Impaired apoptosis in lymphoblasts from Alzheimer’s disease patients: Cross-talk of Ca\textsuperscript{2+}/calmodulin and ERK1/2 signaling pathways

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Abstract. We have analyzed the intracellular signals that allow lymphoblasts from Alzheimer’s disease (AD) patients to escape from serum deprivation-induced apoptosis. The following observations suggested that modulation of ERK1/2 activity by Ca\textsuperscript{2+}/calmodulin (CaM) is involved in preventing apoptosis: (i) ERK1/2 activity seems to support lethality in control cells, as PD98059, the inhibitor of the activating MEK prevented cell death; (ii) control cells show a persistent and higher stimulation of ERK1/2 than that of AD cells in the absence of serum; (iii) CaM antagonists have no effects on control cells, but sensitize AD cells to death induced by serum withdrawal and increased ERK1/2 phosphorylation, and (iv) no apoptotic effects of CaM antagonists were observed in AD cells treated with PD98059. These results suggest the existence of an activation threshold of the ERK1/2 pathway setting by Ca\textsuperscript{2+}/CaM-dependent mechanisms, which appears to be the critical factor controlling cell survival or death decision under trophic factor withdrawal.

Keywords. Alzheimer’s disease, lymphocytes, cell survival, Ca\textsuperscript{2+}/calmodulin, ERKs.

Introduction

Evidence has been accumulating that some neurons degenerate via apoptotic pathways in Alzheimer’s disease (AD). Apoptosis and cell cycle deregulation have been linked with the recurrence of certain types of neuronal cell death. The interpretation of these findings is that a differentiated cell like the neuron is committed to the permanent cessation of cell division, so if for any reason it is forced to enter the cell cycle it dies. Strong support for the role of cell cycle events in neuronal loss in AD comes from the observation that AD neurons contain multiple markers spanning different phases of cell cycle [1–4]. Furthermore, it has been demonstrated [5] that a significant fraction of the hippocampal pyramidal and basal forebrain neurons have fully or partially replicated four independent loci of three different chromosomes. These anomalies were not found in unaffected regions of AD brains or in the hippocampus of non-demented age-matched controls. Moreover, cell cycle regulatory deficit is not restricted to neurons in AD patients [6–8].
Although AD is considered a neurological disease, changes in tissues other than brain in AD patients have been reported, suggesting that it is also systemic, and therefore peripheral tissues such as skin fibroblasts and lymphocytes have been used as a tool to investigate systemic derangement in various neuropsychiatric disorders [9]. Epstein-Barr virus (EBV) infection in vitro causes transformation of B cells and generates B lymphoblastoid cell lines (LCLs) [10]. These LCLs retain the phenotype and functions of mature B cells [11]. LCLs have been widely used as models in various biological and medical studies [12]. Previous work from this laboratory, using EBV-immortalized lymphocytes from late-onset AD patients, demonstrated a Ca\textsuperscript{2+}/calmodulin (CaM)-dependent stimulation of cell proliferation and survival of AD lymphoblasts compared with age-matched non-demented donors [13–15]. The enhanced proliferative activity of the AD cell lines was associated with a high degree of phosphorylation of pRb family proteins and increased activity of the E2F transcription factor, while the Ca\textsuperscript{2+}/CaM-induced increased survival of AD cells was accompanied by diminished NF-xB-DNA binding activity [15].

This work was undertaken to further study the molecular mechanisms involved in the distinct Ca\textsuperscript{2+}/CaM-mediated regulation of cell survival in AD lymphoblasts. Considering the fact that some neuronal populations can survive the accumulating oxidative challenges and degenerative process during the development of AD, an understanding of the molecular mechanisms that can decrease the vulnerability of neurons and thus increase their resistance to stress conditions are of great interest. The results presented here show that lymphoblasts from AD patients are more resistant than those of non-demented subjects to apoptosis induced by serum starvation. The protective mechanism involves an impairment in the Ca\textsuperscript{2+}/CaM-dependent modulation of ERK1/2 signaling pathway and is accompanied by changes in Bcl-2/Bax ratio and caspase activity.

