ELSEVIER

Neuroscience Letters 255 (1998) 13–16

Neuroscience Letters

Alzheimer-specific epitope of AT100 in transfected cell lines with tau: toward an efficient cell model of tau abnormal phosphorylation

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Received 22 July 1998; received in revised form 21 August 1998; accepted 24 August 1998

Abstract

Intraneuronal aggregation of specific hyperphosphorylated tau isoforms in subsets of neurons may explain many neurodegenerative processes. Only some antibodies including AP422 and AT100 are specific to the abnormal phosphorylation of tau proteins in these processes. AT100-immunoreactivity was never observed in cell models with the exception of Sf9 cells. In the present study, we developed a way to induce AT100-immunoreactivity in different cell types including COS and SY5Y cells after tau cDNA transfection and treatment by okadaic acid. This represents a useful model to study abnormal tau phosphorylation in situ. © 1998 Elsevier Science Ireland Ltd. All rights reserved

Keywords: Alzheimer's disease; COS cells; Phosphorylation; SY5Y neuroblastoma cells; tau protein; Transfection

Hyperphosphorylated microtubule-associated tau proteins are the main components of the aggregated filaments found in neurofibrillary tangles (NFT) in Alzheimer's disease (AD) [3,5]. Human tau primary transcript contains 16 exons. Exons 2, 3 and 10 are adult brain-specific alternatively spliced exons. Exon 3 never appears independently of exon 2. Thus, alternative splicing of these three exons allows six combinations (2-3-10-; 2+3-10-; 2+3+10-; 2-3-10+; 2+3-10+; 2+3+10+) [5]. In the brain, tau proteins constitute a family of six isoforms which range from 352 to 441 amino acids. The tau variants differ from each other by either the presence of three (10-) or four repeat-regions (10+) in the carboxy-terminal part of the molecule and one (2+) or two (2+3+) inserts (29 or 58 amino acids) in the amino-terminal part [5].

In AD, the six tau isoforms are hyperphosphorylated and aggregate into paired helical filaments (PHF). By immunoblotting, PHF-Tau are characterized by a tau triplet Tau 55, 64, 69 and a minor variant at 74 kDa [17]. A number of phosphorylation-dependent monoclonal antibodies have been obtained against phosphorylated Ser/Thr sites found on PHF-Tau. Many phosphorylated sites on these aggregated tau proteins are also found on fetal and native adult tau [11,12]. In fact, there is a rapid endogenous dephosphorylation of tau proteins after death. During postmortem delay, normal tau proteins from autopsy-derived materials are dephosphorylated whereas PHF-tau are not, because of the inaccessibility of phosphorylated sites to phosphatases [11]. Another hypothesis may be a decrease in phosphatase activities in AD brain [6].

Despite the fact that many phosphorylation sites are common to aggregated tau proteins, referred to as PHF-tau in AD, and native tau, there are biochemical variations that differentiate them. First, two-dimensional immunoblot analysis reveals that PHF-tau are more acidic than normal tau from biopsy-derived samples [16]. Second, some phosphorylation sites are Alzheimer specific and can be recognized by a few phosphorylation-dependent monoclonal antibodies such as AT100 [11], AP422 [7], PHF-27 [9] or TG3 [19].

Among these antibodies, AT100 was the first described but it was never used in any cell model whereas TG3 and AP422 have already been used [2,19]. Induction of AT100 epitope in cell models may be of particular interest to under-

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PII S0304-3940(98)00693-4

stand the phosphorylation process in neurodegeneration. The present study is the first description of AT100 appearance in a cell model after transfection.

COS-7 cells were grown in 25 cm^2 flasks in Dulbecco's modified Eagle medium (Life Technologies) with 10% fecal calf serum (Boeringher Mannheim) in a 5% CO_2 incubator at 37°C. cDNA of the six human tau isoforms were cloned in pSG5 vector (Stratagene). They are a kind gift of Dr. Michel Goedert (Cambridge, UK). In COS cells, transfection by the DEAE-dextran method was performed using either the six tau cDNAs or one tau cDNA (2+3-10-). Tau isoform (2+3-10-) is one of the main adult tau variants found in human brain [5,17]. Following 42 h transfection, cells were treated or not by 250 nM okadaic acid (OA) (Sigma), a phosphatase 1 and 2A inhibitor, for 6 h [2,13] in serum-free medium. SY5Y neuroblastoma cells were grown as previously described [2]. For transfection in SY5Y cells, the tau cDNA (2+3-10-) was subcloned into pcDNA3.1 Neo (Invitrogen). Transient transfections were performed using the ethyleneimine polymer, ExGen 500 (Euromedex, France) according to manufacturer's instructions. After 42 h, OA treatment was done as described above. Cells were harvested in Tris–EDTA solution at 4°C and centrifuged. Cell pellets were homogenized in Laemmli sample buffer with 0.25% dithiothreitol and boiled for 10 min.

