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Induction of a specific tau Alzheimer epitope in SY-5Y neuroblastoma cells

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HYPERPHOSPHORYLATION of the microtubule-associated tau proteins is one of the main pathological events that leads to neurofibrillary neurodegeneration in Alzheimer's disease. A similar tau phosphorylation pattern may be obtained in SY-5Y neuroblastoma cells after okadaic acid treatment. In this paper, we clearly demonstrate phosphorylation of Ser422 in tau proteins of treated cells as well as in Alzheimer brain homogenates. By contrast, Ser422 was not phosphorylated on native tau proteins from non-treated cells or rapidly processed biopsies. These results confirm that this cell model is still relevant to study neurofibrillary neurodegeneration of the Alzheimer type.

Key words: Alzheimer; AP 422 serum; Hyperphosphorylation; Okadaic acid; SKNSH-SY 5Y cells; Tau protein

Introduction

Pathological tau proteins, also named PHF-tau, are the major components of paired helical filaments (PHF) found in degenerating neurones in brains from patients with Alzheimer's disease.[1–4] For a long time, these proteins were thought to be hyperphosphorylated when compared with tau proteins in normal adult human brain. In fact, native tau proteins found in rapidly processed samples from adult human brain biopsies are more phosphorylated than previously demonstrated and are phosphorylated *in vivo*, but to a lesser extent, on sites that are similar to those found in PHF-tau proteins.[5,6] Common epitopes were thus found in native tau proteins, fetal tau proteins and tau-PHF proteins.[5–9] Other sites on tau proteins are specifically phosphorylated during Alzheimer's disease.[5,10]

We recently described a cellular model in which a shift from fetal-type to Alzheimer-type phosphorylation of tau proteins was induced by okadaic acid (OA) treatment. Indeed, SKNSH-SY 5Y human neuroblastoma cells mainly synthesize the fetal-type tau isoform which is phosphorylated like the fetal brain tau.[11,12] Hyperphosphorylation of these proteins can be induced by adding OA, a potent inhibitor of phosphatase 1 and 2A proteins, to culture medium.[13] This hyperphosphorylation state generates an increase of the apparent mol. wt, acidification of the isoelectric point and modifications in tau detection by phosphorylation-dependent antibodies.[14,15] Tau-1, an antibody which binds to the 192–204 region of unphosphorylated tau proteins,[16] detected cellular tau proteins only before treatment." AT8 and AD2 antibodies, which specifically recognize respectively phosphorylated Ser202–Ser205 and phosphorylated Ser396–Ser404,[17,18] immunolabelled cellular tau before and after treatment. However, small increases in immunodetection by AT8 and AD2 antibodies were observed after OA treatment.[11] This result agrees with the fact that AT8 and AD2 antibodies recognize both rapidly processed adult biopsy and fetal tau proteins.[5,18] Therefore, in this model, tau-1 can be considered as a specific negative marker of Alzheimer-type phosphorylation because the disappearance of the tau-1 epitope is characteristic of the PHF-tau molecules. As a counterpart, however, positive binding to PHF-tau was lacking to validate our cell model.

Recently, polyclonal and monoclonal AP422 antibodies have been described as the most specific anti-tau antibodies available for discriminating PHF-tau from fetal or biopsy-derived tau proteins.[10,19] In this paper, using the polyclonal AP422 serum, we describe the immunochemical analyses of tau proteins from biopsies and cells treated with OA or untreated.

Materials and Methods

Cell culture: SKNSH-SY 5Y cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Boehringer, Mannheim) supplemented with 10% fetal calf serum. For cell differentiation, cells were grown in a serum-free medium containing 10 ng ml^{-1} nerve growth factor (NGF; Sigma, St Louis, MO) as described in Ref. 15. Cells exposed to NGF for 4 days were usually treated with 0.25 μM OA for 6 h.

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Cell and Alzheimer brain extracts: Cells were harvested and centrifuged, and the cell pellet was directly suspended in Laemmli buffer 1:10 (wt/vol) with dithiothreitol 0.25% and heat treated.

Brain tissue was obtained from three subjects (age 55, 64 and 80 years) with confirmed neuropathological (Thioflavin S, immunolabelling by anti-tau and anti-amyloid Aβ) and biochemical (Aβ and tau immunoblotting) evidence of Alzheimer's disease. Diagnosis of Alzheimer's disease was established according to the NINCDS-ADRDA criteria. Postmortem delays were between 8 and 24 h.

Biopsies were obtained in three patients aged 30–55 years who underwent frontal or temporal lobectomy for brain tumour resection. The samples were resected from the normal brain region close to the tumour, as described in Sergeant *et al.*[6] Immediately after excision, samples were rinsed in cold physiological serum (4°C) and snap frozen in liquid nitrogen.

