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Distinct chronology of neuronal cell cycle re-entry and tau pathology in the 3×Tg-AD mouse model and Alzheimer disease patients

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Abstract

Cell cycle re-entry in Alzheimer's disease (AD) has emerged as an important pathological mechanism in the progression of the disease. This appearance of cell cycle related proteins has been linked to tau pathology in AD, but the causal and temporal relationship between the two is not completely clear. In this study, we found that hyperphosphorylated retinoblastoma protein (ppRb), a key regulator for G1/S transition, is correlated with a late marker for hyperphosphorylation of tau but not with other early markers for tau alteration in the 3×Tg-AD mouse model. However, in AD brains, ppRb can colocalize with both early and later markers for tau alterations, and can often be found singly in many degenerating neurons, indicating the distinct development of pathology between the 3×Tg-AD mouse model and human AD patients. The conclusions of this study are two-fold. First, our findings clearly demonstrate the pathological link between the aberrant cell cycle re-entry and tau pathology. Second, the chronological pattern of cell cycle re-entry with tau pathology in the 3×Tg-AD mouse is different compared to AD patients suggesting the distinct pathogenic mechanism between the animal AD model and human AD patients.

Introduction

Alzheimer's disease (AD) is a fatal neurodegenerative disorder characterized by a decline in memory and a debilitating loss of mental function. Prominent pathological hallmarks of AD include amyloid plaques and neurofibrillary tangles (NFTs). Amyloid plaques are formed by aggregation of the amyloid- β peptide (A β) and are recognized as a necessary factor in AD diagnosis. NFTs are primarily composed of tau protein and are the result of tau hyperphosphorylation and subsequent aggregation. Developing within pyramidal neurons in the hippocampus and throughout the cerebral cortex, NFTs are associated with neuronal dysfunction in AD patients [1].

While neurons are known to be post-mitotic and remain in a non-dividing state, it has previously been demonstrated that, in the brains of AD patients, vulnerable neurons re-enter

the cell cycle and attempt to progress through it with aberrant consequences, such as abortive cell death [2, 3]. Once cells progress through the G₁/S checkpoint they are unable to return to the G₁/G₀ phase. Being unable to either return to G₁/G₀ or to complete the cell cycle, it is thought that, as a consequence of re-entering the cell cycle, the neurons ultimately die [2, 4, 5]. Supporting the causal role in neuronal cell death, we and other investigators demonstrated that induction of cell cycle re-entry in neurons caused neuronal cell death in the experimental model systems [6–8]. The hyperphosphorylation of retinoblastoma protein (ppRb) is an essential event that occurs late in the G₁ phase of the cycle, allowing the cell cycle to progress to the S phase by release of E2F transcription factors and subsequent transcription of cell cycle proteins. The induction of ppRb in the vulnerable neurons in AD has been shown to be associated with tau pathology in AD and several other tauopathies [9–11]. It was also found that minichromosome maintenance protein 2 (Mcm2), phosphorylated during the S-phase of the cell cycle and a downstream target of ppRb/E2F pathway, was associated with NFTs, supporting the idea that ppRb can functionally regulate its downstream pathway and regulate cell cycle progress in NFT bearing neurons in AD [12]. Indeed, other cell cycle proteins have also been documented to be abnormally expressed in NFTs, including BRCA-1, various cyclins and cyclin dependent kinases [13–18].

While the pathological mechanism of neuronal cell cycle re-entry in AD is not completely understood, a correlation between tau and the cell cycle has been reported, with cell cycle pathology occurring prior to tau pathology in AD [4, 15]. Supporting the relationship of cell cycle and tau phosphorylation, cell cycle related proteins have been shown to directly phosphorylate tau [19, 20]. Conversely, the manipulation of tau has also been shown to induce cell cycle [21, 22] and, interestingly, Andorfer and colleagues demonstrated the dissociation of cell cycle pathology from tau pathology in their transgenic mouse model overexpressing wild-type tau [22]. Thus much uncertainty remains regarding the causal relationship of cell cycle re-entry relative to the tau pathology which requires further investigation.

