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Original Article

Distinct Regulation of Cell Cycle and Survival in Lymphocytes from Patients with Alzheimer's Disease and Amyotrophic Lateral Sclerosis

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Abstract: Alterations in cell cycle progression seem to be associated with neuronal death in Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS). We previously reported disturbances in the control of cell survival/death fate in immortalized lymphocytes from AD patients. These cell cycle dysfunction and impaired apoptosis were considered systemic manifestations of AD disease. The purpose of this study was to evaluate whether these abnormalities are characteristic of AD, or they may be seen in other neurodegenerative disorders such ALS. Our results indicate that alterations in signaling molecules, Akt and ERK1/2, and in the cyclin-dependent kinase complex inhibitors (CDKis) p21^{Cip1} and p27^{Kip1} are detectable in lymphoblasts from AD patients, but not in ALS patients, suggesting that these variables may be considered for the development of biomarkers of AD. However, lymphocytes from ALS patients do not represent a useful model to study cell cycle-related events associated with neurodegeneration of motoneurons.

Keywords: Alzheimer's disease, amyotrophic lateral sclerosis, lymphocytes, cell proliferation, apoptosis, p21, p27, PI3K/Akt, ERK1/2

Introduction

Neuronal death is involved in the onset of irreversible manifestations of Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS). In AD brain, neuronal loss occurs in the memory system of the association cortex, whereas in ALS the upper and lower motor neurons are affected. Although AD and ALS differ in important ways, they also have common pathogenic features, including neuroinflammation, and oxidative and mitochondrial dysfunction leading to apoptosis.

There is increasing evidence suggesting that regulatory proteins of cell cycle progression are also involved in the pathogenesis of neurodegenerative disorders and in the apoptotic death of injured neurons. Cell cycle disturbances have been observed in a number

of neurological diseases including AD [1, 2] and ALS [3, 4]. In these studies, it has been suggested that cell cycle signaling might affect neuronal death pathway. The cell cycle is associated with the phase specific expression or modification of defined sets of regulatory genes that control proliferation, differentiation or entry into a quiescent state [5]. However, re-entry of quiescent, terminally differentiated neurons into the cell cycle may result in a mitotic catastrophe and cell death [6-9].

Previous work demonstrated that cell cycle regulatory deficit is not only restricted to neurons of AD. It has also been observed in lymphocytes or fibroblasts of AD [10-13], thus providing a useful tool to further study the involvement of cell cycle-related events in the pathogenesis of AD and for the search of treatment strategies. Moreover, lymphocytes from ALS subjects have been shown to exhibit

traits of the disease [14]; however, as far as we know the possible dysfunction of cell survival/death mechanisms has not been studied in peripheral cells from ALS subjects.

Lymphoblasts from AD patients exhibit an enhanced stimulation of proliferation and survival compared with that from control individuals [13, 15-17]. The enhanced proliferative activity of the AD cell lines was associated with a high degree of phosphorylation of Akt and downregulation of the inhibitors of the G1-S checkpoint of the cell cycle, p21 and p27, while the increased survival of serum-deprived AD cells was accompanied by diminished ERK1/2 activation. To investigate whether these abnormalities are characteristic of AD or they are common to neurodegenerative disorders, we generated lymphoblastoid cell lines from ALS patients and compared the cellular response to serum to that of AD lymphoblasts.

Our results indicate that alterations in signaling molecules, Akt and ERK1/2, and in the CDKi p21 and p27 are detectable in immortalized lymphocytes of patients with AD, but not patients with ALS, suggesting that these variables may be considered for the development of biomarkers of AD. However, lymphocytes from ALS patients do not represent a useful model to study cell cycle-related events associated with neurodegeneration of motoneurons.

Materials and Methods

Materials

Polyvinylidene fluoride (PVDF) membranes for western blots were purchased from Bio-Rad (Richmond, CA). Rabbit polyclonal antibodies (pAbs) against human phospho-Akt (Ser473), phospho-ERK1/2, total ERK1/2 were obtained from Cell Signaling (Beverly, MA), and pAbs such as rabbit anti-human p27 (sc-528) and p21 (sc-397), and goat anti-human total Akt (sc-1618) were from Santa Cruz Biotechnologies (Santa Cruz, CA). The enhanced chemiluminescence (ECL) system was from Amersham (Uppsala, Sweden). Tissue culture media and reagents were obtained from Invitrogen (Carlsbad, CA).

