have been identified in fetal and PHF tau include serine 202, serine 235, serine 262, serine 396, serine 404, threonine 231 and threonine 181 (18, 19). With the exception of serine 202 and serine 396, these sites have been found to be partially phosphorylated in normal adult tau (19). In PHF tau, these sites are abundantly phosphorylated (19) and PHF tau can, therefore, be said to be abnormally phosphorylated (i.e. hyperphosphorylated). Hyperphosphorylated tau, i.e. that which is fully phosphorylated at serine 202 and serine 396, is also known as A68 (20).

One possible consequence of hyperphosphorylation of tau is the loss of normal microtubule-binding capacity and, consequently, the destabilization of microtubules (21, 22). As it has been claimed that A68 proteins are essential building blocks of PHF (15), it has been proposed that a second consequence of abnormal hyperphosphorylation is the facilitation of PHF formation. This proposal seems incongruous with the formation of PHF-like polymers from tau fragments containing only the repeat region (23, 24) and full-length non-phosphorylated recombinant tau (25). It could be speculated, therefore, that phosphorylation of serine 202 and/or serine 396 is not obligatory for PHF formation in vivo. The existence, demonstrated here, of intracellular NFT with full-length tau unlabeled (or in large part unlabeled) by phosphorylation-dependent antibodies that otherwise recognize serine 202 and serine 396 suggests that phosphorylation at these sites is not an essential prerequisite for PHF formation.

MATERIALS AND METHODS

Segments of medial temporal lobe containing the hippocampus were obtained from 11 patients (Table 1) with well-documented AD. The diagnosis in each case satisfied NINCDS/ADRDA criteria for definite AD (26). The specimens were fixed in methanol containing 5% acetic acid, embedded in paraffin wax, and sectioned serially at 15 μm as has been described (27). The sections were stained with Congo red (28) and immunolabeled with an anti-tau antibody. They were incubated...
TABLE 1
Numbers of AT8-positive and AT8-negative Intracellular
Tangles per mm² Hippocampus (Area CA1) and Clinical
Characteristics of Patients with Probable Alzheimer’s Disease

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Age</th>
<th>Sex</th>
<th>Dur</th>
<th>PMI</th>
<th>Intracellular tangles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AT8+</td>
</tr>
<tr>
<td>40</td>
<td>76</td>
<td>M</td>
<td>4</td>
<td>un</td>
<td>46.7</td>
</tr>
<tr>
<td>64</td>
<td>91</td>
<td>F</td>
<td>7</td>
<td>6</td>
<td>5.6</td>
</tr>
<tr>
<td>65</td>
<td>78</td>
<td>M</td>
<td>8</td>
<td>10</td>
<td>37.6</td>
</tr>
<tr>
<td>75</td>
<td>72</td>
<td>M</td>
<td>10</td>
<td>33</td>
<td>50.3</td>
</tr>
<tr>
<td>77</td>
<td>75</td>
<td>F</td>
<td>9</td>
<td>14</td>
<td>6.5</td>
</tr>
<tr>
<td>78</td>
<td>67</td>
<td>F</td>
<td>7</td>
<td>50</td>
<td>31.4</td>
</tr>
<tr>
<td>80</td>
<td>84</td>
<td>M</td>
<td>3</td>
<td>11</td>
<td>35.3</td>
</tr>
<tr>
<td>81</td>
<td>84</td>
<td>F</td>
<td>10</td>
<td>15</td>
<td>63.9</td>
</tr>
<tr>
<td>96</td>
<td>un</td>
<td>M</td>
<td>un</td>
<td>un</td>
<td>17.1</td>
</tr>
<tr>
<td>100</td>
<td>92</td>
<td>F</td>
<td>6</td>
<td>12</td>
<td>37.0</td>
</tr>
<tr>
<td>104</td>
<td>76</td>
<td>M</td>
<td>4</td>
<td>17</td>
<td>78.9</td>
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</tbody>
</table>

Age = age at death in years.
Dur = duration of dementia in years.
PMI = Postmortem Interval.
un = Unknown.
( ) = % total intracellular tangles.

overnight with the primary antibody (diluted 1:20 with phosphate-buffered saline, PBS), washed with PBS, and incubated for 1 hour with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin (used with monoclonal primary antibodies) or a FITC-conjugated anti-rabbit immunoglobulin (used with polyclonal antibodies). The sections were mounted on glass slides in Citifluor (Ted Pella, Inc. Redding, CA). The method of immunolabeling has been described (29).

Antibodies

The anti-tau antibodies used were: AT8, AT180, and AT270 (provided by Innogenetics N.V., Ghent, Belgium); SM131 (Stennberger Monoclonals, Baltimore, MD); Alz50 (provided by Dr. Peter Davies); and BR133.