To this end, in this study, we examined the expression of cell cycle markers and hyperphosphorylated and conformationally altered tau, which occurs sequentially during the development of AD, in a transgenic mouse model of AD (3×Tg-AD) [23]. Specifically, extracellular Aβ deposition appears at ages beyond 15 months, while hyperphosphorylated tau is detectable as early as 6 months of age in 3×Tg-AD mouse [24]. Therefore, the progression of tau pathology and cell cycle re-entry across varying ages was analyzed in these mice and compared to hippocampal tissue of AD patients to characterize the pathological relationship between two major AD pathologies in this study.

Methods

Triple Transgenic (3×Tg-AD) Mice

Triple transgenic and non-transgenic littermate mice were kindly provided by Dr. LaFerla at U.C. Irvine and bred in our university animal facility at Case. All animal housing and procedures were performed in compliance with guidelines established by the Institutional Animal Care and Use Committee at Case Western Reserve University. For this study, mice of various ages were perfused by PBS/10% formalin. Brains were dissected out, embedded

in paraffin, cut into 6 μ m sections and mounted on slides for immunocytochemistry. Mice used included 5–7 month (n=3), 11–12 month (n=7), 13–16 month (n=10), and 18–20 month (n=8) 3 $\times$ Tg-AD mice. Age-matched wild type mice were also analyzed.

Human Brain Tissue

Brain tissues from AD patients (n=5, ages 67–87) and one age matched control (age 81) were obtained post-mortem in accordance with a protocol approved by the Case Western Reserve University Institutional Review Board and fixed in formalin or methacarn (methanol: chloroform: acetic acid, 6:3:1). Hippocampus tissues were embedded in paraffin, and cut into 3 μ m sections, with 3 serial adjacent sections placed on each slide, ensuring many of the same neurons would be visualized across multiple sections.

Immunohistochemistry

The slide mounted sections were placed in xylene to dissolve the paraffin and were then hydrated via decreasing concentrations of ethanol. Endogenous peroxidase activity was terminated by incubating the sections in 3% hydrogen peroxide in methanol for 30 minutes. Sections were then incubated in 10% normal goat serum (NGS) in Tris buffered saline (TBS) for 30 minutes to block non-specific antibody binding sites. Primary antibodies diluted with 1% NGS in TBS were then applied, and the slides were kept at 4°C overnight. The primary antibodies used included mouse antibodies against conformational and phosphorylation epitopes of tau: MC1 specifically recognizes conformational epitopes of tau [25], AT8 recognizes tau protein phosphorylated at both serine 202 and threonine 205 [26] (Pierce-Endogen) and CP13 recognizes tau phosphorylated only at serine 202 [27]. Rabbit antibodies against retinoblastoma protein phosphorylated at Serine 807 (ppRb807) (Biosource) [10], phosphorylated minichromosome maintenance protein 2 (pMcm2) [12, 28], and mouse antibodies against BRCA1 (Oncogene Research Products) [18] were also examined.

Species specific secondary antibodies and PAP complexes were added respectively. Slides were then developed with 3'-3'-diaminobenzidine (DAB, Dako) and dehydrated through ascending ethanol to xylene, and then coverslipped. Images were obtained with a Zeiss Axiophot. Double label fluorescent microscopy was also performed using secondary antibodies Alexa 488 and Alexa 568 to further explore colocalization. Fluorescent images were captured using a Zeiss Axiovert.

Quantification

For this study, focus was placed on the tau and cell cycle marker accumulation in the pyramidal neurons of the hippocampus in the 3 $\times$ Tg-AD mice. Using a 20 $\times$ objective, the numbers of neurons positively stained were counted in the entire CA3, CA2, CA1 and subiculum regions of the hippocampus. Over the course of the study, the same antibody was used at different times on sections from the same mouse and no significant difference was found between the different experiments, thus the average value obtained was used for the analysis.

