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HMG-CoA Reductase Inhibitor Simvastatin Inhibits Cell Cycle Progression at the G_1/S Checkpoint in Immortalized Lymphocytes from Alzheimer's Disease Patients Independently of Cholesterol-Lowering Effects

Simone G. Sala, Úrsula Muñoz, Fernando Bartolomé, Félix Bermejo, and Ángeles Martín-Requero

Department of Cellular and Molecular Pathophysiology, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Cientificas, Madrid, Spain (S.G.S., U.M., F.B., A.M.R.); and Hospital Doce de Octubre, Madrid, Spain (F.B.)

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ABSTRACT

Recent work has suggested that statins may exert beneficial effects on patients suffering from Alzheimer's disease (AD). The pharmacological effects of statins extend beyond their cholesterol-lowering properties. Based on the antineoplastic and apoptotic effects of statins in several cell types, we hypothesized that statins may be able to protect neurons by controlling the regulation of cell cycle. A growing body of evidence indicates that neurodegeneration involves the activation of cell cycle machinery in postmitotic neurons. We and others have presented direct evidence to support the hypothesis that the failure of cell cycle control is not restricted to neurons in AD patients, but that it occurs in peripheral cells as well. For these reasons, we found it worthy to study the role of simvastatin on cell proliferation in immortalized lymphocytes from AD patients. We report here that simvastatin (SIM) inhibits the serum-mediated enhancement of cell proliferation in AD by blocking the events critical for G_1/S transition. SIM induces a partial blockade of retinoblastoma protein phosphorylation and inhibition of cyclin E/cyclin-dependent kinase (CDK)2 activity associated with increased levels of the CDK inhibitors $p21^{Cip1}$ and $p27^{kip1}$. These effects of SIM on AD lymphoblasts are dependent on inhibition of the proteasome-mediated degradation of p21 and p27 proteins. The antiproliferative effect of this natural statin may provide a therapeutic approach for AD disease.

Statin therapy is a widely used treatment for hypercholesterolemia, reduces the risk of stroke, and improves cardiovascular functions (Farnier and Davignon, 1998). The statin family of drugs is competitive inhibitors of HMG-CoA reductase, the rate-limiting enzyme in the synthesis of cholesterol (Corsini et al., 1995), which converts HMG-CoA to mevalonate (MEV).

In the last decade, epidemiological, clinical, and experimental evidence has accumulated that links cholesterol to the development of AD, and recent studies showed that statin therapy might be of benefit in treating AD (Wolozin, 2004), although the efficacy of statins at slowing the cognitive decline and the progression of AD remains controversial. The link between cholesterol and AD is not surprising because the brain is the most cholesterol-rich organ, and disturbances in cholesterol homeostasis have been found associated with all major neuropathological features of AD (Shobab et al., 2005). Data from the Canadian Study of Health and Aging revealed a 74% reduced risk of developing AD in statin users compared with the total population (Rockwood et al., 2002). In other studies, a reduction in the risk of AD was observed in patients treated with statins compared with those receiving other medications typically used in cardiovascular disease (Wolozin et al., 2000), suggesting that statins in particular, rather than low cholesterol levels or lipid-lowering agents in general, are responsible for the reduction in the risk of AD.

A number of nonlipid-dependent or pleiotropic effects of statins have been reported (Takemoto and Liao, 2001). By

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S.G.S., Ú.M., and F.B. contributed equally to this work.

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ABBREVIATIONS: AD, Alzheimer's disease; MEV, mevalonate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; EBV, Epstein-Barr virus; Z-VAD-FMK, benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone; pRb, retinoblastoma protein; CDK, cyclin-dependent kinase; SQ, squalene; FBS, fetal bovine serum; PI, propidium iodide; SIM, simvastatin; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal.

preventing the synthesis of isoprenoid intermediates such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) in the mevalonate pathway, statins may alter the subcellular localization and function of multiple proteins, including protein kinases, the subunit of trimeric G proteins, and Ras and Ras-related GTPases (Danesh et al., 2002). Pleiotropic effects of statins include anti-inflammatory properties as well as antiproliferative and proapoptotic effects (Koyuturk et al., 2004), all of these potentially relevant in treating AD. Growing evidence suggests that neuronal cell cycle regulatory failure, leading to apoptosis, may be a significant component of the AD pathogenesis (Herrup et al., 2004; Nagy, 2005). Neuronal changes supporting alteration on cell cycle control in the etiology of AD include the ectopic expression of cell cycle markers, or cytoskeletal alterations (Busser et al., 1998; Copani et al., 2001; Nagy, 2005). Moreover, it was reported that a significant number of hippocampal pyramidal and basal forebrain neurons in AD brain have undergone full or partial DNA replication (Yang et al., 2001). These events occur early in the progression of AD (Yang et al., 2003; Yang and Herrup, 2007), suggesting that cell cycle-induced death is a central mechanistic feature of the disease. There is an expanding body of evidence supporting the ability of some statins to exert direct antiproliferative and proapoptotic effects on various types of human cells (Katz, 2005). On these grounds, we have considered the possibility that the beneficial effects of statin therapy in AD could be related to their ability to interfere with cell cycle machinery. To this aim, we have investigated the effects of simvastatin, a lipophilic statin, on the distinct features of control of cell proliferation in lymphoblasts derived from late-onset AD patients. Previous work from this and other laboratories has demonstrated that cell cycle regulatory deficit is not restricted to neurons in AD; it is also observed in peripheral cells such as lymphocytes or fibroblasts (Tatebayashi et al., 1995; Nagy et al., 2002; de las Cuevas et al., 2003), thus providing a useful tool to study the involvement of cell cycle-related events in the pathogenesis of AD. A number of studies have found AD-specific changes in molecules and signaling pathways in peripheral lymphocytes that mirror changes in the brain (Eckert et al., 1998; Nagy et al., 2002; Muñoz et al., 2007). Moreover, these cells have also been used to study molecular changes in response to therapy in AD (Casademont et al., 2003; Reale et al., 2005). Conversely, Epstein-Barr virus (EBV) infection in vitro causes transformation of B cells and generates B-lymphoblastoid cell lines that resemble activated B cells (Neitzel, 1986). In fact, we have previously demonstrated identical cellular response to serum addition or withdrawal in peripheral lymphocytes or EBV-transformed lymphocytes from control and AD patients (Bartolomé et al., 2007; Muñoz et al., 2007). Taken together, these reports support a rationale for the use of peripheral cells, and in particular EBV lymphoblasts from AD patients as a model to further understand disease biology, progression, and therapeutic actions.

