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The Preventive Effects of Bcl-2 and Bcl-x_L on Lovastatin-induced Apoptosis of C6 Glial Cells

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It has been reported that lovastatin induced cell death and suppressed proliferation in various cell lines. In this study, we examined whether the cytotoxic effects of lovastatin could be prevented by Bcl-2 or Bcl-x_L in C6 glial cells. Overexpression of human Bcl-2 or Bcl-x_L prevented lovastatin (25 μM)-induced changes such as DNA fragmentation, chromatin condensation, disruption of cell membrane, and cleavage of poly (ADP-ribose) polymerase. Lovastatin-induced inhibition of cell proliferation was unaffected by Bcl-2 or Bcl-x_L overexpression. These results suggest that Bcl-2 and Bcl-x_L can prevent lovastatin-induced apoptosis in C6 glial cells, though the inhibition of proliferation remains unaffected by these proteins.

Key Words: Lovastatin, Apoptosis, C6, Bcl-2, Bcl-x_L

INTRODUCTION

Recently, it has been reported that lovastatin, a well-known anti-hypercholesterolemic agent, inhibited proliferation and induced cell death of C6 glial cells (Choi & Jung, 1999). Lovastatin inhibits the 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase that synthesizes mevalonate from HMG-CoA. As a consequence, lovastatin deprives cells of mevalonate, and that was suggested as the cause of lovastatin-induced cytotoxicities (Choi & Jung, 1999). In cells, mevalonate is subsequently converted into various isoprenoids which are required for protein prenylation. Prenylation is a posttranslational modification involving the addition of the farnesyl or geranylgeranyl moiety to the cysteine residue near the carboxyl terminus of proteins, that is needed for activation of various membrane-bound proteins responsible for mediating signal transduction (Zhang & Casey, 1996). Since prenylated proteins such as $p21^{Ras}$-like GTPases are essential for cellular homeostasis including proliferation and viability (Seabra, 1998), inhibition of the prenylation process by deprivation of intracellular mevalonate seems to cause cellular collapse.

It was reported that inhibition of the prenylation process could suppress proliferation or induce cell death in various transformed cell lines. For example, farnesyltransferase inhibitors suppressed the growth of $p21^{Ras}$-dependent tumors in nude mice (Yonemoto et al, 1998). The farnesyltransferase inhibitors were also suggested as a valuable chemotherapeutic candidate against high-grade gliomas (Bredel & Pollack, 1999). HMG-CoA reductase inhibitors also induced cell death in several transformed cells such as C6 glioma (Choi & Jung, 1999), malignant glioma (Jones et al, 1994), malignant mesothelioma (Rubins et al, 1998), and acute myeloid leukemia cells (Dimitroulakos et al, 1999). These reports further suggested the possible value of prenylation inhibitors as chemotherapeutic agents against malignancies (Thibault et al, 1996; Dimitroulakos et al, 1999).

In more than half of all human malignancies, anti-apoptotic proteins, especially Bcl-2, are found at high levels. This is believed to be the cause of inherent multi-drug resistance of cancer cells and is correlated with poor prognosis (Reed, 1997). Since Bcl-2 blocks the execution phase of the apoptotic pathway, it seems possible that it also prevents the cytotoxic effects of prenylation inhibitors. Thus, we investigated the effects of anti-apoptotic genes, *bcl-2* and *bcl-x_L*, on the cytotoxic effects induced by lovastatin. In this study, we showed that the overexpression of either Bcl-2 or Bcl-x_L prevented lovastatin-induced cell death in C6 glioma cells without alleviating the lovastatin-induced inhibition of cell proliferation. These results indicated that the possible anti-tumor chemotherapeutic effect of prenylation inhibitors is correlated with the expressed level of Bcl-2 or Bcl-x_L.

