Phosphatase inhibition in human neuroblastoma cells alters tau antigenicity and renders it incompetent to associate with exogenous microtubules

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Abstract The abnormal cytoskeletal organization observed in Alzheimer's disease has been suggested to arise from hyperphosphorylation of tau and the resultant elimination of its ability to associate with microtubules. This possibility has been supported by a number of studies under cell-free conditions utilizing various kinases, phosphatases and their corresponding inhibitors each, and by treatment of intact cells with kinase and phosphatase activators and inhibitors. However, in studies utilizing intact cells, it remained difficult to attribute microtubule compromise specifically to tau hyperphosphorylation due to potential influence of inhibitors on tubulin and/or other microtubule-associated proteins, which themselves possess assembly-regulatory phosphorylation sites. To address this difficulty, we subjected SH-SY-5Y human neuroblastoma cells to treatment with the phosphatase inhibitor okadaic acid (OA), which has been previously demonstrated to depolymerize microtubules in these cells. OA induced an increase in tau hyperphosphorylation as evidenced by an increase in Alz-50 immunoreactivity and a corresponding decrease in Tau-1 immunoreactivity. When tau-enriched fractions from OA-treated cells were incubated under microtubule assembly-promoting conditions with twice-cycled, tau-free preparations of bovine brain tubulin not exposed to OA, Alz-50-immunoreactive tau isoforms displayed a marked (49%) reduction in ability to co-assemble with bovine microtubules as compared with Tau-1- and 5E2-immunoreactive isoforms. These data indicate that hyperphosphorylated tau has a reduced capacity to associate with microtubules, and support the hypothesis that tau hyperphosphorylation may underlie microtubule breakdown in Alzheimer's disease.

Key words: Tau; Microtubule; Phosphorylation; Phosphatase; Alzheimer's disease; Paired helical filament

1. Introduction

Essential to the development of any treatment to halt or slow the widespread degeneration of neurons that accompanies Alzheimer's disease (AD) is to determine the progression of events, and in particular, the earliest such detectable events, that highlight 'at-risk' neurons in this disorder. A major pathological feature of affected neurons in AD is the presence of neurofibrillary tangles that are comprised in large part of hyperphosphorylated forms of the microtubule-associated protein tau (for review, see ref. [1]).

Several converging lines of evidence point towards tau having a pivotal role in AD neurofibrillary pathology. A marked increase in total tau content is observed in AD brains, and this increase is apparently due to the accumulation of abnormally phosphorylated forms [2]. Paired helical filaments (PHF) that accumulate in affected neurons in Alzheimer's disease are comprised of hyperphosphorylated tau that exhibits electrophoretic and antigenic properties distinct from that of normal adult CNS tau (for reviews, see refs. [3–5]). While most or all of these individual phosphorylation sites are also readily detectable during development and are detectable in rapidly-processed biopsy material from normal adult brain, these sites are apparently phosphorylated at a higher stochiometric ratio in AD brains (for review, see ref. [6]). PHF-tau exhibits a decreased MT binding ability [7], and dephosphorylation dissociates PHFs and restores MT-assembly promoting properties to tau [8–11].