We report here that simvastatin selectively blocked the serum-enhanced proliferation of lymphoblasts from AD patients by regulating critical events of the G_1/S transition, suggesting potential additional molecular targets for the clinical efficacy of this drug in treating AD.

Materials and Methods

Materials

All components for cell culture were obtained from Invitrogen (Carlsbad, CA). Radioactive compounds were purchased from GE Healthcare (Uppsala, Sweden). Simvastatin and the caspase inhibitor benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK) were purchased from Calbiochem (Darmstadt, Germany). Polyvinylidene difluoride membranes for Western blots were purchased from Bio-Rad (Hercules, CA). Mouse monoclonal antibody anti-human
p27 was obtained from BD Biosciences Transduction Laboratories (Erembodegem, Belgium). Rabbit anti-human p27 (sc-528), anti-retinoblastoma protein (pRb) (sc-500), anti-p21 (sc-397), anti-cyclin E (sc-198), and anti-cyclin-dependent kinase (CDK)2 (sc-748) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The enhanced chemiluminescence system and radioactive compounds were from GE Healthcare. Mevalonate, geranylgeranyl pyrophosphate, farnesyl pyrophosphate, squalene (SQ), histone H1, and anti-β-actin antibody were obtained from Sigma-Aldrich (Alcobendas, Spain). All other reagents were of molecular grade.

Source of Cell Lines

EBV-immortalized lymphocytes from 20 late-onset AD patients (mean age $\pm$ S.D., 75.6 $\pm$ 7) and 20 nondemented age-matched individuals were selected from the cell lines present in our cell repository. The diagnosis of probable Alzheimer was made in the Department of Neurology of the University Hospital Doce de Octubre (Madrid, Spain) according to National Institute of Neurological and Communicative Diseases and Stroke-Alzheimer's Disease and Related Disorders Association criteria. The frequency of the apolipoprotein E4 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 of Spain (Ibarreta et al., 1995) and consistent with the late-onset form of AD.

All study protocols were approved by the Spanish Council of Higher Research Institutional Review Board, and they 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.

Culture of Human Lymphoblasts

Cells were grown in suspension in T flasks in an upright position, in approximately 10 ml of RPMI 1640 medium (Invitrogen) medium that contained 2 mM L-glutamine, 100 mg/ml penicillin/streptomycin, and, unless otherwise stated, 10% (v/v) fetal bovine serum (FBS). Flasks were 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

Proliferation was determined by cell counting in a Neubauer chamber. EBV-immortalized lymphoblasts from control and AD individuals were seeded at an initial cell concentration of 1×10^6 cells/ml. Cells were serum-starved for 24 h. The next day, cells were stimulated by adding 10% FBS. Cells were enumerated every day thereafter. Potential toxicity of the reagents used was routinely checked by trypan blue exclusion under inverted phase-contrast microscopy.

Flow Cytometric Analysis

Exponentially growing cultures of cell lines were seeded at an initial concentration of 1×10^6 cells/ml. Forty-eight hours later, cells were analyzed in an EPICS-XL cytofluorimeter (Coulter Científica, Móstoles, Spain). An apoptosis detection kit that measured phosphatidylserine and propidium iodide (PI) was purchased from BD Bio-

sciences PharMingen (San Diego, CA). The assay was conducted following manufacturer directions. Cells were analyzed for phosphatidylserine exposure/PI exclusion by staining with fluorescein isothiocyanate-annexin V and PI.

Immunological Analysis

Cell Extracts. To prepare whole cell extracts, cells were harvested, washed in phosphate-buffered saline, and then lysed in ice-cold lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 50 mM NaF, and 1% Nonidet P-40), containing 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium pyrophosphate, and protease inhibitor Complete Mini Mixture (Roche Diagnostics, Mannheim, Germany). The protein content of the extracts was determined by the Bio-Rad protein assay kit (Bio-Rad).

Western Blot Analysis. Whole cell extracts (50–100 μg) were fractionated on an SDS-polyacrylamide gel and then transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Billerica, MA). The amount of protein and the integrity of transfer were verified by staining with Ponceau-S solution (Sigma-Aldrich). The filters were then blocked with nonfat milk or bovine serum albumin, and they were incubated overnight at 4°C, with primary antibodies from Santa Cruz at the following dilutions: 1:500, anti-pRb; 1:500, anti-p27; 1:500, anti-p21; 1:500, anti-cyclin E; and 1:2000, anti-β-actin. Signals from the primary antibodies were amplified using species-specific antisera conjugated with horseradish peroxidase (Sigma-Aldrich) and detected with a chemiluminescence substrate detection system (ECL; GE Healthcare). Blots were stripped and reprobed with anti-β-actin as a protein loading control. The relative protein levels were determined by scanning the bands with a GS-800 imaging densitometer provided with the QuantityOne 4.3.1 software (Bio-Rad), normalized by that of β-actin. The specificity of anti-p21 and anti-p27 antibodies was checked by using the corresponding blocking peptide obtained from Santa Cruz Biotechnology, Inc. (sc-397-p and sc-528-p, respectively), following manufacturer's directions.

