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Downregulation of extracellular signal-regulated kinase 1/2 activity by calmodulin KII modulates $p21^{Cip1}$ levels and survival of immortalized lymphocytes from Alzheimer's disease patients

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ABSTRACT

Previously, we reported a Ca^{2+}/calmodulin (CaM)-dependent impairment of apoptosis induced by serum deprivation in Alzheimer's disease (AD) lymphoblasts. These cell lines showed downregulation of extracellular signal-regulated kinase (ERK)1/2 activity and elevated content of p21 compared with control cells. The aim of this study was to delineate the molecular mechanism underlying the distinct regulation of p21 content in AD cells. Quantitative reverse transcription polymerase chain reaction analysis demonstrated increased p21 messenger RNA (mRNA) levels in AD cells. The ERK1/2 inhibitor, PD98059, prevented death of control cells and enhanced p21 mRNA and protein levels. The CaM antagonist, calmidazolium, and the CaMKII inhibitor, KN-62, normalized the survival pattern of AD lymphoblasts by augmenting ERK1/2 activation and reducing p21 mRNA and protein levels. Upregulation of p21 transcription in AD cells appears to be the consequence of increased activity of forkhead box O3a (FOXO3a) as the result of diminished ERK1/2-mediated phosphorylation of this transcription factor, which in turn facilitates its nuclear accumulation. Murine double minute 2 (MDM2) protein levels were decreased in AD cells relative to control lymphoblasts, suggesting an impairment of FOXO3a degradation.

1. Introduction

Alzheimer's disease (AD) is a late-onset human neurodegenerative disorder marked by a progressive dementia and a spectrum of behavioral alterations. Whereas the hallmarks of AD, neurofibrillary tangles and amyloid-β–containing plaques are well established, the cause of neuronal loss remains largely elusive. Cumulative evidence has associated the aberrant re-expression of some cell cycle regulatory proteins with neuron vulnerability and neurodegeneration in AD (McShea et al., 2007; Webber et al., 2005). Cell cycle re-entry appears to represent an early and critical event in AD, leading to the development of AD-related pathology such as hyperphosphorylation of tau and amyloid-β deposition and ultimately inducing neuronal cell death (Bonda et al., 2010; Lee et al., 2009). It is believed that certain neurons are able to reactivate cell cycle activity in response to different triggers of neuronal apoptosis, including the withdrawal of growth factors (Park et al., 1998) and other detrimental factors (Kruman et al., 2002; Verdaguer et al., 2002; Zhu et al., 2007). Abnormal cell cycle re-entry leads to neuronal death, however, in AD, the activation of the neuronal cell cycle might result in apoptosis avoidance (Jellinger, 2006; Raina et al., 2000), allowing the cells to arrest in G2, accumulating oxidative damage which in turn would induce its death according to the two-hit hypothesis (Moh et al., 2011; Zhu et al., 2004).

Cell cycle dysregulation is not restricted to neurons, because peripheral cells from AD patients such as lymphocytes or fibroblasts have been shown to display cell cycle-related alterations (Bialopiotrowicz et al., 2011; de las Cuevas et al., 2003; Nagy et al., 2002; Stieler et al., 2012; Tatebayashi et al., 1995; Zhang et al., 2003). It appears therefore that cell cycle disturbances represent a systemic aspect of the disease. Though very limited data exist on the contribution of aberrant cell cycle in lymphocytes to the clinical

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phenotype of AD, there is no doubt that peripheral cells from patients provide convenient material to study cell cycle-related events associated with neurodegeneration.

Previous work from our laboratory indicated that immortalized lymphocytes from AD subjects show enhanced proliferative activity (de las Cuevas et al., 2003; Muñoz et al., 2008) and more resistance to serum withdrawal-induced apoptosis in a Ca^{2+}/calmodulin (CaM)-dependent manner (Bartolome et al., 2007; de las Cuevas et al., 2005). It was also shown that both CaM content and activity were enhanced in AD lymphoblasts (Esteras et al., 2012). Two cell cycle regulatory proteins, the cyclin-dependent kinase (CDK) inhibitors $p27^{Kip1}$ and $p21^{Cip1}$, from now on p27 and p21, are ultimately responsible for the enhanced proliferation and increased resistance to cell death, respectively. Whereas downregulation of p27 induces the enhanced proliferative response of immortalized lymphocytes from AD patients (Muñoz et al., 2008), upregulation of p21 seems to help AD cells to escape from serum deprivation-induced apoptosis (Bartolome et al., 2010).

A number of recent studies pointed out that in addition to being an inhibitor of cell proliferation, p21 might protect cells from apoptosis (Gartel, 2009). For example, it has been reported that upregulation of p21 blocked the oxidative stress-induced death of human myeloma U266 cells (Kim et al., 2001) and rendered resistance to chemotherapy drugs in other types of cancer cells (Gareau et al., 2011). Thus the increase in p21 cellular content in AD lymphoblasts might confer these cells a survival advantage.