It remains impossible, however, to confirm at present whether in AD tau is first hyperphosphorylated, which induces its dissociation from MTs, leading in turn to their collapse, or whether MT collapse represents an initiating phenomenon, and newly-dissociated tau subsequently becomes hyperphosphorylated. Consistent with the former possibility is that phosphorylation of tau at critical MT-binding site(s) prevents proper association of tau with MTs [12–20] and may therefore subject tau to AD-like hyperphosphorylation. That tubulin from AD brains remains assembly-competent is also consistent with the former possibility [8]. By contrast, however, the observation of rapid tau hyperphosphorylation following colchicine- induced MT collapse in the present and in a previous study [21] suggests that the latter possibility (i.e. initial MT collapse) could at least partially underlie tau hyperphosphorylation in AD. An additional, plausible hypothesis for the development of these morphological aspects accompanying AD neuropathology is that both phenomena contribute to the full extent of tau hyperphosphorylation and collapse of the MT system as follows: (1) some phosphorylation of tau at MT-binding sites, perhaps as a consequence of kinase hperactivation during ongoing cycles of tau association–dissociation with MTs, effectively withdraws tau molecules from the binding-competent tau pool; (2) a limited degree of MT destabilization ensues, resulting in additional tau liberation; (3) this newly-dissociated tau is blocked by site-specific phosphorylation from further MT association; (4) additional rounds of these phenomena lead to collapse of the MT system; (5) binding-incompetent tau would be subjected to progressive AD-like hyperphosphorylation. In this regard, a recent examination of MT-promoting activities of tau from AD and control brains suggested that hyperphosphorylated, binding-

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incompetent tau may further exacerbate MT breakdown by scavenging normal tau [22].

A number of kinases have been reported to induce normal tau to exhibit PHF-like characteristics [23–37]. In addition to the potential involvement of altered kinase activities in AD neuropathology, protein phosphatase activities are altered in AD, and have been implicated in the accumulation of hyperphosphorylated tau [38]. This possibility has been substantiated by observation that the phosphatase inhibitor, okadaic acid (OA) increases phosphorylation-dependent, AD-like tau immunoreactivity in cultured neurons and neuroblastoma [39–41], brain slices [42] and brain in situ [43,44]. However, OA treatment may induce MT destabilization by several potential mechanisms. These may include interference with dephosphorylation of both tubulin and tau, since both the ability of α-tubulin to assemble into MTs and the ability of tau to promote MT assembly and stabilize MTs are inhibited by site-specific phosphorylation [12–20]. Whether or not OA-induced phosphorylation events actually dissociate tau from MTs, leading to global MT disassembly, or whether tau can undergo assembly-restrictive OA-induced phosphorylation only following dissociation from MTs during normal association–dissociation cycles is not clear. That increased tau phosphorylation accompanies colchicine-induced MT disassembly as seen herein and in a previous study [18] is consistent with the latter possibility. In the latter case, continued 'scavenging' of tau by OA-induced phosphorylation may eventually be expected to destabilize MTs to the point of collapse of the MT network. Moreover, the relative contribution, if any, of OA- induced tubulin hyperphosphorylation to inhibition of MT assembly remains unclear. While the present analyses cannot differentiate among these possibilities, however, the additional observation of lysosomal accumulation following MT destabilization by OA supports the previous hypothesis [8,9,19], that disruptions in the MT system may represent a critical early event leading to AD neuropathology. Moreover, OA-induced interference with normal phosphorylation of other MAPs [39] and/or other cytoskeletal protein such as neurofilaments [45] could contribute to MT destabilization, since MAP-mediated interactions between MTs and neurofilaments mediate axonal stabilization [46].

In initial efforts to address these issues, we examined whether or not OA-treatment of human neuroblastoma cells reduced the capacity of various tau isoforms to associate with exogenous, non-OA-treated MTs.

2. Materials and methods

2.1. Cell culture and treatment

SH-SY-5Y cells (originally obtained from the stocks of Dr. June L. Biedler, Memorial Sloan-Kettering Cancer Center, Rye, New York) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere containing 10% CO_2. Twenty-four hours later, the medium was replaced with medium containing 1 μM okadaic acid (OA) for 48 h. All reagents and chemicals were obtained from Sigma Chem. Co. (St. Louis, MO).

