medical sciences forum

MDPI

Proceeding Paper

Nuclear Tau as an Early Molecular Marker of Alzheimer's Disease †

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† Presented at the 2nd International Electronic Conference on Biomedicines, 1–31 March 2023; Available online: https://ecb2023.sciforum.net/.

Abstract: Age-related neurodegenerative diseases have drawn the interest of the scientific community due to the gradual increase in the average age in the world's population. Recent studies have indicated an altered cell cycle in the triggering of neurodegenerative diseases such as Alzheimer's disease (AD). This process seems to involve nuclear tau, a protein which we have previously shown to have a central role in neuronal in vitro differentiation. In this work, we studied the role of the nuclear tau protein, specifically of the AT8 epitope, in the onset of AD to evaluate its possible use as an early molecular marker. The immunolocalization in neurons of the CA1 region of the human hippocampus from normal, senile, and AD subjects showed that the AT8 epitope decreases in senile neurons with respect to younger ones, indicating its possible role in the ectopic activation of the cell cycle in differentiated cells. Here, we show data that improve knowledge on the role of nuclear tau in neuronal differentiation and cell degeneration in AD, involving the presence/absence of AT8 in the nucleolus of neurons related to re-entry into the cell cycle. The molecular mechanisms related to the start of AD are not yet clear, so their understanding is relevant if we consider the social impact of this disease in human populations.

Keywords: neurodegenerative diseases; Alzheimer's disease; tau protein; molecular marker; cell cycle; neuronal differentiation; AT8; hippocampus; nucleolus; cell cycle re-entry

Citation: Bruno, F.; Sturiale, V.; Brancato, D.; Gil, L.; Olazabal, I.M.; Pinedo, F.; Rebolledo, A.B.; Saccone, S.; Federico, C. Nuclear Tau as an Early Molecular Marker of Alzheimer's Disease. *Med. Sci. Forum* **2023**, *21*, 28. https://doi.org/10.3390/ECB2023-14131

Academic Editor: Masaru Tanaka

Published: 8 March 2023

1. Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disease characterized by progressive loss of memory and deterioration of cognitive functions. The molecular mechanisms that start neurodegeneration, in AD patients, are still unclear and indeed, even today, there are not early diagnostic methods for AD. The literature data on the degeneration of neurons show that most neurodegenerative diseases are caused by the misfolding of proteins and, consequently, aggregation of proteins in the brain [1,2]. Moreover, recent studies have suggested a predominant role of the altered cell cycle in the triggering of neurodegenerative diseases such as AD [3].

Tau protein has a central role in AD. It is characterized by a high number of phosphorylations that bring about the development of "Paired Helical Filaments" (PHFs), representing the "core" of the "NeuroFibrillary Tangles" (NFTs). The presence of NFTs, highly insoluble fibrillar aggregates, present in the soma of the pyramidal neurons of the hippocampus, are a result of the abnormal hyperphosphorylation of tau protein and represent an important

neuropathological hallmark of AD [4,5]. The tangles lead to progressive neuronal loss in the central nervous system (CNS) correlated with the clinical progression of AD. The NFTs are evident in specific, vulnerable brain areas, and the hippocampus is one of the earliest brain structures to be affected [6].

Although tau protein has been described mainly as a cytoplasmic protein, nuclear localization isoforms in neuronal and non-neuronal cells have been observed, and their function has not yet been elucidated [7,8]. Literature data indicate that tau protein could interact with the genome and, in particular, the nucleolar localization isoforms could be involved in the early events of AD [9]. Nuclear and nucleolar tau isoforms seem to play a protective role in DNA and RNA against damage events such as oxidative and thermal stress, also suggesting its potential role in ribosome biosynthesis and/or in the organization of genes for rRNA [8,10]. Additional studies have proven that nuclear Tau is detected in the NORs (nucleolar organizing regions) of the acrocentric chromosomes and in the fibrillary region of the nucleolus of neuronal and non-neuronal cells, where they may bind rDNA. Moreover, it has been shown that abnormal phosphorylation of tau can lead to its disaggregation from DNA [9,11,12].

