Differential Sensitivity of the Microtubule-associated Protein, Tau, in Alzheimer's Disease Tissue to Formalin Fixation

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Immunohistochemistry of formalin-fixed human Alzheimer's disease (AD) tissue using an anti-tau antibody (Tau-1) reveals staining of neurofibrillary tangles (NFTs) and neuritic plaques (NPs), whereas normal axonal staining is less apparent. In this study, we used a combined biochemical and histochemical approach to assess effects of formalin on immunoreactivity of AD tau. Nitrocellulose blots were treated with fixative to mimic conditions used with tissue sections, a method that might be generally useful for assessing antigen sensitivity to different fixatives. A progressive decrease in Tau-1 immunoreactivity of the tau bands on a Western blot was observed with increasing times of formalin fixation. Phosphatase-digested blots demonstrated an increase in Tau-1 immunoreactivity compared to control blots. These results mimic the phosphatase-sensitive Tau-1 immunohistochemical staining of formalin-fixed AD tissue slices previously reported. Fixation of AD tissue with periodate–lysine–paraformaldehyde (PLP) preserves axonal tau antigenicity. Phosphatase digestion of PLP-fixed AD tissue enhances Tau-1 immunoreactivity of NFTs and NPs but does not alter axonal staining. These results indicate that axonal form(s) of tau are more sensitive to formalin fixation than pathology-associated tau. In addition, a modification of AD tau in pathological structures may protect it from the effects of formalin with regard to Tau-1 antigenicity. (J Histochem Cytochem 36:1117–1121, 1988)

KEY WORDS: Formalin fixation; Microtubule-associated protein; Tau; Alzheimer's disease; Alkaline phosphatase.

Introduction

A modified form of the microtubule-associated protein, tau, is a major component of neurofibrillary tangles (NFTs) and neuritic plaques (NPs) (2,3,10), the pathological structures characterizing Alzheimer's disease (AD) tissue. Tau, or a form of tau, is concentrated in axons (1), whereas NPs and NFTs are found in the neuronal and cell body, respectively, of disease-affected neurons. These immunocytochemical studies attempting to elucidate some of the components of the NFT and NP in Alzheimer's disease tissue were performed on formalin-fixed tissue (2,3,10,11), historically the most commonly employed fixative in neuropathological studies. Axonal immunostaining in AD tissue using an anti-tau antibody is much less intense than NFT or NP staining. One possible explanation of the immunohistochemical results is that white matter tau may be more sensitive to the effects of formalin fixation, whereas the modified form of tau in NFTs and NPs may not be as sensitive. To investigate this difference in tau immunoreactivity between neuronal compartments, we performed a biochemical assessment of the effects of formalin on tau polypeptides enriched from AD tissue. In addition, we compared the effects of formalin fixation of fresh AD tissue with that of PLP (7), a fixative often used to preserve cytoskeletal antigenicity, and performed anti-tau immunohistochemistry.

Materials and Methods

Type VII-L bovine intestinal mucosa alkaline phosphatase, phenylmethylsulfonyl fluoride (PMSF), leupeptin, and 3′,3′-diaminobenzidine (DAB) were purchased from Sigma Chemical Co. (St. Louis, MO). Nitrocellulose paper and electrophoresis grade acrylamide, N,N'-methylene bis-acrylamide, and SDS were purchased from a BioRad (Richmond, CA). All other chemicals were reagent grade. Tau-1 was a gift from Dr. L.I. Binder. The densitometric scan was obtained from a BioRad model 620 video densitometer.

Tau Preparation. Post-mortem tissue was obtained from an 81-year-old patient with pathologically confirmed AD. Frozen tissue was thawed, stripped of the meninges to remove endogenous proteases, and processed for tau. Tau was isolated using a modification of Method 1 from Lindwall and Cole (5). The low-speed pellet was re-homogenized in Buffer A made to 2.5% perchloric acid, for 15 min on ice. The slurry was centrifuged for 20 min at 15,000 rpm to obtain an acid extract. The extract was dialyzed against Buffer A, made of 0.75 M NaCl, 2 mM DTT, and was boiled for 5 min to obtain a heat-stable fraction (HSF). The HSF was treated with 50% NH₄SO₄ for 15 min on ice and was centrifuged at 15,000 rpm. The pellet was then re-suspended in a small volume of Buffer A and dialyzed against the same. Aliquots were frozen at −80°C.

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Electrophoresis. Protein concentrations were determined by the method of Lowry et al. (6). Samples were separated by SDS–PAGE on a 5–12.5% gradient mini-gel and electrophoretically transferred (9) to nitrocellulose paper at 4°C, using 30 V constant voltage for 14 hr.

