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# Research Article

# Impaired apoptosis in lymphoblasts from Alzheimer's disease patients: Cross-talk of $Ca^{2+}$/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 $Ca^{2+}$/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 $Ca^{2+}$/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, $Ca^{2+}$/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].

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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 use 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 $Ca^{2+}$/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 $Ca^{2+}$/CaM-induced increased survival of AD cells was accompanied by diminished NF-κB-DNA binding activity [15].

This work was undertaken to further study the molecular mechanisms involved in the distinct $Ca^{2+}$/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 $Ca^{2+}$/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 benzyloxy-carbonyl-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 Bcl-2 (100) mAb (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 Sigma-Aldrich. 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 (Axix-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 were harvested from the interface and 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 (~$2\times10^5$ 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 Diseases 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 μg/ml penicillin/streptomycin and, unless otherwise stated, 10% (v/v) 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.

**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 fixed with precooled 70% ethanol for 30 min at –20°C and incubated with nucleases following manufacturer's recommendations. 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

value of untreated controls. In some experiments cell survival was determined by direct cell counting in a Neubauer chamber. Potential toxicity of the reagents used was routinely checked by trypan blue exclusion under inverted phase-contrast microscopy.

**Cell cycle analysis.** Exponentially growing cultures of cell lines were seeded at an initial concentration of $1\times10^6$ cells/ml. Cell cycle analysis was performed using a standard method [20]. Cells were fixed in 75% ethanol for 1 h at room temperature. Subsequent centrifugation of the samples was followed by incubation of cells in PBS containing 1 μg/ml RNase at room temperature for 20 min and staining with propidium iodide (PI; 25 μg/ml). Cells were analyzed in an EPICS-XL cytofluorimeter (Coulter Científica, Móstoles, Spain). Estimates of cell-cycle phase distributions were obtained by computer analysis of DNA content distributions. An apoptosis detection kit that measured phosphatidylserine (PS) was purchased from PharMingen (San Diego, CA). The assay was conducted following manufacturer's directions. Cells were analyzed for PS exposure/PI exclusion by staining with FITC-Annexin V and PI.

**Detection of DNA double-strand breaks using TUNEL apoptosis assay.** For detection of DNA strand breaks, the TUNEL (TdT-mediated dUTP nick end labeling) method was applied [21]. The terminal deoxynucleotidyl transferase method, using fluorescein-dUTP, was used to detect 3'-hydroxy ends in genomic DNA produced during apoptotic cell death. Cells were allowed to settle on polyornithine-coated slides. After serum starvation, cells were fixed with paraformaldehyde (4% in PBS) and labeled according to manufacturer's instructions. Cells were analyzed using a Nikon microscope and a digital camera.

**Preparation of whole cell extracts.** To prepare whole cells extracts, cells were harvested and washed in PBS and then lysed in ice-cold lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 50 mM NaF, 1% Nonidet P-40), containing 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM sodium pyrophosphate and protease inhibitor Complete Mini Mixture (Roche, Mannheim, Germany). The protein content of the extracts was determined by the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA).

**Immunoblotting analysis.** For Western blot analysis, 50–100 μg 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 membranes were then blocked with non-fat milk and incubated, overnight at 4°C, with primary antibodies from Santa Cruz at the following dilutions: anti-Bcl-2, 1:2000; anti-Bax, 1:2000; anti-phosphorylated ERK1/2, 1:500; anti-total ERK1/2, 1:2000; anti-phosphorylated, Akt 1:1000; anti-total Akt, 1:1000; anti-phosphorylated p38, 1:1000; and anti-p38, 1:500. Signals from the primary antibodies were amplified using species-specific antisera conjugated with horseradish peroxidase (Sigma) and detected with a chemiluminescent substrate detection system ELC (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 Bio-Rad.

**DNA constructs and transfections.** The pcDNA3 vector containing the constitutively active form of MEK1 was a gift from Dr. Carme Caelles (Biomedical Research Institute IRB-PCB, Barcelona, Spain). The pcDNA3 and pcDNA3-MEK1 were introduced into purified EBV-immortalized lymphocytes with the Nucleofector system (Amaxa Biosystems GmbH, Cologne, Germany). Typically, $2\times10^6$ cells were resuspended in 100 μl Cell Line Nucleofector Solution V and mixed with 5 μg plasmid DNA. To evaluate transfection efficiency, the same number of cells were transfected with the GFP-expressing construct pmaxGFP (Amaxa Biosystems). Nucleofections were performed using the Amaxa Nucleofector II device and the P-16 program. Cell viability and transfection efficiency were evaluated on $5\times10^5$ GFP-nucleofected cells after 16 h by PI staining and flow cytometry analysis. The transfection efficiency was approximately 20%. Serum deprivation was started 16 h postnucleofection. After 24 h, the cells were collected and processed for immunoblotting and cell viability assays.

**Statistical analysis.** Unless otherwise stated, all data represent means ± SE. Statistical analysis was performed on the Data Desk package (version 4.0) for Macintosh. Statistical significance was estimated by the Student's *t*-test or, when appropriated, by analysis of variance (ANOVA) followed by the Fischer's LSD test for multiple comparisons. Differences were considered significant at a level of $p<0.05$.