METHODS

Cell culture and media

C6 cells were maintained in Dulbecco's Minimal Essential Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), non-essential amino acids, penicillin (final concentration, 50 units/liter) and streptomycin (final con-

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ABBREVIATIONS: HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; PI, propidium iodide; LVT, lovastatin; MEV, mevastatin.

centration, 50 μg/liter) at 37°C (all from Sigma Chemical Co., St. Louis, MO). C6 cells were transfected with one of the plasmids, pCMVNeo, pCMVBcl-2, or pCMVBcl-x_L by using lipofectamine (Gibco-BRL, Gaithersburg, MD). pCMVBcl-2 and pCMVBcl-x_L were prepared by subcloning human *bcl-2* or *bcl-x_L* into the EcoRI site of a pCMVNeo expression vector as described previously (Oh et al, 1997). C6/*neo*, C6/*bcl-2* and C6/*bcl-x_L* cells were selected in the medium containing 200 μg/ml of G418 (Gibco-BRL). The expressed level of Bcl-2 or Bcl-x_L was estimated by Western blot analysis. Lovastatin was kindly provided by Choong Wae Pharmaceutical Co. (Seoul, Korea), and was prepared as described by Kita et al (Kita et al, 1980).

Cell proliferation assay

Cells were seeded at a density of 5×10^3 cells/well in 100 μl culture medium in a 96 well plate (Falcon; Becton Dicknson, Franklin Lakes, NJ). Lovastatin and/or mevalonic acid lactone were added to the medium at the appropriate concentrations and cells were cultured for the indicated time periods. Cell proliferation kit II (Boehringer Mannheim; GmbH Biochemica, Mannheim, Germany) was used to measure the number of cells. The number of cells was expressed as mean$\pm$S.E. and statistical analysis of difference was carried out by two-tailed Students' *t* test. All values represent at least three independent experiments performed in triplicate.

DNA fragmentation assay

Internucleosomal DNA fragmentation was examined as described by Lyons et al. (Lyons et al, 1992). Cells were harvested by trypsinization and centrifuged at 800 g for 10 min. After resuspending cells in 250 μl of TE buffer containing 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, 250 μl of lysis buffer (5 mM Tris, pH 8.0, 20 mM EDTA, and 0.5% Triton X-100) was added. The sample was centrifuged at 16,000 g for 15 min in a microcentrifuge (Eppendorf, Hamberg, Germany), and the DNA in the supernatant was ethanol precipitated overnight. The sample was digested with RNase (Boehringer Mannheim, GmbH Biochemica, Mannheim, Germany) at a final concentration of 1.5 mg/ml for 1 hour at 37°C, and with proteinase K (Boehringer Mannheim, GmbH Biochemica, Mannheim, Germany) at a final concentration of 1 mg/ml for an additional 2 hours at 37°C. The sample was analyzed on a 1.5% agarose gel.

Fluorescence-activated cell sorting (FACS) analysis

Cells were cultured in the medium containing lovastatin at a concentration of 25 μM for 36 or 48 hours. The percentage of viable and dead cells was estimated on a FACScalibur™ (Becton Dickinson, San Jose, CA) after staining cells with propidium iodide (PI; Sigma Chemical Co., St. Louis, MO) as described by Hamel et al. (Hamel et al, 1996). After collecting the culture medium, cells were rinsed with PBS and detached by trypsinization. Cells were resuspended in 5 volumes of culture medium and this cell suspension was pooled with medium saved from the corresponding culture that contained detached cells. After centrifugation, cells were resuspended in 1 ml of ice-cold PBS containing PI at a concentration of 5 μg/ml and kept on ice. Then, cells were kept at room temperature for 10~15 min and the fluorescence intensity was measured. The data were analyzed by using Cellquest™ software (Becton Dickinson).

Western blot assay

Cells cultured in a 6-well plate (Falcon) were rinsed once with PBS and were lysed with a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5 mM PMSF. Cell lysate was kept at 4C for 15 min and centrifuged at 16,000 g for 10 min in the microcentrifuge (Eppendorf). The supernatant was collected and the protein content was determined by using the protein assay reagent (Bio-Rad, Hercules, CA). The extract was mixed with one volume of 2 X SDS sample buffer containing 125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 200 mM DTT, and 0.001% bromophenol blue. After heating the samples at 95~100°C for 5 min, approximately 40 μg of protein was electrophoresed on 10~12% SDS-PAGE gel and transferred to PVDF membrane (Bio-Rad). Membranes were blocked overnight in a buffer containing 20 mM Tris, pH 7.6, 0.8% (w/v) NaCl, 0.1% Tween-20, 5% non-fat dry milk. Subsequently, membranes were probed overnight at 4°C with anti-poly (ADP-ribose) polymerase (PARP) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and for 2 hours with anti-rabbit antibody conjugated with horseradish peroxidase. PARP and its cleaved fragment of 89 kDa protein containing the carboxyl terminal were detected by Lumi GLO (New England BioLabs, Beverly, MA) according to the manufacturers instructions. Antibodies against Bcl-2 and Bcl-x_L (Santa Cruz Biotechnology Inc) were used to detect the expressed level of these anti-apoptotic proteins in stably transfected C6 cell lines.

