Colocalization of Tau and Alpha-Synuclein Epitopes in Lewy Bodies

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Abstract. The major protein constituent of Lewy bodies (LBs), the pathological hallmark of Parkinson disease and dementia with Lewy bodies, is considered to be α-synuclein, but other proteins, in particular the microtubule-associated protein tau, have been implicated in the pathogenesis of LBs. Tau is the major structural component of neurofibrillary tangles (NFTs). Both direct immunochemical studies of partially purified LBs and indirect immunohistochemical studies have suggested that LBs may contain tau, but most of these studies were based upon a single tau antibody, and immunologic cross-reactivity was not completely excluded. To gain insight into the relation between tau and α-synuclein in LBs, double immunostaining was performed in Lewy body cases with a rabbit polyclonal antibody to α-synuclein and a panel of monoclonal antibodies to phospho- and nonphospho-tau epitopes (Alz50, CP9, CP13, PG5, TG3, PHF1) that spanned the length of the tau molecule. Tau-immunoreactive LBs were present in the medulla in 80% of the cases, irrespective of Braak stage. All tau antibodies recognized at least some LBs, arguing against nonspecific antibody cross-reactivity. In most lesions the tau immunostaining was present at the periphery of the LB. The phospho-tau antibody, TG3, detected more LBs than any of the other tau antibodies. The proportion of LBs with tau immunoreactivity was greatest in neurons vulnerable to NFTs, such as those in the locus ceruleus and basal nucleus of Meynert, and least in neurons resistant to NFTs, such as the dorsal motor nucleus of the vagus in the medulla. The present results suggest that tau may coaggregate with α-synuclein in LBs, especially in neuronal populations vulnerable to both NFTs and LBs.

Key Words: α-synuclein; Lewy body; Neurofibrillary tangle; Tau.

INTRODUCTION

Lewy bodies (LBs) are intraneuronal granulofilamentous inclusions that occur in selectively vulnerable neuronal populations in a wide range of clinical syndromes, including Parkinson disease (PD) and dementia with Lewy bodies (DLB). The discovery of missense mutations in the α-synuclein gene in familial PD fueled research into α-synuclein (1, 2). Most current evidence suggests that α-synuclein, also known as non-Aβ component of plaques (NACP) (3), is the major structural component of LBs (4). Nevertheless, a number of other molecules have been implicated in the pathogenesis of LBs (5). Most of these studies have been based upon immunohistochemistry, with only a few attempts to directly characterize purified LBs (6, 7). The biochemical studies of purified LBs have suggested that neurofilament and the microtubule-associated protein tau may also be components of LBs.

Tau is the major structural component of neurofibrillary tangles (NFTs), fibrillar neuronal inclusions characteristic of neurodegeneration in Alzheimer disease (AD) as well as other neurodegenerative disorders that have been collectively referred to as the “tauopathies” (8). Tau is a mid-molecular weight, soluble microtubule-associated protein whose functional properties vary depending upon post-translational modification, most notably phosphorylation (9, 10). Missense and splice-site mutations in the gene for tau have been identified in frontotemporal dementia and Parkinsonism linked to chromosome 17 (11), and have been confirmed to cause neurodegeneration with NFTs in transgenic mice that express the mutant human protein (12).

Presence of tau in LBs has been suggested by immunohistochemical (13–16) and electron microscopic studies (17, 18). Most of these studies have been based upon a single or only a few tau antibodies and the possibility of cross-reactivity could not be completely excluded. Moreover, it is unclear if there are epitopes in tau that are preferentially detected in LBs. In fact, there is a study that questioned the presence of tau in LBs of the substantia nigra (19). Consequently, the present study was undertaken to determine the frequency of colocalization of tau and α-synuclein in LBs in neuronal populations that are either vulnerable or resistant to NFTs.

MATERIALS AND METHODS

Case Material

Twenty diffuse Lewy body disease (DLBD) cases with a range of concurrent Alzheimer-type pathology, and 4 AD cases were chosen from the neuropathology files at Mayo Clinic Jacksonville for semiquantitative and quantitative analyses. The demographics and clinical and neuropathologic features of the cases are summarized in Table 1. All DLBD and AD cases underwent a standard neuropathologic assessment for LBs (20) and assessment of Alzheimer-type pathology with thioflavin-S
TABLE 1
Clinical and Neuropathologic Features of Cases