## Results

**Cellular response to serum withdrawal in control and AD lymphoblasts.** Data in Figure 1 summarize the cellular response to serum deprivation of cell lines, used in this study, derived from AD patients and age-matched control individuals. In agreement with previous observations [15], AD lymphoblasts were shown to be more resistant to serum withdrawal-induced cell death. In control cultures, more than 30% of cells died after a 4-day period of serum starvation, whereas less than 10% of AD cells died during the same period of time. To rule out the possibility that differences in the proliferative activity of control and AD lymphoblasts could mask the apparent increased survival of AD cells, we determined the rate of DNA synthesis by assessing the incorporation of BrdU in control and AD cells. As expected, Figure 2 shows that incorporation of BrdU by AD cells was significantly higher than by control cells in the presence of 10% FBS. However, when cells were incubated in the absence of trophic support, no differences in DNA synthesis and hence proliferation between control and AD cells could be detected (Fig. 2). Taken together, our results suggest that increased cell survival after serum withdrawal is a distinct feature of lymphoblasts from AD patients. Data in Table 1 show how the treatment with the CaM antagonist, calmidazolium (CMZ), sensitize AD cells to death triggered by the absence of trophic support, without changing the percentage of surviving control cells (Table 1). Similar results were obtained with KN-62, indicating the involvement of CaMKII in the CaM-mediated regulation of cell survival. Table 1 also shows that cell survival in AD cultures was significantly decreased by the presence of the extracellular $Ca^{2+}$ chelator EGTA, but was not affected by the intracellular $Ca^{2+}$ chelator BAPTA-AM.

**Cell death induced by serum deprivation in fresh isolated human B cells.** Although EBV-transformed LCLs have been widely used as models in various biological and medical studies [12] as they can be easily established and continue proliferating for many generations, we considered it important to study the cellular response to serum deprivation of non-transformed B lymphocytes of control and late-onset AD patients. To address this issue, PBMCs were obtained, and B cells were purified by magnetic sorting. As shown in Figure 2, B cells from AD patients were

**Table 1.** Influence of perturbations of $Ca^{2+}$/calmodulin signaling pathway on the cell death induced by serum withdrawal.

| Additions | Control % of surviving cells | AD |
|---|---|---|
| None | 66±4 | 95±3[a] |
| 1 μM CMZ | 60±3 | 61±6[b] |
| 1 μM KN-62 | 47±5 | 53±7[b] |
| 1 mM EGTA | 48±6 | 64±5[b] |
| 2.5 μM BAPTA-AM | 52±7 | 83±5[a] |

Lymphoblasts from control and Alzheimer's disease (AD) patients were incubated in serum-free RPMI medium for 3 days in the absence or in the presence of the CaM antagonist Calmidazolium (CMZ), the CaMKII inhibitor KN-62 or calcium chelators. The cells were then counted for their survival rate by trypan blue dye exclusion method. Results are expressed as % of the number of cells at day 0, and are the mean ± SE of six independent experiments. Statistical difference: [a] $p<0.05$ from lymphoblasts from control individuals, [b] $p<0.05$ from untreated AD lymphoblasts.

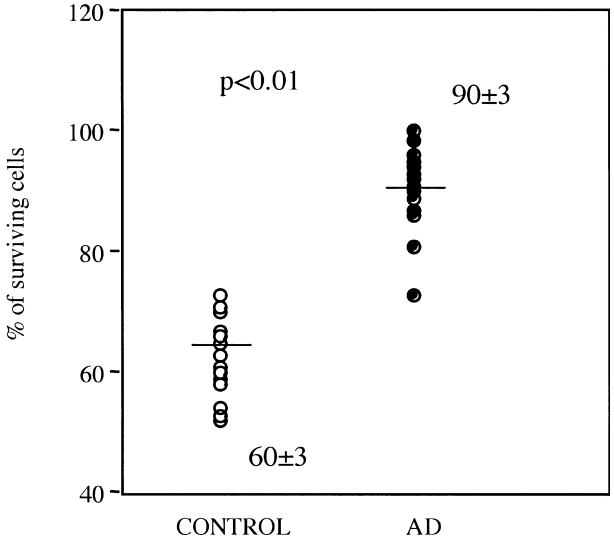


**Figure 1.** Scatter plot comparing cell survival following serum deprivation between lymphoblasts derived from control or Alzheimer's disease (AD) patients. Immortalized lymphocytes from control (open symbols) and AD individuals (filled symbols) were seeded at an initial density of $1\times10^6$/ml and incubated in serum-free RPMI medium for 96 h. 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. Statistical significance was determined by the Student's *t*-test.

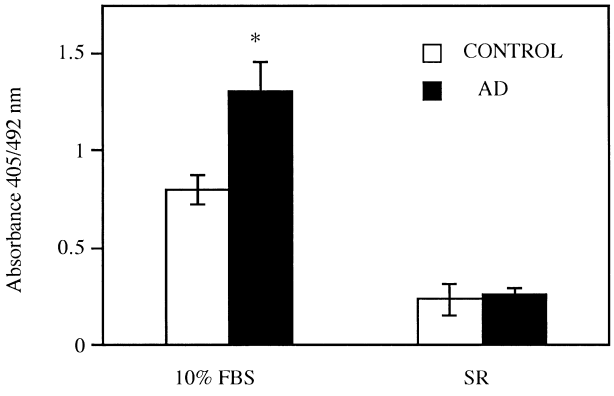


**Figure 2.** Proliferative response of control and AD lymphoblasts. Lymphoblasts from control and AD individuals (5000 cells/well) were seeded in 96-well plates in the presence of 10 % FBS or serum replacement (SR). After 72 h, cells were pulsed with 10 μM BrdU for 4 h. DNA synthesis was assessed by BrdU incorporation method according to the manufacturer's instructions. Proliferation was expressed as absorbance of stimulated minus that of non-stimulated cultures. Each bar represents the mean ± SE of three independent experiments performed in triplicate. * $p<0.05$ significantly different from control cells.