SM131 has been shown to recognize a phosphorylation-dependent site between serine residues 396 and 404 (30). AT8 immunoreactivity depends upon phosphorylation of serine 202 (17). Other phosphorylation-dependent antibodies, AT270 and AT180, recognize epitopes requiring phosphorylation at threo-nine 181 and threonine 231, respectively (19). Part or all of the epitope recognized by Alz50 has been demonstrated in amino acid sequence 2-10 of tau protein and appears not to be phosphorylation-dependent (31), although Alz50 has also been shown to recognize a phasphatase-sensitive epitope (32). BR133 is a polyclonal antibody raised against amino acids 1-16 (33). These antibodies and their epitopes are summarized in Figure 1.

Confocal Microscopy

Sections stained with Congo red and immunolabeled with an anti-tau antibody were analyzed with a confocal microscope. The two fluorescence images, when superimposed, produced red and yellow images corresponding, respectively, to Congo-stained and FITC-labeled objects, the former being more or less red depending on the relative amount of the FITC-labeled epitope present.

Sections were visualized at an initial magnification of 200-600× with a Nikon epifluorescence microscope equipped with 200/75 and 60/1.4 (oil immersion) planapochromatic objective lenses. Selected microscopic fields were then scanned with an MRC-600 laser scanning confocal microscope imaging system (Bio-Rad, Cambridge, MA). The system was equipped with an argon laser and high sensitivity 488 nm and 514 nm exciter filters. The emission spectra from the excited fluorophores were passed to photomultipliers, viewed on a monitor and photographed.

RESULTS

Both intra- and extracellular NFT are stained with Congo red (27), a diazo dye used in neurohistology. When viewed with a fluorescence microscope, intracellular NFT appear as densely packed bundles of intracytoplasmic filaments emitting a bright red fluorescence. These Congo red-labeled intracellular NFT appeared as brightly fluorescent objects in the red fluorophore-visualizing channel (514 nm exciter filter) of the laser scanning confocal imaging system (Fig. 2C, solid arrow). In the green fluorophore-visualizing channel (488 nm exciter filter) they were barely visible (Fig. 2B, solid arrow). Intracellular NFT that are stained with Congo red and not immunolabeled appear red when the two channel images are superimposed (Fig. 2A, solid arrow). Intracellular

Fig. 1. Diagram of the longest human tau protein isoform. Phosphorylation sites are indicated by P. When known, the loci of epitopes recognized by antibodies used in this study are identified.

PHOSPHORYLATION STATUS OF TAU IN TANGLES

Fig. 2. Confocal microscope images of intracellular tangles in sections of hippocampal pyramidal neurons incubated with FITC-tagged AT8 and stained with Congo red. A. Merged image resulting from the superimposition of 488 nm and 514 nm channel images. The red intracellular tangle, not immunolabeled with AT8, is indicated by a closed arrow; the immunolabeled tangle is yellow (open arrow). B. In the 488 nm channel an intracellular tangle, not immunolabeled by AT8, is barely visible (closed arrow); an intracellular tangle immunolabeled by AT8 is clearly visible (open arrow). C. In the 514 nm channel intracellular tangles are clearly visible, brightly fluorescent images (arrows). Bar = 50 µm.

NFT that are stained with Congo red and simultaneously immunolabeled with a FITC-tagged anti-tau antibody had a similar structure but emitted a red-yellow fluorescence when viewed with a fluorescence microscope due to the combined presence of red (Congo red) and green (FITC) fluorophores. The NFT appeared more or less yellow depending upon the relative amounts of the two fluorophores. They appeared with variable brightness when viewed in either the 514 nm (Fig. 2C, open arrow) or 488 nm (Fig. 2B, open arrow) channel of the confocal microscope depending on the relative amounts of Congo red and FITC. When the 514 nm and 488 nm channel images were merged, the original color was captured (Fig. 2A, open arrow). In no case could an intracellular NFT be identified that emitted a green fluorescence, which would be indicative of antibody labeling in the absence of Congo red staining.

In specimens stained with Congo red and immunolabeled with FITC-tagged AT8, SM131, Alz50, AT180, or AT270, intracellular NFT usually emitted a bright yellow fluorescence in the fluorescence microscope. A small percentage of such intracellular NFT emitted a bright red fluorescence, indicating a total, or virtually total, lack of immunolabeling with the above anti-tau antibodies. For example, the absence of immunolabeling of intracellular NFT that had also been stained with Congo red is illustrated in Figure 3. While no immunolabeling with SM131 or Alz50 is seen in the 488 nm channel (Fig. 3C, D), the Congo red-stained NFT are clearly seen in the 514 nm channel (Fig. 3E, F) of the confocal microscope. The composite, merged images are seen in Figure 3A and B.

The numbers of Congo red-stained intracellular tangles that failed to immunolabel with AT8 were counted in area CA1 of the hippocampus and expressed both as the mean value per mm² and as a percentage of the total number of intracellular tangles. As the numbers of neurons that failed to immunolabel with AT8 were similar to those failing to immunolabel with SM131, Alz50, AT180, or AT270, data are presented only for AT8 (Table 1). No significant correlations were found between the neuron or the proportion of AT8-negative neurons and age, sex, duration of dementia, or postmortem interval (PMI).

