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Ectopic localization of phosphorylated histone H3 in Alzheimer's disease: a mitotic catastrophe?

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Abstract Despite their terminally differentiated status, vulnerable neurons in Alzheimer's disease (AD) display evidence of cell cycle activation, suggesting that mitotic dysfunction may be important in disease pathogenesis. To further delineate the role of mitotic processes in disease pathogenesis, we investigated phosphorylated histone H3, a key component involved in chromosome compaction during cell division. Consistent with an activation of the mitotic machinery, we found an increase in phosphorylated histone H3 in hippocampal neurons in AD. However, rather than within the nucleus as in actively dividing cells, activated phosphorylated histone H3 in AD is restricted to the neuronal cytoplasm despite activation of the mitotic machinery. Therefore, the aberrant cytoplasmic localization of phosphorylated histone H3 indicates a mitotic catastrophe that leads to neuronal dysfunction and neurodegeneration in AD.

Keywords Alzheimer's disease · Cell cycle · Phosphorylation · Histone H3 · Mitosis

Introduction

Alzheimer's disease (AD), the leading cause of senile dementia, is characterized by the degeneration of select neuronal populations in the hippocampus and other cortical brain regions (reviewed in [15]). Although the mechanism

of neuronal injury is not completely understood, accumulating evidence indicates that inappropriate cell cycle activation in neurons precedes neuronal death in AD (reviewed in [12, 19]). In this regard, neurons vulnerable to degeneration in AD display ectopic expression of cyclins along with their cognate cell cycle-dependent kinases and cyclin-dependent kinase inhibitors, mitogenic signal transduction pathway components, mitosis-related proteins, and increased transcriptional activation [8, 9, 10, 11]. This neuronal re-entry into the cell division cycle appears to be mediated by external growth stimuli that lead to the sequential activation of proliferative signal transduction cascades [4, 9, 11, 25]. Therefore, AD, like cancer, is a disease characterized by alterations in the cell cycle machinery [13, 23]. However, unlike cancer, there is little compelling evidence for completion of cell division in the neurons of AD patients [2].

Phosphorylation of histone H3 (H3), one of the core histones of the nucleosome, is a key modification in chromosome compaction during cell division [21]. In the nucleus, the highly compact nature of the chromatin fiber severely restricts the interactions of specific proteins with genomic DNA and affects important cellular process such as transcription and chromosome assembly during mitosis and meiosis. The chromatin fiber is composed of repetitive units, known as nucleosomes, which are comprised of an octamer of core histones (two H2A-H2B dimers and one H3-H4 tetramer), around which 146 base pairs of DNA are wrapped. Critical to nucleosome structure, and therefore competent cell division, are histone-histone and histone-DNA interactions, mediated by conserved C-terminal domains present in core histones [6]. Each histone contains a more flexible N-terminal histone tail domain, which is dispensable for the nucleosome formation. These N-terminal domains protrude from the surface of the nucleosome and help to stabilize chromatin fiber [17], and are subjected to multiple post-translational modifications such as acetylation and phosphorylation [22]. Recently, an extremely specific and high-affinity polyclonal antibody was generated against an H3 peptide, phosphorylated at Ser 10 [3, 20]. Thanks to this very valuable tool, it was demon-

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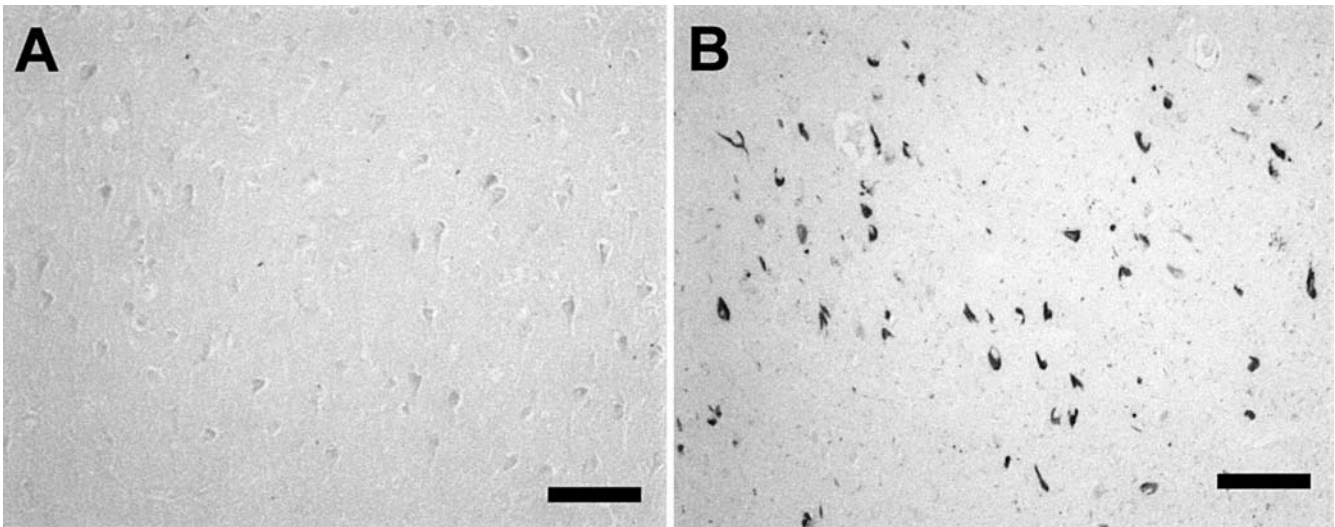