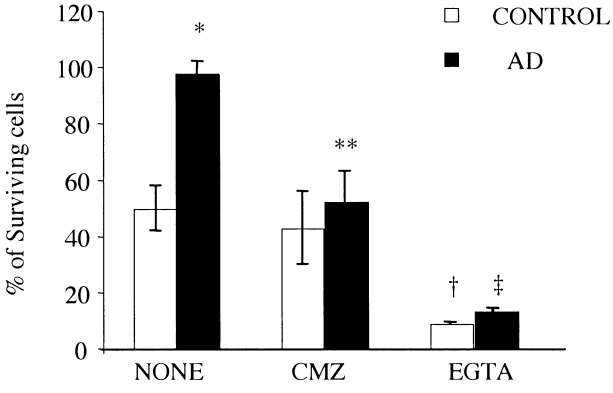


**Figure 3.** Serum deprivation-induced cell loss of human B cells from control and AD patients: Effects of calmidazolium (CMZ) and EGTA. B cells were isolated from peripheral blood mononuclear cells (PBMCs) of five late-onset AD patients and five age-matched control subjects. Lymphocytes were seeded at an initial density of $1\times10^6$/ml and incubated in serum-free RPMI medium for 72 h in the absence or in the presence of 1 μM CMZ or 1 mM EGTA. 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. Statistical significance was determined by ANOVA followed by the Fischer's LSD test for multiple comparisons. * $p<0.01$ significantly different from control cells, ** $p<0.01$ significantly different from AD cells without CMZ, † $p<0.05$ and ‡ $p<0.01$ significantly different from their respective control cells incubated without EGTA.

more resistant to serum deprivation than those from control individuals. As previously shown for EBV-lymphoblasts from AD patients, treatment of B cells with CMZ also results in increased sensitivity to death induced by the absence of trophic support (Fig. 3).

**Serum withdrawal induces apoptosis.** Because cell death can occur via apoptosis or necrosis, it was important to determine which mechanism was involved in serum-starved cells. Apoptosis is characterized by a number of morphological and biochemical events that distinguish it from necrosis. Serum withdrawal-induced cell death was therefore assessed by different procedures. These included (1) flow cytometric analysis of cellular DNA content, (2) detection of DNA breaks by TUNEL, and (3) flow cytometric analysis of phosphatidylserine exposure using Annex-

in V-FITC and PI. Figure 4a shows a representative experiment of cell cycle status after serum deprivation in control and AD lymphoblasts, demonstrating a higher accumulation of hypodiploid nuclei in control cultures than in AD cells. DNA fragmentation was further confirmed by TUNEL staining of control and AD lymphoblasts 4 days after withdrawal of trophic support. Figure 4b shows representative photomicrographs illustrating a higher number of TUNEL-positive cells in control cultures than in lymphoblasts from AD patients. Figure 5 confirmed the externalization of PS in control cells, a characteristic feature of cells entering apoptosis, although there were also 20 % necrotic cells. Treatment of cells with CMZ had no effect on lymphoblasts from control subjects (Fig. 5 upper right panel), but induced a 10-fold increase in the appearance of Annexin $V^+/PI^-$ cells in cultures from AD patients (Fig. 5 lower right panel).

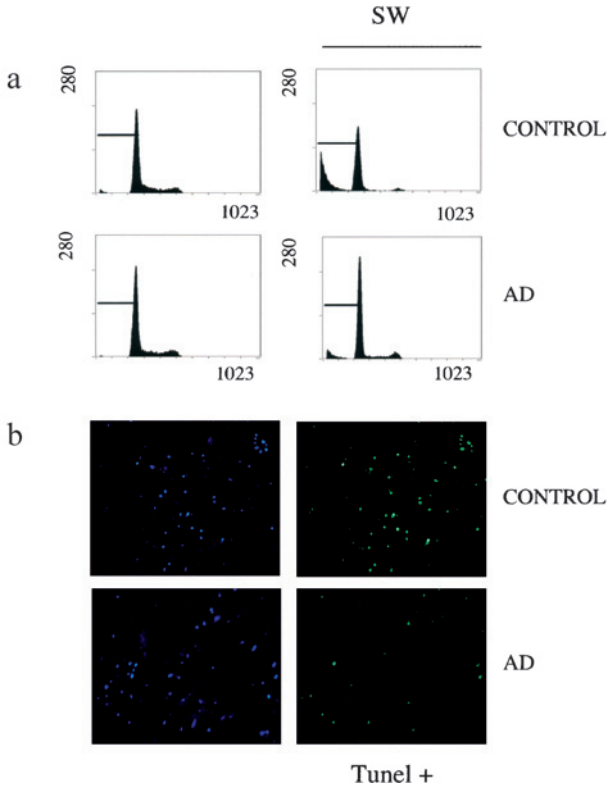


**Figure 4.** Effects of serum withdrawal on distribution of cells in cell cycle and DNA fragmentation of control and AD lymphoblasts. (*a*) Cells were seeded at an initial density of $1\times10^6$/ml and incubated in RPMI medium. After 96 h of serum deprivation, cells were harvested, fixed and analyzed by flow cytometry as described under Materials and methods. Labels show areas of the curve corresponding to sub $G_0/G_1$. (*b*) Control and AD cells were incubated in serum-free medium in polyornithine-coated chambers for 96 h. Cells were visualized by the TUNEL method. Cells positive for DNA strand breaks (TUNEL-positive nuclei) show green fluorescence on fluorescein staining. Representative experiments are shown. 4'6'-Diamino-2-penylindole (DAPI) signal was used to identify nuclei.

