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Neuronal cell cycle re-entry mediates Alzheimer disease-type changes

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Abstract

Evidence showing the ectopic re-expression of cell cycle-related proteins in specific vulnerable neuronal populations in Alzheimer disease led us to formulate the hypothesis that neurodegeneration, like cancer, is a disease of inappropriate cell cycle control. To test this notion, we used adenoviral-mediated expression of c-*myc* and ras oncogenes to drive postmitotic primary cortical neurons into the cell cycle. Cell cycle re-entry in neurons was associated with increased DNA content, as determined using BrdU and DAPI, and the re-expression of cyclin B1, a marker for the G_2/M phase of the cell cycle. Importantly, we also found that cell cycle re-entry in primary neurons leads to tau phosphorylation and conformational changes similar to that seen in Alzheimer disease. This study establishes that the cell cycle can be instigated in normally quiescent neuronal cells and results in a phenotype that shares features of degenerative neurons in Alzheimer disease. As such, our neuronal cell model may be extremely valuable for the development of novel therapeutic strategies.
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Keywords: Alzheimer disease; Cell cycle; Phosphorylation; Tau

1. Introduction

Alzheimer disease (AD) is a chronic degenerative disorder characterized by the intracellular accumulation of highly phosphorylated tau within select neuronal populations [1,2]. While the mechanism(s) that lead to the accumulation of phosphorylated tau are unresolved, an increasing body of work suggests that cell cycle disturbances may play an early role in disease pathogenesis [3–5]. In fact, highly phosphorylated tau is intimately linked to the cell cycle since a near identical phosphorylation of tau also occurs when cells are mitotically active and tau phosphorylation is driven by cyclin-dependent kinases (CDKs) [6–10]. Of potential mechanistic importance, a number of CDKs are ectopically re-expressed in neurons in AD and such CDKs are known to phosphorylate tau in *in vitro* assays in a manner similar to that found in AD *in vivo* [3,4,11–13]. Since cell cycle protein expression in AD precedes the appearance of phosphorylated tau [14], a possible cause–effect relationship is indicated. Although neurons in the normal adult brain are generally quiescent and in the G_0 phase of the cell cycle, neurons vulnerable to degeneration in AD appear to emerge from this postmitotic state and are phenotypically suggestive of cells that have re-entered into the cell cycle [3–5,11–31]. Indeed, the successful duplication of DNA [32] indicates that such neurons successfully complete S phase and precludes the possibility that the re-expression of various cell cycle markers is an epiphenomena of reduced proteosomal activity.

To directly test the effect of neuronal cell cycle re-entry on the initiation of the neuronal alterations observed in AD, we infected primary cortical neurons *in vitro* with a combination of c-*myc* and ras oncogenes to force non-dividing neurons into the cell division cycle. Our observations from this previously unreported methodology support a model of onco-

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genic transformation as an early event in the development of AD.

2. Materials and methods

2.1. Cell culture

Primary cortical neurons were prepared from Sprague–Dawley rat embryos at 17–19 days gestation as described [33]. Briefly, cerebral neocortices were removed and minced in dissociation buffer, treated with trypsin and dissociated by trituration in DNase and trypsin inhibitor. Cells were plated at a concentration of 2×10^5 cells/ml on poly-D-lysine-coated slide chambers or 0.9×10^6 cells/ml on poly-D-lysine coated 10 cm tissue culture plates and infected with the appropriate adenoviral vectors 5 days after plating. Under these conditions, uninfected cultures are viable for 2–3 weeks.

2.2. Adenoviral gene transduction

Recombinant adenoviruses expressing green fluorescent protein (GFP), c-*myc* or ras (H-RAS61L) were constructed and provided by Joseph Nevins at Duke University [34]. Virus stocks were amplified in HEK 293 cells and purified by CsCl banding. Each virus is used as follows: GFP, 5×10^{10} pfu/ml; Ras 1.25×10^9 pfu/ml; Myc 4×10^{10} pfu/ml. Rat embryonic cortical neurons were infected at a multiplicity of infections (MOI) of 50 pfu/cell, and no cytotoxicity was observed at this MOI.

