Immunohistochemical Staging of Neurofibrillary Degeneration in Alzheimer's Disease

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Abstract. Antibodies to different phosphorylated and non-phosphorylated tau epitopes have been used to identify three histologically distinct types of neurofibrillary tangles in Alzheimer's disease. Intracellular tangles (Type 1) were identified by antibodies recognizing epitopes throughout the tau molecule, including the NH$_2$-terminus. Compact extracellular tangles (Type 2) were characterized by the loss of NH$_2$-terminal immunoreactivity and retention of other tau epitopes. Dispersed extracellular tangles (Type 3) were characterized by the presence of epitopes associated with the microtubule binding region and the COOH-terminus. These three types of tangles, found in situ in hippocampus, could be created experimentally by proteolytic treatment of brain sections. These findings suggest that three stages of neurofibrillary degeneration can be understood as a sequential stripping of paired helical filaments in which the loss of amino-terminus epitopes, followed by loss of phosphorylated epitopes, results in the appearance of dispersed extracellular tangles containing PHF-core epitopes.

Key Words: Alzheimer's disease; Neurofibrillary tangles; Neuronal degeneration; Tau protein.

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia in older adults (1). It is characterized microscopically by large numbers of neuritic plaques, dystrophic neurites and neurofibrillary tangles (NFT) in the brain. All three contain paired helical filaments (PHF) (2, 3). These submicroscopic structures, which can be visualized in situ by electron microscopy, consist of two filaments helically wound around each other with a twist about every 65–80 nm. Isolated PHF typically appear composed of a superficial fuzzy coat and a central core (4), although their submicroscopic structure may vary with different isolation procedures (5, 6). A subunit structure of the PHF core consisting of a double stack of C-shaped subunits has been demonstrated by computer-assisted image reconstruction (5).

Paired helical filaments contain the microtubule-associated protein tau (7–9), the NH$_2$-terminus of which is lost with the fuzzy coat when isolated PHF are treated with Pronase (9, 10). The remaining Pronase-resistant PHF core contains a 12 kDa tau fragment, beginning in the vicinity of histidine 268, which encompasses the repeated tubulin-binding domain of tau (11). Both 3- and 4-repeat isoforms of tau have been identified in the Pronase-resistant PHF core (11, 12). Other proteins, namely ubiquitin (13, 14) and B/A4 (10, 15–20), have been associated with PHF but their relationship, if any, to PHF structure is not known.

Although the formation of NFT is closely associated with neuronal degeneration in AD, molecular events underlying neurofibrillary degeneration are unknown. It has been established that tau is abnormally phosphorylated in PHF (21–23), perhaps as a result of a change in intracellular calcium (24). It has been established, also, that the transformation of intracellular to extracellular tangles, in the course of neurofibrillary degeneration, is associated with the loss of fuzzy coat epitopes recognized by antibodies raised against the NH$_2$- and COOH-termini of tau and the recognition of PHF core epitopes by antibodies which recognize epitopes in the vicinity of the repeated tubulin-binding region (10). It has been suggested that the dispersion of extracellular tangles in neuropi may result from invasion by astrocytes (25). Extracellular tangles also undergo changes in tau immunoreactivity (10, 18). They appear to become associated with ubiquitin (18, 26), heparan sulfate proteoglycans (27, 28) and, at least in hippocampus, some become immunoreactive with antibodies against B-amyloid (10, 18, 19). Some may undergo changes in PHF ultrastructure (21, 22).

We have used fluorescence immunohistochemistry and confocal microscopy to track NFT changes during the course of neurofibrillary degeneration in postmortem brain specimens from AD patients. These changes in NFT microscopic structure can be interpreted as reflecting biochemical changes in PHF demonstrated by means of antibodies against specific phosphorylated and non-phosphorylated tau epitopes.