2.2. Immunocytochemistry

Treated cultures and untreated controls were rinsed in Tris-buffered saline (TBS; pH 7.4), fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 15 min at room temperature, and rinsed 3× in TBS. Immunocytochemistry was carried out on fixed cultures using 1:100 dilutions of monoclonal antibody Alz-50 (raised against a neurofibrillary tangle preparation from AD brains [47,48] or monoclonal antibody Tau-1 (which reacts only with tau that has not been phosphorylated at a site that is hyperphosphorylated in AD; obtained from Boehringer-Mannheim, Indianapolis, IN) [49] followed by incubation with the appropriate peroxidase- (Sigma) or alkaline phosphatase-conjugated (Boehringer-Mannheim) secondary antibody and visualization by standard methodologies as described previously [50].

2.3. Enrichment of tau from SH-SY-5Y lysates

Cells were scraped from the plate and homogenized (50 strokes in a glass-Teflon homogenizer) in 1% Triton X-100 in 50 mM Tris-HCl (pH 7.4). Tau-enriched fractions were prepared as described [51]: The Triton-soluble fraction was heated at 95°C for 15 min then the above centrifugation was repeated; the resulting supernatant, containing heat-stable, Triton-soluble proteins, contained virtually all of the tau as confirmed by immunoblot analyses (e.g. Fig. 2).

2.4. Isolation of bovine brain tubulin

Bovine brain was homogenized in PEM buffer (100 mM PIPES (pH 6.6) containing 1 mM EGTA and 1 mM $MgCl_2$) at 4°C and centrifuged at 40,000 × *g* for 30 min. GTP was added to the resulting supernatant at final concentration of 1 mM and the supernatant was incubated for 30 min at 37°C to promote MT assembly, followed by centrifugation at 40,000 × *g* for 30 min. The crude MT pellet was resuspended in PEM buffer at 4°C and this latter suspension and assembly procedure was repeated for a total of 3 cycles. The final pellet of purified MTs was resuspended in 1/10 volume of PEM buffer and stored at −80°C. For dissociation of bovine MAPs from MTs, this preparation was made 0.7 M NaCl in PEM, and centrifuged, first at 40,000 × *g* at 4°C (to sediment any denatured tubulin-containing aggregates) and the resulting supernatant at 40,000 × *g* for 30 min at 37°C; the MAP-free MT pellet was utilized for further analyses [52].

2.5. MT assembly assays

Bovine brain tubulin and heat-stable Triton-soluble fractions from untreated and OA-treated SH-SY-5Y cells were incubated separately and together in an excess of 0.1 M in PEM buffer for 30 min at 30°C in the presence of 10 μM taxol (generous gift of Dr. M. Mercken, McLean Hospital, Belmont MA), then centrifuged at 100,000 × *g* for 15 min at the respective temperature.

2.6. Electrophoresis and immunoblot analyses

Heat-stable Triton-soluble fractions (100 μg of total protein) from OA-treated and untreated cells, bovine brain microtubules (10 μg of total protein), and supernatants and pellets obtained from sedimentation analyses were electrophoresed on SDS 7% polyacrylamide gels and transferred to nitrocellulose using a Hoefer Transphor apparatus as described [50]. The proteins were visualized by sequential reaction of the replica in TBS overnight at room temperature with Tau-1, Alz-50, monoclonal antibody 5E2 (which reacts with tau regardless of phosphorylation state; generous gift of Dr. K. Kosik, Brigham and Women's Hospital, Boston, MA) and with a monoclonal antibody directed against all forms of α-tubulin (Sigma), followed by the appro-

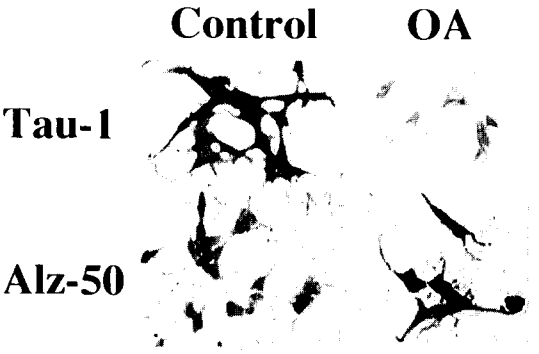

Fig. 1. Okadaic acid increases hyperphosphorylated tau immunoreactivity in SH-SY-5Y neuroblastoma cells. Cells were treated with 1 μM okadaic acid for 0 (Control) or 48 h (OA) then processed for immunocytochemistry with Tau-1 or Alz-50. Note the marked diminution of Tau-1 immunoreactivity and corresponding increase in Alz-50 immunoreactivity following OA treatment.