2.3. Immunocytochemistry

Cells were fixed in 1% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min and permeabilized with 0.5% NP-40 for 5 min at room temperature, washed with PBS, then blocked with 5% non-fat milk in PBS for 1 h. Antibodies were incubated in 1% non-fat milk in PBS overnight at 4 °C. Secondary antibodies were incubated for 40 min at room temperature, washed three times in 1% non-fat milk in PBS and samples counterstained with DAPI (1 μg/ml in methanol for 15 min at 37 °C) to localize all nuclei before mounting in Vector shield (Vector Laboratories, Burlingame, CA). Samples were analyzed under epi-fluorescence on a Nikon Inverted microscope with Infinity optics and images captured with Metamorph software (Meridian Instruments, Kent, WA). Omission of primary antibodies showed no positive fluorescent signals.

2.4. Antibodies

Hybridoma supernatants of TG3 and Alz50 antibodies (gift of Peter Davies) were used at 1:50 and 1:20 respectively. The TG3 monoclonal antibody was raised against purified PHF's [3] and recognizes tau phosphorylated at Thr 231. Alz50 was raised against AD brain homogenates [35] and recognizes a conformational epitope requiring both N terminal residues (7–9) and residues

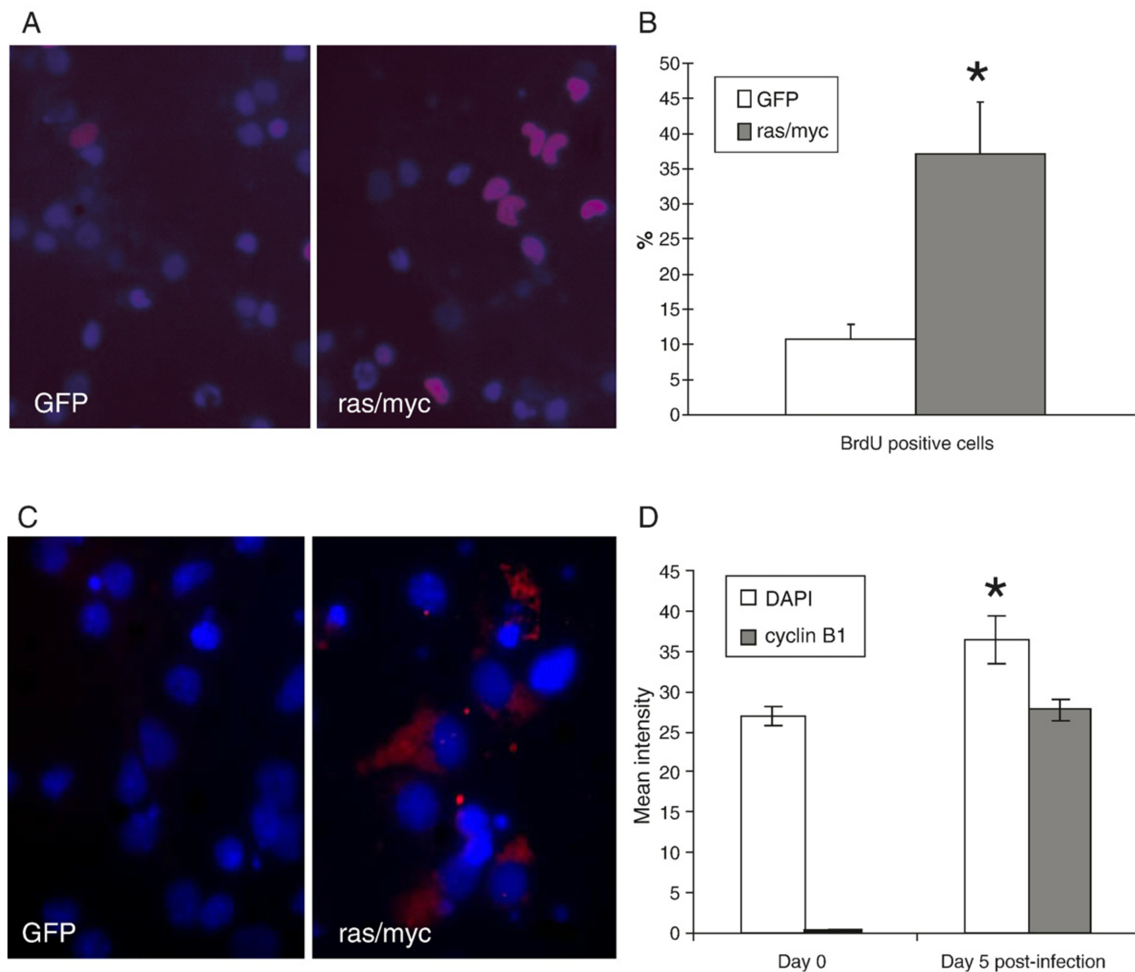

Fig. 1. Cell cycle re-entry in ras/myc adenoviruses infected primary cortical neurons. (A) BrdU incorporation (red) in primary cortical neurons infected with either control GFP or ras/myc containing adenoviruses. Quantification (B) shows a significant increase in BrdU incorporation in ras/myc-infected cells ($p<0.001$) over GFP-infected cells. Cyclin B1 (red) is only expressed in ras/myc-infected cells (C) along with a significant concomitant increase in DNA ($p<0.001$) (D), visualized with DAPI (blue).

312–342 in the third microtubule binding domain [36]. Cyclin Bl (Santa Cruz, Santa Cruz, CA, 1:500) is a rabbit polyclonal raised against full-length recombinant cyclin B. Fluorescent secondary reagents used were goat anti-mouse CY3 or goat anti-rabbit FITC at dilution of 1:500 (Jackson Laboratories, West Grove, PA). Isotype specific biotin-conjugated and anti-streptavidin FITC/CY3 (Southern Biotech, Birmingham, AL) were also used.

