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RESEARCH ARTICLE

Simvastatin overcomes the resistance to serum withdrawal-induced apoptosis of lymphocytes from Alzheimer's disease patients

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Abstract Statins may exert beneficial effects on Alzheimer's disease (AD) patients. Based on the antineoplastic and apoptotic effects of statins in a number of cell types, we hypothesized that statins may be able to protect neurons by controlling the regulation of cell cycle and/or apoptosis. A growing body of evidence indicates that neurodegeneration involves the cell-cycle activation in postmitotic neurons. Failure of cell-cycle control is not restricted to neurons in AD patients, but occurs in peripheral cells as well. For these reasons, we studied the role of simvastatin (SIM) on cell survival/death in lymphoblasts from AD patients. We report here that SIM induces apoptosis in AD lymphoblasts deprived of serum. SIM interacts with PI3K/Akt and ERK1/2 signaling pathways thereby decreasing the serum withdrawal-enhanced levels of the CDK inhibitor $p21^{Cip1}$ (p21) and restoring the vulnerability of AD cells to trophic factor deprivation.

Keywords Alzheimer's disease · Lymphocytes · Simvastatin · p21 · PI3K/Akt · ERK1/2

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Introduction

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is the rate-limiting enzyme in the conversion of HMG-CoA to mevalonate (MEV), a fatty acid intermediate in the de novo synthesis of cholesterol [1]. Several lipid isoprenoid intermediates such as geranylgeranyl pyrophosphate (GGPP), and farnesyl pyrophposphate (FPP) are also enzymatically generated from MEV through this pathway. These compounds may post-translationally modify small GTP-binding proteins (G proteins), including Rho, Rab, Rac, and Ras, that play pivotal roles in normal and pathological cell signaling [2].

Statins are small-molecule inhibitors of HMG-CoA reductase that effectively reduce low-density lipoprotein cholesterol plasma levels and exhibit additional pleiotropic effects on the vasculature [3, 4]. Therefore, statins are widely used in the prevention and treatment of hypercholesterolemia, atherosclerosis, and cardiovascular and cerebrovascular diseases [5–7]. They have also been associated in some epidemiologic studies with reduced risk of AD [8, 9] and a link between cholesterol and late-onset AD has been documented [10]. However, the evidence from the epidemiological studies on the benefit of statin therapy for AD remains to be solved. Findings from randomized clinical trials evaluating the effects of statins on AD patients had yielded conflicting results [11, 12].

Moreover, a recent population-based study involving 135 elderly people treated with statins and 411 age-matched control individuals living in central Spain, failed to show a benefit in cognition [13]. On the other hand, while some studies examining the role of statins in established AD had shown a potential benefit in cognitive decline [14], a recent large-scale randomized controlled trial evaluating statin therapy for mild to moderate Alzheimer's (the LEADe study) did not find significant benefit on cognition or global function [15]. The beneficial effect of statins beyond lowering cholesterol might, in part, be due to the ability of statins to inhibit the synthesis of FPP and GGPP. It has been recently reported that FPP and GGPP levels are significantly increased in human AD brain [16], suggesting that protein prenylation may contribute to AD pathophysiology. Statins were found to effectively inhibit protein prenylation of a subset of GTPases involved in APP processing, thereby limiting the production of $A\beta_{1-42}$ [17]. On the other hand, it is known that protein prenylation interferes with G protein-mediated cancer survival pathways, and results in the induction of apoptosis in various cancer cells [18, 19]. Therefore, the possible benefit of statins in AD could also be related to the effects of statins modulating cell cycle and apoptosis, as growing evidence suggests that neuronal cell-cycle regulatory failure, leading to apoptosis, may be a significant component of the AD pathogenesis [20, 21].

Aberrant expression of many cell cycle-related proteins and direct evidence for DNA replication in vulnerable neuronal population indicate that, at least, some neurons reentered the cell cycle and entirely passed through a functional interphase having achieved successfully the S phase of cell cycle [22, 23]. However, it is still not clear why neurons then become stuck at the G2 phase, which leads to apoptosis in AD [24]. A number of recent studies have pointed out that in addition to control cell cycle progression by inhibiting the activity of cyclin-dependent kinase complexes (CDKs), p21 can modulate multiple biological functions, including DNA synthesis, stress response, and apoptosis [25]. p21 has been found to be expressed in the cytoplasm of neurons from AD patients [26, 27], often in tangle-bearing neurons and dystrophic neurites, indicating a loss of function as CDK inhibitor and suggesting a possible role of p21 in neuronal apoptosis.

We, and others, have presented evidence that cell-cycle regulatory failure is not restricted to neurons, since peripheral cells from AD patients such as fibroblasts or lymphocytes show altered proliferative activity compared to age-matched control individuals [28–31]. Moreover, we have recently reported that SIM, a lipophilic statin, inhibits cell-cycle progression at the G1-S checkpoint in immortalized lymphocytes from AD patients [32]. These cell lines were also found to be more resistant to serum withdrawal [33]. On these grounds, we found it interesting to study the influence of SIM treatment on the control and AD cell fate upon serum deprivation and in the regulation of cellular content of p21. The results presented here indicate that SIM sensitize AD lymphoblasts to serum deprivation-induced apoptosis. SIM blunted the serum withdrawal-mediated enhanced p21 levels in AD cells by modulating ERK1/2 and PI3K/Akt signaling pathways.