MATERIALS AND METHODS

Specimens of hippocampus were obtained after death from the brains of ten patients who had satisfied NINCDS-ADRDA...
Fig. 1. Diagram of the amino acid sequences of tau protein showing the relative positions of epitopes recognized by antibodies used in this study.

criteria (29) for definite AD. The brains had been removed 2–
12 hours (h) after death, and specimens of the temporal lobe
were excised and fixed overnight in 5% acetic acid in methanol.
Tissues were embedded in paraffin and sectioned at 15 μm.
Sections were deparaffinized in xylene, hydrated in the conven-
tional manner, and washed in phosphate buffered saline (PBS).
They were treated with 2% nonfat milk in PBS to block non-
specific labeling before being treated (2 h at room temperature)
with a primary antibody diluted 1:10 in PBS. The sections were
washed in PBS and treated (1 h at room temperature) with an
appropriate fluorochrome-labeled secondary antibody. FITC-
conjugated (or Texas Red-conjugated) anti-mouse immuno-
globulin (used with monoclonal primary antibodies) or
FITC-conjugated (or Texas Red-conjugated) anti-rabbit immu-
globulin (used with polyclonal primary antibodies) were
obtained commercially (FITC from Sigma Chemical Co., St.
Louis, MO; Texas Red from Molecular Probes, Eugene, OR).
The sections were washed in PBS, mounted on glass slides in
Citifluor (Ted Pella, Inc., Redding, CA), and stored in the dark
at 4°C until used. Selected sections were incubated for 2–30
minutes (min) at 37°C in Pronase (0.5 mg/ml; Tris-HCl, 50 mM,
pH 7.0) and washed with PBS to stop the reaction, prior to
blocking and incubation with a primary antibody. Pronase
(Pronase E derived from S. griseus, 2.05 units in 0.5 mg/ml,
was obtained from Sigma Chemical Co.). The specificity of
antibodies used was established by demonstrating that their
absorption with tau abolished immunolabeling of tangents and
neurons.

Antibodies

Three polyclonal primary antibodies (BR133, BR135, BR134)
were raised, respectively, against amino acids 1–16, 323–335,
and 428–441 of human tau protein, numbered according to the
longest tau isoform (30). Non-phosphorylated epitopes recog-
nized by these antibodies have been shown to correspond, re-
spectively, to the NH2-terminal, repeat, and COOH-terminal
regions of tau (30). Monoclonal antibody (mAb) 423, which is
not phosphorylation-dependent, was raised against a fraction
isolated from PHF and was shown to recognize tau protein that
is incorporated in the PHF core and truncated at residue 391
(31, 32). SM131 (obtained from Sternberger Monoclonals, Baltimore,
MD) has been shown to recognize a phosphorylation-dependent
site between serine residues 396 and 404 (33). As with SM131,
mAb 11.57 has been shown to recognize hyperphosphorylated
tau protein sensitive to dephosphorylation on immunoblots and
isolated PHF by immune-electron microscopy, but its epitope
has not been mapped. These anti-tau antibodies and the epitopes
they recognize are summarized in Figure 1.

Confocal Microscopy

Sections double-labeled with FITC- and Texas Red-labeled
antibodies were examined with a confocal microscope. The two
fluorescence images, when superimposed, produced green, red
and yellow images corresponding, respectively, to FITC-labeled,
Texas Red-labeled and double-labeled objects, the last being
more or less red depending upon the relative intensity of the
Texas Red label.

Sections were visualized at an initial magnification of 200–
600× with a Nikon epifluorescence microscope equipped with
20/0.75 and 60/1.4 (oil immersion) planapochromatic objective
lenses. Selected microscopic fields were then scanned with an
MRC-600 confocal microscope imaging system (Bio-Rad, Cam-
bridge, MA). The system was equipped with an argon laser and
high sensitivity filters: a blue excitation filter set containing a
488 nm exciter filter; and a green excitation filter set containing
a 514 nm exciter filter. The emission spectra from the excited
fluorophores were passed to photomultipliers, viewed on a mon-
tor and photographed.

RESULTS

Three types of NFT were identified with the antibodies
used in this study. The first and third are well known and
correspond to intracellular tangles and a form of extra-
cellular tangles (sometimes referred to as “ghosts”) in
which the component fibrils are loosely distributed. We
have described both previously in immunoperoxidase
preparations (10).