<table>
<thead>
<tr>
<th>Case</th>
<th>Clinical Dx</th>
<th>Pathological diagnosis</th>
<th>Age</th>
<th>Gender</th>
<th>Braak stage</th>
<th>TG3-positive LBs (score: 0–2+)</th>
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<td>DLBD/PA</td>
<td>89</td>
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</tr>
<tr>
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<td>PD</td>
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</tr>
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<td>F</td>
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<tr>
<td>24</td>
<td>NA</td>
<td>AD</td>
<td>NA</td>
<td>M</td>
<td>6</td>
<td>N</td>
</tr>
</tbody>
</table>

* Indicates 4 cases used in quantitative analyses; AD = Alzheimer disease; DLB = dementia with Lewy body; DLBD = diffuse Lewy body disease; FTD = frontotemporal dementia; PD = Parkinson disease; PSP = progressive supranuclear palsy; NA = not available.

TABLE 2
Antibody Specifications

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Isotype</th>
<th>Epitope</th>
<th>Dilution</th>
<th>Ref.</th>
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<td>monoclonal</td>
<td>IgG1</td>
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<td>(23)</td>
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<td>IgG1</td>
<td>phospho-serine 202</td>
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<td>(25)</td>
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<tr>
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<td>IgG1</td>
<td>phospho-threonine 231</td>
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<td>IgM</td>
<td>conformation; phospho-threonine 231 and phospho-serine 235</td>
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<td>(24)</td>
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<tr>
<td>PHF1</td>
<td>monoclonal</td>
<td>IgG1</td>
<td>phospho-serines 396 and 404</td>
<td>1:100</td>
<td>(22)</td>
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<tr>
<td>PG5</td>
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<td>IgG3</td>
<td>phospho-serine 409</td>
<td>1:100</td>
<td>(25)</td>
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<tr>
<td>NACP</td>
<td>polyclonal</td>
<td>IgG</td>
<td>α-synuclein</td>
<td>1:1000</td>
<td>(21)</td>
</tr>
</tbody>
</table>

* Indicates 4 cases used in quantitative analyses; AD = Alzheimer disease; DLB = dementia with Lewy body; DLBD = diffuse Lewy body disease; FTD = frontotemporal dementia; PD = Parkinson disease; PSP = progressive supranuclear palsy; NA = not available.
nitro blue tetrazolium (0.15 mg/ml) for 10 min to detect tau. The sections were then washed, dehydrated, and cover slipped.

For immunofluorescent microscopy, deparaffinized and rehydrated sections were incubated in a cocktail of TG3 and NACP after blocking nonspecific antibody binding with normal goat serum, followed by a cocktail of Cy2-conjugated and Cy3-conjugated isotype specific secondary antibodies (Cyanine dyes, 1:100; Jackson Immunoresearch, West Grove, PA) for 2 hours at room temperature. The sections were washed and coverslipped in Aquamount (Lerner Laboratories) and observed with a confocal fluorescent microscope (Fluoview Version 2.0, Olympus).

**Frequency of Tau-Immunoreactive LBs**

For semiquantitative analysis, we scored the number of TG3/NACP double-positive LBs in the medulla of the 20 DLBD cases under a light microscope as follows: 0 = 0; 1+ = a few (<1 and <5); and 2+ = many (5 or more). For quantitative studies of the number and type of double-positive LBs, sections of medulla, LC, and nbM from 4 representative cases that spanned the range of Alzheimer-type pathology were chosen. In the quantitative study, we counted the number of double-positive LBs (also noting their morphologic type) and calculated the ratio of double-positive LBs to single-positive LBs. For this quantitative analysis, images were captured under a microscope (Olympus, BX40) at 100 magnification (0.33 mm²; Matrox Intelicam for Windows Version 2.05, Matrox Electronic Systems, Ltd, Quebec, Canada) in each of the following regions: medulla (30–50 fields); nbM (3 fields); and LC (3 fields). The number of NACP single-positive neurons and tau/NACP double-positive neurons were counted in the images. The ratio of the double-positive neurons to single NACP-positive neurons was calculated.

**Statistical Analysis**

Statistical analyses were performed with SigmaStat 2.03 (SPSS, Inc., Chicago, IL). The correlation between Braak stage and the double-positive LB score in medulla was analyzed with Spearman rank order correlation. The differences between the ratios were examined with 1-way ANOVA or Kruskal-Wallis 1-way ANOVA on ranks. All pair wise multiple comparison procedures (Duncan’s method) were applied when significant differences were observed. A probability value of p < 0.05 was regarded as significant for all analyses.