Table 1 Demographic characteristics of all subjects enrolled in the study

	Control ($n = 20$)	AD ($n = 20$)
Age (years)	73 ± 3	74 ± 2
Gender (F/M)	11/9	5/15
Duration of dementia (years)	–	3.6 ± 3
ApoE 4/3 (No. of cases)	1	6

This diagnosis was made according to the criteria developed by the National Institutes of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ARDA). Control individuals with no sign of cognitive disorders. Values are expressed as mean ± SE

AD patients with a diagnosis of probable AD, *F* female, *M* male, *n* number of patients

Materials and methods

Materials

All components for cell culture were obtained from Invitrogen (Barcelona, Spain). The kinase inhibitors PD98059, Ly294002, and the caspase inhibitor benzyloxy-carbonyl-Val-Asp-fluoromethylketone (z-VAD-fmk) were obtained from Calbiochem (Darmstadt, Germany). Poly (vinylidene) 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, and goat polyclonal anti total Akt were obtained from Cell Signaling (Beverly, MA USA). Rabbit-anti human p21 antibody (sc-397) was from Santa Cruz Biotechnologies (Santa Cruz, CA). The enhanced chemiluminescence (ECL) system was from Amersham (Uppsala, Sweden). All other reagents were of molecular biology grade.

Study samples and cell lines

A total of 40 subjects were recruited for this study. These included: (1) 20 AD patients (Table 1), with moderate to severe disease. Patients were diagnosed in the department of Neurology of the University Hospital Doce de Octubre (Madrid, Spain) according to the National Institute of

Neurological and Communicative Disorders Association criteria [34]. Cognitive status was quantified using the Mini-Mental State Examination (MMSE); patients were diagnosed of moderate AD (DSM-III-R, MMSE score between 10 and 18) or severe AD (MMSE < 10). They were all considered sporadic, late-onset AD cases (onset of symptoms >65 years; family history negative for neuropsychiatric disorders). All AD patients presented a 1–6 year history of progressive cognitive impairment predominantly affecting memory; (2) 20 healthy subjects, matched for age distribution, without history of degenerative or cerebrovascular diseases, and without cognitive impairment or other neurological disorders. A summary of demographic characteristics of all subjects enrolled in the study is reported in Table 1.

Establishment of lymphoblastoid cell lines was performed in our laboratory as previously described [35], by infecting peripheral blood lymphocytes with the Epstein-Barr virus [36]. Cells were grown in suspension in T flasks in an upright position, in approximately 10 ml of 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. The medium was routinely changed every 2 days.

Cell survival assay

The cell suspension was mixed with a 0.4% (w/v) Trypan Blue solution, and the number of live cells was determined using a hemocytometer. Cells failing to exclude the dye were considered nonviable. In some experiments, cell viability was checked by the MTT assay [37], obtaining similar results.

Assessment of apoptosis and caspase activity

An apoptosis-detection kit that measured phosphatidylserine (PS) was purchased from Pharmingen (San Diego, CA). The assay was conducted following the manufacturer's directions. Cells were analyzed for phosphatidylserine (PS) exposure/propidium iodide (PI) exclusion by staining with FITC-Annexin V and PI. The activation of executive caspases was investigated using the Vybrant FAM Caspase-3 and 7 Kit (Invitrogen) including FLICA reagent that is retained within the cell, if bound to the active caspase molecule. Control and AD lymphoblasts were resuspended in 300 μl of RPMI containing 10 μl of FLICA reagent and incubated in 5% CO_2 at 37°C for 60 min. The cells were then washed with, and suspended in, wash buffer provided with the kit. The samples were analyzed on the flow cytometer.

Immunoblotting analysis

For Western blot analysis, 50–100 μg of protein from 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 1% BSA and incubated overnight at 4°C with primary antibodies at the following dilutions: 1:500 anti-phosphorylated ERK1/2, 1:2,000 anti-total ERK1/2, 1:500 anti-phosphorylated Akt, 1:1,000 anti-total Akt, 1:500 anti-p21 anti 1:5,000 β-actin. 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 BioRad.

Statistical analysis

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

Results

Effect of SIM on serum deprivation-induced cell death in lymphoblasts from control or AD subjects

Figure 1 shows a time-course analysis of the effect of increasing doses of SIM on rates of cell death, upon serum deprivation, of lymphoblasts from control and AD patients. In agreement with previous reports from this laboratory, it was found that lymphoblasts from AD patients were more resistant to cell death induced by serum deprivation [33, 38]. Here, we show that SIM treatment sensitizes AD lymphoblasts to cell death. The highest concentration tested had little effect in enhancing the death of control cells induced by serum deprivation (Fig. 1, upper left panel). However, in AD lymphoblasts, there is a dose–response effect of SIM inducing cell death in lymphoblasts from AD patients (Fig. 1, upper right panel).

For determining the role of various isoprenoids derived from MEV in regulating the modulatory effect of simvastatin on survival of serum-deprived AD lymphoblasts,