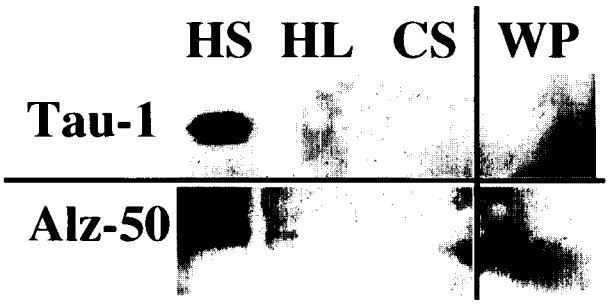

Fig. 2. Distribution of tau during fractionation of SH-SY-5Y cells. Cells were homogenized in the presence of 1% Triton, the Triton-insoluble cytoskeleton (CS) was sedimented, and the resulting Triton-soluble material was heated at 56°C as described in section 2, then centrifuged to yield a (Triton-soluble) heat-labile (HL) pellet and a heat-stable (HS) supernatant. Immunoblot analysis revealed the quantitative recovery of both Tau-1 and Alz-50 immunoreactive tau isoforms in the heat-stable supernatant fraction. This fraction was not assembly-competent in the absence of exogenous tubulin, since incubation of this fraction at 37°C in the presence of 10 μM taxol but in the absence of exogenous tubulin followed by high speed centrifugation did not result in sedimentation (WP) of either Tau-1 or Alz-50 immunoreactive tau isoforms.

priate alkaline phosphatase-conjugated secondary antibody and visualized as described [50]. Immunoblots were subjected to densitometric analysis as described previously [53].

3. Results

As has been demonstrated in other neuronal and neuroblastoma culture systems [39–41], treatment of SH-SY-5Y cells with OA markedly reduced Tau-1 immunoreactivity and increased Alz-50 immunoreactivity (Fig. 1). To enrich for tau, the Triton-soluble fraction from OA-treated cells was subjected to heating at 95°C followed by centrifugation to yield a supernatant containing heat-stable proteins [51]. Immunoblot analyses of fractions derived from OA-treated (Fig. 2) and untreated control cells (not shown) demonstrated, as has been shown for tau derived from brain [51] that both Tau-1- and Alz-50-immunoreactive tau isoforms were quantitatively recovered within the heat-stable cytosolic fraction.

To ascertain the ability of differentially-immunoreactive tau isoforms from OA-treated SH-SY-5Y cells to co-assemble with MTs, this fraction was incubated under MT-assembly-promoting conditions with and without tubulin purified from bovine brain by two assembly/disassembly cycles. The assembly-competence of this tubulin preparation was first demonstrated by sedimentation by high-speed centrifugation following incubation for 30 min in MT assembly buffer at 30°C in the presence of taxol (Fig. 3; Table 1). Immunoblot analyses confirmed the absence of detectable 5E2, Tau-1, Alz-50 immunoreactivity within the bovine brain MT preparation prior to the addition of the tau-enriched SH-SY-5Y fraction (Fig. 3).