Fig. 1 Immunohistochemical labeling of phosphorylated H3 in age-matched control (**A**) and AD (**B**). Immunoreactivity of phosphorylated H3 in the cytoplasm of pyramidal neurons in the hippocampus in AD (**B**) is increased in comparison to similar neurons in age-matched control brain (**A**) (AD Alzheimer's disease). Bars **A**, **B** 100 μ m

strated that in mitosis in organisms as divergent as *Tetrahymena thermophila*, *Aspergillus nidulans*, *Caenorhabditis elegans*, plants and vertebrates, chromatin condensation is accompanied by phosphorylation of H3 on Ser 10 [18, 20, 21]. In this study, to further delineate the role of mitotic processes in the pathogenesis of AD, we investigated phosphorylated H3 (Ser 10) in the brains of individuals affected by AD and age-matched control cases.

Materials and methods

Tissue

Hippocampal tissues from patients with AD ($n=17$, ages 69–96 years), along with age-matched controls ($n=9$, ages 71–93 years) with similar postmortem intervals (AD, 3–24 h; controls, 6–36 h), were fixed in methacarn (methanol:chloroform:acetic acid; 60:30:10) at 4°C overnight. Following fixation, tissue was dehydrated through ascending ethanol, embedded in paraffin, and 6- μ m sections placed on silane-coated slides. All AD cases met CERAD criteria [7] for definite AD corresponding to Braak stages V–VI. Control cases were also assigned by CERAD and Braak criteria and, in some cases, showed age-related neurofibrillary pathology identified with mouse monoclonal AT-8 antibody to phosphorylated cytoskeletal tau protein [24]. Control cases otherwise showed no significant neuropathological changes.

Immunohistochemistry

Tissue sections were deparaffinized in xylene, hydrated through descending ethanol and endogenous peroxidase activity was abolished with a 30-min incubation in 3% H_2O_2 in methanol. Nonspecific binding sites on the tissue sections were blocked by a 30-min incubation in 10% normal goat serum. Tissue sections were immunostained with rabbit polyclonal antibody to phosphorylated H3 (Ser 10) (Upstate Biotechnology, Lake Placid, N.Y.; 1:200) followed by the peroxidase-anti-peroxidase (PAP) method with 3, 3'-diamino-

benzidine (DAB) as co-substrate. Adjacent sections were immunostained with AT-8 to identify the location of pathological structures. For some experiments sections were double-labeled using our two antibodies. In this case, phosphorylated H3 was localized using the PAP method with DAB as a chromogen, and phosphorylated tau was localized using the alkaline phosphatase anti-alkaline phosphatase method using Fast Blue as a chromogen [24]. Control experiments, including omission of primary antisera and absorption with antigen of the primary antibody, were performed to verify the specificity of antibody binding. For the latter, the immunostaining protocol was repeated, except using absorbed antibody produced by incubation of primary antibody with purified phosphorylated H3 peptide (100 μ g/ml) overnight at 4°C.