Immunoprecipitation and Cyclin E/CDK2 Kinase Assay

Lymphoblasts from control and AD individuals were seeded at an initial cell density of 1×10^6 cells/ml and incubated for 24 h. Protein extracts (500 μg) were incubated with an antibody against cyclin E for 2 h at 4°C, followed by an incubation with 20 μl of protein G-Sepharose for 2 h. Samples were washed in kinase buffer (50 mM KCl, 8 mM $MgCl_2$, 1 mM dithiothreitol, 3 mM ATP, and 50 mM HEPES, pH 7.4). The immune complexes were resuspended in 40 μl of kinase buffer containing 0.2 μg/μl histone H1. [γ-^{32}P]ATP (10 μCi) was added, and after shaking for 1 h at 37°C, the reaction was stopped by addition of 10 μl of 6× SDS sample buffer. After boiling, the samples were resolved in a 12% SDS-polyacrylamide gel electrophoresis, and the phosphorylated histone H1 was visualized by autoradiography. The amounts of histone H1 were detected by gel staining with Coomassie.

Statistical Analysis

Unless otherwise stated, all data represent means ± S.E. 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 followed by the Scheffé post hoc analysis. Differences were considered significant at a level of $p < 0.05$.

Results

Simvastatin Causes Both Cell Growth Inhibition and Apoptotic Cell Death. Figure 1 shows a time course analysis of the effect of increasing doses of simvastatin on rates of proliferation, upon serum stimulation, of lymphoblasts from control and AD patients. In agreement with previous reports

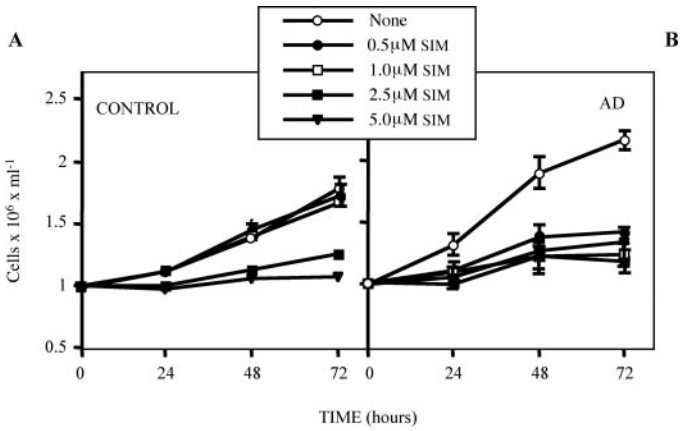

Fig. 1. Effect of increasing concentrations of SIM on proliferation of lymphoblasts from normal and AD individuals. Lymphoblasts from control (A) and AD subjects (B) were seeded at an initial density of $1 \times 10^6 \times ml^{-1}$ in RPMI 1640 medium containing 10% FBS, in the presence of increasing concentrations of SIM, and they were cultured for 3 days. Every day thereafter, samples were taken for cell counting. Values shown are the means ± S.E. for six to eight independent experiments carried out with different individuals.

from this laboratory (de las Cuevas et al., 2003; Muñoz et al., 2005, 2007), the proliferative activity of AD lymphoblasts was significantly higher than that of control cells. Treatment of cells with simvastatin resulted in a significant inhibition of cell growth of both control and AD lymphoblasts in a dose-dependent manner. However, the sensitivity toward SIM inhibitory action was higher in cells derived from AD subjects. In these cell lines, the inhibitory action was observed at the lowest concentration tested (0.5 μM) (Fig. 1B), whereas a 5-fold increase in the concentration of simvastatin is needed to decrease the total cell number in control cultures (Fig. 1A).

We next assessed the percentage of cells showing characteristics of apoptosis/necrosis following treatment with SIM. To this end, we performed a comparative analysis of annexin V exposure/PI exclusion by flow cytometry. Only high concentrations of SIM, 5 μM, induced a significant increase in the percentage of apoptotic and necrotic cells, both in control and AD lymphoblasts (Fig. 2, right). In contrast, 1 μM SIM did not have significant apoptotic or necrotic effects on AD cells. Figure 2 (middle and left) shows that approximately 80% of cells remained viable after 72 h of serum stimulation in the absence and in the presence of 1 μM SIM. These results suggest that the blockade of the serum-induced increase in the cell number of AD lymphoblasts, by low doses of SIM, is due to inhibition of cell proliferation rather than the consequence of cytotoxic or apoptotic effects.

Simvastatin Inhibits the Serum-Mediated Enhanced Proliferation of AD Lymphoblasts. To further study the antiproliferative action of SIM, 1 μM concentration was chosen in subsequent experiments to obtain maximal differences in the effect of SIM in AD cells without affecting proliferation of control cells (Fig. 3). As shown in Fig. 3, the inhibitory effect of simvastatin was time-dependent.