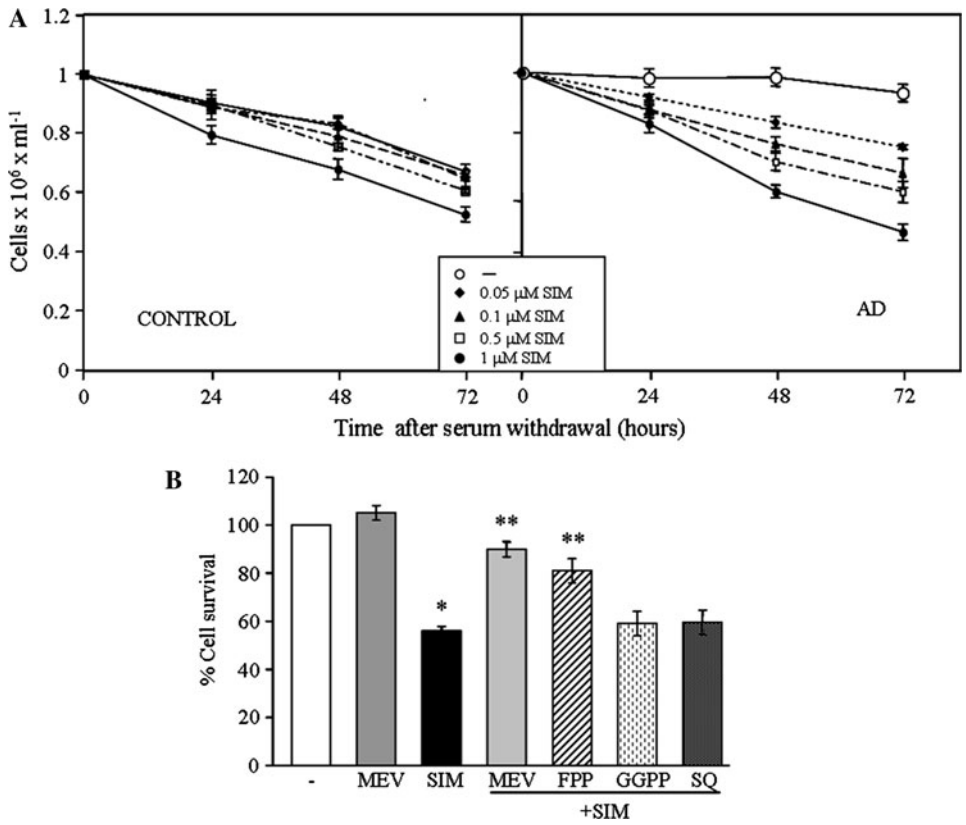

Fig. 1 Effect of SIM on cell survival following serum deprivation in lymphoblasts derived from control or AD patients. **a** Immortalized lymphocytes from control and AD individuals were seeded at an initial density of 1×10^6 ml^{-1} and incubated in serum-free RPMI medium for 72 h in the absence or in the presence of increasing concentrations of SIM. Every day thereafter, cell viability was determined by Trypan Blue exclusion under inverted phase-contrast microscopy. Data shown are the mean ± SE of four to ten independent experiments carried out with cell lines from different individuals. **b** Lymphoblasts from AD subjects were seeded at an initial density of 1×10^6 ml^{-1} and cultured for 3 days in the absence or presence of 1 μM SIM alone or in combination with 200 μM MEV, 5 μM FPP, 5 μM GGPP, or 5 μM SQ. Cell survival was determined by enumeration of cells excluding Trypan Blue. The percent of cell survival is shown after setting the survival of untreated cells as 100%. Values shown are the mean ± SE for four to ten independent experiments carried out with cells derived from different individuals. *$p < 0.01$ significantly different from untreated cells; **$p < 0.01$ significantly different from SIM-treated cells

cells were cotreated with 1 μM SIM and MEV or various isoprenoid intermediaries GGPP, FPP, and SQ. Figure 1b shows that MEV reversed the effect of SIM on cell death. The cotreatment of AD cells with FPP, but not GGPP or squalene (SQ), prevented the SIM-induced apoptosis (Fig. 1b), suggesting that the effect of SIM is independent of cholesterol biosynthesis, and the involvement of farnesylated proteins in the process.

SIM induced apoptosis in AD lymphoblasts

The cell death induced by SIM in serum-deprived AD lymphoblasts showed characteristics of apoptosis. First, a FACS analysis using FITC-AnnexinV/PI double staining showed that the addition of SIM to AD lymphoblasts mainly increased the percentage of cells in early apoptosis (Fig. 2). It is also shown that the effect of SIM is prevented by the pan-caspase inhibitor z-VAD-fmk (Fig. 2). Moreover, fluorescent cell distribution using FLICA green fluorescent probe indicates that SIM increased the activity of executive caspase 3 and 7 (Fig. 3) as FLICA binds irreversibly to these enzymes when are activated, thus increasing the fluorescence signal in apoptotic cells.

Serum deprivation-induced apoptosis is accompanied by changes in p21 protein levels

p21 has been shown to play an important role in regulating apoptosis in a number of cell types [25, 26]. For this reason, we first evaluated whether serum deprivation induces changes in the cellular levels of p21 in control and AD lymphoblasts, and second, we investigated the effect of SIM on p21 content. Figure 4a shows a time-course analysis of serum withdrawal-induced changes in p21 levels. p21 content increased transiently, with a peak level at 24 h of the serum deprivation and returning to basal levels at