Electron microscopic assay

After trypsinization, suspended cells were centrifuged at 800 g for 10 min and fixed in Karnovsky solution containing 0.1 M cacodylate, pH 7.4, 2% glutaraldehyde, 2% paraformaldehyde, and 0.5% $CaCl_2$. Samples were postfixed in cacodylate-buffered osmium tetroxide (1.33%), dehydrated sequentially using 50~100% ethanol, immersed in propylene oxide for 10 min, and embedded in EPON mixture. Thin sections were stained with uranyl acetate and lead citrate, and then examined using a Philips CM-10 electron microscope (Philips Electron Optics, Eindhoven, Holland).

RESULTS

Protective effect of Bcl-2 and Bcl-x_L for lovastatin-induced cell death

The cytotoxic effects of lovastatin were examined in C6 cells stably transfected with human *bcl-2* or *bcl-x_L*. When cells were cultured in the medium containing lovastatin at a concentration of 25 μM, cell numbers at 24 hours after lovastatin treatment were significantly suppressed compared to the control regardless of the expression of Bcl-2 or Bcl-x_L (Fig. 1). Such inhibitory effect of lovastatin was completely prevented by adding mevalonate to the medium at a concentration of 300 μM (Fig. 1), showing that lovastatin-induced cytotoxicity was induced by mevalonate deprivation. The decrease in cell number may have resulted from either cell death or proliferation inhibition. To determine viability, C6 cells were examined by PI staining,

which is selectively permeable to cells with disrupted plasma membrane. At 48 hours after lovastatin treatment, FACS assay showed that the occurrence of PI-permeable cells in C6/*bcl-2* and C6/*bcl-x*$_L$ was greatly reduced compared to mock-transfected C6/*neo* cells (Fig. 2). These results indicated that the overexpression of Bcl-2 or Bcl-x$_L$ prevented lovastatin-induced cell death, while failing to restore the suppressed proliferation in lovastatin-treated cells.

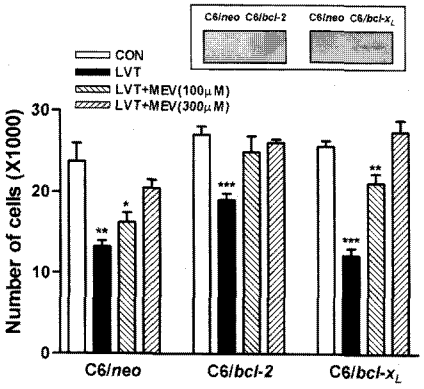

Fig. 1. Lovastatin-induced inhibition of cell proliferation in C6/*neo*, C6/*bcl-2*, and B6/*bcl-x*$_L$. Cells were cultured for 24 hours in the media containing 25 μM lovastatin without mevalonate (LVT) or with 300 μM mevalonate (LVT+MEV). CON, control. At 24 hours after treatment, cell numbers were determined. The figures in the inlet show the expressed levels of Bcl-2 and Bcl-x$_L$ in C6/*bcl-2* and C6/*bcl-x*$_L$, respectively.