Phosphorylation-dependent monoclonal antibodies (AD2 and AT100) were used to characterize tau proteins. AD2 is directed against phosphorylated Ser396 and 404 [1]. AT100 binds to a conformational epitope including phosphorylated Thr212 and Ser214 [21]. It was used at a 0.1 μg/ml concentration. M19G is a polyclonal antibody directed against the first 19 amino-acids of the tau sequence encoded by exon 1 [1]. To ascertain the specificity of the antibodies, biopsy-derived materials from control cases and brain homogenates from AD patients, described in previous works [1,16], were also used in the present study.

Electrophoresis and immunoblotting were performed as previously described [1]. Briefly, samples were loaded onto 10% SDS-PAGE (Pharmacia Biotech). Transfer was performed either on nitrocellulose or polyvinylidene difluoride (PVDF) membranes (Amersham). Different blocking protocols were also used: (1) 5% skim milk, (2) 2% bovine serum albumin and (3) 0.5% Tween 20 in 50 mM Tris buffer (pH 8). After transfer and blocking, membranes were incubated with the primary antibody 90 min at room temperature. Horseradish peroxidase-conjugated antibody (Sigma) was used as secondary antibody and reaction product was detected using the Amersham ECL Western blotting system.

Co-transfection of the six tau cDNAs in COS cells followed by OA treatment led to the formation of Tau 55, 64, 69 and 74 whereas tau cDNA (2+3-10-) transfection in COS and SY5Y cells led to the formation after OA treatment of only one tau variant at 64 kDa (Fig. 1).

Using AD2 antibody, the typical tau triplet Tau 55, 64, 69 and the minor variant at 74 kDa were found in AD and biopsy-derived materials from control brains. However, Tau 74 was poorly detected since it resulted from the hyperphosphorylation of the tau isoform (2+3+10+) that is present at very low levels in human brain [17]. Conversely, since tau cDNA was added in similar amounts for all six tau isoforms in COS cells, AD2-immunoreactivity of hyperphosphorylated tau (Tau 55, 64, 69 and 74) was high including that of the 74 kDa tau variant (Fig. 1A). Transfection of the tau isoform 2+3-10- cDNA in COS and SY5Y cells led to the formation after OA treatment of a tau variant co-migrating on SDS-PAGE with the 64 kDa tau species found in AD (Fig. 1A). In SY5Y transfection experiments, a 55 kDa tau variant corresponding to the endogenous fetal tau isoform was also weakly detected (Fig. 1A). This was confirmed using the polyclonal antibody M19G (data not shown).

The same samples were used for immunoblotting using AT100 antibody (Fig. 1B). Using transfer onto nitrocellulose sheets and blocking with skim milk as performed for AD2, no AT100 immunoreactivity was observed with the exception of a poor staining of the AD sample. Strong background was obtained. Another blocking agent, bovine serum