Materials and methods

Materials. All components for cell culture were obtained from Invitrogen (Barcelona, Spain). Serum replacement was obtained from Sigma-Aldrich (Alcobendas, Spain). The kinase inhibitors PD98059, SB202190, LY294002, and the caspase inhibitor benzoyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) were obtained from Calbiochem (Darmstadt, Germany). Radioactive compounds were purchased from Amersham (Uppsala, Sweden). Polyvinylidene fluoride (PVDF) membranes for Western blots were purchased from Bio-Rad (Richmond, CA). Rabbit polyclonal antibodies against human phospho-Akt (Ser473), phospho-ERK1/2 (Thr202/Tyr204), total ERK1/2, p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), and goat polyclonal antibody anti-total Akt were obtained from Cell Signaling (Beverly, MA). Mouse anti-human Bel-2 (100) Ab (SC-509) and rabbit anti-human Bax (N-20) pAb (SC-493) were from Santa Cruz Biotechnologies (Santa Cruz, CA). The enhanced chemiluminescence (ECL) system was from Amersham. 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), calmidazolium (CMZ), and the CAMKII inhibitor KN-62 were obtained from Calbiochem. All other reagents were of molecular biology grade. Collection of peripheral blood and isolation of mononuclear cells. Peripheral blood (15 ml) was collected by venipuncture from seven AD patients and seven age-matched non-demented individuals. The AD cases were all considered to have sporadic late-onset AD (onset of symptoms >65 years; family history negative for neuropsychiatric disorders). Peripheral blood mononuclear cells (PBMCs) were isolated on Lymphoprep™ density-gradient centrifugation according to the instructions of the manufacturer (Axis-Shield Po CAS, Oslo Norway). Cells were washed twice with phosphate-buffered saline (PBS), counted, and resuspended at the desired concentration. PBMCs were stimulated with a B cell mitogen ( pokeweed mitogen, PWM). B cells were prepared by magnetic sorting using a B cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Briefly, PBMCs that were harvested from the irradiated blood were washed twice in PBS and incubated with anti-CD19 for 15 min at 4°C. Unlabeled cells were then isolated by elution from magnetic columns. B cells (~2×10\textsuperscript{6} cells) were incubated in 0.2 ml RPMI for 3 days in the absence of serum and mitogens. In all cases, peripheral blood samples were taken after written informed consent of the patients or their relatives.

Cell lines. Twenty patients diagnosed in the department of Neurology of the University Hospital Doce de Octubre (Madrid, Spain) as probable AD cases according to NINCDS-ADRDA (National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer’s Disease and Related Disorders Association) criteria were used in this study. The average age of onset of the disease was 74±2 years. The frequency of the ApoE 4 allele was found to be 3% in the control group and 39% in the AD group in agreement with values previously reported for the normal and AD population in Spain [16], and were consistent with the late-onset form of AD. A group of 20 non-demented age-matched individuals was used as control.

Establishment of LCLs was performed in our laboratory as previously described [17], by infecting peripheral blood lymphocytes with the EBV [18]. Cells were grown in suspension in T flasks in an upright position, in approximately 10 ml RPMI 1640 (Gibco, BRL) medium that contained 2 mM L-glutamine, 100 mg/ml penicillin/streptomycin and, unless otherwise stated, 10% (v/v) fetal bovine serum (FBS) and maintained in a humidified 5% CO\textsubscript{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.

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 (5000 cells/well) were seeded in 96-well microtiter plates. Four hours prior to the end of the interval of measurement, BrdU (10 μM) was added. The cells were then treated for 30 min at 37°C with peroxidase-conjugated anti-BrdU antibody. Excess antibody was removed by washing the cells three times, followed by the addition of substrate solution. Absorbance was measured at 405 nm with a reference wavelength of 492 nm using a microplate reader.

MTT colorimetric survival assay. Active mitochondria of living cells can cleave MTT to produce formazan, the amount of which is directly proportional to the living cell number. Cell survival was assessed essentially as described [19]. Cells were incubated with 1 mg/ml MTT in a reaction volume of 200 μl. After the incubation DMSO was added to dissolve formazan crystals. Dye absorbance in viable cells was measured at 570 nm with 630 nm as a reference wavelength. Cell survival was estimated as the percentage of the