**RESULTS**

**Morphology of Tau-Positive LBs**

Several patterns of tau immunoreactivity were detected in α-synuclein-immunoreactive LBs. To be included in the analysis, LBs had to be at least 10 μm in diameter and included both brainstem-type LBs (dense hyaline cytoplasmic inclusions) and cortical-type LBs (noncompact cytoplasmic inclusions). Intra-axonal LBs that were at least 10-μm wide and 20-μm long were also counted, while Lewy neurites and small punctate α-synuclein-immunoreactive structures were not included in the analysis. Tau immunoreactivity was detected in the cytoplasm of neurons with LBs, most often in NFTs that were also present in the same neuron, at the periphery of LBs, with α-synuclein immunoreactivity in the core of the LB, or throughout the LB, in both the periphery and the central core, overlapping with α-synuclein immunoreactivity.

Consequently, tau-positive LBs could be classified into 4 types. Type 1 had a “rim” or “peripheral” staining pattern and has been described in previous papers (17, 28) (Fig. 1A–C). The peripheral tau immunoreactivity was granular or diffuse and distinctly different from fibrillar tau immunoreactivity in NFTs. Neurons with this pattern of tau immunoreactivity, in fact, consistently lacked any evidence of NFTs. Type 2 were LBs in neurons with concurrent NFTs and distinct fibrillar tau immunoreactivity (Fig. 1D–F). In some neurons with both NFTs and LBs, tau immunoreactivity was present in both the LBs and the NFTs (Fig. 1D, F), while in others the tau immunoreactivity was limited to the NFTs (Fig. 1E). Type 3 was the least common and characterized by diffuse colocalization of tau and α-synuclein immunoreactivity in both the central and peripheral domains of the LB (Fig. 1G–I). Type 4 was specifically recognized as LBs in neuritic processes, consistent with so-called “intra-neuritic” LBs. In this type of LB, virtually all of the tau immunoreactivity was at the periphery or rim, as in type 1 lesions (Fig. J–L). As in type 1 lesions, the tau immunoreactivity in intra-neuritic LBs was granular or diffuse rather than fibrillar (Fig. 1K, L).

In the quantitative analysis not only the number or tau-positive LBs, but also the type of staining as classified above was recorded. The analysis was conducted on neuronal populations that are vulnerable to NFTs (nbM and LC) and neurons that are resistant to NFTs (medulla). In any given region the staining of various types of LBs was heterogeneous, with some LBs of the various types showing tau immunoreactivity and others showing no evidence of tau colocalization, which added confidence to the specificity of the observed immunoreactivity (Fig. 1M–O). To further confirm the specificity of the immunoreactivity, confocal microscopy with fluorochromes that have nonoverlapping emission spectra (Cy 2 and Cy 3) was performed with TG3, the antibody that labeled the most LBs. The various patterns of immunoreactivity observed with the peroxidase/phosphatase labeling at the light microscopic level were also detected with confocal microscopy (Fig. 2).

**Frequency of Tau-Positive LBs in the Medulla in DLBD**

To determine the frequency of tau-positive LBs, analysis was performed in the medulla, a region of the brain generally resistant to NFTs. The results of the semiquantitative analysis of the 20 DLBD cases are shown in Table 1. Not all DLBD cases had tau-immunoreactive LBs. Of the 16 cases (80%) that had tau-immunoreactive LBs, 6 had only a few and 10 had many. To determine if the severity of concurrent Alzheimer-type pathology might
account for the presence of tau in some LBs a correlation analysis was performed between the TG3/NACP double labeling scores and Braak Stage. There was no significant correlation between the score and Braak stage ($r = 0.372, p > 0.05$). In the 4 AD cases examined there was no α-synuclein-immunoreactivity and only a few scattered tau-immunoreactive NFTs in the medulla.

Frequency of Tau-Positive LBs in Medulla, LC, and nbM with a Panel of Tau Antibodies

Medulla: The average proportion of LBs labeled by the various tau antibodies ranged from less than 4% to more than 35% with the following rank order: TG3 > CP13 > CP9 > PG5 > Alz50 > PHF-1 (Fig. 3a). The proportion of TG3- and CP13-immunoreactive LBs was significantly higher than the other tau antibodies ($p < 0.05$, Duncan’s method). Not only in the medulla, but also in the other areas studied, TG3, an antibody that recognizes a complex epitope in NFTs that depends upon both phosphorylation and a specific conformation of tau (26), labeled the most LBs. Virtually none of the LBs in the medulla were in neurons that had NFTs (see below).