Cell Lines

20 patients diagnosed in the Department of

Neurology of the University Hospital Doce de Octubre (Madrid, Spain) of probable Alzheimer according to NINCDS-ADRDA (National Institute of Neurological and Communicative Diseases and Stroke-Alzheimer's Disease and Related Disorders Association) criteria [18]. Of the 20 patients, 7 had mild AD (DSM-III-R, Mini Mental State Examination (MMSE) score between 18-24), 5 had moderate AD (MMSE:10-18), and 8 had severe AD (MMSE:<10). 10 patients suffering from ALS (diagnosed based on the revised El Escorial criteria were used in this study [19]. A group of 20 healthy individuals was used as control. A summary of demographic and clinical characteristics of all subjects enrolled in this study is reported in **Table 1**.

Table 1 Summary of study population

	AD	ALS	HC
Age	75±2	63±3	71±2
Age range	59-89	46-79	43-82
Gender			
Male	9	5	8
Female	11	5	12
Total	20	10	20

HC, healthy control individuals, no sign of neurological disease; AD, patients with a diagnosis of probable AD; ALS, patients with a diagnosis of amyotrophic lateral sclerosis. Values are expressed as mean±SE.

All study protocols were approved by the Spanish Council of Higher Research Institutional Review Board and are in accordance with National and European Union Guidelines. In all cases, peripheral blood samples were taken after written informed consent of the patients or their relatives.

Establishment of lymphoblastic cell lines was performed in our laboratory as previously described [20] by infecting peripheral blood lymphocytes with the Epstein Barr virus (EBV) [21]. Cells were grown in suspension in T flasks in an upright position, in approximately 10 ml of RPMI-1640 (Invitrogen) medium that contained 2 mM L-glutamine, 100 µg/ml streptomycin and, unless otherwise stated, 10 % (v/v) fetal bovine serum (FBS) and maintained in a humidified 5% CO₂ incubator at 37°C. Fluid was routinely changed every two days by removing the medium above the settled cells and replacing it with an equal volume of fresh medium.

Determination of Cell Proliferation

Proliferation was determined by cell counting in a Neubauer chamber. EBV immortalized lymphocytes from control and AD individuals were seeded at an initial cell concentration of 1×10^6 cells/ml. Cells were enumerated everyday thereafter. Potential toxicity of the reagents used was routinely checked by trypan blue exclusion under inverted phase-contrast microscopy.

Preparation of Cell Extracts

To prepare whole cell extracts, cells were harvested and washed in PBS and then lysed in ice-cold lysis buffer (20mM Hepes pH 7.9, 25% glycerol, 0.4M NaCl, 50mM NaF, 1mM EDTA, 1mM EGTA, 1mM DTT), containing 1mM sodium orthovanadate, 1mM PMSF, 1mM sodium pyrophosphate and protease inhibitor complete mini mixture (Roche, Mannheim).

Western Blot Analysis

50-100 μ g of whole cell extracts were fractionated on a SDS polyacrylamide gel, and transferred to PVDF membrane (Immobilon-P). The amount of protein and the integrity of transfer were verified by staining with Ponceau-S solution (Sigma). The filters were then blocked with non-fat milk and incubated at 4°C overnight, with primary antibodies from Santa Cruz at the following dilutions: 1:500 anti-p27, 1:500 anti-p21, 1:1000 anti-phospho Akt, 1:1000 anti-Akt, 1:500 anti-phospho ERK1/2, 1:2000 anti-ERK1/2, and 1:2000 anti-actin. Signals from the primary antibodies were amplified using species-specific antisera conjugated with horseradish peroxidase (Sigma) and detected with a chemiluminiscent substrate detection system ECL (Amersham). The relative protein levels were determined by scanning the bands with a GS-800 imaging densitometer provided with the Quantity One 4.3.1. software from BioRad.