Immunoblotting

Gray matter tissue from temporal cortex taken from AD ($n=3$) and control ($n=3$) cases were homogenated in 10 volumes of lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol), and then acid solution protein extraction was carried out as described by the protocol of Upstate Biotechnology (<http://www.upstatebiotech.com>). Briefly, H_2SO_4 was added to a final concentration of 0.2 M (0.4 N) and left for 60 min on ice. Supernatant fractions were transferred to fresh microcentrifuge tubes after centrifugation at 14,000 rpm/10 min and precipitated on ice for 45 min with 50% trichloroacetic acid to a final concentration of 20% trichloroacetic acid. These tubes were centrifuged at 14,000 rpm/10 min at 4°C, and the pellets were washed once with acidic acetone and once with acetone, respectively. Acid-soluble proteins (10 μ g) were loaded and separated by polyacrylamide gel electrophoresis (PAGE) and transferred onto Immobilon-P (Millipore, Bedford, Mass.). Blots were then incubated sequentially with blocking agent (10% nonfat milk in TBS-Tween) and probed with rabbit polyclonal antibody to phosphorylated H3 (Ser 10) (Upstate Biotechnology) and followed by affinity-purified goat anti-rabbit immunoglobulin peroxidase conjugate pre-adsorbed to eliminate human cross-reactivity and developed using ECL (Santa Cruz Biotechnology Inc., Santa Cruz, Calif.) according to the manufacturer's instruction.

Results

Immunoreactivity of phosphorylated H3 (Ser 10) was present in the cytoplasm of neurons in the hippocampus of AD cases, and was significantly increased in comparison with control (Fig. 1A, B). The highest levels of phosphor-

Fig. 2 Double-immunostaining of pyramidal neurons in the hippocampus of an AD patient with AT-8 and phosphorylated H3. *Inset* shows a higher magnification of the area marked by an *asterisk*. Most neurofibrillary tangles in AD contain both AT-8 and phosphorylated H3 (*arrows*). *Bar* 100 μ m

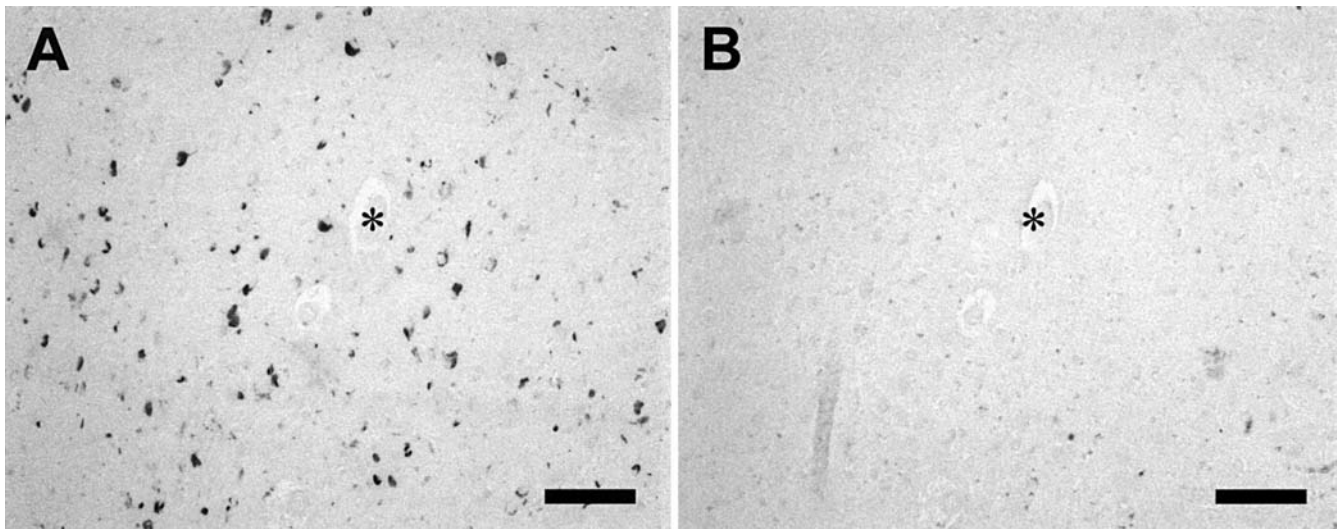
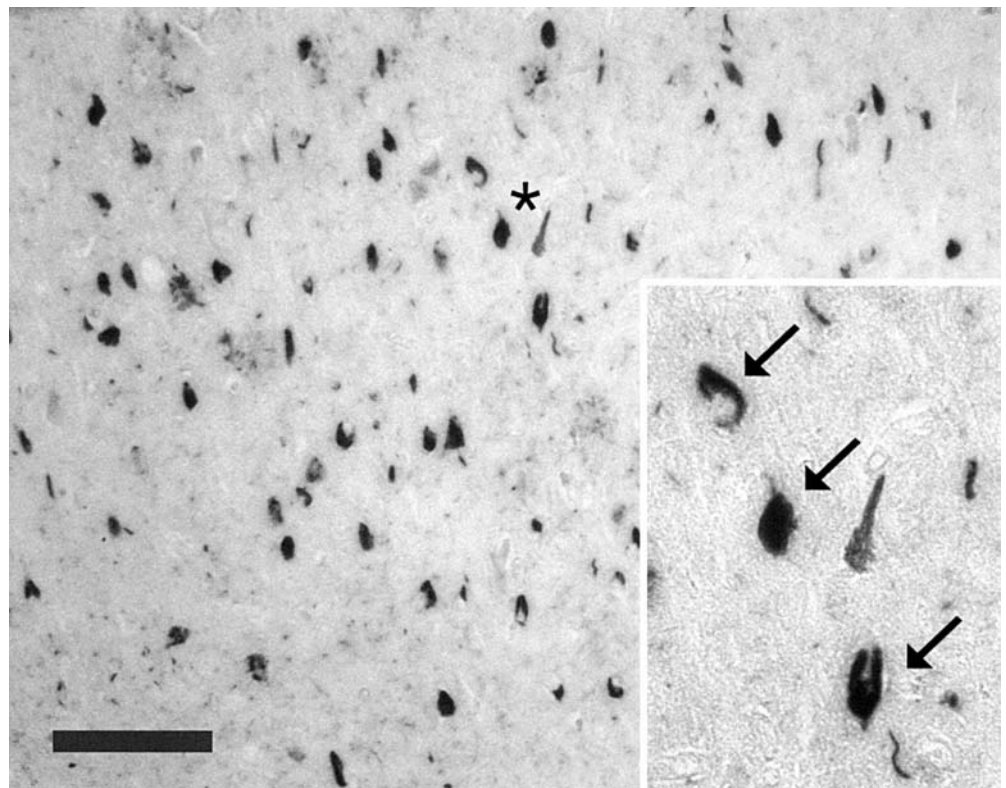