As recently demonstrated in "in vitro" models, the nucleolar phosphoepitopes of tau protein, Tau-1 (unphosphorylated Pro189/Gly207 region) and AT8 (phosphorylated Ser202/Thr205 region), with a mechanism that would involve direct interaction with the DNA and/or the RNA in the nucleolus, could play a role in the early events of AD. In particular, the shorter isoform of tau 0N3R was detected in the human neuroblastoma cell line SK-N-BE during neuronal differentiation, precisely the epitopes Tau-1 and AT8. The Tau-1 epitope was observed in replicative and differentiated cell line SK-N-BE, in a spot-like distribution and colocalizing with UBTF (upstream binding transcription factor); alternatively, the AT8 epitope is not observed in replicative neuroblastoma cells, while appearing during neuronal cell differentiation. Moreover, the AT8 epitope is observed in the nucleolus when the transcriptional activity is blocked or highly reduced, such as after cell exposition to Actinomycin-D [9].

Some tau hyperphosphorylated epitopes have been observed in the neurons of the hippocampus region in the brains of patients with AD and, moreover, the close relationship between nuclear tau in the structure and stabilization of the global heterochromatin blocks [13–15], pericentromeric heterochromatin [16] and perinucleolar heterochromatin [11] and its direct involvement in the development of AD have been verified. The phosphoepitope AT100, a specific nuclear tau epitope (pThr212/Ser214), detected in the more compact chromatin of human neurons, was observed to increase during aging in the nucleolus of neurons from the DG and CA1 regions of the human hippocampus, indicating its possible protective role for chromatin. However, it progressively decreases from AD-I up to disappearing in the more severe AD stages, with a consequent progressive decrease in interaction between AT100 and global chromatin. Therefore, the AT100 epitope can be considered a molecular marker of neuronal aging, and its disappearance from the nucleus can be considered a signal of the onset of Alzheimer's disease [15].

Over the years, many studies that have led to the most recent biological hypothesis of Alzheimer's disease, namely the "Cell cycle hypothesis", are also interesting, according to which AD could be considered a disease caused by neuron cell cycle deregulation and that it is possible that the aberrant drivers of cell cycle re-entry reported in the literature deregulate the neuronal cell cycle machinery, trigger cell cycle checkpoints, and prevent cell cycle completion [3,17]. Indeed, several lines of evidence indicate a predominant role of cell cycle malfunction in the pathogenesis of AD. It has been proposed that tau-induced heterochromatin loss may be related to cell cycle re-entry and to the degeneration of postmitotic neurons [13].

Here, we present results concerning the presence of AT8 epitope in hippocampal neurons during the life of normal humans, and in subjects at the first stage of Alzheimer's disease. The rationale of the study was to evaluate the implication of AT8 in the early events leading to AD.

2. Materials and Methods

2.1. Brain Tissue Samples

Human brain sections, of the hippocampus CA1 region, were obtained from the Institute of Neuropathology HUB-ICO-DIBELL Biobank. All experiments performed in the present study were in accordance with the ethical standards of the institutional and/or national research committees and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The brain samples were prepared from normal human subjects at different ages: fetus, young (between 20 to 40 years), and senile (older than 60 years). Moreover, samples from subjects with AD at the first stage (AD-I) were also used.

2.2. Indirect Immunofluorescence

Paraffin-embedded human brain sections, used for the indirect immunofluorescence (IIF) experiments, were prepared as previously described by Scuderi et al. [18]. Briefly, slides with the tissue sections were placed at 55 °C for 20 min, deparaffined in xylene, and rehydrated in graded alcohols. To reduce auto-fluorescence, the sections were placed in citrate buffer (10 mM sodium citrate, pH 6), brought to a boil, and finally treated with Sudan Black B for 30 min at room temperature [19]. Pre-incubation with blocking solution was performed (non-fat dry milk or bovine serum albumin) before incubation with the primary antibody.

Immunodetection of the AT8 epitope was achieved through overnight incubation at 4 °C with the specific primary antibody AT8 (Thermo Fisher Scientific, Foster City, CA, USA, Cat. MN1020) to detect pSer202/Thr205. After, the slides were rinsed in PBS solution, and the specimens were incubated at 37 °C for 1 h with an FITC-conjugated sheep anti-mouse secondary antibody (Sigma-Aldrich, Darmstadt, Germany, 1:100 dilution in blocking solution).

The experiments were repeated at least three times. Images were recorded with a confocal laser scanning microscope (CLSM) Zeiss LSM700 (Zeiss, Gottingen, Germany) equipped with 40× and 63× objectives. ZEN version 2010 software (Zeiss) was used for image acquisition. Cell counting and statistical analyses were performed as previously described [14]. In detail, the cells were counted on 0.5 µm scanned images for the immunofluorescence data obtained with CLSM. Three to six cases per group were analyzed.