Statistical Analysis

Unless otherwise stated, all data represents mean \pm SE. Statistical analysis was performed on the Data Desk package (version 4.0) for Macintosh. Statistical significance was estimated by analysis of variance (ANOVA) followed by the Scheffe test for multiple comparisons. Differences were considered significant at a level of $p < 0.05$.

Results

Proliferation of Immortalized Lymphocytes from Control and AD or ALS Patients

Data in **Figure 1** confirms and extends our previous finding [13, 15, 17, 22] by showing that the serum-induced proliferation of lymphoblasts from AD patients is enhanced compared with that of cells from healthy controls. In contrast, no significant differences were observed between control and ALS lymphoblasts (**Figure 1**).

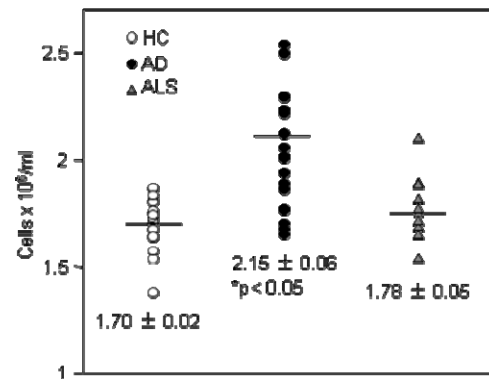


Figure 1 Scatter plot comparing proliferative activity between control and AD or ALS lymphoblasts. Immortalized lymphoblasts from control, AD, and ALS individuals were seeded at an initial density of 1×10^6 /ml and cultured for 3 days in RPMI medium containing 10% FBS. Everyday thereafter, samples were taken for cell counting. Values shown are from day 3 of culture, and are the mean \pm SE. Statistical significance was determined by ANOVA.

Since the mean age of ALS patients was lower than that of control or AD subjects, we investigated whether the proliferative activity of control cells was affected by aging. As shown in **Figure 2A**, there were no differences in proliferation of cells derived from subjects younger or older than 70 years old. Moreover, cell proliferation of lymphoblasts from AD patients was not affected by the degree of disease progression, as cell lines from mild, moderate or severe AD patients show similar rates of cell growth (**Figure 2B**).

Cellular Response to Serum Withdrawal in Control and AD or ALS Lymphoblasts

Data in **Figure 3** summarizes the cellular response to serum deprivation of all cell lines used in this study, derived from AD and ALS patients and control individuals. In agreement with previous observations [16], it is shown

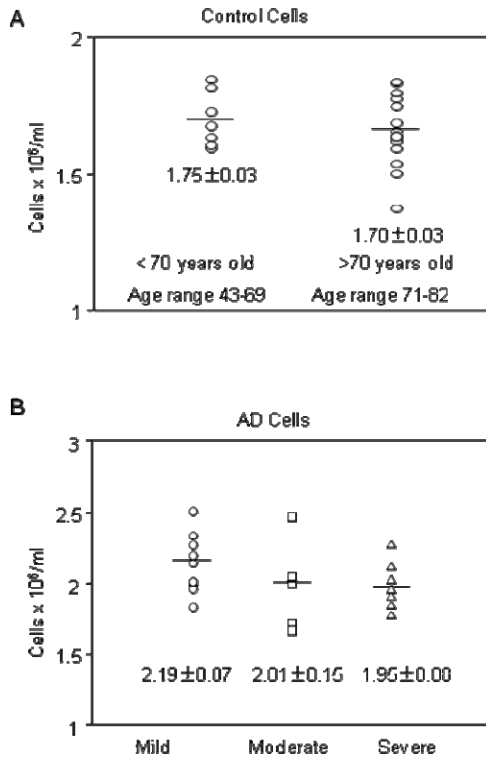


Figure 2 Aging does not affect the proliferative activity of control cells. Neither AD progression influences cell proliferation. **A.** Scatter plot comparing the influence of aging on the proliferation of lymphoblasts from control individuals. Immortalized lymphocytes from control individuals were seeded at an initial density of 1×10^6 /ml and cultured for 3 days in RPMI medium containing 10% FBS. **B.** lymphoblasts from mild, moderate or severe AD were cultured as above. Values shown are the mean \pm SE.

that AD lymphoblasts were more resistant to serum withdrawal-induced cell death than control cells. The cellular response of ALS lymphoblasts did not differ from that of control cells. In control, as well as in ALS cultures, more than 30% of cells died after 3-day period of serum starvation, whereas less than 10% of AD cells died during the same period of time.