For determining the role of various isoprenoids derived from MEV in regulating the inhibitory effect of simvastatin on proliferation of AD lymphoblasts, cells were cotreated with 1 μM SIM and MEV or various isoprenoid intermediaries GGPP, FPP, and SQ. Figure 4 shows that MEV reversed the inhibitory effect of SIM on cell proliferation. The cotreatment of AD cells with GGPP and FPP, but not SQ, prevented

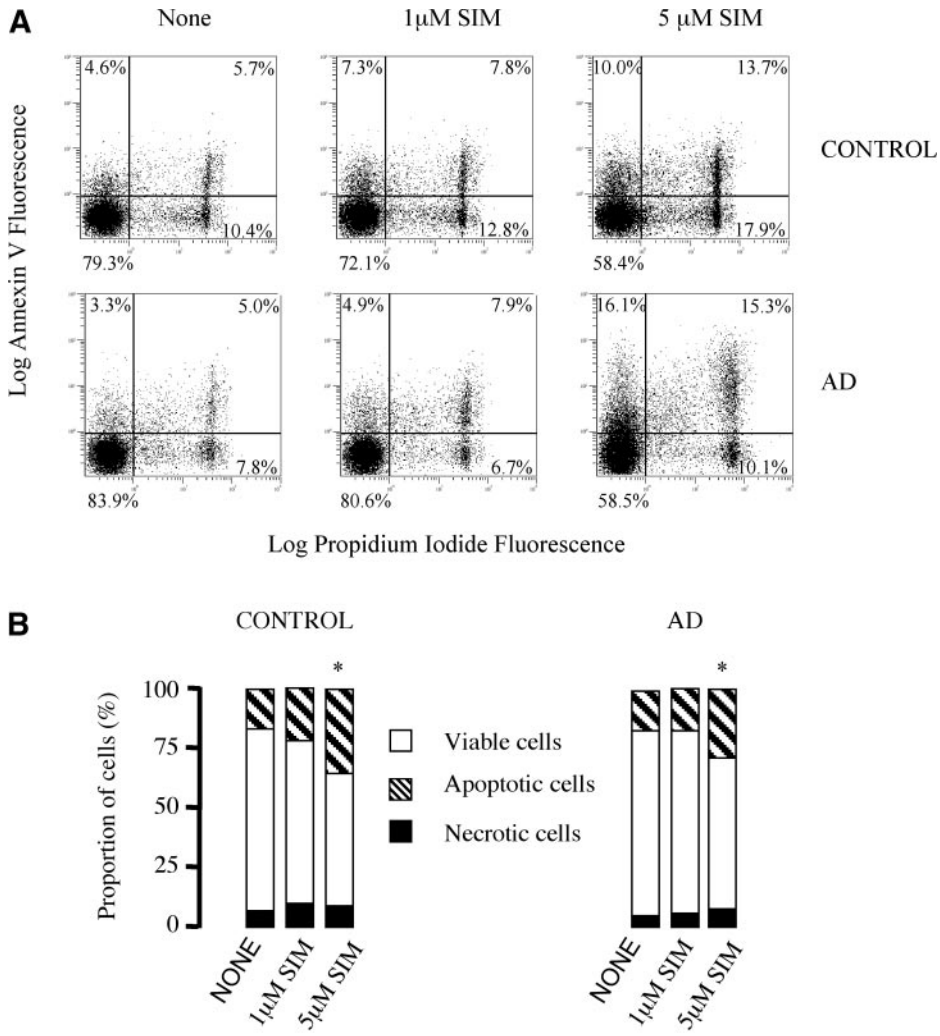

Fig. 2. Flow cytometric analysis of the fraction of viable, apoptotic, and necrotic cells after treatment of AD lymphoblasts with SIM. A, lymphoblasts from AD subjects were seeded at an initial density of $1 \times 10^6 \times ml^{-1}$, and they were cultured for 2 days in the absence (Me_2SO) or in the presence of 1 or 5 μM SIM. Then, they were stained with fluorescein isothiocyanate-annexin V and propidium iodide. A representative experiment is shown. B, means of five experiments carried out with cells derived from different individuals are shown. $*$, $p < 0.05$, significant difference in the proportion of apoptotic cells compared with untreated cultures.

the action of SIM, suggesting that the modulatory effect of SIM is dependent on GGPP and FPP and independent of cholesterol synthesis.

Effects of Simvastatin on the Expression of Key Cell Cycle Regulatory Proteins. Previous work from this laboratory demonstrated that the serum-mediated enhancement of AD cell proliferation is the consequence of altered G_1/S transition (de las Cuevas et al., 2003; Muñoz et al., 2005). It was shown that serum induced an increase in the expression levels and phosphorylation status of the pRb family proteins in AD lymphoblasts. For this reason, we investigated the effect of SIM on the activity of the cyclic E-dependent kinase and on the phosphorylation of pRb in control and AD lymphoblasts. Data in Fig. 5 confirm and extend our previous finding (de las Cuevas et al., 2003), showing that both levels and phosphorylation of pRb were increased in AD lymphoblasts (Fig. 5). The SIM treatment did not alter significantly the phosphorylation status of pRb in control cells, but it partially prevented the serum-induced enhanced phosphorylation of pRb in lymphoblasts from AD patients (Fig. 5). SIM inhibited the serum-mediated enhanced cyclin E/CDK2-associated kinase activity in AD cells (Fig. 5).

The SIM-induced inhibition of cell proliferation and cyclin E/CDK2 kinase in AD cells was not due to changes in the expression levels of CDK2 and cyclin E (Fig. 6A), but it was associated with an accumulation of the CDK inhibitors $p27^{Kip1}$ and $p21^{Cip1}$ (hereafter p27 and p21, respectively). SIM addition increased the content of these two proteins in AD lymphoblasts, without changing the expression levels of p27 and p21 in control cells (Fig. 6B).

Effect of SIM on Degradation of p21 and p27 Proteins. As in other cell types, p21 is a short-lived protein in human lymphoblasts (Fig. 7). Inhibition of protein synthesis with cycloheximide leads to a rapid decrease in p21 levels, with less than 50% remaining after 2 h. It is shown that p21 disappears faster in AD cells than in control lymphoblasts, showing a shorter half-life of p21 (0.9 ± 0.04 versus 1.7 ± 0.12 h in control cells). Treatment of cells with SIM had no effect on control cells, but it significantly reduced the rate of decay of p21 levels on AD cells (Fig. 7). The cellular content of β-actin was not affected by SIM treatment in control and in AD cells (Fig. 7), thus making unlikely the possibility of a direct interference with the activity of cellular proteases. In agreement with a recent report from this laboratory (Muñoz et al., 2007), p27 degradation was enhanced in AD lymphoblasts (Fig. 7). This protein has a longer half-life than p21 in AD cells (approximately 12 h). As shown in Fig. 7, treatment of AD cells with SIM increased the half-life of p27 protein to values found for control cells (approximately 22 h).