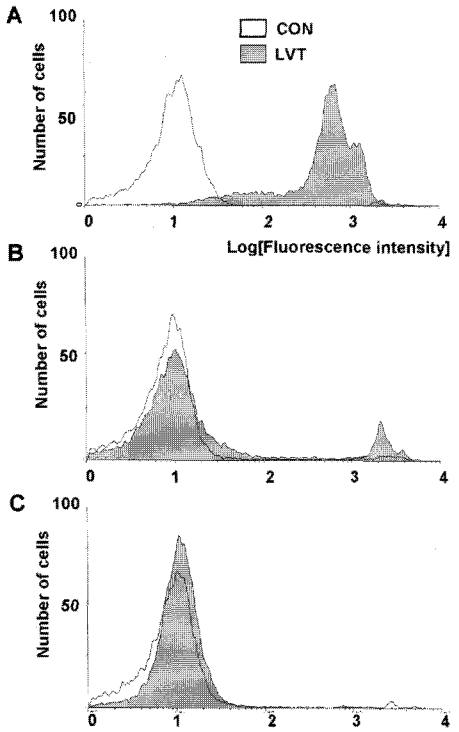

Fig. 2. Overexpression of Bcl-2 or Bcl-x$_L$ prevented cell death in lovastatin-treated C6 cells. Cells were cultured in the media containing 25 μM lovastatin. At 48 hours after lovastatin treatment, the viability of cells was determined by FACS assay after staining cells with PI. A, C6/*neo*. B, C6/*bcl-2*. C, C6/*bcl-x*$_L$.

Preventive effects of Bcl-2 and Bcl-x$_L$ *for lovastatin-induced chromatin condensation and internucleosomal DNA fragmentation*

In C6 cells treated with lovastatin for 36 hours, the electron microscopic assay showed nuclear chromatin clumps that represented the chromatin condensation at the nuclear edge, one of the characteristics of apoptosis (Fig. 3). During apoptosis, activation of the nuclease responsible for DNA fragmentation is induced by caspase-mediated cleavage of I^{CAD}/DFF45, a nuclease inhibitor (Enari et al, 1998). In this study, overexpression of Bcl-2 or Bcl-x$_L$ prevented DNA fragmentation completely at 24 hours after lovastatin treatment (Fig. 3). These results indicated that these anti-apoptotic proteins could prevent the caspase-mediated process in mevalonate-deprived cells.

Protective effect of Bcl-2 and Bcl-x$_L$ *for lovastatin-induced cleavage of PARP*

As the internucleosomal DNA fragmentation, cleavage of PARP into two fragments of molecular mass 89 kD and 24

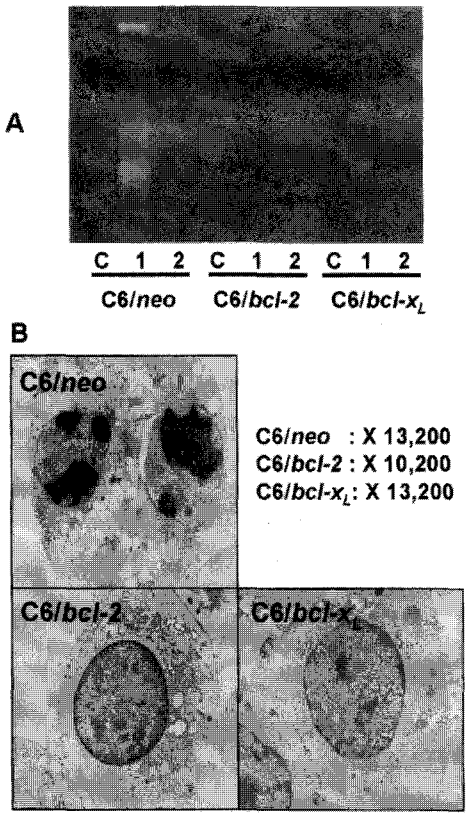

Fig. 3. Overexpression of Bcl-2 or Bcl-x$_L$ prevented lovastatin-induced DNA fragmentation and chromatin condensation in C6 cells. Cells were cultured in the media containing 25 μM lovastatin. A, DNA fragmentation was examined at 24 hours after lovastatin treatment. B, cellular structures were examined at 36 hours after lovastatin treatment.