Both normal biopsy and Alzheimer autopsy samples were homogenized in Laemmli sample buffer and heat-treated, as described by Delacourte *et al.*[2]

The autopsy cases were obtained according to French Caillavet Law No. 76-1181 (December 22, 1976) and in accordance with the ethics committee of the CH&U de Lille (October 19, 1994).

Polyacrylamide gel electrophoresis and Western blotting: Proteins, extracted from 5×10^5cells, were resolved on 10% SDD–PAGE gel. Proteins were electrophoretically transferred. The samples were laid onto a 10% polyacrylamide gel, in a large well. After transfer onto nitrocellulose membranes (Schleicher & Schuell) using a semi-dry blotting device (Pharmacia), each lane was cut into three pieces to allow simultaneous detection (and then a comparison) of the same sample with three different antibodies.

Polyclonal amino-terminal tau serum M19G (against the first 19 amino acids of the N terminus of the tau molecule) was prepared and characterized in our laboratory.[15] On immunoblots, this serum detects all the tau variants from control and Alzheimer brains. Tau-1 was purchased from Boehringer Mannheim and used at a dilution of 1/1000. Tau-1 is a well-characterized monoclonal antibody which recognizes an unphosphorylated site on normal tau proteins of control brains.[16] AD2, a monoclonal antibody which binds to phosphorylated tau Ser396 and 404, was used at a dilution of 1/10 000.[18] AP422 was described by Moroshima-Kawashima *et al.*[10] and diluted to 1/500. The anti-rabbit or anti-mouse conjugated with peroxidase (Diagnostic Pasteur) were used as second antibodies at a dilution of 1/2000. Visualization was performed using the ECL (enhanced chemiluminescence) detection kit from Amersham.

Results

Specific detection of Alzheimer tau proteins by AP422 serum: Brain homogenates from biopsy samples were analysed by Western blotting. Only faint bands were detected with AP422 antibody but the pattern was quite different from the tau triplet, characteristic of biopsy-obtained tau or PHF-tau. This tau triplet was effectively visualized with M19G and AD2 antibodies (Fig. 1).

In brain homogenates from AD patients, all three antibodies studied here (AD2, M19G and AP422) detected the same pathological PHF-tau triplet (Fig. 1).

Epitope analysis of cellular tau proteins: Normal tau proteins of SKNSH-SY 5Y cells were analysed with the same three antibodies. When cells were not treated with OA, M19G and AD2 antibodies bound to cellular tau proteins but AP422 failed to do so (Fig. 2). Tau proteins which were hyperphosphorylated as a result of OA treatment were detected with the three antibodies (Fig. 2). Appearance of the AP422 epitope corresponded with the phosphorylation of the corresponding site.

Phosphorylation of the AP422 site resulting from OA treatment was independent of the cell differentiation because the same results were obtained with non-differentiated or NGF differentiated cells (not shown).

Discussion

In the present work, using polyclonal M19G and monoclonal AD2 antibodies, we confirmed the presence of a tau triplet in rapidly processed biopsy homogenates, as previously described in Refs 5 and 6. This agrees with a phosphorylated state of native tau protein. Conversely, in the same biopsy samples, polyclonal AP422 serum faintly detected some bands which migrated in the tau triplet region but did not correspond to the triplet pattern (Fig. 1). The faint staining cannot be explained by a low antibody titre since PHF-tau proteins in Alzheimer homogenates were labelled equally strongly by mAb AP422 and AD2 (Fig. 1). Thus, the AP422 site on normal tau proteins were only slightly phosphorylated compared with the other sites such as AD2. Such comparison of electrophoretic patterns was not performed in Ref. 19 probably because of too weak a detection in biopsy samples. Then, the near absence of tau detection by AP422 serum can be considered as specific for native tau proteins.

By contrast, the AP422 serum detected the tau-PHF triplet from Alzheimer homogenates as effectively as the M19G and AD2 antibodies. The