Figure 8 shows that the effects of SIM on p21 and p27

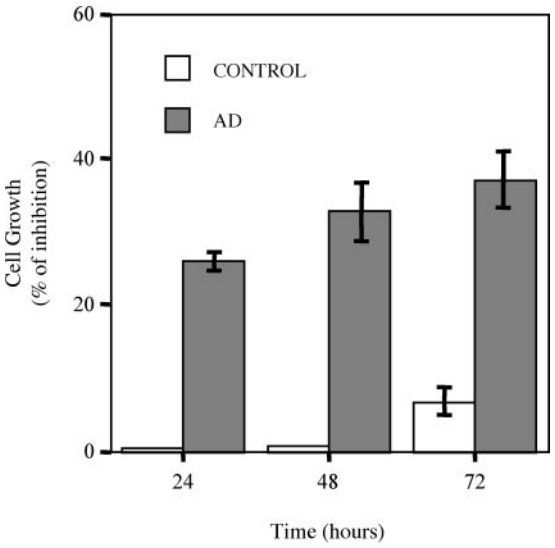

Fig. 3. Effect of SIM on the serum-mediated proliferative activity of control and AD lymphoblasts. Lymphoblasts from control and AD subjects were seeded at an initial density of $1 \times 10^6 \times ml^{-1}$, and they were cultured for 3 days in the absence (Me_2SO) or in the presence of 1 μM SIM. Every day thereafter cells were enumerated. The percentage of cell growth is shown after setting the growth of untreated cells as 100%. Values shown are the means ± S.E. for six to eight experiments carried out with cells derived from different individuals. The mean values of cell proliferation ranged from 1.7 to 1.92 and from 1.6 to 1.99 cells $\times 10^6 \times ml^{-1}$ in control cells in the absence or in the presence of 1 μM SIM, respectively. For AD cells, the corresponding values ranged from 2.02 to 2.43 and from 1.1 to 1.8 cells $\times 10^6 \times ml^{-1}$ in the absence or in the presence of 1 μM SIM.

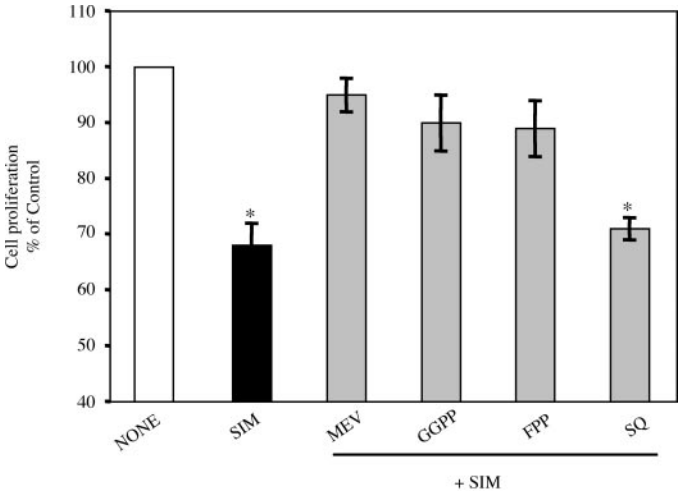

Fig. 4. Modulatory effect of SIM on serum-induced cell proliferation in AD lymphoblasts. Lymphoblasts from AD subjects were seeded at an initial density of $1 \times 10^6 \times ml^{-1}$, and they were cultured for 3 days in the absence (Me_2SO), or in the presence of 1 μM SIM alone or in combination with 200 μM MEV, 5 μM GGPP, 5 μM FPP, or 5 μM SQ. Cell proliferation was determined by enumeration of cells excluding trypan blue. The percentage of cell proliferation is shown after setting the proliferation of untreated cells as 100%. Values shown are the means ± S.E. for four experiments carried out with cells derived from different individuals. *, $p < 0.01$, significantly different from untreated cells.

levels were completely prevented by cotreatment with cycloheximide, indicating de novo protein synthesis is required for its effects. SIM and the proteasome inhibitor MG132 had similar effect on p21 and p27 accumulation, and no additive effects were observed when added together (Fig. 8). In addition, the caspase inhibitor Z-VAD-FMK did not prevent the