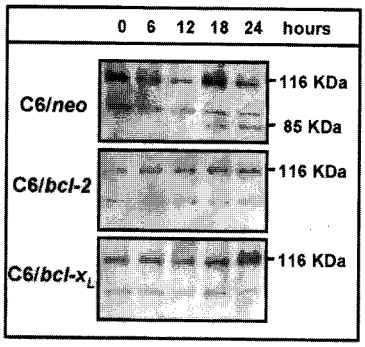

Fig. 4. Overexpression of Bcl-2 or Bcl-x_L prevented lovastatin-induced cleavage of PARP. Cells were cultured in the media containing 25 μM lovastatin. At the indicated time points, cells were harvested and the cleaved fragment of PARP was detected by Western blot assay.

kD is also one of the earliest events mediated by proteases during apoptosis. In this study, PARP cleavage was observed at 18 hours after lovastatin treatment in C6/*neo* cells, which was prevented by the overexpression of Bcl-2 or Bcl-x_L (Fig. 4).

DISCUSSION

It has been reported that lovastatin exerted two different cytotoxic effects on C6 glioma cells: induction of cell death and suppression of proliferation (Choi & Jung, 1999). In this study, we showed that the overexpression of Bcl-2 or Bcl-x_L protected C6 glioma cells from lovastatin-induced cell death. In general, apoptosis can be classified into two categories: death receptor-mediated apoptosis and the apoptosis mediated by cytotoxic insults such as γ- and ultra-violet irradiation, cytokine withdrawal, and cytotoxic drugs (Adams & Cory, 1998). The former is usually initiated by processed caspase-8 and the latter is initiated by processed caspase-9. It is generally acknowledged that Bcl-2 and Bcl-x_L can prevent the caspase-9-mediated apoptosis, while it still remains controversial whether or not death receptor-mediated apoptosis can bypass the Bcl-2 inhibitory step (Thornberry & Lazebnik, 1998). As shown in figures 3 and 4, both Bcl-2 and Bcl-x_L prevented DNA fragmentation and PARP cleavage at 24 hours after lovastatin treatment. In addition, FACS assays showed that Bcl-2 and Bcl-x_L kept the cellular membrane intact at 48 hours after lovastatin treatment. DNA fragmentation and PARP cleavage by activated caspases are widely accepted as sensitive markers of the early apoptotic phase (Edpraetere & Golstein, 1998; Thornberry & Lazebnik, 1998). As lovastatin-induced apoptotic changes could be prevented by adding mevalonate to the medium, these data indicate that the deprivation of intracellular mevalonate in lovastatin-treated cells activated the effector pro-caspases, such as caspase-3 and caspase-7, which could be counteracted by Bcl-2 or Bcl-x_L.

In this study, lovastatin inhibited cell proliferation in C6/*neo*, C6/*bcl-2* and C6/*bcl-x*$_L$ cells. Lovastatin has been shown to arrest the cell cycle by either inactivating $p21^{Ras}$ (Howe et al, 1993) or increasing the levels of $p27^{Kip1}$ (Hengst & Reed, 1996). Lovastatin has been reported to inhibit cell proliferation by hindering $p21^{Ras}$-activated, MAP kinase-dependent pathway in renal tubular cells (Vrtovsnik et al, 1997). In HeLa cells, it has been reported that lovastatin increased the synthetic rate of $p27^{Kip1}$ which was implicated in the negative regulation of G_1 progression during the cell cycle (Hengst & Reed, 1996). In addition, the inhibition of geranylgeranylation might be responsible for the lovastatin-induced inhibition of cell proliferation. Protein geranylgeranylation, but not farnesylation, was shown to be essential in cell cycle progression through G_1 in mouse fibroblasts (Vogt et al, 1996). In this study, overexpression of Bcl-2 or Bcl-x_L could not restore the inhibited proliferation in lovastatin-treated C6 cells.

This study demonstrates that the overexpression of Bcl-2 or Bcl-x_L prevented lovastatin-induced apoptosis in C6 glioma cells. As lovastatin treatment can inhibit both farnesylation and geranylgeranylation, our data suggest that the overexpression of Bcl-2 or Bcl-x_L can also prevent cell death induced by farnesyltransferase inhibitors or geranylgeranyltransferase inhibitors. It seems that the possible anti-tumor chemotherapeutic effects of lovastatin and prenyltransferase inhibitors are correlated, at least partly, with the expressed level of Bcl-2 or Bcl-x_L.

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