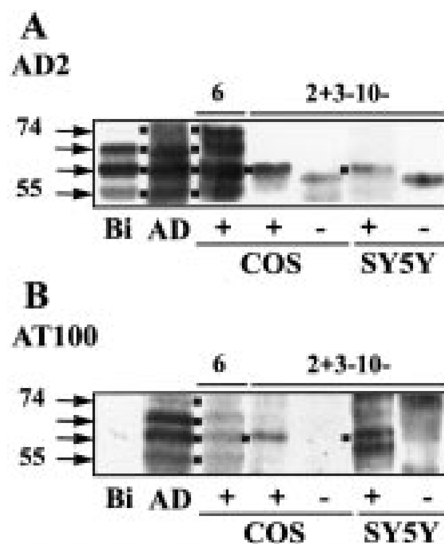

Fig. 1. Immunoblotting with phosphorylation-dependent monoclonal antibodies AD2 (A) and AT100 (B) of total brain homogenates and lysates of COS and SY5Y cells treated (+) or not (–) by okadaic acid and transfected by either all six tau cDNAs (6) or the 2+3-10- tau cDNA. AD2 labels phosphorylated tau proteins in both biopsy-derived material from control cases (Bi) and autopsy-derived materials from AD patient (AD) (A) whereas AT100 only labels hyperphosphorylated tau proteins in AD (B). The six hyperphosphorylated overexpressed tau isoforms in COS cells (6) run on SDS-PAGE as four variants at 55, 64, 69 and 74 kDa and are labeled by AD2 (A) and AT100 (B). Transfection of the 2+3-10- tau cDNA in both cell lines (COS and SY5Y) led after OA treatment (+) to the formation of a 64 kDa tau variant that is labeled by AD2 (A) and AT100 (B). In non-treated cells (–), there is no AT100 labeling. Molecular weight (55, 64, 69 and 74 kDa) are indicated on the left part of each immunoblot (arrows). Black dots indicate the position of labeled hyperphosphorylated tau variants.

albumin, was used and a weak staining was visualized for some samples (data not shown). Finally, AT100-immunoreactivity was clearly obtained when proteins were transferred onto PVDF membranes using Tween 20 as a blocking agent (Fig. 1B). The characteristic tau triplet was observed in AD whereas no staining was visualized in biopsy-derived samples (Fig. 1B). In transfected COS cells treated by OA, the electrophoretic profile of hyperphosphorylated tau proteins were similar (Fig. 1B) to that observed with AD2 labeling (Fig. 1A). However, in SY5Y cells, the low amount of endogenous tau proteins cells was not detected even after OA treatment. Only the hyperphosphorylated overexpressed tau isoform (2+3-10-) was detected in OA-treated transfected SY5Y cells at 64 kDa (Fig. 1B). Furthermore, numerous AT100 cross-reactive bands were also visualized and some show electrophoretic mobility close to that of tau proteins. For instance, a 50 kDa band was detected in OA-treated COS cells samples and also one between 64 and 55 kDa in OA-treated SY5Y cells. Nevertheless, these bands were not related to tau proteins since neither M19G (data not shown), nor AD2 (Fig. 1A) bound to these molecules.

The present study demonstrates at the molecular level that the tau electrophoretic profiles encountered in neurodegenerative disorders may be obtained in cell models. Alzheimer-type tau phosphorylation (i.e. Thr212 and Ser214 phosphorylation) may be reproduced and thus may allow a better understanding of tau phosphorylation in situ.

Tau cDNAs from each of the six tau isoforms were previously transfected into COS cells and in every case, more than one peptide was observed [13]. In the present study, after transfection of all six tau isoforms, a cell treatment by OA was performed and hyperphosphorylated tau isoforms were obtained allowing to replicate the tau electrophoretic profile found in AD. These data are similar to those describing tau phosphorylation by glycogen synthase kinase-3β (GSK3β) [14].

OA, a phosphatase 1 and 2A inhibitor, is a common agent used to induce tau hyperphosphorylation in numerous cell lines including COS and SY5Y [2,13]. OA treatment in SY5Y also leads to microtubule depolymerization [18]. Altogether, these data support the hypothesis that tau hyperphosphorylation may underlie microtubule breakdown in AD [18]. Tau hyperphosphorylation may involve stress-activated protein kinases [4] and GSK3β [14] independently of mitogen-activated protein kinase activation [8]. Since GSK3β and the combination GSK3β followed by protein kinase A (pKA) may phosphorylate tau proteins at AD2 and AT100 epitopes, respectively [14,15,21], we conclude that OA treatment should lead to the appearance of both epitopes. Thus, it is not surprising to visualize their immunoreactivity in the present cell models. However, AT100-immunoreactivity was previously described in transfected Sf9 but never in other cell models [21]. On one hand, lack of specific staining may be related to numerous technical difficulties including AT100 weak affinity, non-specific AT100 binding after OA treatment, types of transfer membranes used and blocking agent. In fact, in the present study, a real improvement of AT100 staining was observed after transfer onto PVDF membranes and use of Tween 20 as blocking agent. On the other hand, OA is usually added to either medium supplemented with fetal calf serum or defined medium containing insulin. In fact, insulin and insulin-like growth factor 1 may activate 3-phosphoinositide-dependent protein kinase that then activates protein kinase B, which in turn inactivates GSK3β [10]. In the present study, OA was added to serum-free medium. Thus, GSK3β was not inactivated. Conversely, when serum (that contains insulin) is present, GSK3β may be temporary inactivated. At the same time, pKA is likely to be activated following OA treatment even in presence of fetal calf serum or insulin [20]. This differential kinase activation may modify the sequential phosphorylation (GSK3β followed by pKA) responsible for appearance of AT100 epitope [21]: pKA may first phosphorylate tau proteins at Ser214 and the resulting conformation may inhibit further GSK3β phosphorylation at Thr212 and thus, AT100 epitope appearance [21]. Such phenomena would explain lack of AT100 staining in a number of cell models.