This work was undertaken to unravel the molecular mechanisms underlying the Ca^{2+}/CaM-mediated resistance of AD lymphoblasts to cell death induced by serum deprivation and the role of Ca^{2+}/CaM in the regulation of p21 cellular levels. We tested the hypothesis that enhanced Ca^{2+}/CaM signaling would protect AD cells from apoptosis via upregulation of p21. Our data suggest that CaMKII indirectly regulates the forkhead box O3a (FOXO3a)-mediated activation of p21 transcription by preventing its phosphorylation mediated by extracellular signal-regulated kinase (ERK)1/2 and subsequent translocation and degradation of this transcription factor via an murine double minute (MDM2)-mediated ubiquitin-proteasome pathway.

2. Methods

2.1. Materials

All components for cell culture were obtained from Invitrogen (Carlsbad, CA, USA). Calmidazolium (CMZ), KN-62, pifithrin-α (PFT-α), and Serum Replacement were obtained from Sigma Aldrich (Alcobendas, Spain). LY294002 and PD98059 were obtained from Calbiochem (Darmstadt, Germany). Polyvinylidene difluoride membranes for Western blot analysis were purchased from Bio-Rad (Richmond, CA, USA). Rabbit anti-human polyclonal antibodies, such as FOXO3a, ERK1/2, CaMKII, p53, and phospho-p53 (Ser 15), and rabbit anti-human monoclonal antibodies such as p21 and phospho-ERK1/2 were from Cell Signaling. Rabbit anti-human phospho-CaMKII (Thr286) (sc-12886-R), and mouse anti-human α-tubulin (sc-23948) were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Rabbit anti-human β-actin antibody was from Sigma. Rabbit anti-human MDM2 was from Millipore (Darmstadt, Germany). Mouse anti-human Lamin B was from Calbiochem. The enhanced chemiluminiscence system was from Amersham (Uppsala, Sweden). All other reagents were of molecular grade.

2.2. Cell lines

A total of 34 patients from the department of Neurology of the University Hospital Doce de Octubre (Madrid, Spain) with a diagnosis of probable Alzheimer according to National Institute of Neurological and Communicative Diseases and Stroke-Alzheimer's Disease and Related Disorders Association criteria were used in this study. Of the 34 patients, 20 had mild AD (*Diagnostic and Statistical Manual of Mental Disorders*-III-R, Mini Mental State Examination [MMSE] score between 18 and 24), 7 had moderate AD (MMSE: 10–18), and 7 had severe AD (MMSE: <10). A group of 23 nondemented age-matched individuals was used as control. In all cases peripheral blood samples were obtained after written informed consent of the patients or their relatives. A summary of demographic characteristics of all subjects enrolled in this study is reported in Table 1.

Establishment of lymphoblastic cell lines was performed in our laboratory as previously described by infecting peripheral blood lymphocytes with the Epstein-Barr virus (Koistinen, 1987). Cells were grown in suspension in T flasks in an upright position, in approximately 8 mL of RPMI-1640 (Gibco, BRL) medium that contained 2 mM L-glutamine, 100 μg/mL penicillin/streptomycin and, unless otherwise stated, 10% (vol/vol) fetal bovine serum (FBS) and maintained in a humidified 5% CO_2 incubator at 37 °C. Fluid was routinely changed every 2 days by removing the medium above the settled cells and replacing it with an equal volume of fresh medium.

2.3. Cell survival assay

The cell suspension was mixed with a 0.4% (wt/vol) Trypan Blue solution (Sigma), and the number of live cells was determined using a hemocytometer. Cells failing to exclude the dye were considered nonviable. In addition, apoptosis was characterized by chromatin condensation/fragmentation, as determined by cell fixation followed by 4′,6-diamidino-2-phenylindole (DAPI) staining and fluorescence microscopy examination.

2.4. Determination of cell proliferation

Cell proliferation was assessed by the 5-bromo-2′-deoxyuridine (BrdU) incorporation method, using an enzyme-linked immunoassay kit procured from Roche (Madrid, Spain). Cells (100,000 cells per well) were seeded in 96-well microtiter plates. Four hours before the end of the interval of measurement, BrdU (10 μM) was added. The cells were fixed with precooled 70% ethanol for 30 minutes at −20 °C and incubated with nucleases following manufacturer's recommendations. Cells were then treated for 30 minutes at 37 °C with peroxidase-conjugated anti-BrdU antibody. The excess of antibody was removed by washing the cells 3 times, followed by the addition of substrate solution. Absorbance was measured at 405 nm with a reference wavelength of 490 nm.

2.5. Preparation of whole-cell extracts and subcellular fractionation

To prepare whole-cell extracts, cells were harvested, washed in phosphate-buffered saline (PBS) and then lysed in ice-cold buffer (50 mM Tris pH 7.4, 150 mM NaCl, 50 mM NaF, 1% Nonidet P-40),

Table 1
Summary of study population

	Control, $n = 23$	AD, $n = 34$
Age, y	75 ± 1	76 ± 5
Age range, y	(60–83)	(59–89)
Sex		
Male	8	15
Female	15	19

Key: AD, patients with a diagnosis of probable Alzheimer's disease; Control, healthy control individuals, no sign of neurological disease.