Fig. 3 Immunolabeling of phosphorylated H3 in AD brain (A) is almost completely abolished by absorption of the antibody with phosphorylated H3 peptide (B). *Asterisks* represent landmark blood vessel in adjacent sections (A, B). *Bars* A, B 100 μ m

ylated H3 were observed in the perikaryal cytoplasm of pyramidal allocortical neurons that contained neurofibrillary tangles (most numerous in CA-1 of hippocampus and subiculum). Indeed, phosphorylated H3 immunostaining overlapped almost completely those identified by phosphorylated tau using double immunostaining (Fig. 2A, B). On the other hand, neuropil threads and degenerating neurites marked by phosphorylated tau did not show eleva-

tions of phosphorylated H3, indicating that the phosphorylation of H3 is specific for the cell body of vulnerable neurons. To demonstrate the specificity of phosphorylated H3 (Ser 10) detection, several control experiments were performed in parallel. First, absorption of the phosphorylated H3 (Ser 10) antibody with the immunizing H3 peptide essentially abolished immunostaining (Fig. 3A, B). Second, we found no specific labeling of sections if primary antibody was omitted from the protocol (data not shown). Third, we found an intense immunoreactivity of phosphorylated H3 in the nucleus of M17, a human neuroblastoma cell line, which is mitotically active (data not shown). Fourth, in a dot blot assay, phosphorylated H3

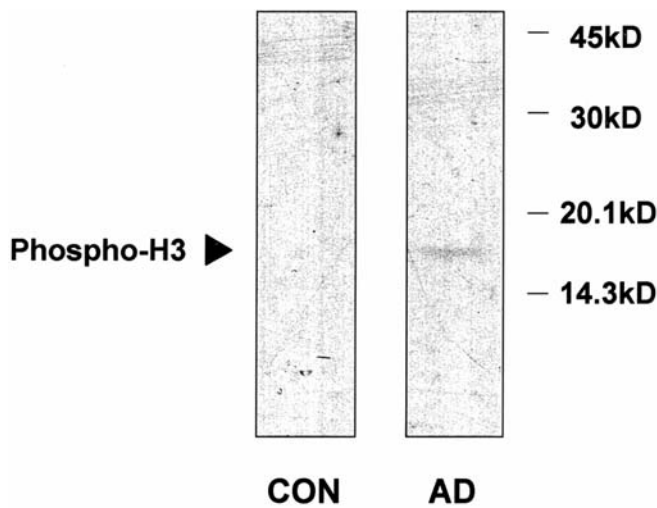


Fig. 4 Representative immunoblot of cortical gray matter. Samples were homogenized in lysis buffer, and proteins acid extracted and probed with antisera against phosphorylated H3 (Ser 10). A strong band at the expected molecular mass of 17kDa is seen in AD cases (*AD*) which is much weaker in control cases (*CON*)