Locus Ceruleus: While the overall proportion of tau-positive LBs increased in the LC for all tau antibodies, the proportion of LBs recognized by the various tau antibodies showed a similar trend (Fig. 3b). Both TG3 and CP13 labeled more LBs than the other tau antibodies; however, TG3 was the only antibody that was significantly greater than the other tau antibodies ($p < 0.05$, Duncan’s method). Some of the increased tau immunoreactivity in LBs was clearly due to the presence of NFTs in some of the neurons with LBs.

Basal Nucleus of Meynert: Similar to the medulla and LC, the average proportion of tau-positive LBs showed the same trend for the tau antibodies. The proportion of positive LBs was about 10% higher in nbM than medulla for all but TG3. No antibody, however, detected significantly more LBs than the others in the nbM ($p > 0.05$, Kruskal-Wallis 1-way ANOVA on ranks). Like the locus ceruleus, some of the tau immunoreactivity was due to NFTs in neurons with LBs.

Frequency of Tau-Positive LBs with Respect to Braak Stage

Given that tau is associated with NFTs and NFTs increase in the LC and nbM with increasing Alzheimer-type pathology, it was of interest to know if the proportion of tau-positive LBs increased in nuclei vulnerable to NFTs with increasing Braak stage. While the overall average proportion of LBs labeled with all the tau antibodies increased in neurons vulnerable to LBs, namely the LC and nbM, there was no apparent increase in the proportion of tau-positive LBs in cases with increasing Braak stage (data not shown).

Proportion of Tau-Positive LBs by Histopathologic Type

To investigate the relationship of tau immunoreactivity of LBs with respect to tau epitope and the morphology of LBs and coincidence of NFTs, we examined the proportion of LBs of each type detected by the panel of tau antibodies. In the medulla, the majority of tau-positive LBs were located within neurites (type 4) and had either a diffuse (type 3), or more often, peripheral/rim pattern of tau immunoreactivity (Fig. 4a). No LBs were found in neurons with NFTs (type 2), which is consistent with the fact that neurons in the medulla are relatively resistant to NFTs. In contrast to the medulla, type 2 immunoreactivity accounted for approximately 20% to 40% of the tau-positive LBs in the LC and nbM (Fig. 4b, c). In other words, a high proportion of LBs with tau immunoreactivity were in neurons that also had fibrillar tau inclusions in neurons that are known to be vulnerable to both LBs and NFTs. Tau-positive intra-neuritic LBs were more frequent in the nbM than the LC. Diffuse overlapping tau and α-synuclein immunoreactivity (type 3) was most common for LBs in the medulla and nbM. The proportion of LBs of various types was nearly the same in the LC.

DISCUSSION

The present study documents tau immunoreactivity in LBs with a panel of tau antibodies recognizing epitopes that span the length of the tau molecule. The proportion of double-labeled LBs, however, varied markedly from one antibody to the next, with the most LBs labeled with some phosho-tau antibodies (e.g. TG3 and CP13) and the least with antibodies to nonphosphorylated epitopes (e.g. Alz50). Moreover, not all DLBD cases had tau-positive LBs, at least in the medulla, a region resistant to NFTs. The number of double-labeled LBs also varied from region to region, with the highest frequency in the nbM and LC (20%–40% tau-positive) and the lowest in the medulla (10%–30% tau-positive). There was morphologic diversity in pattern of double staining. In a minority of LBs the tau epitopes overlapped completely with α-synuclein. More often, tau was detected at the periphery
Fig. 2. Confocal images of LBs immunostained with α-synuclein (Cy 3, red) and tau (TG3) (Cy 2, green); with merged image in far right panel. LBs in nbM (A–C, G–I, J–L) and LC (D–F). Arrows show TG3-immunoreactive LBs, and arrowheads show TG3-negative LBs. Note the mixed population of LBs in all regions with respect to TG3 immunoreactivity. Scale bars: A, D, G = 25 μm; J = 40 μm)
of the LB. In some cases, especially in the LC and nbM, this was merely due to the fact that LBs were present in neurons that also had NFTs. In the medulla, almost no NFTs were detected, and the colocalization of tau with α-synuclein in LBs in the medulla is unlikely to be an artifact due to coincident NFTs. Moreover, the presence of immunoreactivity with a panel of tau antibodies makes antigenic cross-reactivity a very unlikely explanation for presence of tau epitopes in LBs in the medulla.