Appearance of AT100 epitope may be particularly useful to monitor drug screening in cell models in order to visualize abnormal tau phosphorylation. For instance, lithium is of particular interest since it is a good GSK3β inhibitor [15]. In the same way, insulin derivatives are very promising since they have been shown to regulate tau phosphorylation in cultured human neurons [10]. Thus, SY5Y neuroblastoma cell is a relevant cell model to study tau hyperphosphorylation since both AT100 and AP422 epitopes may be induced ([2], and the present study).

In AD, aggregation and hyperphosphorylation of tau isoforms may lead to the degeneration of subsets of neurons containing particular kinases/phosphatases characterized by the appearance of AT100 epitope. AT100-immunoreactivity was obtained in our cell models after tau overexpression suggesting that the phosphorylation responsible for AT100 epitope formation occurs in COS and SY5Y cells. Since transduction signals involved in kinases activation/phosphatases inhibition are much more complex than a single hyperphosphorylation process, such models should allow to better dissect the pathological processes leading to tau aggregation into filaments in neurodegenerative disorders.

The authors thank Dr. Michel Goedert (Cambridge, UK) for tau isoforms cDNAs and Dr. Eugeen Vanmechelen (Innogenetics) for AT100 antibody. AD2 was developed through a collaboration between UMR 9921 CNRS, Sanofi/Diagnostic Pasteur and INSERM. We thank Drs. Jean-Claude D'Halluin and Laetitia Dupont-Wallois for technical advice. This study was supported by CNRS, INSERM and a grant from Conseil Régional Nord-Pas de Calais (Pôle Neurosciences). C.M. is a recipient of a scholarship from the French Research Ministry. T.B. is spon-

sored by a fellowship from both Conseil Régional Nord-Pas de Calais and CHRU-Lille.

[1] Buée-Scherrer, V., Condamines, O., Mourton-Gilles, C., Jakes, R., Goedert, M., Pau, B. and Delacourte, A., AD2, a phosphorylation-dependent monoclonal antibody directed against tau proteins found in Alzheimer's disease, Mol. Brain Res., 39 (1996) 79–88.

[2] Caillet-Boudin, M.L. and Delacourte, A., Phosphorylated Ser422, a specific Alzheimer epitope is expressed by SKNSH-SY5Y cells after okadaic acid, NeuroReport, 8 (1996) 307–310.

[3] Delacourte, A. and Buée, L., Normal and pathological tau proteins as factors for microtubule assembly, Int. Rev. Cytol., 171 (1997) 167–224.

[4] Goedert, M., Hasegawa, M., Jakes, R., Lawler, S., Cuenda, A. and Cohen, P., Phosphorylation of microtubule-associated protein tau by stress-activated protein kinases, FEBS Lett., 409 (1997) 57–62.

[5] Goedert, M., Spillantini, M.G., Jakes, R., Rutherford, D. and Crowther, R.A., Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles, Neuron, 3 (1989) 519–526.

[6] Gong, C.X., Shaikh, S., Wang, J.Z., Zaidi, T., Grundke-Iqbal, I. and Iqbal, K., Phosphatase activity toward abnormally phosphorylated tau: decrease in Alzheimer's disease, J. Neurochem., 65 (1995) 732–738.