Type 1 tangles (intracellular) were identified in
immunofluorescent preparations by the tightly packed ar-
rangement of intensely fluorescent fibrils within a pyra-
midal neuron. These NFT are usually associated closely
with autofluorescent lipofuscin granules, a non-fluores-
cent plasma membrane and the outline of an unlabeled,
barely visible nucleus (Fig. 2A). Type 2 tangles had the
appearance of a pack of intensely fluorescent fibrils shaped
like an enlarged neuron without a clearly defined nuclear
outline or plasma membrane (Fig. 2B). The component
fibrils were more loosely packed than in Type 1 tangles,
but more compact than in Type 3 tangles, and appeared
to represent an early stage of the extracellular NFT. Type
3, dispersed, extracellular tangles were identified as less
Fig. 2. Confocal micrographs of NFT in a section of hippocampus (area CA1) pretreated with Pronase (5 min), immunolabeled with mAb 423, and visualized with FITC-labeled immunoglobulin. (A) Type 1, intracellular tangle. (B) Type 2, compact extracellular tangle. (C) Type 3, diffuse extracellular tangle. Arrows indicate location of nucleus.

intensely fluorescent, diffusely distributed packs of fibrils, often suggesting a grossly swollen neuron. They were never associated with a nucleus or lipofuscin granules (Fig. 2C).

Each of these three types of NFT had a characteristic immunoreactivity (Table 1). Type 1 tangles were immunolabeled by BR133, BR134, BR135, SM131, and mAb 11.57. An identical pattern of immunoreactivity was equally characteristic of neurites in both the peripheral domain of senile plaques and in the neuropil. Type 2 tangles, which showed no BR133 immunoreactivity,

TABLE 1

<table>
<thead>
<tr>
<th>Antigens</th>
<th>SM131</th>
<th>BR135</th>
<th>BR133</th>
<th>mAb 11.57</th>
</tr>
</thead>
</table>

NFT types
- Type 1 + + + ++
- Type 2 – + + +*
- Type 3 – – + +

* Positive in other fixation conditions or in methanol/acetic acid-fixed tissues after 2–5 min Pronase pretreatment.

were labeled by BR134, BR135, SM131, and mAb 11.57. Type 3 tangles, which were not labeled by SM131, mAb 11.57 or BR133, were immunolabeled by BR134, BR135 and mAb 423.

By means of double-labeling and confocal microscopy, the three types of NFT could be identified in the same section. With FITC- and Texas Red-tagged secondary antibodies, sites of BR135 (FITC-labeled) and SM131 (Texas Red-labeled) immunoreactivities could be visualized simultaneously (Fig. 3A). Type 1 NFT, immunolabeled with BR135 (FITC-labeled) and SM131 (Texas Red-labeled), appeared yellow. Type 3 NFT, labeled only with BR135, appeared green. Type 2 NFT, labeled with SM131 and BR135, but more intensely with SM131 (presumably because the SM131 epitope is more available), appeared reddish-yellow (Fig. 3A).

Type 2 and Type 3 NFT, although often difficult to differentiate by morphological criteria alone, were readily differentiated in sections double-labeled with BR135.
(FITC-labeled) and SMI31 (Texas Red-labeled). While morphologically similar due to the plane of section, the Type 2 NFT (immunolabeled by SMI31 and BR135) was reddish-yellow; the Type 3 NFT (immunolabeled by BR135) was green (Fig. 3B).

The three types of NFT characterized in situ could be created experimentally by Pronase treatment prior to immunolabeling. After a brief (2–5 min) pretreatment of sections with Pronase, Type 1 NFT were no longer immunolabeled by BR133. It was, therefore, no longer possible to identify Type 1 NFT on the basis of their characteristic immunoreactivity, as Type 1 NFT were immunohistochemically indistinguishable from Type 2 NFT. After 20 min Pronase pretreatment, Type 1 and Type 2 NFT essentially lost SMI31 and mAb 11.57 immunoreactivities. They remained immunoreactive with mAb 423, BR134 and BR135, that is, they retained the immunoreactivity profile characteristic of Type 3 NFT. This Type 3 immunoreactivity persisted through 20 min of Pronase incubation. These effects of Pronase pretreatment on the immunoreactivities of the three types of NFT are summarized in Table 2.

Although all NFT can be immunolabeled with mAb 423 after formalin fixation (34), mAb 423 immunolabeling of Type 1 and Type 2 NFT required brief Pronase pretreatment (2–5 min) under conditions of tissue preparation used here (Table 2). This mAb 423 immunoreactivity typically survived Pronase incubation of 20 min.