Results

Brain tissue sections from a series of different aged 3×Tg-AD and wild type mice were immunostained for MC1, CP13, AT8, BRCA1, ppRb807 and pMcm2. In the hippocampus the neurons in the wild-type mice had neither detectable levels of tau (CP13, MC1, AT8) nor any cell cycle marker (BRCA1, ppRb807, pMcm2) (data not shown). Conversely, in the 3×Tg-AD mice at 5 months, the earliest age studied here, some neurons were positive for phosphorylated tau (CP13) (data not shown), and conformationally altered tau (MC1), epitopes which are known to occur in the early phase of tau pathology development in AD. Neurons detected by MC1 in the CA1 of the hippocampus became progressively copious with increasing age (Figure 1A, D, G). After 18 months, intensely stained tau pathology was abundant and spread thoroughly throughout the entire pyramidal layer of the hippocampus. On the other hand, AT8-positive neurons with hyperphosphorylated tau began to appear consistently in the hippocampus at 12–14 months (Figure 1E), as do ppRb807 positive neurons (Figure 1F). The expression pattern for both AT8 and ppRb807 was remarkably similar, in that positive neurons first showed up in the subiculum between 12 and 14 months, then progressed into CA1 by 18 months, and further into the hippocampus beyond 18 months (Figure 1H, I). Indeed, AT8 and ppRb807 were co-expressed in the majority of neurons (insets Figure 1).

The analysis of serial adjacent sections clearly showed that many of the same neurons were positive for both AT8 and ppRb807 across all ages. Even in a 14 month mouse with very few AT8 positive neurons, ppRb807 was always present and co-localized with AT8 (Figure 1E, F and insets). Quantification found that correlation between the number of neurons positive for AT8 and ppRb807 in each mouse was highly significant across all animals ($R=0.99$, $p<0.0001$). Double label immunofluorescence microscopy confirmed the complete co-localization of AT8 and ppRb807 in the same neurons even in the older mice (Figure 2D–F). However, not all MC1 positive cells contained ppRb807 (Figure 2A–C) further demonstrating a specific correlation of ppRb807 with a later development stage of tau pathology (AT8) but not with early stage of tau pathology (MC1).

Other cell cycle related proteins are also expressed in the pathological neurons in the aged 3×Tg-AD mice. While MC1 is present in the most neurons (Figure 3A), serial adjacent sections find neurons stained with ppRb807 (Figure 3B) and to a lesser extent other cell cycle proteins pMcm2 (Figure 3C) and BRCA1 (Figure 3D) in a 19 month old 3×Tg-AD mouse, a finding which supports the notion that ppRb807 is functionally active and can induce its downstream cell cycle pathways in a subset of neurons.

Upon quantification of the changes in tau pathology and cell cycle expression in the aging 3×Tg-AD mice, it was found that the number of neurons in the entire hippocampus region expressing tau phosphorylation and conformational changes steadily increase with age (Figure 4). MC1 neuronal staining significantly correlates with age in a linear fashion from 5–20 months ($R=0.71$, $p<0.05$). However, the appearance of AT8, ppRb807, BRCA1, and pMcm2 neuronal staining is delayed and does not increase significantly until after 18 months of age (Figure 4).

We then used these same antibodies to see if the relationship between tau hyperphosphorylation and cell cycle reactivation found in the 3×Tg-AD mice is indeed recapitulated in cases of human AD. Using hippocampus tissues from 5 AD cases and one aged control, we analyzed the correlative pattern of ppRb807 expression with MC1, CP13, or AT8. First, using very thin 3 µm adjacent serial sections, immunostaining of MC1 and ppRb807 was compared in the same neurons. In the CA1 region, most NFT contain both MC1 and ppRb807 (Figure 5A,B, blue arrows). Some neurons exhibiting relatively lower immunoreaction with MC1, suggestive of neurons often referred to as “pretangles”, are strongly immunoreactive for ppRb807 (Figure 5A,B, arrowheads). However, in the CA3 and CA4 regions, many neurons that contain only ppRb807 lack any MC1 immunostain (Figure 5C,D). Using a similar approach, two adjacent sections were first both stained for ppRb807 detected using DAB to ensure that each neuron stained with ppRb807 was present and visualized in both sections. Then subsequent application of either AT8 or CP13 was detected with Alexafluor 588-labelled secondary antibody. In most NFT bearing neurons, ppRb807 co-localized with both AT8 (Figure 5E–G) and CP13 (Figure 5H–J). Moreover, some neurons were stained with ppRb807 alone, and did not contain either tau epitope (asterisks, Figure 5F,I) suggesting human AD presents with a distinct pattern of pathology compared to the pathology observed in the 3×Tg-AD mouse.