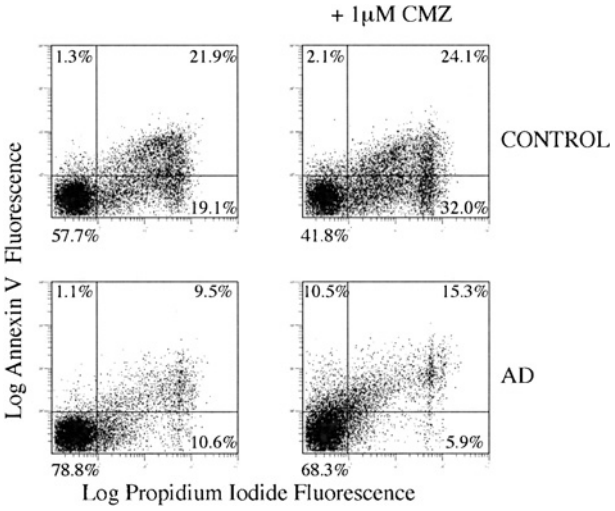


**Figure 5.** Flow cytometric analysis of apoptotic and necrotic cells from control and AD subjects resulting from serum deprivation in the absence or in the presence of CMZ. Representative results of flow cytometric measurements of the fraction of viable (bottom left), apoptotic (top left) and necrotic (top right) lymphoblasts from AD patients incubated for 72 h in serum-free RPMI medium, in absence (left panels) or in the presence of 1 μM CMZ (right panels). Cells were analyzed for phosphatidylserine exposure/propidium iodide (PI) exclusion by staining with FITC-Annexin V (y-axis) and PI (x-axis).

To address whether or not the activity of caspases is also essential for the observed increase in apoptosis after serum withdrawal, control and AD cells were treated with a general caspase inhibitor z-VAD-fmk. Figure 6a shows that this compound prevented apoptosis in control cells without affecting survival of lymphoblasts from AD patients. Bcl-2 and related proteins are important regulators of mitochondrial-mediated apoptosis [22]. To provide a mechanistic view of how serum withdrawal induced apoptosis, the protein expression levels of anti- or pro-apoptotic proteins, Bcl-2 and Bax, respectively, were determined by Western blotting. Figures 6b and c show a significant increase in AD lymphoblasts of Bcl-2/Bax ratio as compared with control cultures.

**Involvement of PI3-K/Akt and MAPK activation on cell survival of control and AD lymphoblasts.** In most cell types, the processes of survival and proliferation are dependent on the PI3-K/Akt and MAPKs (*i.e.*, ERK1/2 and p38), the downstream mediators in the signaling cascade activated via trophic ligand receptor interactions [23–25]. The potential participation of these signaling pathways was analyzed using pharmacological inhibitors. Cells were treated with LY294002, inhibitor of PI3-K/Akt [26]. Likewise, the involvement of MAPK pathways in the serum withdrawal-induced apoptosis was addressed using SB202190, a p38 MAP kinase inhibitor [27] and

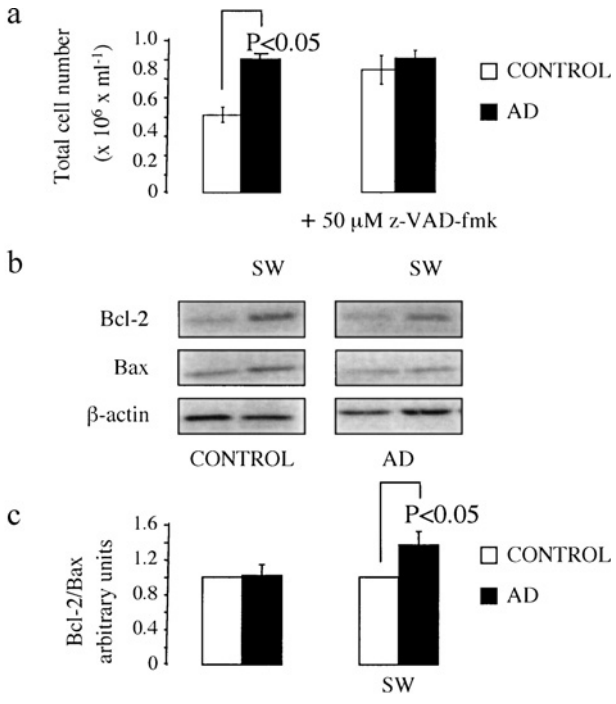


**Figure 6.** Serum withdrawal induced apoptosis. (*a*) Protective efficacy of the caspase inhibitor z-VAD-fmk on serum withdrawal-induced apoptosis. Lymphoblasts from non-AD and AD patients were incubated onto 96-well dishes, at an initial density of $1\times10^6$/ml, in serum-free RPMI medium (SW) for 72 h in the absence or in the presence of 50 μM of the broad-spectrum caspase inhibitor z-VAD-fmk. Cell viability was assessed by trypan blue exclusion. Six independent experiments, carried out with different cell lines, were carried out in duplicate. Values shown are the mean ± SE and expressed as percentage of the value of untreated controls. (*b*) Expression levels of Bcl-2 and Bax proteins from control and AD lymphoblasts. After 72 h of serum deprivation, cell extracts were prepared for Western blot analysis. To ensure equal loading of proteins, the membranes were reprobed with anti-β-actin. Representative immunoblots are shown. (*c*) Densitometric analysis of the ratio Bcl-2/Bax. Values shown represent the mean ± SE for eight independent experiments with cells derived from different individuals.

PD98059, an inhibitor of MEK 1/2, the upstream activator of ERK1/2 [28]. The effectiveness of PD98059 and LY294002 was confirmed by reduced phosphorylation of ERK1/2 or Akt, respectively (Fig. 7a). However, under our experimental conditions, phosphorylated p38 was below detection levels in our immunoblot assays even in the absence of SB202190. As shown in Figure 7b, treatment of control cells with SB202190 or LY294002, had no effect on cell survival after serum deprivation. The lack of effect of the PI3-K inhibitor in AD lymphoblasts contrasts with the inhibitory action of this drug on the enhanced proliferative response of AD cell lines to serum [29]. Treatment of AD cells with LY294002 prevented the down-regulation of the levels of the CDK inhibitor $p27^{kip1}$ induced by serum [29]. However, treatment of control cells with PD98059 prevented cell death induced by serum starvation (Fig. 7b). This inhibitor had no effect in AD cells, but prevented the effects of CMZ inducing apoptosis in these cell lines (Fig. 7b). These findings indicate that ERK1/2 pathway supports lethality in control cells, and that this mechanism is altered in a $Ca^{2+}$/CaM-dependent manner in AD cells.