antibody showed no immunoreactivity with purified tau (PHF-tau) from AD cases although PHF-1, an established antibody against PHF-tau, showed strong immunoreactivity, indicating that there is no cross-reactivity between phosphorylated H3 antibody and PHF-tau (data not shown). Finally, immunoblot analysis of cortical brain homogenates demonstrated an anti-phosphorylated H3-immunoreactive band at the expected molecular mass of 17,000 Da in AD with much less immunoreactivity in control (Fig. 4).

Discussion

In this study, we demonstrate increased levels of phosphorylated H3 (Ser 10) immunoreactivity in neuronal cytoplasm in AD compared to controls, suggesting that neurons in AD are mitotically activated. This finding is in line with other studies that indicate that the cell cycle is activated in neurons in AD (reviewed in [12, 19]). Particularly striking is that elevations of phosphorylated H3 are virtually identical to the distribution of cell body-associated phosphorylated tau. Since tau phosphorylation is thought to be an early event in AD pathogenesis, these data indicate a proximal role for phosphorylated H3 in the disease process. Although factors that stimulate the neurons to re-enter the cell division cycle remains unclear, a likely explanation is the inappropriate expression, or release, of growth factors, and, in this regard, it is noteworthy that elevated levels of nerve growth factor (NGF), transforming growth factor-1 (TGF) and basic fibroblast growth factor (bFGF) are found in AD [12]. Supportive of this notion, in AD, there are significant alterations in signal transducing pathways that respond to growth factor stimulation [4, 9, 11, 25].

Phosphorylation of H3 at Ser 10 plays a crucial role in chromatin compaction during cell division, and is a highly conserved event among eukaryotes [21]. A mutant *Tetra-*

hymena thermophila strain, containing non-phosphorylatable H3, exhibited perturbed chromosome condensation and abnormal segregation, further confirming the important role of phosphorylation of H3 at Ser 10 in cell division [21]. During chromosome assembly, global phosphorylation of H3 occurs in a step-wise and ordered manner [3]. In mammalian cells in late G2 phase, phosphorylation is first detected in pericentromeric heterochromatin and, as mitosis proceeds, spreads throughout the entire chromosome. In light of the normal localization of histones in the nucleus, it is notable that, in AD, the increase in phosphorylated H3 is restricted to the cytoplasm in vulnerable neurons. Therefore, while the necessary signals of late G2 may be present, these signals lead to cytoplasmic, rather than nuclear, phosphorylation of H3. This suggests that neurons in AD are mitotically restricted despite activation of the proliferative machinery. Alternately, while H3 phosphorylation at Ser 10 is traditionally viewed as a marker for mitosis, the rapid and transient phosphorylation of a minute fraction of H3 at Ser 10 occurs during the transcription of a subset of immediate early response genes such as *c-fos* and *c-jun*, both of which are reportedly up-regulated in AD [1]. Recent reports have identified two related H3 kinases as likely mediators in these pathways; ribosomal S6 kinase-2 (RSK-2), which is downstream of p42/p44 MAPK/ERK, and mitogen- and stress-activated protein kinase-1 (MSK-1), which is downstream of both MAPK/ERK and p38 MAPK [14, 16]. Interestingly, we and others have shown that these MAPK pathways are up-regulated in neuronal populations in AD with close association with neuronal pathological alterations [4, 11, 25], similar to the pattern we find here for phosphorylated H3, suggesting that MAPK may partly participate in the phosphorylation of H3 in AD.

In conclusion, we demonstrate that phosphorylated H3, a key regulator in chromatin compaction during cell division, is increased in neuronal populations vulnerable to degeneration in AD. The aberrant expression and localization of phosphorylated H3, together with other cell cycle dysregulation, indicates that a mitotic catastrophe plays a role in the pathogenesis of AD. Furthermore, it is notable that the presence of phosphorylated H3 in the neuronal cytoplasm may not be unique to AD. Indeed, a previous study showed increased levels of histones in the neuronal cytoplasmic pool in Huntington's disease brain [5]. Therefore, changes in the distribution of histones, likely as a consequence of mitotic events, may be a common alteration in neurodegenerative disorders.

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