When heat-stable Triton-soluble fractions from OA-treated cells were co-incubated with bovine brain tubulin in an excess of MT assembly buffer, significantly more Tau-1-immunoreactive tau was sedimented than Alz-50-immunoreactive tau (Fig. 4; Table 1). As was observed with the MT preparation alone (Fig. 3), significantly more of each tau isoform was sedimented when cellular fractions were co-incubated with the MT preparation at 30°C in the presence of taxol, however, Alz-50-immunoreactive isoforms demonstrated a markedly reduced ability to co-assemble with exogenous MTs as compared to that of Tau-1- or 5E2-immunoreactive isoforms within the same cellular fraction. By contrast, neither Tau-1- and Alz-50-immunoreactive tau isoforms from OA-treated cells were capable of sedimenting when the heat-stable fraction from SH-SY-5Y cells was incubated in an equivalent amount of MT assembly buffer at 30°C in the presence of 10 μM taxol but in the absence of exogenous bovine brain tubulin (Fig. 2). Although the relative amounts of Tau-1 and Alz-50-immunoreactive tau differed substantially between non-OA-treated and OA-treated cells (Fig. 1), Tau-1 and Alz-50-immunoreactive tau isoforms enriched from non-OA-treated cells exhibited identical distribution and MT assembly behavior as those presented herein for OA-treated cells (not shown).

4. Discussion

Compromise of the MT system has been suggested to represent an antecedent event in the development of AD neuropathology which may underlie PHF formation [9,22] and, by disruption of MT-dependent membrane cycling, foster the accumulation of lysosomal hydrolases that accompany the early stages of neurodegeneration in AD [54,55]. It remains impossible to confirm at present whether in AD tau is first hyperphosphorylated, which induces its dissociation from MTs, leading in turn to their collapse, or whether MT collapse represents an initiating phenomenon, and newly-dissociated tau subsequently becomes hyperphosphorylated. Accordingly, experimental alteration in kinase and phosphatase activities in neuronal cell culture systems represent a useful approach towards unraveling antecedent events. In previous studies, phosphatase inhibition by OA treatment has been shown to disrupt neuroblastoma MTs [50,56] and increase hyperphosphorylated tau immunoreactivity in cultured neurons, neuroblastoma [39–41,57], brain slices [39] and brain in situ [40,41]. While tau that has been dissociated from MTs is susceptible to hyper-

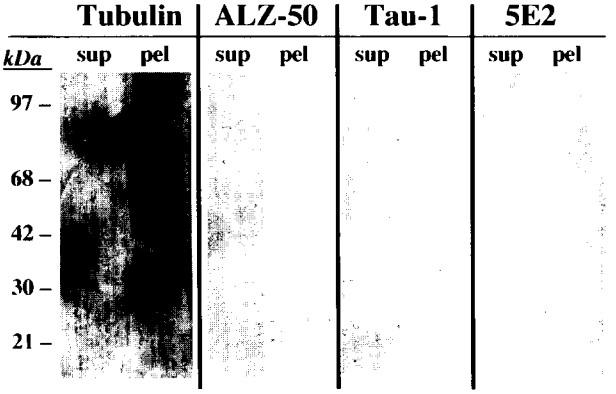

Fig. 3. Assembly of bovine brain tubulin. Tubulin purified from bovine brain by two assembly/disassembly cycles was incubated for 30 min at 4°C in the absence of taxol and at 30°C in the presence of 10 μM taxol, then subjected to high-speed centrifugation and separated from endogenous MAPs as described in section 2. Immunoblot analysis of resulting pellets and supernatants demonstrated MT assembly as evidenced by sedimentation of tubulin immunoreactivity; see Table 1 for quantitation. Immunoblot analyses also confirmed the absence of detectable Alz-50, Tau-1 and 5E2 immunoreactivity within the bovine brain MT preparation prior to the addition of the tau-enriched SH-SY-5Y fraction.