In specimens stained with Congo red and immunolabeled with BR133 all intracellular NFT emitted a bright yellow fluorescence in the fluorescence microscope. In no case was it possible to demonstrate an intracellular NFT emitting only a red fluorescence. In the confocal microscope, 488 nm (Fig. 4A) and 514 nm (Fig. 4B) channel images were virtually identical and the merged images (Fig. 4C) were essentially yellow.

DISCUSSION

These findings suggest the existence of a small population of intracellular NFT that stain with Congo red but fail to immunolabel with antibodies against specific phosphorylation-dependent epitopes. Two of these epitopes, which are typically recognized by AT8 and SM131, have been demonstrated in fetal tau but are not detectable (in postmortem brain) in normal adult tau (reviewed in 34). They typically occur in intracellular PHF and are characteristic of hyperphosphorylated tau (A68) proteins (15). Similarly, a small percentage of Congo red-stained intracellular NFT fail to label with Alz50 (Fig. 3D). Because Alz50 has been shown to recognize a non-phosphorylated epitope in the amino acid sequence containing residues 2-10 (31), the lack of Alz50 immunolabelling might suggest the loss of the N-terminal region of the Tau molecule. Alternatively, it is possible that unidentified conformational changes to the molecule might make the N-terminal epitope inaccessible to Alz50. Additionally, an unidentified sequence(s) or conformation not contained in residues 2-10 might contribute to the epitope recognized in intracellular NFT (35).

Because all intracellular NFT are immunolabeled by BR133 (Fig. 4), an antibody that recognizes an epitope within amino acid sequence 1-16 (33), our findings suggest that loss of the amino terminus of tau is not responsible for the failure of a small population of intracellular NFT to label with Alz50 or AT8. This would imply that conformational changes associated with the absence of AT8 immunoreactivity may also be responsible for the loss of Alz50 immunoreactivity. It has been demonstrated that phosphorylation can produce conformational changes in normal tau (36).

In excisional biopsy specimens of human brain obtained during surgery (37) and in fresh rat brain (38), PHF tau, fetal tau, and normal adult human tau appear to be phosphorylated at similar phosphorylation sites including serine 202, serine 396, threonine 181, and threonine 231. Because these sites are more likely to remain phosphorylated in PHF tau than in normal adult tau in postmortem specimens, it has been suggested that hyperphosphorylation of PHF tau results from the downregulation of certain protein phosphatases in AD (37). It might also be suggested that variations in the immunoreactivity of phosphorylation-dependent epitopes of PHF tau, as reported here, might reflect variations in PMI. However, we failed to find statistically significant correlations between PMI and the number or proportion of unlabeled intracellular NFT (Table 1), suggesting, instead, the presence of a small population of NFT that are not phosphorylated at sites usually phosphorylated in PHF tau.

Our findings suggest that hyperphosphorylation of tau need not be an obligatory prerequisite for the formation of NFT. Although the metabolic process(es) underlying the phosphorylation of tau in intracellular NFT is not known, we would speculate that the accumulation of non-hyperphosphorylated tau into at least some PHF is consistent with our findings. There is no evidence of dephosphorylation of tau after being accumulated into PHF in vivo, although this has been demonstrated in vitro (17) and might contribute to the pool of normal tau from which PHF are formed. It seems unlikely that tau is dephosphorylated after NFT formation because the intracytoplasmic phosphatases would be expected to com-
Fig. 4. Confocal microscope images of intracellular tangles immunolabeled with FITC-tagged BR133 and stained with Congo red. A. 488 nm channel. B. 514 nm channel. C. Merged image. Bar = 50 μm.

mence their action at the surface of the NFT prior to acting on internal filaments; image reconstruction by confocal microscopy gives no evidence of layers with different degrees of phosphorylation in any NFT.

The demonstration of NFT composed of little or no hyperphosphorylated tau argues against the necessity of abnormal phosphorylation for PHF assembly in AD (15, 17). It is, however, consistent with recent findings showing that only a minority of PHF are hyperphosphorylated in AD and that hyperphosphorylation inhibits the binding of tau to itself in the formation of tau complexes (39, 40). Our findings suggest that hyperphosphorylation of PHF tau can be seen as a variable secondary event that occurs after PHF begin to accumulate in the somatodendritic compartment of affected neurons.

The physical properties of tau depend upon its state of phosphorylation (36), and hyperphosphorylation has been shown to result in a decreased capacity to bind tubulin and stabilize microtubules (21, 22, 41–43). Indeed, it is conjectured that hyperphosphorylation of tau leads to a loss of microtubular integrity and failure of intracellular transport systems and that this is associated with neurofibrillary pathology and neuronal death in AD (44, 45).

Hyperphosphorylation of tau may also promote PHF formation in AD (15, 23, 24, 41). However, our results from this and other studies (39, 40) indicate that it may
not be a necessary prerequisite of PHF formation. The results of recent studies showing only minor abnormalities in tau-deficient mice (46) also point to the improbability that hyperphosphorylation of tau in PHF contributes directly to the process of neuronal death in AD.

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REFERENCES


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