Effect of PI3K/Akt and MAPK Activation on the Survival of Control and AD Lymphoblasts

We have recently reported that alterations of PI3K/Akt and ERK1/2 signaling pathways underlined the abnormal cellular response of AD lymphoblasts to the presence or the absence of trophic support [15, 16]. Enhanced proliferation of AD lymphoblasts was associated with increased activation of

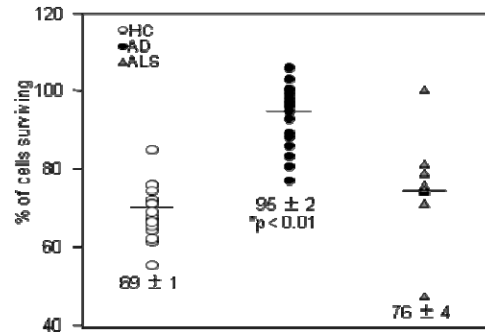


Figure 3 Scatter plot comparing cell survival following serum deprivation between lymphoblasts derived from control, AD or ALS patients. Immortalized lymphocytes from control, AD, and ALS individuals were seeded at an initial density of 1×10^6 /ml and incubated in serum-free RPMI medium for 72 hours. Cell viability was determined by Trypan blue exclusion under inverted phase-contrast microscopy, and expressed as percentage of the initial number of cells at day 0. Values shown are the mean \pm SE. Statistical significance was determined by ANOVA.

PI3K/Akt as monitored by the stimulation of phosphorylation of Akt [15]. Data in **Figure 4** shows, in consonance with the lack of stimulation of proliferation of ALS lymphoblasts, no changes in the cellular content of phosphorylated Akt, compared with levels of control cells. As expected, AD cells show increased phosphorylation of Akt relative to the levels of phospho Akt observed in control or ALS cells (**Figure 4**). PI3K/Akt signaling appears to regulate the G1-S checkpoint of cell cycle by downregulating the levels of two CDK inhibitors p27 and p21 [15, 17]. It was suggested that PI3K/Akt modulates the rate of protein degradation by the proteasome, after phosphorylation of specific residues of these proteins. Therefore, the levels of these proteins were determined in control, AD or ALS lymphoblasts. Only cells from AD patients exhibit significant lower content of p21 and p27 (**Figure 5**).

The ERK1/2 pathway seems to support lethality in immortalized lymphoblasts, as the specific inhibitor PD98059 prevented the serum withdrawal-induced cell death by apoptosis [16]. Moreover, it was shown that deprivation of trophic support induced a sustained increase in the ERK1/2 phosphorylation of significantly lower intensity in AD lymphoblasts [16]. In this work, we have compared the activation of this pathway in