Formalin Fixation of Blots. Nitrocellulose strips were incubated in 10% phosphate-buffered formalin for 2 days–2 weeks at room temperature, with agitation. The blots were washed with at least two to three changes of Tris-buffered saline (TBS) to remove excess fixative before alkaline phosphatase digestion.

Alkaline Phosphatase Digestion of Tissue Sections and Blots. Alkaline phosphatase digestion was performed as previously reported (10), using type VII-L bovine intestinal mucosa alkaline phosphatase in the presence of 10 μg/ml leupeptin and 2 mM PMSF. Concentrations of 300 μg/ml alkaline phosphatase were used for tissue sections and 75 μg/ml for blots. Digestion times were 2 hr for tissue sections and 5 hr for blots. Alkaline phosphatase was heat-inactivated by boiling for 2 min and was used as a control.

Immunohistochemistry and Immunoblotting. Tissue sections for immunohistochemistry were prepared as previously reported (10). In all experiments, tissue sections or blot strips were processed simultaneously after alkaline phosphatase digestion. Tau-1 was used at a dilution of 1:1000. Both tissue sections and blots were processed using the avidin–biotin base Vectastain kit (Vector Labs, Burlingame, CA) and DAB as the chromogen.

Figure 1. Western blot of the tau polypeptides and their proteolytic fragments, highly enriched from AD tissue, showing decreased Tau-1 immunoreactivity with increasing times of formalin fixation. Each lane was loaded with 35 μg of protein. Lanes B, C, D and E were fixed in 10% neutral-buffered formalin for 4, 12, 24, and 48 hr, respectively. As a control, lane A did not receive any formalin fixation.

Figure 2. Formalin-fixed Western blot of tau isolated from a single case of Alzheimer's disease tissue, showing increase Tau-1 immunoreactivity with alkaline phosphatase digestion (upper panel). Equivalent amounts of protein were loaded in each lane. Nitrocellulose strips, fixed in formalin for 2 weeks, were digested with either heat-inactivated alkaline phosphatase (lane A) or active enzyme (lane B). The lower panel is a histogram showing the differences in peak areas, obtained from a densitometric scan, between lane A and lane B. Scans were normalized to the tracking dye at the gel front (peak in position 124).

Results
Tau antigenicity on immunoblots decreases in a time-dependent manner with increasing exposure to formalin (Figure 1). Not only do the tau polypeptides lose immunoreactivity but so do the immunonegative proteolytic fragments. These fragments are inherent to sample preparations from post-mortem human tissue, despite the presence of protease inhibitors included during the isolation procedure. Although the decrease in tau immunoreactivity indicates that tau is sensitive to the effects of formalin, it does not differentiate between the modified and unmodified forms of tau. Complete biochemical separation of these two forms of tau from AD tissue requires further investigation. We know that a large population of NFTs and NPs are Tau-1 immunoreactive in formalin-fixed tissue after alkaline phosphatase digestion. One biochemical approach to separately visualizing modified tau from unmodified tau is to digest formalin-fixed nitrocellulose strips with alkaline phosphatase and then immunoblot against Tau-1.
The upper panel in Figure 2 shows a formalin-fixed Western blot of tau isolated from a sample of AD tissue. Fixed nitrocellulose strips were digested with either heat-inactivated or active alkaline phosphatase and processed simultaneously using Tau-1. With equivalent protein loadings per lane, there was enhanced immunoreactivity of the tau bands and their proteolytic fragments in the lane treated with active alkaline phosphatase, (lane B) as compared to the heat-killed control (lane A). A densitometric scan of this immunoblot (lower panel), normalized to the tracking dye, shows on average a 100% increase in Tau-1 immunoreactivity of the tau bands in lane B as compared to lane A. These biochemical results mimic the in situ immunohistochemistry previously reported (10), and this approach may be generally useful for differential biochemical detection of modified and unmodified forms of tau in AD tissue.