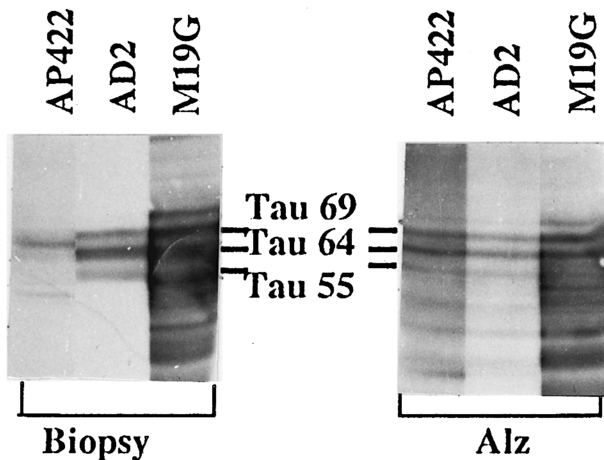

FIG. 1. Immunodetection of tau proteins from homogenates of normal brain obtained at biopsy or at autopsy of patients with Alzheimer's disease by dependent- (AP422 and AD2) or independent-phosphorylation (M19G) tau protein antibodies. In Alzheimer samples, the PHF-tau triplet was revealed by the three antibodies while, in biopsy-derived samples, only AD2 and M19G. The PHF-tau bands (Tau 55, Tau 64, Tau 69) are indicated by bars. The faint bands detected with AP422 serum in biopsy samples did not correspond to this PHF-tau triplet.

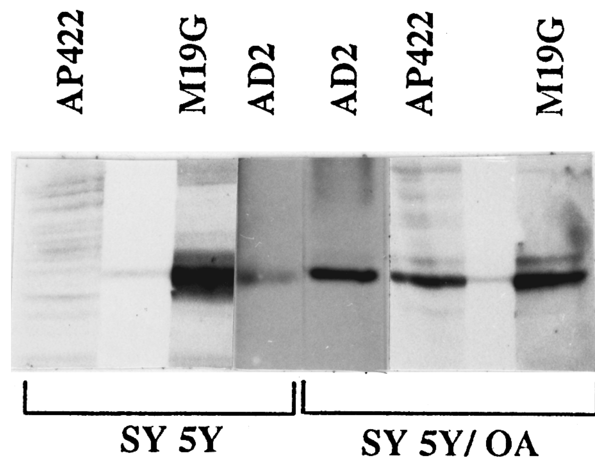

FIG. 2. Immunodetection of tau proteins from normal or OA-treated SKNSH-SY 5Y cell lysates by dependent (AP422 and AD2) or independent (M19G) tau protein antibodies. Note that the AP422 epitope is only present on OA-modified tau proteins.

three bands revealed by these antibodies co-migrate exactly (Fig. 1).

These experiments were performed on samples from three different biopsy and Alzheimer autopsy-derived homogenates (see Materials and Methods) and the AP422 serum was always reactive with PHF-tau triplet from Alzheimer autopsy homogenates and not with tau from biopsy-derived samples. Thus, the positive immunodetection of PHF-tau triplet by AP422 serum seems to be specific for Alzheimer pathology rather than the ageing process. This result agrees with Hasegawa's report[19] and supports the pathological phosphorylation of tau Ser422 during Alzheimer's disease.

Thus, only three antibodies are specific for either normal tau or PHF-tau. Tau-1 binds tau proteins in normal biopsy[5] or normal autopsy tissue[4,20] but does not bind to PHF-tau except when they are dephosphorylated.[4,20] AT100 and AP422 antibodies (Refs 5 and 19, and present study) are reported as being specific for PHF-tau rather than tau in biopsies. Other antibodies reported to be specific for PHF-tau do not depend on phosphorylation but are more specific for a particular conformation, such as Alz 50[21] and tau-2.[22]

We have tested the immunoreactivity of SKNSH-SY 5Y cell tau proteins with AP422 serum. The AP422 epitope was not detected on non-treated cell tau proteins, although these cellular proteins were phosphorylated at other sites, as demonstrated by the AD2 blotting (Fig. 2) and reported in Refs 11 and 12. Thus, the phosphorylation state of normal cellular tau proteins seemed similar to that observed in normal biopsies. However, the OA treatment of the cells was able to induce the appearance of the AP 422 epitope on tau proteins. The presence of the AP422 epitope on OA-modified tau proteins proved that the induced hyperphosphorylation described in previous reports[11,15] occurred at specific sites recovered in pathological proteins. The hyperphosphorylation induced by OA treatment corresponded not only to an amplification of phosphorylation processing on sites normally present on both normal cellular and biopsy-derived tau but also to the appearance of pathological phosphorylated sites detected on Alzheimer PHF-tau.

Conclusion

This paper demonstrates that tau phosphorylated Ser422 is not present on native cellular or biopsy-derived tau proteins. Cell treatment by OA not only amplified the phosphorylation of normal sites of tau proteins but also induced Ser422 phosphorylation in tau-PHF. Both an increase in the degree of phosphorylation on normal sites and appearance of new sites are characteristic of Alzheimer PHF.[5,10] Thus, this cell model is likely to be relevant for the study of Alzheimer-type neurodegeneration.

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