2.5. DNA measurements

DNA content was measured following counterstaining fixed samples with DAPI. DNA replication was quantified by labeling primary neuronal cultures with 10 μM bromodeoxyuridine (BrdU) coincident with viral infection. Nuclear BrdU accumulation was assayed by in situ immunofluorescence with an anti-BrdU monoclonal antibody [37] (Becton Dickinson, San Jose, CA). DNA content (mean intensity) and BrdU incorporation (% positive cells) measurements were carried out by measuring 5 random fields using Metamorph Image Analysis Software (Meridian Instruments, Kent, WA). All experiments were performed at least in triplicate and data presented is the mean of all experiments. The student's *t* test was used to determine significance.

3. Results

3.1. Cell cycle entry

Cultured primary rat cortical neurons (E18) were infected with adenovirus vectors encoding two different oncogenes, myc and a mutationally active ras (H-RAS61L), in an attempt to force the post-mitotic neurons into the cell cycle. Primary cortical neurons were infected 5 days after plating and fixed for immunostaining after either 2 or 5 days.

To determine if cells had re-entered the cell division cycle and initiated DNA replication (i.e., entered S-phase), cultured neurons infected with GFP or ras/myc were incubated with BrdU and immunolabeled with anti-BrdU antibody (Fig. 1A). Levels of BrdU incorporation in approximately 30 and 40% of cells expressing myc/ras were significantly elevated (Fig. 1B, $p<0.001$) compared to cells expressing GFP, suggesting that infected neurons synthesize DNA and re-enter into the cell cycle. A mean incorporation frequency (background) of about 10% (Fig. 1B) in GFP control infected cultures was consistent with the post-mitotic neuronal population representing >90% of the culture [38]. To further characterize this cell cycle-related phenomena, we localized cyclin B1, a cell-cycle marker specifically induced in the late S/G_2 phase of the cell cycle but not associated with cells in the G_1 phase of the cell cycle, and found increased cyclin B1 in ras/myc adenovirus infected neurons (Fig. 1C).