[7] Hasegawa, M., Jakes, R., Crowther, R.A., Lee, V.M.Y., Ihara, Y. and Goedert, M., Characterization of mAb AP422, a novel phosphorylation-dependent monoclonal antibody against tau proteins, FEBS Lett., 384 (1996) 25–30.

[8] Ho, D.T., Shayan, H. and Murphy, T.H., Okadaic acid induces hyperphosphorylation of tau independently of mitogen-activated protein kinase activation, J. Neurochem., 68 (1997) 106–111.

[9] Hoffmann, R., Lee, V.M.Y., Leight, S., Varga, I. and Otvos, L. Jr., Unique Alzheimer's disease paired helical filament specific epitopes involve double phosphorylation at specific sites, Biochemistry, 36 (1997) 8114–8124.

[10] Hong, M. and Lee, V.M.Y., Lithium reduces tau phosphorylation by inhibition of glycogen synthase kinase-3, J. Biol. Chem., 272 (1997) 19547–19553.

[11] Matsuo, E.S., Shin, R.W., Billingsley, M.L., Van de Voorde, A., O'Connor, M., Trojanowski, J.Q. and Lee, V.M.Y., Biopsy-derived adult human brain tau is phosphorylated at many of the same sites as Alzheimer's disease paired helical filament tau, Neuron, 13 (1994) 989–1002.

[12] Mawal-Dewan, M., Henley, J., Van de Voorde, A., Trojanowski, J.Q. and Lee, V.M.Y., The phosphorylation state of tau in the developing rat brain is regulated by phosphoprotein phosphatases, J. Biol. Chem., 269 (1994) 30981–30987.

[13] Medina, M., Montejo de Garcini, E. and Avila, J., The role of tau phosphorylation in transfected COS-1 cells, Mol. Cell Biochem., 148 (1995) 79–88.

[14] Mulot, S.F.C., Hughes, K., Woodgett, J.R., Anderton, B.H. and Hanger, D.P., PHF-Tau from Alzheimer's brain comprises four species on SDS-PAGE which can be mimicked by an in vitro phosphorylation of human brain tau by glycogen synthase kinase-3β, FEBS Lett., 349 (1994) 359–364.

[15] Munoz-Montano, J.R., Moreno, F.J., Avila, J. and Diaz-Nido, J., Lithium inhibits Alzheimer's disease-like tau protein phosphorylation in neurons, FEBS Lett., 411 (1997) 183–188.

[16] Sergeant, N., Bussière, T., Vermersch, P., Lejeune, J.P. and Delacourte, A., Isoelectric point differentiates PHF-Tau from biopsy-derived human brain Tau proteins, NeuroReport, 6 (1995) 2217–2220.

[17] Sergeant, N., David, J.P., Goedert, M., Jakes, R., Vermersch, P., Buée, L., Lefranc, D., Wattez, A. and Delacourte, A., Two-dimensional characterization of paired helical filament-Tau from Alzheimer's disease: demonstration of an additional 74 kDa component and age-related biochemical modifications, J. Neurochem., 69 (1997) 834–844.

[18] Shea, T.B. and Fisher, I., Phosphatase inhibition in human neuroblastoma cells alters tau antigenicity and renders it incompetent to associate with exogenous microtubules, FEBS Lett., 380 (1996) 63–67.

[19] Vincent, I., Rosado, M. and Davies, P., Mitotic mechanisms in Alzheimer's disease?, J. Cell Biol., 132 (1996) 413–425.

[20] Wadzinski, B.E., Wheat, W.H., Jaspers, S., Peruski, L.F. Jr., Lickteig, R.L., Johnson, G.L. and Klemm, D.J., Nuclear protein phosphatase 2A dephosphorylates protein kinase A-phosphorylated CREB and regulates CREB transcriptional stimulation, Mol. Cell Biol., 13 (1993) 2822–2834.

[21] Zheng-Fischhöfer, Q., Biernat, J., Mandelkow, E.M., Illenberger, S., Godemann, R. and Mandelkow, E., Sequential phosphorylation of Tau by glycogen synthase kinase-3β and protein kinase A at Thr212 and Ser214 generates the Alzheimer-specific epitope of antibody AT100 and requires a paired-helical-filament-like conformation, Eur. J. Biochem., 252 (1998) 542–552.