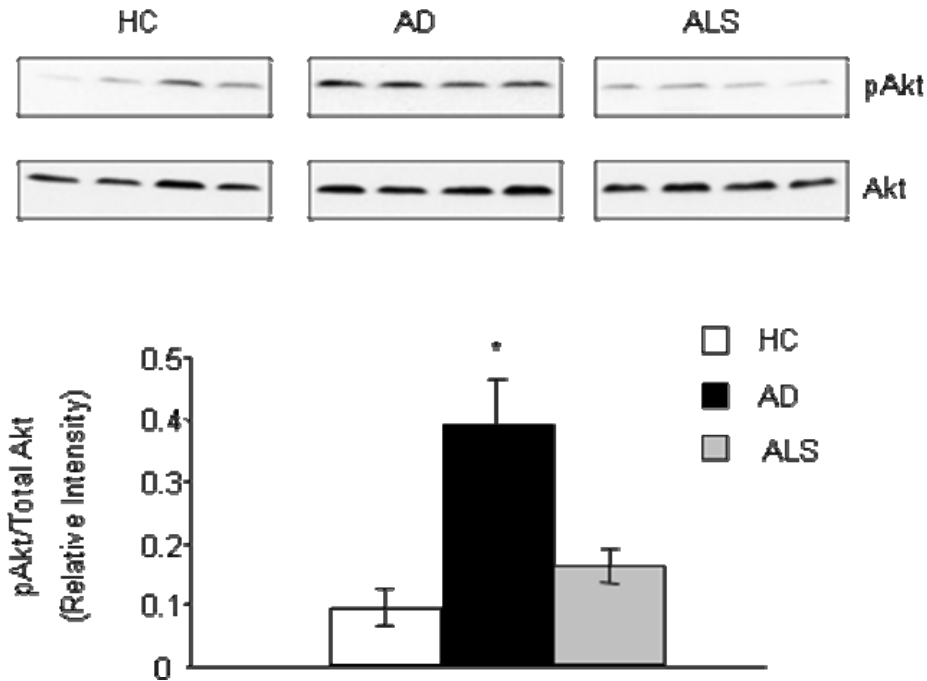


Figure 4 PI3K/Akt activation in control, AD and ALS lymphoblasts. Control, AD, and ALS lymphoblasts from 4 different individuals, were incubated in RPMI containing 10% FBS. Whole cell extracts were prepared 24 h thereafter, and were immunoblotted with antibodies anti-phospho-Akt (Ser473) and total Akt. The densitometric data represent the mean \pm SE. * $p < 0.05$, significantly different from control cells.

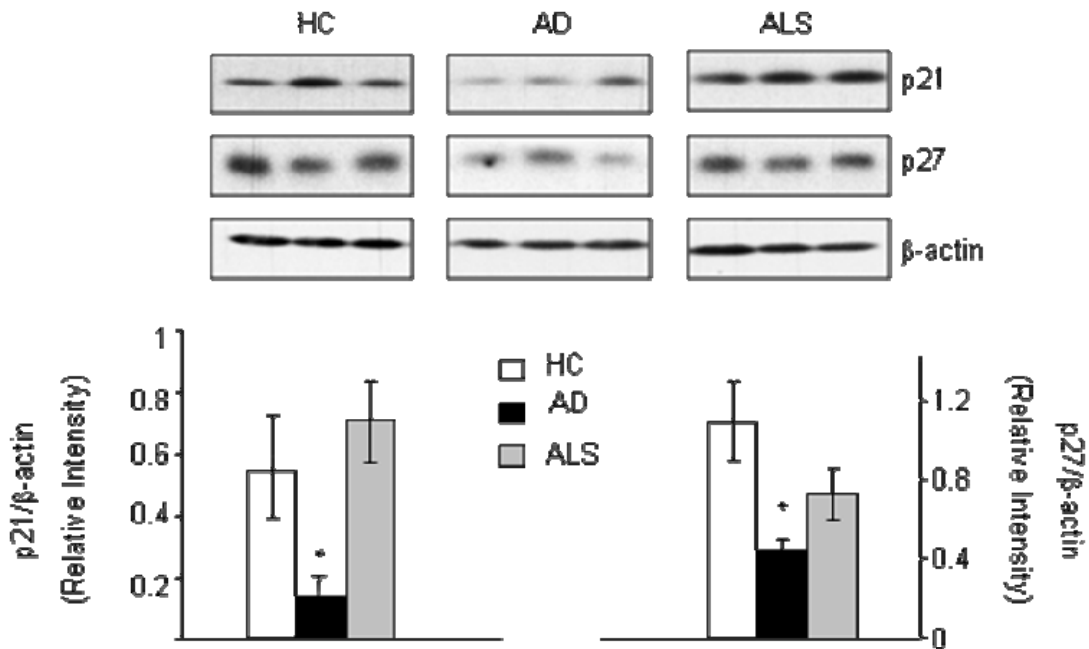


Figure 5 p21 and p27 protein levels in control, AD and ALS lymphoblasts. Lymphoblasts from control, AD or ALS subjects were seeded at an initial density of $1 \times 10^6 \times \text{ml}^{-1}$ and cultured for 24 h in RPMI medium containing 10% FBS. Thereafter aliquots were taken to prepare cell extracts. Western blots of p21 and p27 proteins in 3 different cell lines from control, AD, and ALS individuals are shown. Densitometric analysis of these proteins are shown below. Results are means \pm SE. * $p < 0.05$ significantly different from control cells.