To examine if the oncogenically-infected neurons had become mitotically active (i.e., entered M-phase), the neurons were counterstained with DAPI to identify mitotic figures and no mitotic figures were found (Fig. 1C). Therefore, while the BrdU data (Fig. 1A) indicates progression into S-phase, nuclear morphology (Fig. 1C) indicates that the mitotic index was not increased in oncogene-infected neurons.

These data suggest that infected neurons arrest at the beginning of mitosis but fail to complete DNA synthesis and progress to cell division. Such a notion is consistent with the significant increase in DAPI labeling of nuclei ($p<0.001$) (Fig. 1D) and absence of mitotic figures. Nuclear translocation of cyclin B1 is typically the mechanism by which mitosis is initiated and cytoplasmic cyclin B1 expression is associated with cells in the G_2 phase of the cell cycle or cells that are arrested at the earliest stages of the G_2/M checkpoint. Therefore, our data suggest that primary neurons that are forced to re-enter the cell cycle do not overcome the G_2/M check point and therefore do not progress to M phase (i.e., analogous to findings in AD) [32].

3.2. Tau expression

To examine whether ras/myc adenoviruses-induced cell cycle re-entry of neurons was associated with AD-like tau changes, we used two different antibodies that detect abnormalities in tau: tau phosphorylation (phospho-threonine 231;TG3 antibody) and tau conformational changes (Alz50 antibody).

On average, 30±7.4% of the ras/myc adenovirus infected neurons (Fig. 2B), but not GFP infected controls (Fig. 2A)

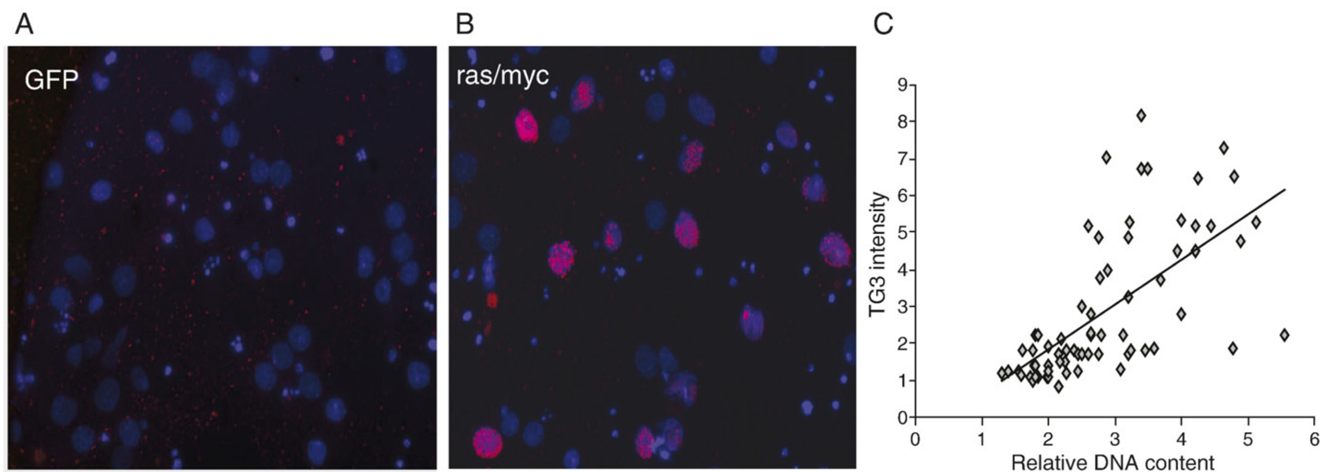

Fig. 2. Increased phospho-tau expression in ras/myc adenoviruses-infected primary cortical neurons. Phospho-tau, detected with antibody TG3 (red), is expressed in cell infected with ras/myc (B) and not in GFP-infected cells (A) after 2 days post-infection. DNA content visualized with DAPI (blue) shows significant positive correlation with TG3 epitope expression ($p<0.00001$) (C).