Discussion

In this study, we demonstrate the increase of cell cycle markers in neurons of the 3×Tg-AD mouse and their correlation with tau pathology development. In addition, our data suggests distinct chronological patterns of cell cycle re-entry related with tau pathology in human AD and the 3×Tg-AD mouse. The mouse model provides us with the ability to test the appearance of each protein with chronology, something that is much more difficult in human disease. In our analysis of the 3×Tg-AD mice, tau pathology was readily detectable with the antibodies specific to early tauopathy (CP13 and MC1) in hippocampal neurons even in the 5 month old mice and progressively developed further in the older age groups (Figure 1G,J). The selective population of those neurons with early stage of tau pathology especially in the CA1 and subiculum eventually progressed to the development of relatively later stage of tau pathology (AT8) and to cell cycle re-entry. Even in the earliest ages of mice with AT8 tau pathology (i.e., after 11–12 months approximately), there was almost complete overlap of AT8 and ppRb807, even when only 1 or 2 neurons with AT8 positive tau pathology were present (Figure 1E,F, arrows). Indeed, when all animals were quantified, a nearly 100% overlap was found.

Previously, inconsistent with our findings, Lopes and colleagues failed to detect the induction of cell cycle in the 3×Tg-AD mouse [29]. However, their study focused on cerebral cortex and no data for hippocampus was provided. In addition, the level of AT8 was measured by western blot with brain homogenates which makes it difficult to compare with our data. As shown in our data, the expression of cell cycle markers is limited to relatively small number of neurons containing AT8 positive tau pathology in selected regions (i.e., CA1, subiculum) at the specific pathologic stage. Thus, while it will require further study to fully explain the discrepancy between both studies, such limited expression pattern of cell

cycle marker with different experimental approaches might be one of the main reasons for such discrepancy.

We have previously reported the pathological accumulation of cell cycle proteins ppRb and pMcm2 in many different tauopathies including progressive supranuclear palsy, Pick disease, Guam Parkinson and others [11]. While there is considerable overlap of the cell cycle changes with the pathological forms of tau within pathology, the overlap is not complete, with some cells expressing ppRb and not tau suggesting ppRb precedes tau pathology in the disease pathogenesis. In the present study, we further confirmed such chronological pattern in AD hippocampus. In the CA1, a small number of NFT contained only ppRb807, and neither CP13, MC1, nor AT8. In the CA3 and CA4, a greater number of neurons contained ppRb807 only. Notably, some neurons expressing a low level of tau accumulation with a diffuse cytoplasmic distribution of MC1, suggestive of pretangles or immature tangles [30], demonstrated a strong immunoreactivity with ppRb807. These findings are in contrast to the 3×Tg-AD mouse model studied here which demonstrates exclusive overlap between tau accumulations detected by AT8 and cell cycle marker expression than is found in human disease. Neurons from the younger mice with early tau accumulations of both CP13 and MC1 epitopes in the cell body and axonal processes did not express ppRb807.