3. Results

Indirect immunofluorescence (IIF) experiments were performed on sections of human brain tissue from the CA1 region of the hippocampus at different ages, from the fetal brain to the senile brain, and further to brain tissue sections of subjects with AD at the first stage. IIF analysis, to detect cells with the AT8 epitope, was performed by using confocal laser scanning microscopy (CLSM). The brain sections detected and more precisely the CA1 region of the hippocampus were analyzed by counting the number of neurons showing the presence of AT8 in the nucleolus with respect to the total number of neurons. This should define the percentage of undifferentiated neurons, owing to these neurons not showing AT8, as we previously demonstrated in the replicative neuroblastoma SK-N-BE cells or after differentiation through retinoic acid treatment. In the case that the degenerative neuronal cells correspond to the cells with an ectopic restart of the cell cycle, the degenerated neurons in the CA1 region of the brain from AD subjects should be identified by the absence of the AT8 epitope.

Our data indicated an increasing percentage of AT8-positive cells from the fetus to young samples. On the contrary, the percentage of AT8-positive cells largely decreases from young to senile or AD-I brain tissue sections. The number of neurons with AT8 epitope showed a statistically significant decrease from the young to the senile brain. The same result was obtained in the AD-I samples, with the percentage of positive AT8 cells in the nucleolus decreasing from young to AD-I and from senile to AD-I. These data indicate a

significant decrease in differentiated cells from young to senile or in the AD-I brain (CA1 region of the hippocampus).

4. Discussion

We previously described, in the human neuroblastoma SK-N-BE cell line, specific expression of the AT8 epitope in replicative vs. differentiated cells [9]. Indeed, AT8 is absent in the SK-N-BE replicative cells and present in the nucleolus of these cells after differentiation induced by retinoic acid treatment. The disappearance of AT8 from the nucleus of differentiated cells is related to the activation of the replicative cell cycle, a condition that seems one of the first events leading to AD, as previously suggested by other authors [3,17], namely, neurons degenerate because an ectopic cell cycle starts in the differentiated cells.

To demonstrate this hypothesis, we analyzed the presence of AT8 in the neurons of the region involved in AD, namely the CA1 region of the hippocampus. Coherent with this hypothesis, AT8 disappears in a significant number of neurons from young to senile and to AD-I brain, indicating that a statistically significant number of neurons from the CA1 region tried to re-enter in replicative status, starting cell degeneration.

The AT8 epitope corresponds to the pSer202/pThr205 region of the tau protein, a region of tau protein that seems relevant in its interaction with DNA, and conformational changes related to this phosphorylation can induce chromatin alteration and cell degeneration [9,15]. Indeed, the presence of phosphorylation in the AT8 region is related to the nucleolus and cell differentiation functionality. Here, we showed the Tau-1 epitope located in the nucleolus of neuronal cells of the hippocampus CA1 region in all ages analyzed and in AD cases and that AT8 gradually disappears in old age as well as in the replicative neuroblastoma cell line.

The present results improve knowledge on the role of nuclear tau in the neuronal differentiation and cell degeneration that happens in AD, a role that seems to involve the presence/absence of the AT8 epitope in the nucleolus of neurons and re-entry into the cell cycle which seems to have a central role in the start of AD. This event seems to happen very early during neuronal degeneration, and understanding when and how this occurs in neuronal cells can be considered relevant progress not only for the use of AT8 as an early AD biomarker but also in the search for natural or synthetic compounds that are pharmacologically active against ectopic cell cycle restart related to the AT8 epitope.

Supplementary Materials: The presentation materials can be downloaded at: https://www.mdpi.com/article/10.3390/ECB2023-14131/s1.

Author Contributions: F.B., C.F. and S.S. conceived the research and planned the experiments; F.B., V.S. and D.B. performed the immunofluorescence experiments; C.F., S.S. and L.G. performed the immunofluorescence analyses, and S.S. performed the confocal microscopy analyses; A.B.R. and F.P. prepared and provided the brain slices; I.M.O. and F.P. contributed to the interaction analyses; F.B. wrote the original draft, and S.S., C.F. and D.B. performed the manuscript review and editing; L.G., S.S. and C.F. were responsible for supervision; C.F. was responsible for project administration and funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by a Research Plan PIACERI L.3 Starting Grant from the Dept. Biological, Geological and Environmental Sciences, University of Catania, to C.F.

Institutional Review Board Statement: All procedures performed were in accordance with the ethical standards of the institutional and national research committees and the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed Consent Statement: The brain samples were obtained from the Institute of Neuropathology HUB-ICO-IDIBELL Biobank.

Data Availability Statement: IIF data are available from the corresponding authors upon request.

Conflicts of Interest: The authors declare that they have no conflict of interest.

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