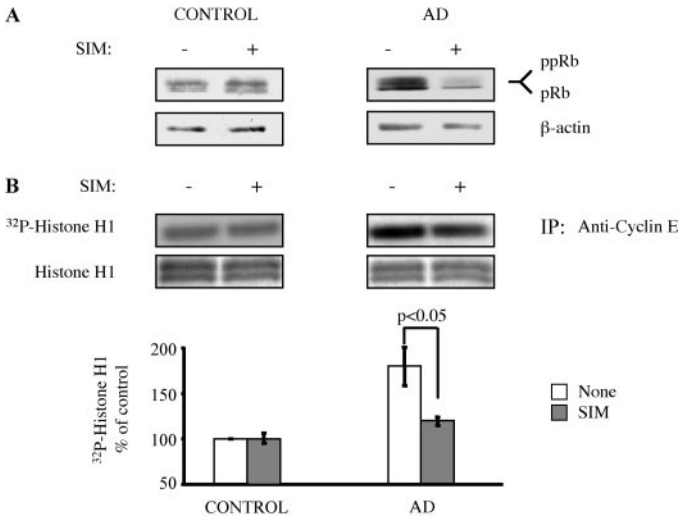

Fig. 5. Effects of SIM on phosphorylation status of pRb and cyclin E/CDK2 kinase activity in control and AD lymphoblasts. A, lymphoblasts from AD subjects were seeded at an initial density of $1 \times 10^6 \times ml^{-1}$ and incubated for 24 h in the absence (Me_2SO) or in the presence of 1 μM SIM. Hypo- and hyperphosphorylated pRb (ppRb) levels were determined by Western blot. Representative Western blots are presented. B, control and AD lymphoblasts were incubated in RPMI 1640 medium containing 10% FBS for 24 h. Cell extracts were immunoprecipitated using anti-cyclin E antibody, and they were assayed for kinase activity using histone H1 as substrate. Phosphorylated histone H1 was visualized using autoradiography. Levels of H1 were determined by gel staining with Coomassie. A representative experiment is shown, whereas below the densitometric analysis is presented. Data shown represent the means ± S.E. for four different experiments.

effect of SIM on p21 and p27 accumulation in AD cells. Taken together, these results suggest that SIM inhibits the proteasome-mediated degradation of p21 and p27 proteins.

Discussion

Recent evidence suggests that statin use is associated with decreased risk for AD, although the mechanisms underlying the apparent risk reduction are poorly understood. In the present work, we have considered the possibility that the benefit of statins treatment for AD patients is related to the ability of these drugs to inhibit cell proliferation. This issue has been addressed by carrying out a comparative study of the influence of statin treatment on the proliferative activity of EBV-immortalized lymphocytes from late-onset AD patients and age-matched nondemented individuals. Previous work from this laboratory indicates that differences in the proliferative activity and cell cycle regulatory proteins are also found between untransformed lymphocytes from control and AD subjects (Bartolomé et al., 2007; Muñoz et al., 2007).

Incubation of lymphoblasts from AD patients and control subjects, with increasing concentrations of SIM inhibited cell growth and induced apoptosis in a dose-response-dependent manner. AD cells were more sensitive to SIM than control cells. At low doses (≤1 μM), SIM selectively blunted the serum-mediated enhancement of proliferation of AD lymphoblasts, without altering the normal basal rates of proliferation. It is known that statin induced cell growth inhibition is tumor cell-specific. Micromolar concentrations of statins were very effective in inhibiting cell proliferation of human hepatocarcinoma cells, but they had little effect on normal hepatocytes (Kubota et al., 2004). Moreover, primary myeloid

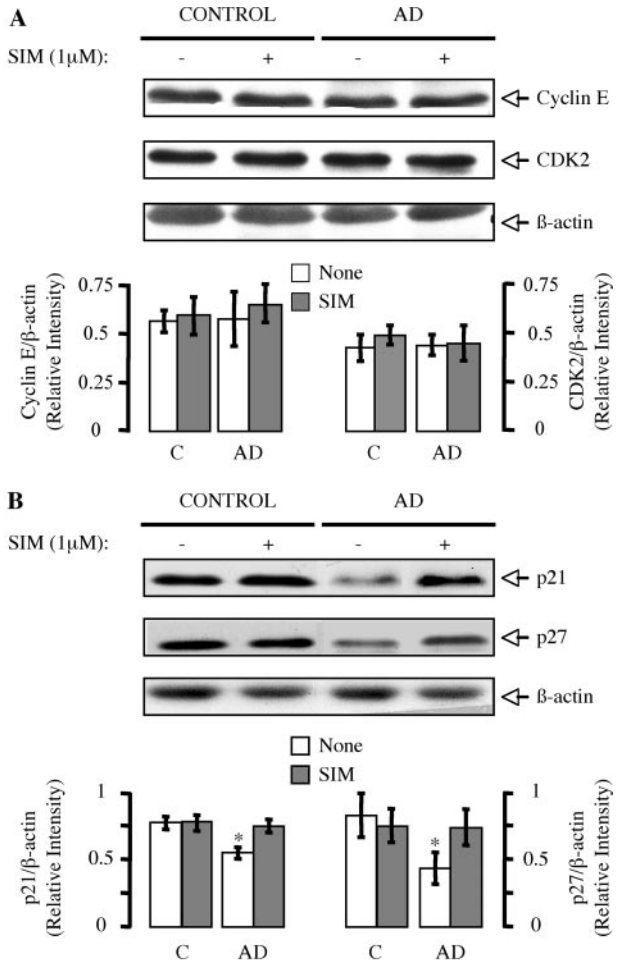

Fig. 6. Effects of SIM on CDK2, cyclin E, and p21 and p27 protein levels in control and AD lymphoblasts. A, lymphoblasts from AD subjects were seeded at an initial density of $1 \times 10^6 \times ml^{-1}$, and they were cultured for 24 h in the absence (Me_2SO) or in the presence of 1 μM SIM. Representative Western blots of cyclin E and CDK2 proteins in control and AD lymphoblasts are shown. Band intensities were determined and normalized by that of β-actin. Results are means ± S.E. for four to six independent experiments. B, representative Western blots of p21 and p27 proteins. The densitometric data represent the means ± S.E. for six different experiments. *, $p < 0.05$.