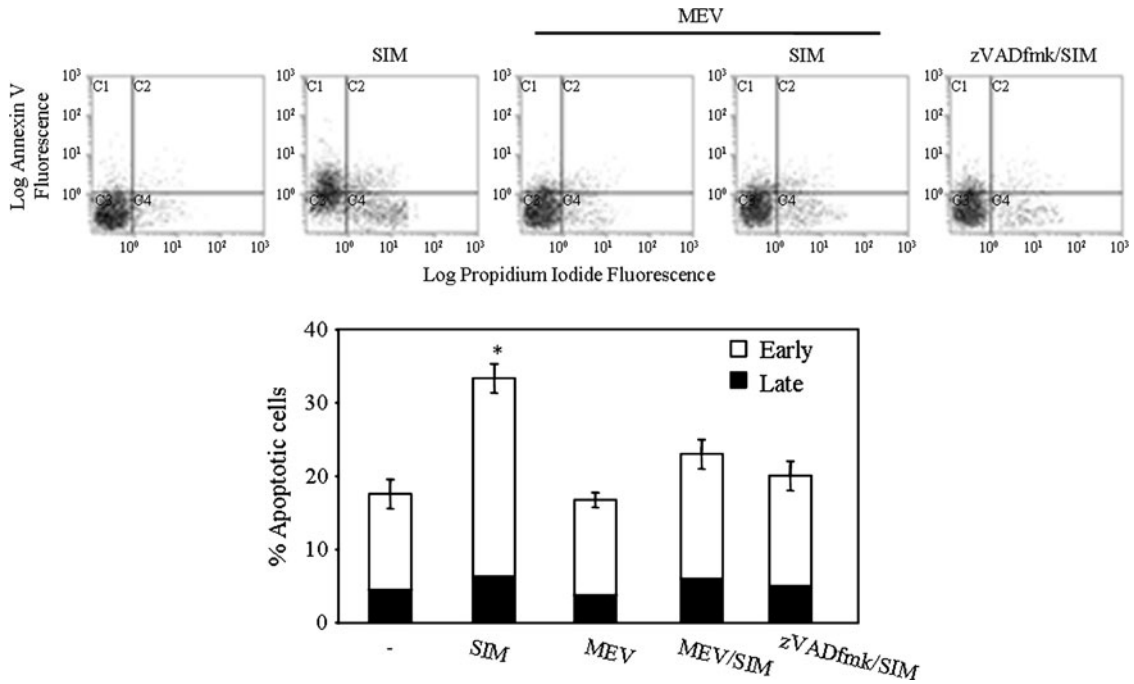

Fig. 2 Flow cytometric analysis of the fraction of viable, apoptotic, and necrotic cells after treatment of AD lymphoblasts with SIM. Lymphoblasts from AD subjects were incubated as described in the legend to Fig. 1, and then stained with FITC-Annexin V and PI. A representative experiment is shown. When present, the concentration of z-VAD-fmk was 1 μM. The means of eight independent experiments carried out with cells derived from different individuals are shown below. *Statistically significant ($p < 0.02$) difference of the proportion of apoptotic cells compared to untreated cultures

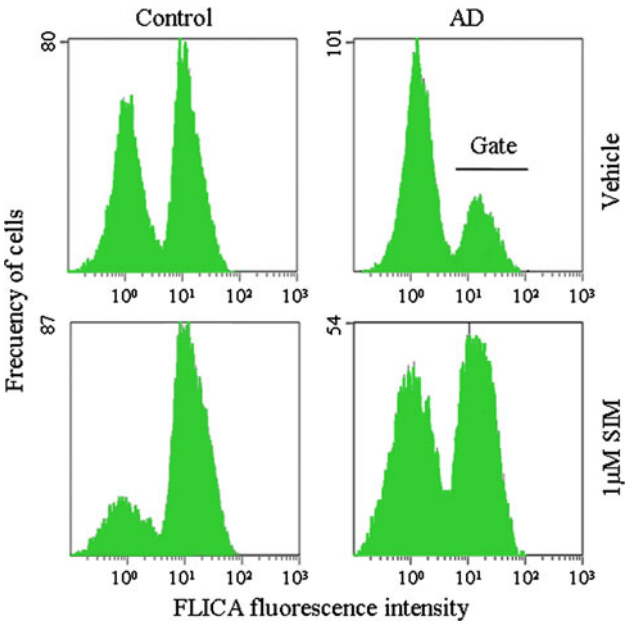

Fig. 3 Caspase activation in serum-deprived lymphoblasts from control and AD subjects. Effect of SIM treatment. Lymphoblasts from control and AD subjects were incubated in serum-free medium for 72 h, in the absence (*upper*) or presence (*lower*) of 1 μM SIM. Then, cells were labeled with the FLICA reagent, following the manufacture's recommendations to detect its binding to active caspases-3 and 7. A representative flow cytometric analysis of the frequency distribution of cells according to their green fluorescence intensity is presented

72 h. The response of control and AD cells was qualitatively identical, but in the latter more p21 accumulated. Thereafter, we focused our subsequent experiments at 48 h of serum deprivation to compare differences in p21 content and cell survival between control and AD lymphoblasts treated with SIM. Figure 4b shows that while SIM slightly decreased p21 levels in control cells, it strongly inhibited the content of p21 in AD lymphoblasts. Addition of MEV to AD cells, in the presence of SIM, partially prevented the effects of the statin, decreasing p21 cellular content. Under these conditions, AD cells treated with SIM undergo significant apoptosis as they do in control cells (Fig. 2). These results suggest that overexpressed p21 may play a role in the resistance of AD lymphoblasts to serum withdrawal-induced cell death, and that SIM is able to overcome this feature by decreasing p21 levels.

Time course of intracellular signaling pathway activation following serum withdrawal

PI3K/Akt and ERK1/2 pathways play a central role in cell death and survival [39, 40]. Both pathways are activated after serum deprivation in the control cells as well as in the AD cells (Fig. 5). Activation was assessed by determining the phosphorylation status of Akt and ERK1/2, by Western-blot analysis. PI3K/Akt activity increased transiently, reaching a peak 24 h after serum withdrawal. Enhanced