**Role of serum on ERKs activation.** In an initial series of experiments, we examined the effect of serum on the activation of the ERK1/2 pathway in control and

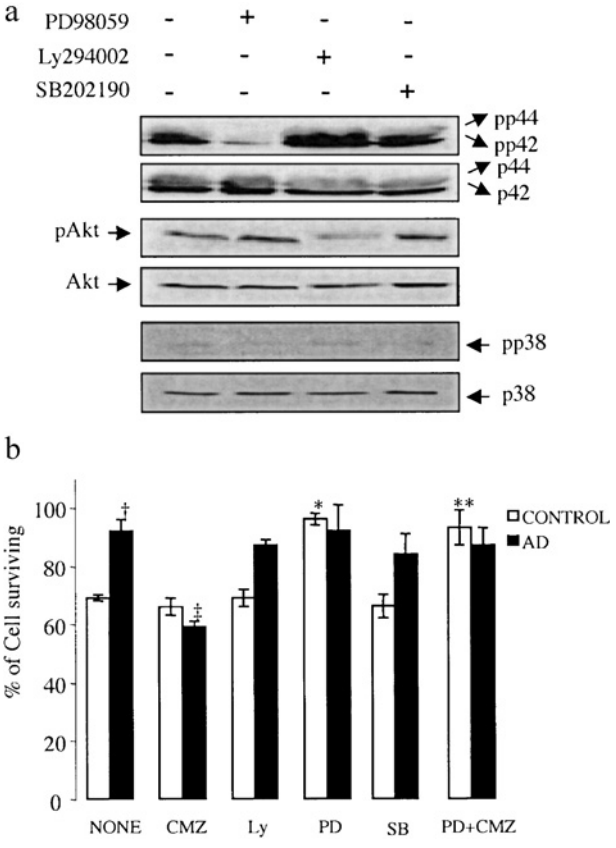


**Figure 7.** Effects of kinase inhibitors on serum withdrawal-induced loss of cell viability. (*a*) Lymphoblasts from control individuals were incubated at an initial density of $1\times10^6$/ml in serum-free RPMI medium, for 72 h in the absence or in the presence of 20 μM PD98059, 10 μM Ly294002, 10 μM SB202190, or 1 μM CMZ. The relative levels of activation of p42/p44 ERKs, p38 and Akt were assessed by Western blot analysis using phospho-specific antibodies. Representative immunoblots are shown. (*b*) Lymphoblasts from control and AD patients were incubated on 96-well plates in the absence and in the presence of different kinases inhibitors as above. Cell viability was assessed by measuring the MTT reduction and by counting cells excluding trypan blue. Independent experiments, with different cell lines, were carried out in duplicate. Values shown are the mean ± SE for three to nine experiments, and expressed as percentage of the value of untreated controls. Statistical significance was determined by ANOVA followed by the Fischer's LSD test for multiple comparisons. * $p<0.001$ and ** $p<0.05$ significantly different from control cells in the absence of inhibitors; † $p<0.001$ significantly different from control cells; ‡ $p<0.01$ significantly different from untreated AD cells.