**DISCUSSION**

In 1907, Alzheimer (35) described neurofibrillary degeneration as progressing from the appearance of coarsened intracellular fibrils, to the formation of distinct intracellular NFT, to the disintegration of neurons and their replacement by extracellular tangled bundles. We show here that three types of NFT can be immunohistochemically identified: intracellular tangles (Type 1), extracellular tangles with diffusely distributed filaments (Type 3), and an intermediate extracellular type in which the component filaments are more compactly distributed (Type 2). While variables of sectioning sometimes interfered with the identification of these NFT types by histological criteria, the three types could be identified by their characteristic patterns of immunohistochemical reactivity, which were due to the presence or absence of various sequences of the tau molecule.

We have shown previously that intracellular tangles are immunolabeled by BR133 and that the epitope recognized by BR133 is lost in the transformation of intracellular NFT to extracellular NFT (10). We have also shown that extracellular NFT, but not intracellular NFT, are immunolabeled (in methanol-acetic acid-fixed tissues) by mAb 423 (10) and that this specific pattern of immunohistological reactivity appears to reflect the immunoreactivity of isolated PHF as demonstrated in vitro (9, 11).

While recent high resolution electron microscopic studies question the paired helical structure of NFT filaments (6), the filamentous components of NFT are conventionally referred to as PHF. The fine structure of PHF and its relationships in situ with adjacent PHF, cytoplasmic ground substance and extracellular substance are uncertain, but PHF isolated from the brains of AD patients are composed of a Pronase-resistant core and a Pronase-sensitive fuzzy coat. Biochemical analyses indicate that the PHF is composed largely, if not exclusively, of tau protein, and that at least some of this PHF-tau is hyperphosphorylated (21–23, 26, 30, 36–38). It has been shown that only the repeated, microtubule-binding domain of tau is present in the Pronase-resistant PHF core (9, 11, 39, 40) and that an expressed fragment of tau spanning only the repeat region can assemble into PHF-like structures in vitro (38, 41). Electron microscopic immunohistochemical studies have shown that the amino-terminal half of tau, with some contribution from the carboxyl-terminal region, is contained in the fuzzy coat (11). It is, however, not known how the tau protein is distributed in the fuzzy coat.

BR133 recognizes the first 16 amino-terminal residues of human tau which are not phosphorylated (30). Because all immunolabeling with BR133 was abolished by brief pre-incubation of sections with Pronase, it is likely that BR133 decorates epitopes in the superficial, Pronase-sensitive fuzzy coat of PHF (see Fig. 4), which is lost during the transformation of intracellular to extracellular NFT in the course of neurofibrillary degeneration (10). This conclusion is supported by studies demonstrating loss of the NH2-terminus of tau protein from some PHF isolated without the use of Pronase treatment (10).

The failure of mAb 423 to immunolabel Type 2 NFT in paraffin-embedded, methanol-acetic acid-fixed tissues without prior Pronase treatment suggests that the labeling of Type 2 NFT depends upon the exposure, by Pronase.
of immunoreactive sites in the PHF core, that the three types of NFT are labeled by BR135 and mAb 423 in formalin-fixed tissue without Pronase pretreatment suggests that these “deep” sites can also be exposed by certain methods of fixation and tissue preparation. That immunolabeling of Type 2, but not Type 3, NFT requires Pronase pretreatment suggests that the epitope decorated by mAb 423 is more deeply located in Type 2 NFT than in Type 3 NFT. This is consistent with earlier studies showing the epitope recognized by mAb 423 to be in juxtaposition to the repeat region of tau in the Pronase-resistant core of the PHF (32).

Amino acid residues 396 and 404, both of which are phosphorylated, are contained in the epitope recognized by SMI31 (33). We find here that the immunoreactivity of NFT with SMI31 resists up to 10 min of Pronase pretreatment but is largely destroyed by 20 min pretreat-

ment (Table 2). The immunoreactivity of NFT with mAb 11.57 and its resistance to Pronase pretreatment in tissue sections is identical to that of SM131. In addition, the immunoreactivities of isolated PHF with mAb 11.57 and SM131 are identical. Although the epitope recognized by mAb 11.57 is not known, these immunohistochemical findings suggest a close proximity to the epitope recognized by SM131.