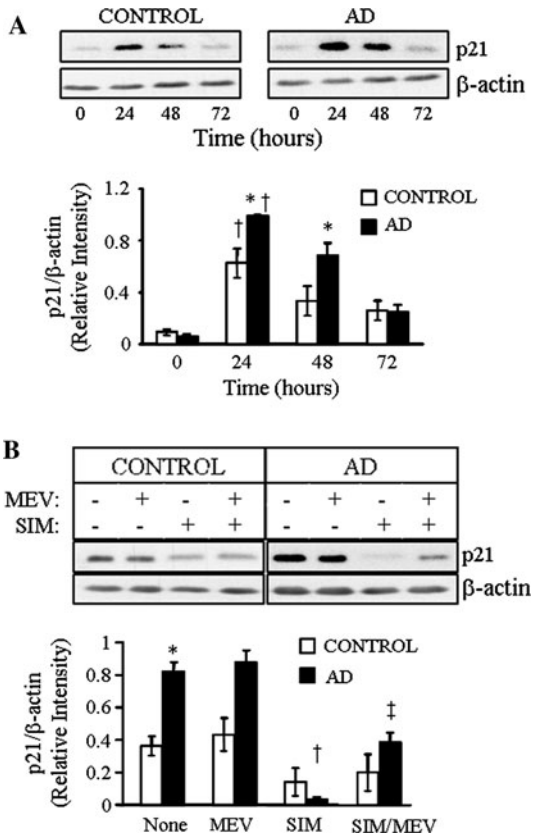

Fig. 4 Serum withdrawal induced a transient increase in p21 levels. Effects of SIM and MEV treatments. **a** Immortalized lymphocytes from control and AD individuals were seeded at an initial density of 1×10^6 ml^{-1} and incubated in serum-free RPMI medium for 72 h. At the times indicated, aliquots were taken to prepare cell extracts. The relative levels of p21 were assessed by Western-blot analysis using anti-p21 antibody. The same membranes were then stripped and reprobed with antibody against β-actin. The immunoreactive bands were quantified by densitometric analysis. Results shown below are the mean ± SE of five determinations. **b** Lymphoblasts from control and AD patients were incubated for 48 h in the absence or presence of 1 μM SIM alone or in combination with 200 μM MEV. Results shown are the mean ± SE of six independent experiments carried out in cell lines from different individuals. $^*p < 0.01$ significantly different from control cells; $^\dagger p < 0.01$ significantly different from both control or AD cells before serum deprivation; $^\ddagger p < 0.01$ significantly different from AD cells incubated in the absence of SIM after 72 h of serum deprivation

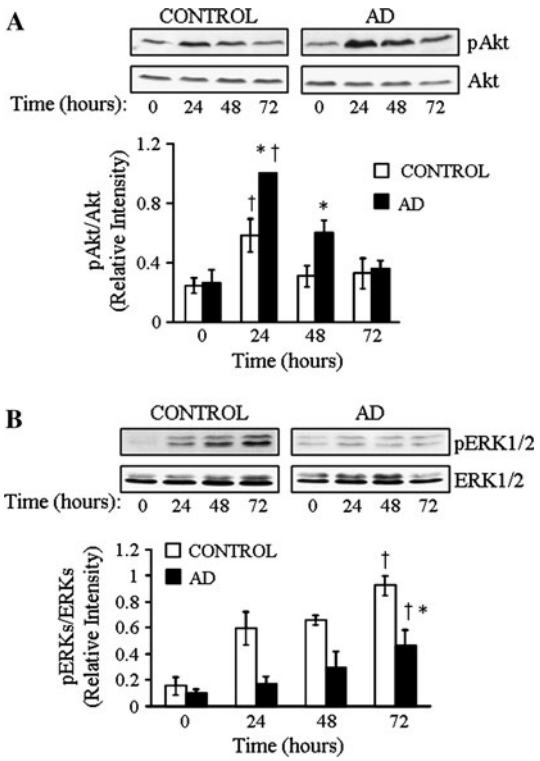

Fig. 5 PI3K/Akt and ERK1/2 signaling pathways are activated by serum deprivation in control and AD lymphoblasts. **a**, **b** Immortalized lymphocytes from control and AD individuals were seeded at an initial density of 1×10^6 ml^{-1} and incubated in serum-free RPMI medium for 72 h. At the times indicated, aliquots were taken to prepare cell extracts. The relative levels of activation of PI3K/Akt and that of ERK1/2 were assessed by Western-blot analysis using phosphospecific antibodies. The same membranes were then stripped and reprobed with antibodies against total Akt or ERK1/2. Representative immunoblots are shown, while densitometric analysis are the mean ± SE of independent experiments carried out in cell lines from different individuals. $^*p < 0.05$ significantly different from control cells harvested at the same time point, $^\dagger p < 0.01$ significantly different from both control or AD cells before serum deprivation

PI3K/Akt activation was found in AD cells compared to control cells (Fig. 5). On the other hand, as previously reported [33], ERK1/2 activity increased progressively in response to serum deprivation in control and AD lymphoblasts, although the phosphorylation of the ERK1/2 was significantly lower in AD cells (Fig. 5).

To evaluate the relationship between alterations in PI3K/Akt and ERK1/2 and apoptosis of control and AD lymphoblasts in serum-free medium in the absence or in the presence of SIM, we use the selective PI3K/Akt and ERK1/2 pathway inhibitors, Ly294002 and PD98059, respectively [41, 42]. Despite the activation of PI3K/Akt following serum deprivation, the treatment of cells with Ly294002 did not change the cell response to serum withdrawal-induced cell death in control cultures (Fig. 6a). In contrast, the inhibitor of the ERK1/2 pathway, PD98059, prevented serum deprivation-induced apoptosis in control cells, which is in agreement with previous work from our laboratory [33]. The effect of SIM-inducing cell death in AD lymphoblasts is not affected by ERK1/2 inhibition (Fig. 6a). However, Ly294002 blunted the effect of SIM-inducing apoptosis in serum-deprived AD cells. Ly294002 slightly increased death of AD lymphoblasts following serum withdrawal (Fig. 6a). We confirmed the effect of these inhibitors on the phosphorylation status of their respective kinases (Fig. 6b). PD98059 decreased ERK1/2 phosphorylation but did not modify Akt phosphorylation, whereas Ly294002 inhibited the Akt phosphorylation