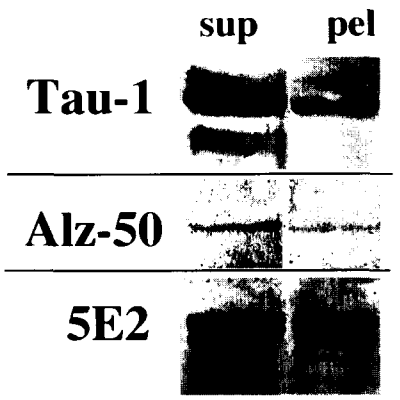

Fig. 4. Alz-50-immunoreactive tau isoforms demonstrate a reduced ability to co-sediment with exogenous microtubules. Heat-stable Triton-soluble fractions from OA-treated cells were co-incubated for 30 min at 30° C in the presence of 10 μM taxol with bovine brain tubulin in an excess of MT assembly buffer and subjected to high-speed centrifugation as described in section 2. Immunoblot analysis demonstrated that significantly more Tau-1- and 5E2-immunoreactive tau was sedimented than Alz-50-immunoreactive tau following incubation with assembling MTs.

phosphorylation, and such hyperphosphorylation will prevent its subsequent association with MTs [1,9,12,16], it remains unclear in such studies whether OA treatment fosters MT destabilization by interference with phosphorylation/dephosphorylation cycles of tau, tubulin or both, since both the ability of α-tubulin to assemble into MTs and the ability of tau to promote MT assembly and stabilize MTs are inhibited by site-specific phosphorylation [12–17]. Moreover, OA-induced interference with phosporylation/dephosphorylation cycles of other MAPs [39] and/or other cytoskeletal protein such as neurofilaments [45] could contribute to MT destabilization, since MAP-mediated interactions between MTs and neurofilaments mediate axonal stabilization [46]. To address this issue, in the present study we monitored the ability of phosphorylation-dependent and nonphosphorylation-dependent immunoreactive tau isoforms derived from OA-treated cells to co-assemble with exogenous (non-OA-treated) assembly-competent MTs.

We observed in the present study that tau isoforms displaying Alz-50 immunoreactivity, indicative of an altered configuration generated by hyperphosphorylation, exhibited a markedly reduced ability to associate with exogenous MTs than did isoforms displaying non-phosphorylated (Tau-1) or total (5E2) immunoreactivity. Despite that there may be overlap between the populations of tau molecules displaying the phospho-dependent ALZ-50 and Tau-1 epitopes, these antibodies apparently represent useful markers of 'hyperphosphorylated' and 'hypophosphorylated' tau, since Alz-50-immunoreactive tau increased, and Tau-1-immunoreactive tau decreased, following inhibition of phosphatase activity. While these results do not eliminate that possibility that the MT system is comprised independently of effects on tau, they confirm that alteration of tau phosphorylation/dephosphorylation cycles within intact cells can render tau incompetent to associate with MTs. The findings of the present study are largely consistent with those of a previous examination of the assembly-competence of tau from untreated SH-SY-5Y cells [58]. In the present study, cells were treated with OA merely to increase the levels of Alz-50-positive tau, which, although present, is relatively low in SH-SY-5Y cels in our hands in the absence of additional treatments; the (low levels of) Alz-50-positive tau and Tau-1-positive tau exhibited the identical abilities to coassemble with exogenous MTs as did tau isoforms enriched from OA-treated cells.

The findings of the present study are consistent with previous hypotheses [9,22,58] that tau hyperphosphorylation contributes to the breakdown of MTs in AD.

Table 1
Alz-50-positive tau has a reduced ability to co-sediment with exogenous microtubules

Antigen	Sample	Relative density*	% Sedimented**
α-Tubulin	Supernatant	1	–
	Pellet	66	99%
Tau-1	Supernatant	109	–
	Pellet	87	44%
Alz-50	Supernatant	31	–
	Pellet	9	23%
5E2	Supernatant	85	–
	Pellet	42	49%

*Calculated as the staining intensity obtained following densitometric analysis using NIH Image software as described in section 2 of the appropriate band minus background of adjacent areas of replicas of similar density; values presented represent the average obtained for two replicas from separate experiments for each antigen.
**Calculated as the (average amount in the pellet/(average amount in the pellet + average amount in the supernatant) × 100 for each condition.

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