While animal models of disease are important tools for studying the pathogenesis of human disease, it has been debated if the mouse models of AD accurately represent human AD pathogenesis appropriately. The mutant APP overexpressing models successfully develop human-disease like amyloid plaques and many of the cellular changes that accompany plaques in human AD such as oxidative damage and increased gliosis. For many years however, these models have been criticized for not incorporating the neuronal cell loss that correlate with the cognitive decline featured in AD [31]. Even so, tremendous effort and resources were put forth to develop therapies to combat the plaque accumulations, which showed some promise in reversing the disease process in the animal model, however proved unsuccessful in thwarting human disease, suggesting inadequacy of our current animal models for AD. In this vein, our study indicates incongruence in the temporal relationship between tau pathology and cell cycle re-entry between AD patient samples and the transgenic animal model, and raises a concern to translate the animal model data to human AD.

In conclusion, our study clearly demonstrated that neuronal cell cycle re-entry can be detected in the 3×Tg-AD mouse but such pathology is limited to highly selective neurons containing later stage of tau pathology. Such chronological correlation between tau pathology and cell cycle re-entry in the 3×Tg-AD mouse suggests that cell cycle re-entry is a late pathological event and might be caused by tau pathology. However, the appearance of cell cycle re-entry is not limited to such specific type of tau pathology and can be even found in neurons without tau pathology in AD patients. Therefore, these observations suggest that, despite significant overlapping pathology between the 3×Tg-AD mouse and human AD, the animal model do not recapitulate the disease pathogenesis completely and, especially, further use of AD animal models for studying the cell cycle re-entry in AD should proceed with caution.

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Figure 1.

With increasing age, neurons in the CA1 and subiculum express increasing levels of tau pathology, detected with MC1 (A,D,G). While no tau pathology have yet developed at 7 months of age (A–C), small numbers of tau pathology bearing neurons, detected with AT8, are found in a 14 month mouse (E), and the numbers of such neurons increase dramatically in the 18–20 month animals (H). A similar pattern to AT8 is seen using the cell cycle marker ppRb807 (C,F,I). In fact, even at the earliest age of tau pathology development, both AT8 and ppRb807 are present in all tau pathology bearing neurons (inset of boxed areas E and F show the same neurons from adjacent serial sections). Scale bar= 100 μ m.

Figure 2.

Double fluorescence immunostaining of the 3×Tg-AD mice find MC1 stains many neurons in a 19 month old mouse (A), with considerable, but not total overlap, with ppRb807 (B and merged image C). However, essentially all tau positive neurons contain both hyperphosphorylated tau stained with AT8 (D) and ppRb807 (E and merged image F). Scale bar= 100 μm.

Figure 3.

In 3xTg-AD mice over 18 months of age, many neurons and neurofibrillary tangles are prominent in the CA1 region of the hippocampus stained with MC1 (A). In adjacent sections of the same mouse, antibodies used to detect cell cycle proteins also stain some of the NFT including ppRb807 (B), BRCA-1 (C), and pMcm2 (D). Counterstain with hematoxylin. Scale bar= 100 μ m.

Figure 4.

Quantification of the large aging series of the 3×Tg-AD mice, found the percentage of neurons in the entire hippocampus expressing tau correlated significantly with age using MC1 ($p < 0.05$). However, antibodies that detect only the neurons that contain NFT including AT8 and the cell cycle markers BRCA-1, pMcm2, and ppRb807 show variable numbers in the mice between 11 and 16 months of age, and then only increase dramatically after 18 months of age, a delayed increase relative to early tau accumulation detected by MC1.

Figure 5.

Human AD serial adjacent hippocampus sections were stained with MC1 (A,C) and ppRb807 (B,D). In the CA1 (A,B), many of the same NFT contain both tau and cell cycle (arrows) and some pretangles or immature NFT with only diffuse weaker MC1 staining, still show strong ppRb807 (arrowheads). In the CA3, there are many ppRb807-positive NFT that have no increased MC1 staining (C,D). Adjacent serial sections stained for ppRb807 detected using DAB to ensure the same stained cells were visualized in both sections (E,H), were then stained using either AT8 or CP13 detected with Alexafluor 588-labelled secondary

antibody. In most NFT, ppRb807 co-localized with both AT8 (F,G) and CP13 (I,J). Some neurons positive for ppRb807 did not contain hyperphosphorylated tau (asterisks, F,I).

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