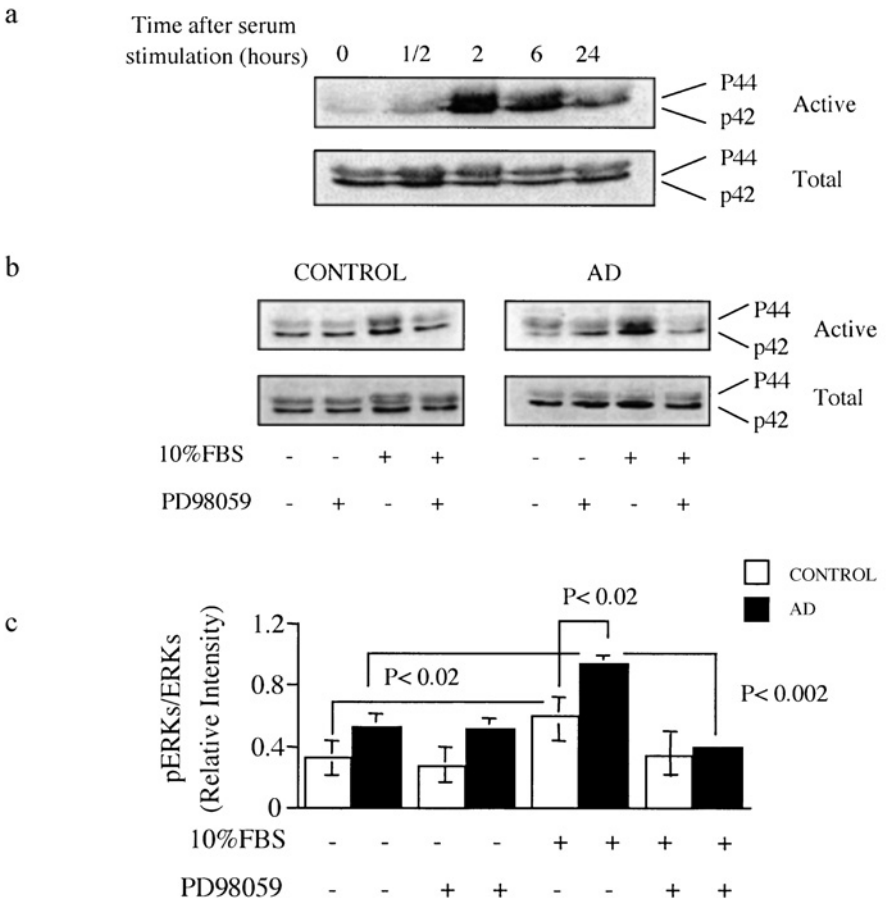


**Figure 8.** ERK1/2 pathway is transiently activated by serum in control and AD lymphoblasts. (*a*) Lymphoblasts from AD patients were seeded at an initial density of $1\times10^6$/ml in serum-free medium for 24 h. Then 10% FBS was added and cells were harvested at the time periods indicated. The relative 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. A representative immunoblot is shown. (*b*) Lymphoblasts from control and AD patients were seeded as above in the absence or in the presence of 10 μM PD98059, and then stimulated by 10% FBS for 1 h. (*c*) The immunoreactive bands were quantified by densitometric analysis. Results shown below are the mean ± SE of three to five determinations made in experiments using cells from different individuals. Statistical significance was determined by ANOVA followed by the Fischer's LSD test for multiple comparisons.

AD lymphoblasts. This was accomplished by Western blot analysis using phosphorylation-specific antibodies to detect levels of active (*i.e.*, phosphorylated ERK) *versus* phosphorylation-independent antibodies to quantitate the total for each of the kinases. Addition of 10% FBS to cell cultures resulted in a clear activation of ERK1/2 (Fig. 8a). The FBS-induced ERK1/2 phosphorylation is transient, the effect being maximum 2 h after serum addition and declined afterwards. The increase in the phosphorylated form of ERKs did not result from increased expression of ERKs as total ERKs levels were not altered (Fig. 8a). The addition of serum activated the ERK1/2 pathway in both control and AD, although the response was enhanced in lymphoblasts from AD patients (Fig. 8b, c). As expected, treatment of cells with PD98059 blocked the serum-induced ERK phosphorylation (Fig. 8b, c). These results correlate with the enhanced proliferation of AD cells following serum stimulation [15]. Similar response was obtained when untransformed lymphocytes from control and AD patients were stimulated with the plant lectin PWM, which preferentially activates B cells [30]. Figure 9 shows that 24 h after PWM stimulation, incorporation of BrdU by AD cells was higher than by control cells. The increased proliferation of AD lymphocytes was associated with enhanced phosphorylation of ERK1/2 (Fig. 9).

It is known from previous studies [31, 32] that ERK phosphorylation may produce different outcomes in the same cell type as proliferation or apoptosis, depending on the duration of ERK activation. For this reason, we studied the kinetics for ERK1/2 activation upon serum withdrawal. Figure 10a shows that deprivation of trophic support induced a sustained increase in the ERK1/2 phosphorylation that

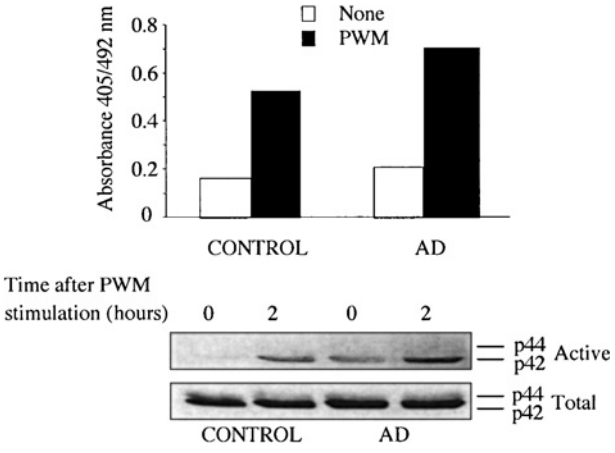


**Figure 9.** PWM–induced cell proliferation and ERK1/2 activation in PBMCs from control and AD individuals. PBMCs were isolated from two control individuals and two AD patients and kept in culture for 3 days. They were then resuspended in fresh medium at a density of $1\times10^6$/ml and cultured in the absence or in the presence of 5 μg/ml B cell mitogen, pokeweed mitogen (PWM) for 24 h. The proliferative response to PWM was determined by BrdU incorporation in the last 4 h. Results are the mean of two experiments performed in triplicate. Protein extracts for Western blot analysis were prepared 2 h after PWM stimulation. A representative immunoblot is shown.

lasted for at least 96 h in both control and AD cells, although a considerable reduced ERK1/2 activation was observed in lymphoblasts from AD patients (Fig. 10a, b). The lower response of ERK pathway to serum withdrawal in AD cells seems to protect them from the apoptosis induced by serum withdrawal. When control cells were treated with PD98059 to inhibit ERK1/2 phosphorylation, they became resistant against apoptosis (Fig. 7b). On the other hand, treatment of cells with either CMZ or the CaMKII inhibitor KN-62 produced an increase in ERK1/2 phosphorylation in AD cells without affecting significantly the phosphorylation status of ERK1/2 in control cells (Fig. 10c). Under these experimental conditions, AD cells underwent apoptosis as did control cells (Fig. 7b). Thus, these observations suggest that the enhanced signaling through the $Ca^{2+}$/CaM pathway in AD cells decreased the activation of ERK1/2 by serum deprivation, resulting in a sustained, but lower than in control cells, activation of ERK pathway compatible with cell survival. To further investigate this possibility, we transiently transfected AD cells with constitutively active MEK1, the upstream activator of ERK. pcDNA3 and pcDNA3-MEK1 vectors were introduced in AD lymphoblasts by nucleofection. Although the transfection efficiency was no higher than 20%, the increased expression of MEK1 was sufficient to activate ERK1/2 and sensitize AD cells to death in response to serum withdrawal (Fig. 11).