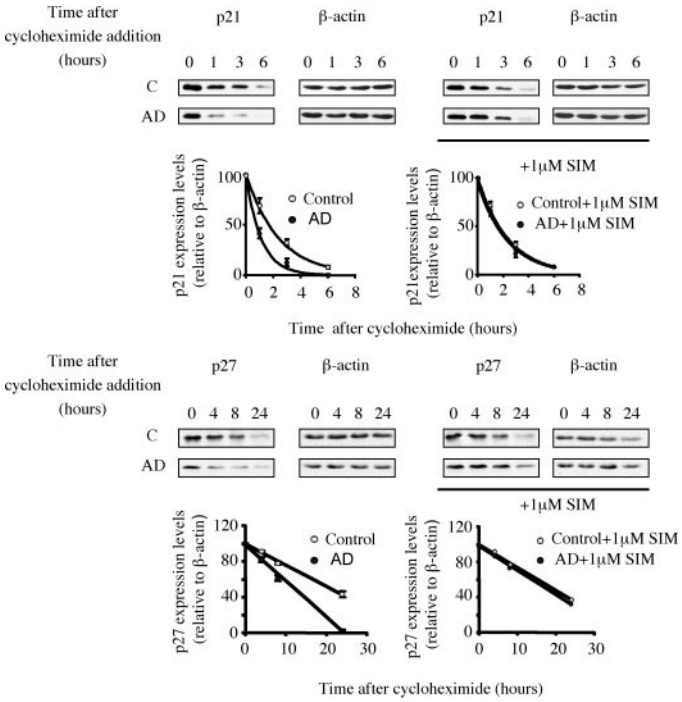

Fig. 7. Effect of SIM on degradation of p21 and p27 proteins in control and AD lymphoblasts. A, cells were incubated for 24 h in RPMI 1640 medium containing 10% FBS in the absence (Me_2SO) or in the presence of 1 μM SIM, and then 20 μg/ml cycloheximide was added. Cells were harvested 1, 3, and 6 h thereafter, and p21 was detected by immunoblotting. The decay of the p21 signal is graphed as a function of time post-cycloheximide addition. Data from five different experiments were used to calculate the half-life of the protein. B, experimental conditions are identical to that described above, except that for p27 detection, aliquots of cells were taken at 4, 8, and 24 h after cycloheximide addition. The experiment was performed twice with different cell lines with similar results, and one of the experiments is shown.

B leukemic and myeloma cells undergo apoptosis with statins, whereas their normal counterparts are resistant to statin effects (van de Donk et al., 2002). Thus, the higher sensitivity of AD cells to SIM may represent other neoplastic-like feature of these cell lines, as suggested previously (de las Cuevas et al., 2005).

The antiproliferative effect of SIM in AD cells is directly related to HMG-CoA reductase inhibition because cell proliferation was completely or partially rescued by MEV or FPP and GGPP, respectively, but not by SQ, an intermediate of cholesterol synthesis. These results suggest that prenylation of small G proteins could be involved in the serum-enhanced proliferation of AD cells.

SIM inhibits the serum-mediated increased proliferation of AD cells by attenuating the activity of several key cell cycle regulators that control G_1/S progression. It particularly prevented the enhanced phosphorylation of pRb in response to serum in AD cells. Mitogenic stimuli induce hyperphosphorylation of pRb and related "pocket" proteins from mid-G_1 to mitosis. pRb hyperphosphorylation releases E2F transcription factors, thus contributing to the expression of several growth and cell cycle regulatory genes with functional E2F binding sites in their promoters (Stevaux and Dyson, 2002). In agreement with previous reports (de las Cuevas et al., 2003; Muñoz et al., 2005, 2007) lymphoblasts from AD patients showed a higher degree of phosphorylation of pRb. The addition of SIM had no effect on the phosphorylation status of pRb in control cells, but it significantly reduced the levels of the hyperphosphorylated form of pRb in AD lymphoblasts. This effect was due to inhibition of the kinase activity of cyclin E/CDK2 complex, and it was found to be associated with increased levels of the CDK inhibitors p21 and p27.

Accumulating evidence has suggested the involvement of p21 and p27 in statins-induced antiproliferative effects in a number of cell types (Efuet and Keyomarsi, 2006; Takeda et al., 2007). However, the molecular mechanisms implicated in up-regulation of these proteins by statins treatment are not fully elucidated.

p21 and p27 abundance are regulated at the levels of transcription and protein turnover. The ubiquitin-proteasome pathway is thought to be the prevalent mechanism of p27 regulation in many cellular systems (Pagano et al., 1995). The regulation of p21 levels is very complex. Although transcriptional regulation, by p53-dependent (el-Deiry et al., 1993) and p53-independent mechanisms (Parker et al., 1995), is well established, recent studies suggest that p21 can also be regulated by posttranslational mechanisms (Na-