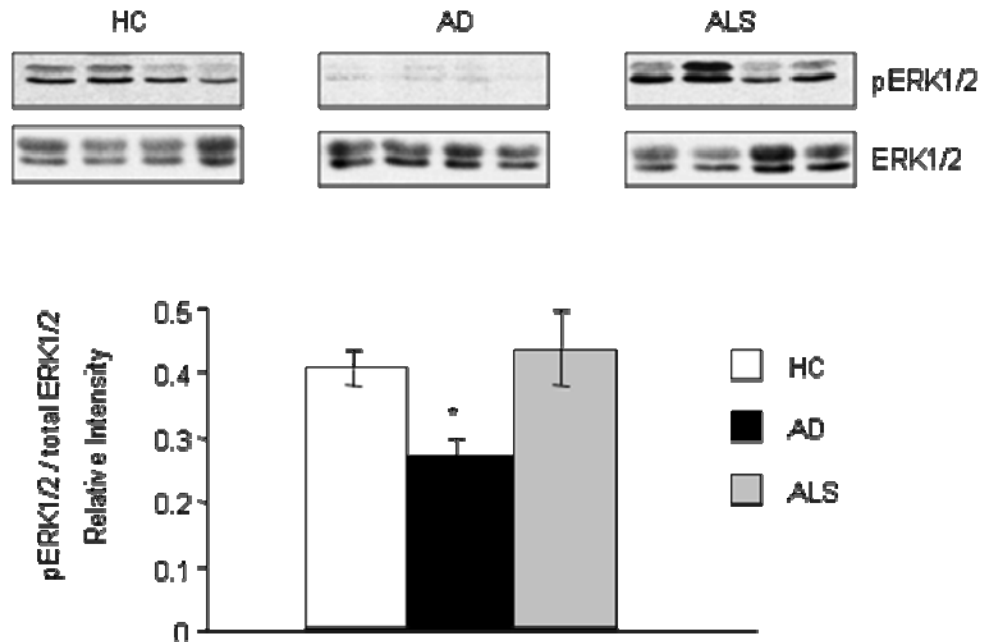


Figure 6 Activity of ERKs in serum deprived control, AD and ALS lymphoblasts. Lymphoblasts from control, AD, and ALS lymphoblasts were serum deprived for 72 h. Thereafter aliquots were taken to prepare cell extracts. The relative levels of activation of p42/p44 ERKs were assessed by western blot analysis using phospho-specific antibodies. The same membranes were then stripped and reprobed with antibodies against total ERKs. Representative Western blots in 4 different cell lines from control, AD and ALS patients are shown. The immunoreactive bands were quantified by densitometric analysis. Results shown below are the mean \pm SE of 8 different experiments. * $p < 0.05$ significantly different from control cells.

control, AD and ALS cells 72 hours after serum deprivation. The activity of the ERK1/2 pathway was assessed by western blotting, using phospho-specific antibodies. **Figure 6** shows the state of activation of the ERK1/2 pathway in control and AD or ALS lymphoblasts. As expected, after 72 hours of serum deprivation, the phosphorylation status of ERK1/2 in AD cells is significantly reduced compared with that of control or ALS lymphoblasts. Under these experimental conditions, ALS cells undergo apoptosis as control cells do (**Figure 3**). Thus, these observations suggest that the sustained, but reduced signaling through the ERK1/2 pathway in AD lymphoblasts protects them from the serum withdrawal-induced cell death.

Discussion

Recent work has highlighted the important role of neuronal vulnerability in the instigation and progression of neurodegenerative diseases including AD and ALS [23]. Moreover, a number of published studies indicated that cell cycle status significantly influence neuron

vulnerability and neurodegenerative pathways [8, 24-26]. The ability to control cell cycle has been considered a critical factor in preventing neurons entering a vulnerable state with high risk for instigation of neurodegenerative mechanisms [27].