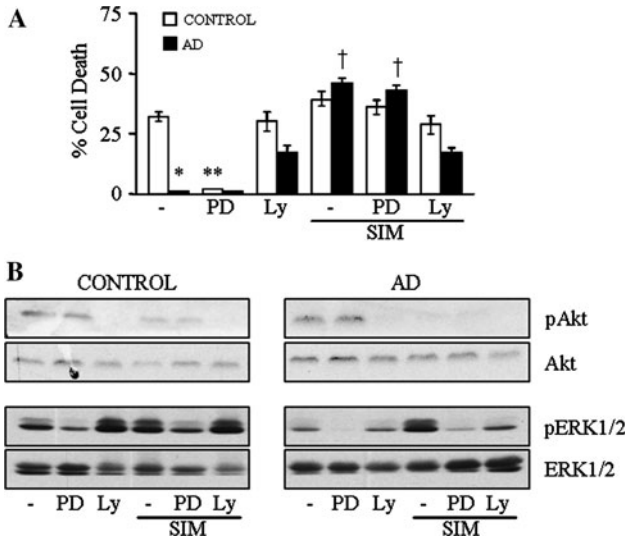

Fig. 6 Effects of SIM on cell viability in the absence and presence of kinase inhibitors. **a** Immortalized lymphocytes from control and AD individuals were seeded at an initial density of 1×10^6 ml^{-1} and incubated in serum-free RPMI medium for 72 h, in the absence or presence of 1 μM SIM with or without 10 μM Ly294002 or 20 μM PD98059. Cell viability was determined by Trypan Blue exclusion. Values shown are the mean ± SE of seven independent experiments carried out in cell lines from different individuals. $^*p < 0.01$ significantly different from control cells; $^{**}p < 0.01$ significantly different from control cells in the absence of PD98059; $^{\dagger}p < 0.01$ significantly different from AD cells in the absence of SIM. **b** The relative levels of activation of ERK1/2 and Akt were assessed by Western-blot analysis using phosphospecific antibodies in cells extract prepared 48 h after serum deprivation. Representative immunoblots are shown

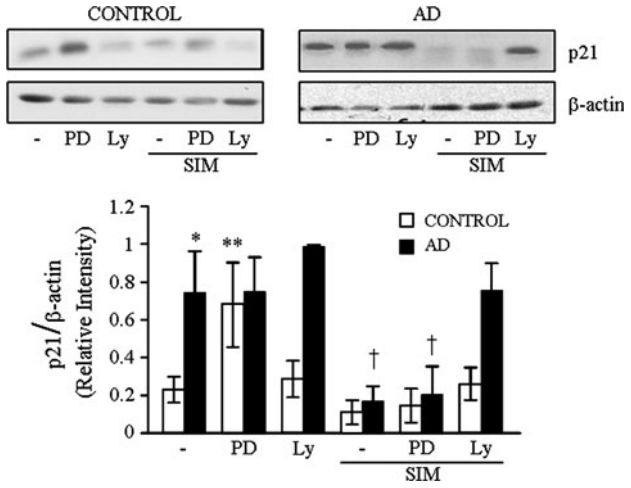

Fig. 7 Effects of SIM on p21 content in the absence and presence of kinase inhibitors. The experimental conditions are identical to those described in the legend to Fig. 6. The cellular levels of p21 were assessed by Western-blot analysis in cell extracts prepared 48 h after serum deprivation. Immunoblots shown are representative of four separate experiments carried out in cell lines from different individuals. Densitometric analyses are shown below. $^*p < 0.05$ significantly different from control cells; $^{**}p < 0.05$ significantly different from control cells incubated in the absence of PD98059; $^{\dagger}p < 0.05$ significantly different from AD cells in the absence of inhibitors

without affecting ERK1/2 phosphorylation status. In addition, we observed that SIM decreased Akt phosphorylation in both control and AD lymphoblasts and it rescued the decreased ERK1/2 activation in AD cells following serum withdrawal (Fig. 6b). SIM did not affect the levels of total ERKs or Akt. The effects of these inhibitors alone or on combination with SIM in the cellular content of p21 are presented in Fig. 7. Inhibition of ERK1/2 activity by PD98059 in control cells caused an increase in p21 levels (Fig. 7). However, the presence of PD98059 did not block the effect of SIM-reducing p21 content (Fig. 7), and impaired the protective effect of PD98059 on the serum withdrawal-induced apoptosis in control cells (Fig. 6a). As mentioned, SIM reduced the levels of p21 in AD cells in the absence or in the presence of PD98059 and sensitized AD lymphoblasts to apoptosis (Fig. 6a). The effect of SIM-reducing p21 levels was prevented by Ly294002 (Fig. 7), in consonance with the blockade of the SIM-induced ERK1/2 activation and cell death in these conditions (Fig. 6).

To gain insight into the role played by the PI3K/Akt and ERK1/2 signaling pathways in regulating the cellular content of p21, we compared the kinetics of changes in the activation of these pathways and that of p21 levels following the treatment of cells with the selective inhibitors Ly294002 and PD98059. The results of these time-course experiments are presented in Fig. 8. Treatment of control or AD cells with Ly294002 rapidly inhibited Akt phosphorylation, and modestly decreased the p21 content (Fig. 8a). However, PD98059 progressively inhibited ERK1/2 phosphorylation in control cells, with simultaneous increase in p21 content (Fig. 8b, left panel). Treatment of AD cells with PD98059 had no consequences in the levels of p21 in these cell cultures (Fig. 8b, right panel). Taken together, these results suggest that cell fate (survival or death) ultimately relies on p21 levels, which appear to be controlled by both the PI3K/Akt and the ERK1/2 signaling pathway in an opposite manner. The ERK1/2 pathway seems to play an indispensable proapoptotic role in the serum deprivation-induced apoptosis, while the PI3K/Akt pathway may act as a survival signal.