BR134, which readily labels Type 1 and Type 2 NFT, also labels Type 3 NFT, especially after Pronase pretreatment. Labeling is not abolished by 20 min pre-incubation in Pronase. Because BR134 recognizes amino acid residues associated with the carboxy-terminus of tau (30), this suggests that the carboxy-terminus may be protected within the PHF core as suggested by immunohistochemical analyses of isolated PHF (42).

The apparent transformation of NFT, from Type 1 to Type 2 to Type 3, suggests that neurofibrillary degeneration proceeds through a sequence of at least three stages, each marked by the presence of an immunohistologically identifiable NFT type. The first stage is characterized by the appearance of Type 1, intracellular, NFT immunolabeled with anti-tau antibodies which recognize the NH2- and COOH-termini and tandem repeat regions of tau. The second and third stages are characterized by the appearance of extracellular tangles that fail to label with antibodies recognizing NH2-terminal tau epitopes in the most superficial part of the PHF fuzzy coat. The immunolabeling characteristics of the second stage, associated with the appearance of Type 2 NFT, also include prominent immunolabeling of epitopes in the deeper regions of the fuzzy coat by SM131 and mAb 11.57. Type 3 NFT, indicative of the third stage of neurofibrillary degeneration, are immunolabeled only by antibodies against the repeat region and COOH-terminus in the PHF core. These relationships are summarized diagrammatically in Figure 4.

Our findings suggest that the immunoreactivity characteristic of Stage 1 is due to the presence of an NH2-terminally intact tau molecule. Our findings are in agreement with those showing that at least some dispersed PHF, isolated without Pronase and immunolabeled by BR133, contain the whole tau molecule (23). These findings indicate that at least some of the tau molecules labeled by BR133 are intrinsic to PHF in intracellular NFT. Because A68 protein, recognized by ALZ50 (23, 43), has been shown to contain the amino-terminus of tau, recent reports that ALZ50 immunolabels only intracellular tangles (18, 43) are consistent with this interpretation.

The loss of BR133 immunolabeling in Stage 2 accompanies loss of the NH2-terminus of tau and is associated with the transformation of intracellular to extracellular tangles (10). The appearance, in Stage 3, of more loosely packed, dispersed extracellular NFT that retain immunoreactivity associated with the tandem repeat (BR135)
and neighboring mAb 423 epitopes, suggests the presence of only the PHF core in these extracellular tangles (Type 3). The recent report of Dickson et al (44) that an antibody against the microtubule-binding domain of tau (AH-1) labels extracellular and not intracellular NFT is consistent with this interpretation. It is congruent, also, with findings of Brion et al (45) who found extracellular (“ghost”) tangles to be labeled only by antisera raised against synthetic sequences in the most carboxy-terminus portions of tau protein. It has been reported previously that extracellular tangles tend to be more intensely labeled by mAb 423 than intracellular tangles and that immunoreactivity is enhanced by trypsin pretreatment (34).

We show here that the stages of neurofibrillary degeneration described originally by Alzheimer and the three stages identified here can be understood as a sequential stripping away of the fuzzy coat of PHF concomitant with the progressive degeneration of neurons. The denuded PHF core that remains extracellularly is comparable to that which remains when isolated PHF are treated with Pronase in vitro. While no PHF core sites of abnormal phosphorylation have been identified, serine 202 (one of the sites of phosphorylation that distinguishes fetal from normal adult tau protein) has recently been identified as a site of abnormal phosphorylation in PHF-tau (46). It is thought to play a role in the process of NFT formation (46). Our finding that serine 396, another site of abnormal phosphorylation in AD (22, 33), is a marker for Type 2 NFT suggests that abnormal phosphorylation may also play a role in neurofibrillary degeneration.

Characterization of the epitopes recognized by antibodies used in this study and antibodies raised against other phosphorylated and non-phosphorylated epitopes of tau protein will be important in defining more precisely the stepwise changes that occur during the course of neurofibrillary degeneration. Our findings suggest that further exploration of the stages of neurofibrillary degeneration by the analysis of changing patterns of immunohistochemical reactivity may elucidate the molecular events underlying neuronal death in AD.

REFERENCES


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