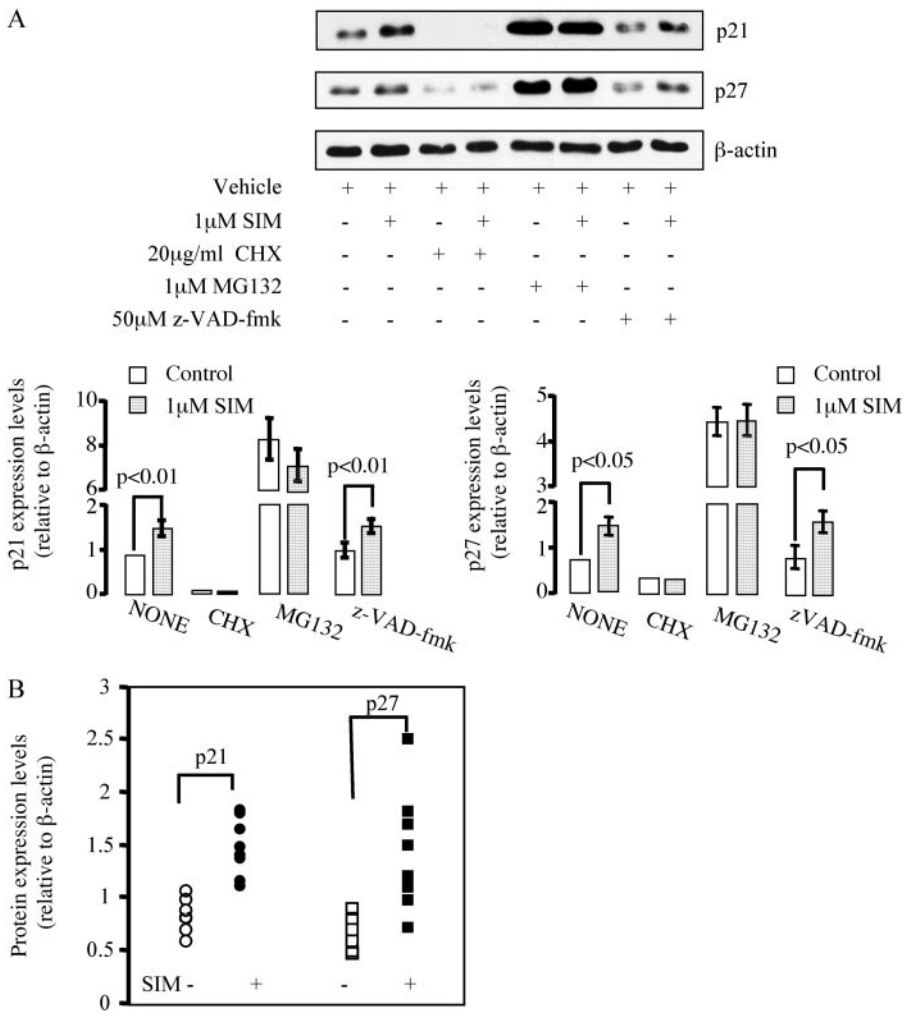

Fig. 8. Effects of cycloheximide, MG132, and Z-VAD-FMK on SIM-induced p21 and p27 accumulation in AD lymphoblasts. A, cells from AD patients were incubated for 24 h in RPMI 1640 medium containing 10% FBS, in the absence (Me_2SO) or in the presence of 1 μM SIM alone or with 20 μg/ml cycloheximide, 1 μM MG132, or 50 μM Z-VAD-FMK. Representative Western blots of p21 and p27 are presented. Densitometric analysis of these proteins is shown below. Results are means ± S.E. for six to eight independent experiments. B, scatter plots comparing p21 and p27 levels between untreated (open symbols) or treated with 1 μM SIM (filled symbols) lymphoblasts derived from eight different patients.

kayama and Nakayama, 2006). Our results suggest that SIM induced the accumulation of p21 and p27 proteins in AD lymphoblasts, at least partially, by inhibiting the rate of degradation of these two proteins. First, the half-lives of p21 and p27 were increased up to the values found in control cells, following treatment of AD lymphoblasts with SIM, and second, the effects of SIM were dependent on de novo protein synthesis. The ubiquitin-proteasome pathway seems to be implicated in the impaired degradation of p21 and p27 proteins induced by SIM. This asseveration is supported by the observations that SIM mimicked the effect of the proteasome inhibitor MG132 on the cellular content of p21 and p27 proteins and that the effect of SIM was not influenced by cotreatment with the caspase inhibitor Z-VAD-FMK. Whether SIM is able to modify the activity of the proteasome machinery or the ubiquitination of p21 and p27 proteins cannot be ascertained from the present work. We had recently reported that global proteasome activity and the accumulation of ubiquitin-tagged proteins are not impaired in lymphoblasts from AD patients (Muñoz et al., 2007) and thus cannot be the explanation for the down-regulation of p21 and p27 in AD cells. It was also shown that enhanced degradation of p27 protein in AD lymphocytes was dependent on signaling through phosphatidylinositol 3-kinase/Akt pathway (Muñoz et al., 2007). Whether this pathway is also implicated in the control of the cellular content of p21 protein and the possible modulation of the phosphatidylinositol 3-kinase/Akt-mediated signaling pathway by statins is currently under investigation in our laboratory. The possibility should also be considered that differences in the cellular content of the CDK inhibitors between control and AD cells may be due to changes in the redox balance in AD cells, taking into account the sensitivity of p27 and p21 protein levels to redox status (Hwang et al., 2004).

In summary, the present work indicates that SIM could effectively suppress the serum-mediated enhanced proliferation of AD cells, and this effect was associated with substantial elevation of CDK inhibitors p21 and p27, leading to inactivation of the kinase activity of the cyclin E/CDK2 complex during G_1/S progression. Considering that changes in the abundance of CDK inhibitors had also been detected in AD brain (Griffin et al., 2005) and that cell cycle regulatory failure has been linked to the disease pathogenesis, our results could provide new insight into one of the multiple potential mechanisms through which statins may exert neuroprotective actions in AD brain. This effect of SIM, upregulating specifically p21 and p27 levels may lead to vulnerable neurons that had entered the cell cycle to arrest at G_1/S restriction point.

Regarding the pathophysiological relevance of our findings, it is worth mentioning that recent evidence in AD patients and in animal models (Yang et al., 2003; Yang and

Herrup, 2007) supports the hypothesis 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 (Yang et al., 2003), and cell cycle disturbances have also been reported in lymphocytes from mild cognitive impairment patients (Nagy et al., 2002). These observations suggest that cell cycle-induced death is a central mechanistic feature of the disease, and therefore alterations in cell cycle regulatory proteins may serve as disease markers. On these grounds, the demonstration of shared alterations in p21 and p27 protein levels in peripheral lymphocytes and AD brain, suggest that these cells, easily obtainable, may be potential useful surrogate for early diagnosis and therapeutic monitoring of AD.

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Address correspondence to: Dr. Ángeles Martín-Requero, Centro de Investigaciones Biológicas, Ramiro de Maeztu 9, 28040 Madrid, Spain. E-mail: amrequero@cib.csic.es