Discussion

It was previously reported that EBV-immortalized lymphocytes from AD patients from late-onset AD patients show enhanced proliferative activity [28, 43] and appear to be ill-equipped to survive to serum deprivation [33, 44]. These tumor-like features of lymphoblasts from AD

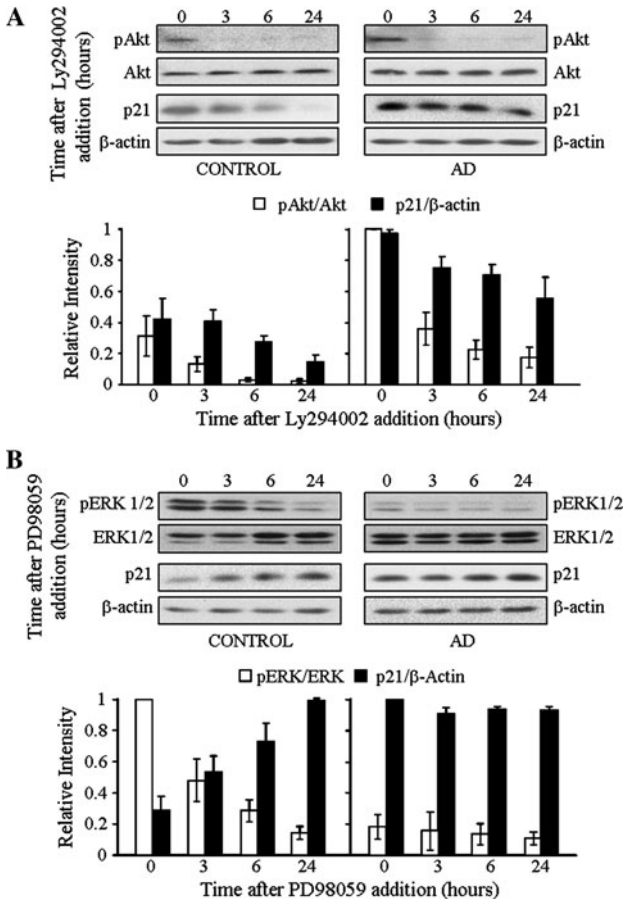

Fig. 8 Comparative kinetics of either PI3K/Akt or ERK1/2 inhibition and changes in cellular p21 levels. **a** Control and AD cells deprived of serum during 24 h, were incubated in the presence of 10 μM Ly294002 for the indicated periods of time. **b** Control and AD cells were incubated in serum-free medium in the presence of 20 μM PD98059 for the indicated periods of time. The immunoblots show the time-dependent effect on Akt phosphorylation or ERK1/2 phosphorylation and p21 content after 3, 6, and 24 h of treatment. The blots are representative of four to eight separate experiments carried out with cell lines form different individuals. Densitometric analyses are shown below

patients were considered as systemic manifestations of the proposed relationship between cellular stress and unscheduled cell-cycle entry observed in susceptible neurons in AD [45]. Moreover, we could demonstrate that the immortalization procedure did not alter the cellular response of fresh obtained lymphocytes to addition or withdrawal of mitogenic factors [33, 43]. Thus, it was considered that EBV-transformed lymphocytes represent a suitable model to study cellular or molecular perturbations of pathophysiological significance.

The present work aimed at studying the influence of statins on the cell fate under serum deprivation of lymphoblasts from late-onset AD patients as compared to that obtained from age-matched non-demented individuals.

The results herein reported confirm and extend our previous work by showing that the survival of AD lymphoblasts is associated with enhanced p21 content upon serum deprivation in comparison to the levels found in control cells. Increased levels of p21 and higher resistance to oxidative stress-induced apoptosis were also found in AD fibroblasts [46]. Although there seems to be a selective impairment of mechanisms involved in cell death in peripheral cells from AD patients [46–49], contradictory results of whether cells from AD patients are more resistant or more vulnerable to situations that promote cell death had been reported. These discrepancies may be due, in part, to the fact that fibroblasts, T or B lymphocytes or EBV-immortalized lymphocytes, from either sporadic or familial AD, exposed to a number of cell-death-inducing conditions have been considered.

A number of recent studies pointed out that in addition to being an inhibitor of cell proliferation, p21 may protect cells from apoptosis [50]. For example, it has been reported that up-regulation of p21 blocked the oxidative stress-induced death of human myeloma U266 cells [51] and that inducible expression of exogenous p21 render glioblastomas resistant to chemotherapy drugs [52]. Thus the increase in p21 cellular content in AD lymphoblasts may confer these cells a survival advantage to these cells as described for cancer cells [53].

SIM treatment slightly increases the serum deprivation-mediated death of control lymphoblasts while sensitizing AD cells to serum withdrawal-induced cell death. In contrast with the effect of SIM increasing the levels of CDK inhibitors, p21 and p27 in AD lymphoblasts under proliferative conditions [32], SIM drastically decreased the p21 content of serum-deprived lymphoblasts from AD. Cell death induced by SIM showed characteristics of apoptosis, since it was prevented by a pan-caspase inhibitor, and showed dependence on caspase-3 and 7 activation.

The higher sensitivity of AD cells to SIM, compared to that of control lymphoblasts, is in line with reports indicating that B leukemic and myeloma cells undergo apoptosis with statins treatment, whereas their normal counterparts are resistant to statin effects [54]. Moreover, statins inhibit the growth of variant human embryonic stem cells and cancer cells in vitro but not the growth of normal human embryonic stem cells [55]. The results described herein are, however, in contrast with the reported neuroprotective effects of statins in neuronal cells [56–58]. These effects of statins, protecting neuron from certain noxious stimuli, involve alteration in the ratio of pro and anti apoptotic proteins and inactivation of caspases [59]. Although lower concentrations of statins (<1 μM) were used in treating cortical neurons [58], the little effect of concentrations of SIM (up to 1 μM) in control lymphoblasts rules out a cytotoxic effect of the drug. Taken

together, it seems that the effects of SIM, inducing or preventing apoptosis, are cell type-specific, and perhaps dependent on the nature of the stimulus.