Our hypothesis is that formalin decreases the antigenicity of the axonal form(s) of tau and that phosphatase treatment unmasks an epitope on the modified form of tau. It is necessary to show that phosphatase treatment does not change the immunoreactivity of the axonal form of tau, but this has been difficult to demonstrate because axonal staining is weak in formalin-fixed tissue. To address this question, we fixed fresh AD tissue in periodate-lysine-paraformaldehyde (PLP) (7), a fixative often used for preservation of cytoskeletal antigenicity. Figure 3 illustrates the difference between fixation procedures for preservation of axonal tau antigenicity. Figures 3A–3D show hippocampal white matter axons cut in cross-section, whereas Figures 3E and 3F show fascia dentata granule cell axons cut longitudinally. White matter immunostaining in PLP-fixed AD tissue sections was prominent (Figures 3A, 3B, and 3E), compared to formalin-fixed AD tissue sections (Figures 3C, 3D, and 3F). In support of our hypothesis, there was no apparent difference in immunostaining intensity of axons after alkaline phosphatase treatment (Figures 3B and 3D) as compared to controls (Figures 3A and 3C).

We examined hippocampal tissue sections from two PLP-fixed (Figures 4A–4D) and one formalin-fixed (Figures 4E and 4F) AD cases for phosphatase-sensitive Tau-1 immunoreactivity of axonal and pathology-associated tau. On the basis of the number of NFTs per field, the severity of the two PLP-fixed cases differs. Despite this difference, in all three cases the number of immunoreactive NFTs and NPs increased with alkaline phosphatase digestion (Figures 4B, 4D, and 4F), compared to heat-inactivated controls (Figures 4A, 4C, and 4E). The background axonal staining in the PLP-fixed tissue (Figures 4A–4D) was more discrete and defined compared to the diffuse background axonal staining in the formalin-fixed tissue (Figures 4E and 4F). In contrast to the immunostaining of
the NFTs and NPs, the axonal staining was not enhanced with alkaline phosphatase digestion. These results further support our previous observation that tau associated with NFTs and NPs is modified, since axonal staining in the same field does not change.

Discussion

Using a variety of monoclonal and polyclonal anti-tau antibodies, several investigators have reported tau antigenicity in the pathological structures of AD tissue (2,3,8,10,11). In all of these studies, it is unclear why normal axonal staining of unaffected neurons has not been prominent when antibodies directed against a protein apparently concentrated in axons were used. In this study, we have used two different fixation methods (formalin and PLP) to show that the lack of strong axonal staining in formalin-fixed AD tissue is due to the fixation method, commonly employed in neuropathology.

Formalin functions as a fixative by binding its reactive aldehyde group to amine groups on the polypeptide backbone and amino acid side chains. The sites of fixation, therefore, are also the potential antigenic sites of the protein. PLP was first described as a fixative directed towards carbohydrate moieties in glycoproteins (7). In theory, PLP functions by periodate-oxidizing the carbohydrates to aldehyde groups and the amine groups of lysine cross-linking the aldehydes. Antigenicity is preserved, therefore, because the glycoprotein is stabilized through the carbohydrate moiety while the protein is presumably unaffected (7). Although tau is not a glycoprotein, it may be that PLP is fixing the protein by interacting with hydroxyl groups of amino acids, such as serine, threonine, and/or tyrosine, leaving the polypeptide backbone relatively unaltered for antibody recognition.

The antigenicity of the microtubule-associated protein, tau, is sensitive to formalin fixation, both on the Western blot and in situ. It should be noted that the lack of strong axonal staining in situ may also be due to endogenous proteolysis or to sensitivity of tau to other steps in the tissue-processing procedure. The decrease in antigenicity of the isolated protein on a Western blot, however, indicates that non-proteolized tau is sensitive to formalin fixation. Tissue sections fixed with PLP show strong axonal staining. With the better preservation of axonal tau antigenicity, the neuroplastic pathology in AD tissue, which is characterized by short, curly, dystrophic neurites, is quite remarkable, and supports the aberrant localization of tau during the disease process (4).

A major problem in understanding the extent of modification of tau in the NFTs and NPs is the difficulty of biochemically separating the modified form of tau from the unmodified form of tau.
in AD tissue. By phosphatase treatment of formalin-fixed nitrocellulose strips containing tau isolated from a case of AD tissue, we were able to enhance Tau-1 immunoreactivity. Since axonal tau immunoreactivity does not change with alkaline phosphatase digestion, we believe that the phosphatase-sensitive immunoreactivity on the Western blot represents the modified form of tau in this AD tissue. It is unclear why these forms of tau are differentially affected by formalin fixation with regard to Tau-1 antigenicity. One possible explanation, however, is that the abnormal phosphorylation of tau may protect the Tau-1 epitope from the effects of formalin. The sensitivity of cytoskeletal antigens to certain fixatives seems to be particularly relevant to interpretation of immunohistochemical studies of human post-mortem tissue. Fixation of nitrocellulose blots may provide a generally useful method for determining antigen sensitivity to different fixatives.

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Literature Cited