A number of previous studies have focussed on the occurrence of α-synuclein-immunoreactive lesions (most often resembling cortical-type LBs) in neurons with NFTs. This phenomenon is most common in the amygdala, where α-synuclein has been demonstrated in neurons with NFTs in sporadic AD (15–18, 29), Down syndrome (30), and familial AD (31). Colocalization of α-synuclein in neurons with NFTs most often occurs in the setting of advanced AD, consistent with secondary α-synuclein deposition in neurons with pre-existing NFTs. In a study of α-synuclein in NFTs, Arima et al categorized colocalization of the 2 antigens into 4 types, including a type with complete overlap of tau and α-synuclein (17), but the observed lesions in their study resembled NFTs, not LBs. Similarly, Takeda et al showed that α-synuclein was present in lesions in a range of neurodegenerative tauopathies that were also immunoreactive with tau (32), but these lesions also resembled NFTs, not LBs. In contrast to these studies, the present study is one of only a very few investigations on the role of tau in histologically typical LBs.

One of the first studies to suggest that tau might be a component of some LBs was that of Galloway et al who showed that some LBs in the substantia nigra from DLBD, but not PD, had tau immunoreactivity, with a peripheral pattern of immunostaining (13). More recently, tau has been demonstrated in LBs in a case of familial PD (Contursi kindred) due to mutations in α-synuclein (33). The present study extends these observations to sporadic Lewy body disease.

Since only a minority of LBs were recognized with tau antibodies, it would seem highly unlikely that tau is crucial to the pathogenesis of LBs. Moreover, given that the most common location of the tau epitopes is at the periphery of the LB, it would suggest that tau deposition in LBs is a secondary process. The present findings would suggest the intriguing possibility that α-synuclein might serve as a nidus or seed for tau aggregation. When α-synuclein is detected in NFTs in the amygdala, there are clearly 2 different filament types (tau-immunoreactive paired helical filaments and 10-nm-diameter α-synuclein filaments) (17). It remains to be determined if tau filaments are present in tau-immunoreactive LBs, and immunoelectron microscopic studies are in progress to determine if the tau in LBs is filamentous. The diffuse or granular immunoreactivity observed at the light microscopic level would suggest that tau in LBs might not be fibrillar, analogous to the nonfibrillar abnormal tau immunoreactivity in so-called “pre-tangles” (34, 35).

Another possibility that needs to be considered, especially for phospho-tau antibodies, is that they are actually detecting neurofilament epitopes within LBs. It has been known for years that neurofilament antibodies that stain NFTs cross-react with phospho-epitopes in tau (36). Since neurofilaments are likely to be another constituent of LBs (37) and there are shared phospho-epitopes between neurofilament and tau, it is possible that some of the observed tau immunoreactivity in LBs is due to cross-reactivity with phospho-epitopes in neurofilaments. On
the other hand, not all of the tau antibodies in this study recognized phospho-epitopes. Interestingly, these were also the tau antibodies that recognized the fewest LBs. Thus, even accounting for possible cross-reactivity with phospho-epitopes in neurofilaments, there remains a small proportion of LBs that almost certainly have co-deposition of tau in addition to α-synuclein.

Although it remains to be determined from in vitro studies whether α-synuclein can act as a seed for tau filament formation, it has already been shown that α-synuclein can bind to tau and that this interaction promotes protein kinase A-catalyzed phosphorylation of tau (28). It is of more than passing interest that both tau and α-synuclein are phospho-proteins. Phosphorylation clearly plays a significant role in the normal functional properties of tau and may even be critical to filament formation (9, 10), but the role of phosphorylation of α-synuclein is less clear. There are only a limited number of phosphorylation sites in α-synuclein, but numerous sites in tau (38). Serine 87 and 129 are potential phosphorylation sites and only a few kinases have been implicated in α-synuclein phosphorylation (39, 40). In contrast, many kinases, including cyclin dependent kinase-2, cyclin dependent kinase-5 (Cdk 5), and glycogen synthase kinase-3β have been implicated as tau protein kinases (41–43). Cdk 5 is especially interesting since it has been detected not only in NFTs, but also in LBs (44). The molecular basis for susceptibility of select populations of neurons to both NFTs and LBs is unknown, but the complement of kinases in the vulnerable neurons may play a role in their selective vulnerability. Additional studies are needed to determine the significance of interactions between tau and α-synuclein and molecular processes that converge in the formation of LBs with tau immunoreactivity, as well as the presence of LB-like inclusions in neurons with NFTs.

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