Reports from our and other laboratories had presented evidences indicating that, while the predominant clinical expression arises from the SNC, AD and ALS have systemic expression at the cellular and molecular levels [12, 15, 28-31]. Although these alterations appear to have no clinical consequences outside the central nervous system, their parallel expression in peripheral cells and in the brain, provide a plausible pathophysiological model to explain partly the clinical manifestations. Of particular relevance to this work is the fact that dysfunction of cell cycle is a more general phenomenon affecting cells other than neurons in AD patients [10, 11, 13, 15, 22].

In the present study, we addressed the question whether easily peripheral cells from patients affected with ALS show altered cell

survival/death mechanisms comparable to the previously reported for lymphoblastoid cells lines from AD patients [13-16, 32]. We found that AD cells distinctively proliferate at higher rates and showed a decreased vulnerability to serum deprivation-induced cell death than EBV-immortalized lymphocytes from age-matched control individuals. In addition, we demonstrated that the cellular response to serum addition or deprivation was not affected by the viral transformation [15, 16], indicating that lymphoblastoid cell lines are a useful tool to study the involvement of cell cycle-related events in the neurodegenerative diseases.

Despite the fact that recent literature has shown data regarding systemic manifestation in ALS [30, 33], we did not observed changes in the proliferative capacity of lymphoblasts from ALS patients compared with that of cells from healthy individuals. In contrast, lymphoblasts from AD patients show an enhanced proliferative activity after serum stimulation, in agreement with previous reports from this laboratory [13, 15, 22]. It was reported that overactivation of PI3K/Akt signaling pathway in AD cells, was likely responsible for the enhanced proliferative activity by down-regulating cellular levels of the CDKis, p21 and p27 [15]. In contrast, no significant change in Akt activity was observed in ALS lymphoblasts. Accordingly, p21 and p27 levels were found to decrease only in cells from AD patients. This finding is not in agreement with earlier reports showing deregulation of CDKis associated with neurodegeneration in ALS [4, 34, 35]. A possible explanation for this divergence may be the use of peripheral cells versus motoneurons. At present, we cannot ascertain whether the lack of changes in cell cycle regulators in peripheral cells from ALS patients reflects the absence of cell cycle related events associated with neurodegeneration in ALS, or, on the contrary, cell cycle disturbances thought to occur in motoneurons [3], do not have systemic manifestations. In this regard, it is worth to mention a recent report showing no evidence for oxidative stress in fibroblast from ALS patients [36], despite the fact of impaired stress response in affected motoneurons [37].

Other distinct feature of AD cells is to be less vulnerable than control lymphoblasts to cell death induced by serum deprivation [16, 32]. A sustained, but lower, activation of ERK1/2 in AD cells, as compared with the control group

seems to protect AD lymphoblasts from death. We report here that lymphoblasts from ALS patients behave as control cells in response to serum withdrawal by showing similar phosphorylation of ERK1/2 and equal susceptibility to the induced cell death.

Collectively, our results show no major alterations in the proliferative capacity or vulnerability to serum deprivation-induced cell death in ALS lymphoblasts compared with cells from control individuals. These observations suggest that these cells do not represent a useful model to study cell cycle-related events associated with motoneurons degeneration.

On the other hand, our findings that cell cycle progression, cell survival and their molecular regulators are distinctively altered in lymphoblasts from AD patients add further support in favor of considering AD as a systemic disease, underlying as possible etiopathogenic mechanism altered responsiveness to cell activation agents. Considering that changes in the abundance of CDK inhibitors had also been detected in AD brain [38], and that perturbation in the activity of PI3K/Akt and ERK1/2 signaling pathways had been detected in AD brains [39-41], our results highlight the usefulness of peripheral cells from AD patients as potential surrogate for diagnosis and therapeutic monitoring of AD.

The cell cycle disturbances and alteration of the apoptotic response found in AD lymphoblasts did not correlate with progression of the disease. They seem to be early manifestations of the disease. This observation is in consonance with recent evidence in AD patients and in animal models [42, 43] indicating that cell cycle dysfunction is an early event in AD pathogenesis. Cell cycle proteins have been found in brains of individuals with mild cognitive impairment (MCI) [42], and cell cycle disturbances have also been reported in lymphocytes from MCI patients [11]. These observations suggest that cell cycle-induced death is a central mechanistic feature of AD, and therefore alterations in cell cycle/apoptosis regulatory proteins may serve as markers of AD disease.

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