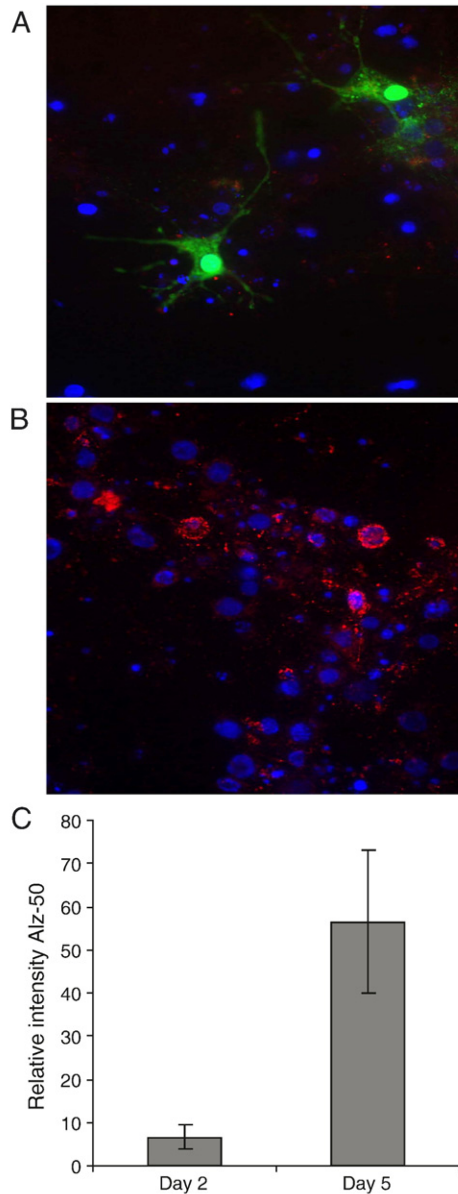

Fig. 3. Conformational change of tau protein by ras/myc adenoviruses infection in primary cortical neurons. Alz50 antibody staining (red) in primary cortical neurons infected with either control GFP (green) adenovirus (A) or ras/myc (B) for 5 days and stained with DAPI (blue). There is over an 8-fold increase of Alz50 epitope expression from day 2 to day 5 (C).

were TG3 positive. The appearance and increase of TG3 immunoreactivity directly correlated with DNA content determined using DAPI ($p<0.00001$) (Fig. 2C).

Alz50 is an antibody that is considered an early pathological marker for AD [35] and, in contrast to TG3, recognizes a higher order conformational epitope of tau [36,39]. Less then 1% of cells were positive for Alz50 in the cultures infected with GFP adenovirus at either 2 days or 5 days post-infection (Fig. 3A). In marked contrast, in ras/myc-infected cells, 5% of neurons were found to be Alz-50 positive at 2 days post-infection (data not shown), but, by 5 days, around 50% become positive (Fig. 3B, C) and there was an 8-fold increase in immunoreactivity.

In summary, the coincidence of the cell cycle markers (BrdU and cyclin B1) and the appearance of key AD specific PHF antigens (TG3, Alz50) suggest that the introduction of ras/myc oncogenes into cultured neurons leads to changes in cell cycle dynamics and specific elements of AD neuropathology.

4. Discussion

In this study, we have driven non-dividing, differentiated primary neurons into the cell cycle using adenovirus vectors carrying two powerful oncogenes, c-*myc* and a mutationally active ras (H-RAS61L). Expression of these oncogenes has been shown to cause fibroblasts to exit G_0, overcome the G_1/S checkpoint and re-enter the cell cycle [34]. It was expected that this stimulus would cause an aberrant cell cycle response in the neurons and our data suggests that the oncogene-expressing neurons re-enter the cell cycle and arrest at the G_2/M phase of the cell cycle as determined by the appearance of cyclin B1 and increased DNA replication (BrdU and DAPI). Notably, although the BrdU data suggests progression into S-phase in oncogene-infected neurons, nuclear morphology indicates that the mitotic index was not increased. Therefore, although neurons initiate DNA synthesis, they do not appear to complete mitosis (i.e., divide) consistent with our previous notion of cell cycle dysfunction [40]. Indeed, the neurons do not show any progression from the G_2/M phenotype and prolonged culture (>5 days post infection) leads to a steady decrease in the overall DNA content and DNA condensation consistent with cell death.