Despite the pro-apoptotic effect of SIM on AD lymphoblasts, a neuroprotective effect of SIM in AD brain cannot be fully discarded. In this regard, it is worth mentioning that SIM addition to AD cells was able to restore the "normal" cell response to serum stimulation [32] or withdrawal (this manuscript), by blunting the enhanced proliferative activity of AD cells or sensitizing cells to apoptosis in the absence of serum. In both situations, SIM was able to increase [32] or decrease the levels of p21 of AD lymphoblasts to reach those of control cells. It remains to be demonstrated whether SIM would protect neurons in AD brain from apoptosis by modulating p21 content.

The proapoptotic effect of SIM in AD cells is directly related to HMG-CoA reductase inhibition because the effects of SIM on cell viability and p21 content were completely or partially reversed by MEV. It is well known that MEV acts as a precursor to lipid moieties covalently attached to isoprenylated proteins, such as small GTP-ases. By preventing isoprenylation of certain proteins, SIM may regulate essential signaling pathways such as PI3K/Akt or MAPK, which are involved in cell proliferation and survival [2]. The modulatory action of SIM on the serum withdrawal-induced apoptosis reflects interference with farnesylation rather than with geranygeranylation of proteins.

Our results show that serum deprivation induces a transient increase in PI3K/Akt activity together with a sustained activation of the ERK1/2 pathway in both control and AD lymphoblasts. However, important quantitative differences were observed. While PI3K/Akt activation was enhanced in AD cells, the activity of ERK1/2 was downregulated with the net result of increased p21 levels and higher resistance to cell death. SIM treatment inhibited PI3K/Akt activation in control and AD cells, whereas in the latter, it reversed the activity of the ERK1/2 pathway to levels similar to those found in control cells. This shift away from the PI3K/Akt signaling pathway towards sustained ERK1/2 activation seems to favor cell death over survival. Indeed the ERK inhibitor PD98059 is able to overcome the serum deprivation-induced apoptosis in control cells, however inhibition of PI3K/Akt by Ly294002 had only a minor effect on the survival of AD cells. The SIM-induced apoptosis of AD cells was blocked in the presence of Ly294002 and, on the other hand, SIM prevented the effect of PD98059 protecting control cells from apoptosis induced by serum deprivation. These results suggest that both pathways cooperate to cell decision. Cell survival was always associated with decreased ERK1/2 phosphorylation levels, together with little change or even inhibition of PI3K/Akt provided that p21 content was maintained over certain levels. The proposed scenario is represented schematically in Fig. 9. The interaction of SIM with the ERK1/2 and PI3K/Akt signaling pathways results in a significant reduction of p21 content and apoptosis of AD lymphoblasts following serum withdrawal. In control cells, the balance between ERK1/2 and PI3K/Akt following serum deprivation is tipped in favor of ERK1/2 activation-induced p21 decreased levels and apoptosis. In these conditions, the inhibition of PI3K/Akt by SIM had only a moderate increase in the cell death induced by serum withdrawal.

Overexpressed p21 could protect cells from apoptosis at different levels. First, p21 is shown to induce the expression of antiapoptotic proteins [26]. Second, p21 interacts with several caspases [25, 60]. Third, it is possible that p21 can protect against oxidative stress-induced toxicity and thus promote survival of cells [61]. Which of these mechanisms predominates in lymphocytes is not yet known and needs further experimentation.

It is worth mentioning that overexpressed p21 has also been detected in the frontal cortex of AD brains [62]. It is known that monocytes can protect themselves from oxidative stress by upregulating the cytosolic levels of p21 [63]. It is therefore tempting to speculate that neurons in AD brain facing conditions of oxidative stress could initiate a similar compensatory response to protect the injured cells from death.

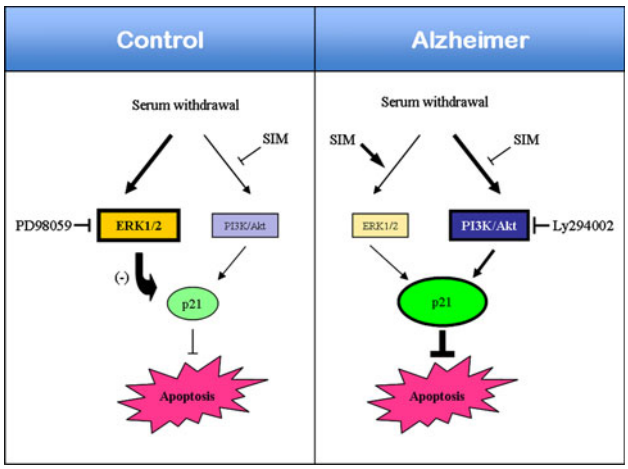

Fig. 9 Diagram summarizing the interaction of SIM with ERK1/2 and PI3K/Akt pathways in control and AD lymphoblasts under conditions of serum deprivation. **a** In control cells, serum withdrawal promotes apoptosis by inducing sustained overactivation of ERK1/2 which in turn reduces p21 accumulation. **b** In AD cells, in the absence of SIM, the ERK1/2 pathway is downregulated, and the activity of PI3K/Akt is enhanced relative to control cells, as well as it is the p21 cellular content. Treatment of AD cells with SIM prevented serum deprivation-dependent overactivation of PI3K/Akt, increased ERK1/2 activity, normalized p21 levels, and sensitized AD cells to the serum-induced apoptosis

Finally, alterations in PI3K/Akt and ERK1/2 signaling pathways and changes in the abundance of p21 similar to those described in immortalized lymphocytes from AD patients, has been detected in brain from affected individuals [62, 64–66], thus suggesting that peripheral cells from patients may be a potential useful surrogate for diagnosis, prognosis, and therapeutic monitoring of AD.

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