## Discussion

The results presented here indicate that lymphocytes from AD patients are less vulnerable to cell death induced by serum deprivation than those derived from age-matched control individuals (Figs 1 and 3, Table 1). A selective impairment of mechanisms involved in cell death in peripheral cells from AD patients has been previously reported [33, 34], although there are contradictory results as to whether cells from AD patients are more resistant [35] or more vulnerable to different cell stressors [36, 37].

Cell death induced by serum deprivation showed characteristics of apoptosis (Figs 4–6). The lower sensitivity of AD cells to trophic factor withdrawal was due to changes in the balance of pro- and anti-apoptotic proteins (Fig. 6).

AD cells treated with CaM antagonists undergo significant apoptosis in the absence of serum in the culture medium as they do in control cells (Fig. 5), suggesting a role of the $Ca^{2+}$/CaM signaling pathway in protecting AD cells from apoptosis. A role for CaM mediating cell survival has been previously reported in a number of cell types including neurons [38–40]. CaM antagonists, however, had no effect in control cells, suggesting a threshold for CaM activation as the survival signal. Our results are in consonance with previous reports indicating that CaM antagonists specifically resulted in apoptosis in tumorigenic mammary carcinoma cells, but did not affect normal mammary gland-derived epithelial cells [41]. $Ca^{2+}$/CaM regulation of cell survival appears to be mediated by CaMKII, as its specific inhibitor KN-62 mimics the effects of CaM antagonists. Therefore, it seems unlikely that CaM-dependent activities other than CaM kinases (*i.e.*, calcineurin) are recruited by serum withdrawal to accomplish its trophic effects in AD cells. In agreement with this observation, it was reported that inhibition of this kinase rendered resistant glioma cells sensitive to FAS-mediated apoptosis [42]. The protective effect of CaM against serum withdrawal-induced apoptosis requires extracellular $Ca^{2+}$, indicating that $Ca^{2+}$ influx is critical for survival under serum deprivation. In contrast, the presence of the intracellular $Ca^{2+}$ chelator, BAPTA, did not influence the survival of either control or AD cells; however, it has to be taken into account that the buffering capacity of BAPTA may be overwhelmed with time [43].

On the other hand, inhibition of ERKs protected control cells from serum withdrawal-induced apoptosis and blocked the apoptotic effects of CaM antagonists in AD cells (Fig. 7). Therefore, it seems that CaM has a critical role as mediator of cell survival by regulating the activity of the ERK pathway.

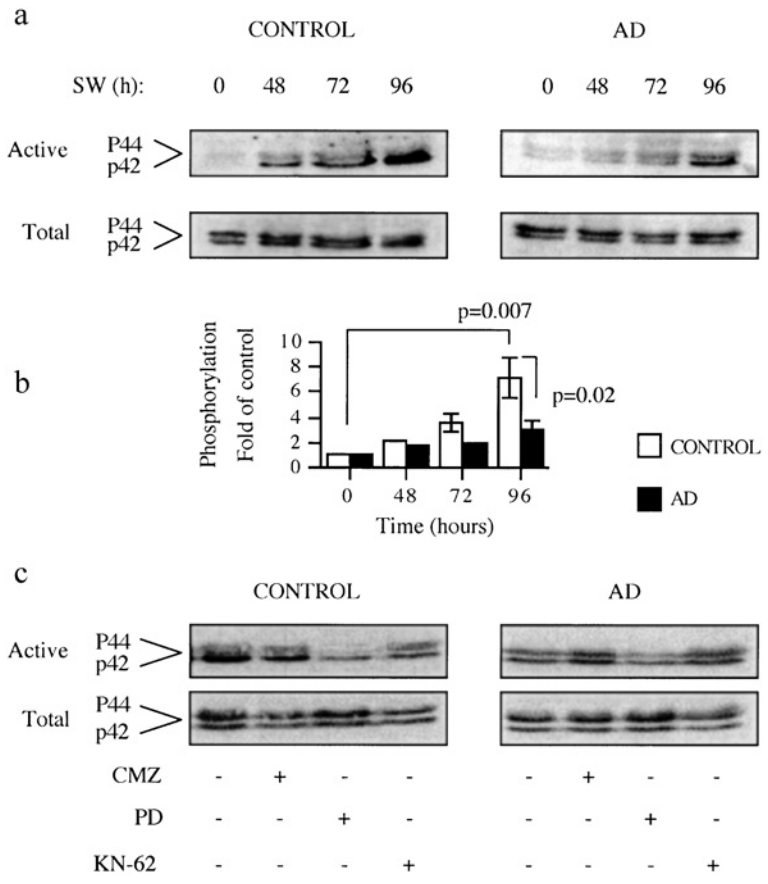


**Figure 10.** Persistent activation of ERKs by serum deprivation in control and AD lymphoblasts. Effects of PD98059, CMZ, and KN-62 on ERKs phosphorylation. (*a*) Lymphoblasts from control and AD lymphoblasts were serum deprived (SW) for 96 h. At the times indicated, aliquots were taken to prepare cell 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. (*b*) The immunoreactive bands were quantified by densitometric analysis. Results shown below are the mean ± SE of three to five determinations made in different experiments. (*c*) Cells were incubated in the absence of serum for 72 h in the presence of 20 μM PD98059, 1 μM CMZ or 1 μM KN-62. Representative experiments are shown.