The TG3 phosphoepitope of tau is present in both mitotic cells at G_2/M and NFT in AD [3,41] and other neurodegenerative disorders [42]. In mitotic cells, the TG3 phosphoepitope appears just prior to the onset of prophase and is maximal during metaphase [3,41]. In AD, the TG3 epitope is observed in vulnerable neurons prior to NFT formation [14]. In this report, we show that myc/ras stimulation of neuronal cultures leads to the appearance of the TG3 epitope. Of note is the observation that TG3-positive staining precedes Alz50 staining by at least 24 h. This is not unexpected and recapitulates the temporal

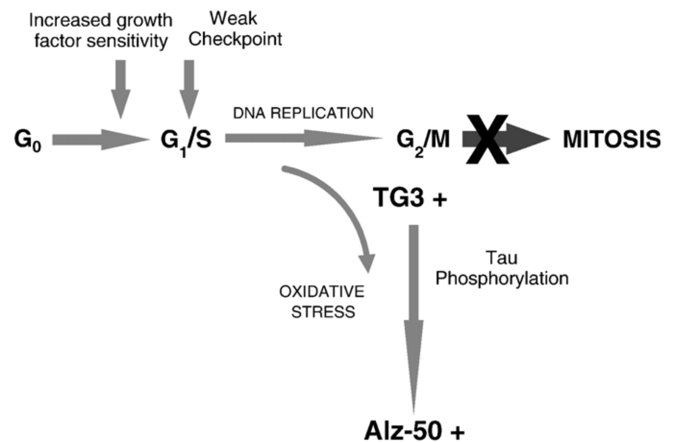

Fig. 4. Schematic representation of possible stages of the cell cycle and tau expression. Cell cycle re-entry (i.e., G_0 to G_1/S and beyond) is associated with tau phosphorylation and oxidative stress. Unable to enter mitosis, cells enter “phase stasis” and further conformational changes in tau result.

appearance of these antigenic markers in AD [43] and, further, suggests that formation of the Alz50 epitope, which is created by the association of two distinct regions of the tau molecule, is more complex than initiation of a mitotic event and may require secondary events [30], i.e., oxidative stress. Indeed, previous studies have shown that the Alz50 epitope can be created by reactive oxidative intermediates produced during neuronal oxidative stress, coincident with the induction of heme-oxygenase-1, and that the Alz50 conformation may result from oxidative damage to an already phosphorylated form of tau [44].

Although various mitotic markers are upregulated in the vulnerable neurons in AD brain, no evidence of actual mitosis has ever been found, suggesting that oncogenically-infected neurons become arrested at a point(s) prior to the actual event of cellular division. However, it is well known that once cyclin A is expressed, the arrested cells lack the ability to return to G_0 and therefore must either complete the cycle or die. Given the lack of evidence for successful completion of the cell cycle, it is likely that the re-activation of cell cycle machinery in post-mitotic neurons leads to their death. In support of this, when a powerful oncogene, SV40 T antigen, is expressed specifically in maturing Purkinje cells in transgenic mice, the cells replicate their DNA (i.e., initiate cell cycle) but then subsequently degenerate and die [45]. Similarly, the expression of SV40 T antigen by the rhodopsin promoter causes photoreceptor degeneration, again associated with cell cycle reactivation and DNA synthesis [46].

This report represents the first *in vitro* model for the initiation of cell-cycle mediated neurodegeneration that mimics several features of AD neurodegeneration (Fig. 4). Specifically, reentry into the cell cycle is associated with DNA replication and tau phosphorylation/conformational changes. Obviously, we would anticipate that other approaches to promote cell cycle re-entry (e.g., SV40T, overexpression of CDK family members or transcription factors) would induce the same phenotype in primary neurons. In any case, the availability of the *in vitro* oncogenic model presented here will allow us to focus on early steps leading to neurodegeneration. This, in turn, might allow us to bring the large arsenal of anti-proliferative compounds developed over the last decades to bear on the problem of neurodegeneration [47].

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