Previous reports indicated that the kinetics and duration of ERK activation are important factors in determining the cellular response [30–32]. In fact, we observed an enhanced and transient activation of ERK in AD lymphoblasts in the presence of serum (Fig. 8), whereas serum deprivation induced a sustained phosphorylation (although lower than in control cells) of ERKs (Fig. 10). Whether there is serum or not, AD cells proliferate at higher rates or show a decreased degree of cell death [15]. Our results are in consonance with previous work in which a persistent activation of ERK was associated with cell cycle arrest and apoptosis in different cell types [44–46]. Both the transient and the sustained activation of ERK1/2 in the presence or in the absence of serum are sensitive to CaM antagonists or CaMKII inhibitors. Under trophic factor deprivation, CaM appears to down-regulate the sustained ERK activation. Several lines of evidence support this idea: (i) ERK1/2 phosphorylation is markedly decreased in AD cells, in which enhanced CaM signaling has been previously reported [13, 14]; (ii) CaM antagonists restored ERK activity of AD lymphoblasts to levels similar to those of control cells; and (iii) CaM antagonists failed to sensitize AD cells to apoptosis induced by serum withdrawal in the presence of PD98059. Thus, this study reveals that the $Ca^{2+}$/CaM and ERK pathways interact and, together, regulate the cellular response to the trophic support availability.

Interactions between these two pathways have been previously described in multiple cell systems. For example, it was reported that pharmacological inhibition of CaM decreased EGF-induced MAPK activity [47], or that CaMKII modulates integrin-stimulated ERK activation in thyroid TAD-2 cells [48]. It has also been described that $Ca^{2+}$/CaM may down-regulate ERK phosphorylation in 3T3 Swiss cells [49]. Therefore, it is clear from these observations and the results of this study that $Ca^{2+}$/CaM signal may produce opposite effects depending on the cell type or the cell status context.

Several mechanisms for $Ca^{2+}$/CaM modulation of ERK activity have been reported. For example, it was shown in thyroid cancer cells that CaMKII associate and phosphorylate Raf-1, contributing to ERK activation [48]. On the other hand it was reported that CaM negatively regulate the Ras/Raf/MEK/ERK pathway in fibroblasts [50]. *In vitro*, CaMKII can activate synGAP [51], a Ras inhibitory GTPase expressed in neurons [52], which may inhibit ERK activation. Whether there are similar mechanisms operative in human lymphocytes and

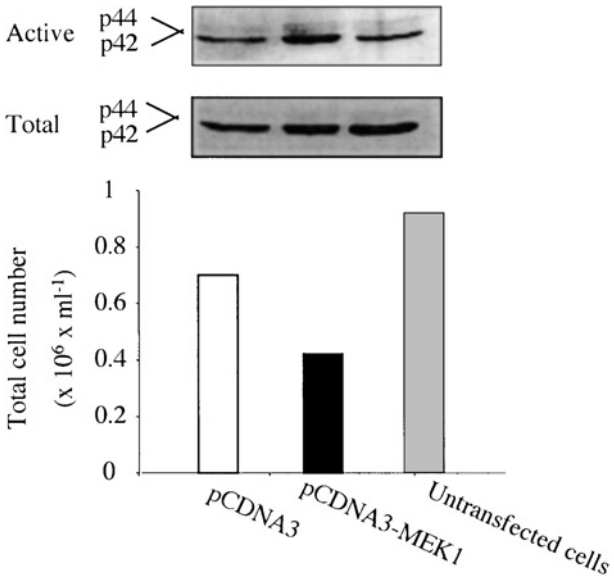


**Figure 11.** Serum deprivation-induced death of AD lymphoblasts following transfection with constitutively active MEK1 construct. AD lymphoblasts were transiently transfected with pcDNA3 or pcDNA-MEK1 constructs as described in the Methods. At 16 h after nucleofection, cells were seeded at a density of $1\times10^6$/ml, deprived of serum and incubated for 24 h. Cell viability was then determined by trypan blue exclusion under inverted phase-contrast microscopy. The relative levels of activation of p42/p44 ERKs were assessed by Western blot analysis. The experiment was performed twice with different cell lines with similar results, and one of the experiments is shown.

impaired in AD patients will need further investigation.

Alternatively, the $Ca^{2+}$/CaM signaling pathway may also regulate cell death/survival by increasing the activity of the $Na^+/H^+$ exchanger as previously reported [13]. An increase in intracellular pH is known to be protective against apoptosis [53].

Previous reports indicated that ERK1/2 pathway is actively involved in the pathogenesis of AD [54]. This signaling pathway is known to play a critical role in hippocampal synaptic plasticity and the processes of learning and memory [55]. Thus, the abnormal activated pathway in the hippocampus in AD may specifically impair hippocampal functions and contribute to the memory deficits of AD patients. ERK1/2 activation has been shown in degenerative neurons [56], and an association between phosphorylated ERK1/2 and neurofibrillary tangles has been demonstrated in human brain [57]. *In vitro*, ERK1/2 can be activated by β-amyloid and phosphorylate tau protein [58, 59].

Interestingly, deregulation of ERK1/2 activation has been also detected in fibroblasts from AD patients [60, 61]. Thus, together, these observations and our results showing a reduced activation of ERK pathway by serum deprivation in AD lymphoblast add further support to the idea that AD has systemic expression at cellular and molecular levels.

In summary, this study reveals a functional relationship between $Ca^{2+}$/CaM and ERKs in serum-induced signaling in immortalized lymphocytes, controlling cell fate (death or survival) depending on growth factor availability. The enhanced signaling through $Ca^{2+}$/CaM in AD cells seems to protect them from apoptosis by down-regulating the persistent activation of ERK1/2 induced by serum deprivation.

This mechanism might represent an adaptative response for AD cells that are exposed to chronic stress. It has been considered that susceptible neurons in AD survive for long time in a compromised way by delaying the apoptotic process, a mechanism termed abortosis or abortive apoptosis [62, 63].

Although AD-associated changes detected in peripheral cells might not reflect exactly those in the AD brain, a $Ca^{2+}$/CaM-dependent modulation of ERK1/2 pathway in response to both mitogenic stimuli or cellular stressors, could provide an explanation for the relationship between cellular stress and unscheduled cell cycle entry observed in susceptible neurons in AD [64]. In fact these two events seem to be necessary and invariant features of AD as proposed by the "two-hit" hypothesis [64, 65].

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