

Mistletoe Lectin Induces Apoptosis and Telomerase Inhibition in Human A253 Cancer Cells through Dephosphorylation of Akt

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Mistletoe lectin has been reported to induce apoptosis in different cancer cell lines *in vitro* and to show antitumor activity against a variety of tumors in animal models. We previously demonstrated the Korean mistletoe lectin (*Viscum album* var. *coloratum*, VCA)-induced apoptosis by down-regulation of Bcl-2 and telomerase activity and by up-regulation of Bax through p53- and p21-independent pathway in hepatoma cells. In the present study, we observed the induction of apoptotic cell death through activation of caspase-3 and the inhibition of telomerase activity through transcriptional down-regulation of hTERT in the VCA-treated A253 cells. We also observed the inhibition of telomerase activity and induction of apoptosis resulted from dephosphorylation of Akt in the survival signaling pathways. In addition, combining VCA with the inhibitors of phosphatidylinositol 3-kinase (PI3-kinase) upstream of Akt, wortmannin and LY294002 showed an additive inhibitory effect of telomerase activity. In contrast, the inhibitor of protein phosphatase 2A (PP2A), okadaic acid inhibited VCA-induced dephosphorylation of Akt and inhibition of telomerase activity. Taken together, VCA induces apoptotic cell death through Akt signaling pathway in correlated with the inhibition of telomerase activity and the activation of caspase-3. From these results, together with our previous studies, we suggest that VCA triggers molecular changes that resulting in the inhibition of cell growth and the induction of apoptotic cell death of cancer cells, which suggest that VCA may be useful as chemotherapeutic agent for cancer cells.

Key words: Mistletoe lectin, A253, Telomerase, hTERT, Akt, Caspase-3, Apoptosis

INTRODUCTION

The aqueous extract of European mistletoe (*Viscum album*, L.) has been used in conventional cancer therapy for decades, mainly in Europe (Büssing, 2000). D-galactose- and/or N-acetyl-D-galactosamine-specific lectins (*Viscum album*, L. agglutinins, VAA-I, II, III) are considered to be major active components in European mistletoe ((Ribereau-Gayon *et al.*, 1997; Büssing, 2000). A galactose- and N-acetyl-D-galactosamine-specific lectin (*Viscum album* L. *coloratum* agglutinin, VCA) was isolated from Korean mistletoe, a subspecies of European mistletoe, possessing anticancer activities (Lyu *et al.*, 2000, 2001, 2002). Mistletoe lectin induces cell death in various cancer cells

(Büssing, 2000; Lyu *et al.*, 2002). The antitumor effect of lectins is thought to be induction of the death of tumor cells *via* binding of B-chain to the cell surface and inhibition of protein synthesis by A-chain (Büssing, 1996, 2000; Bantel *et al.*, 1999).

Recent works suggested that mistletoe lectin may induce apoptosis by activating death pathways, possibly through activation of the caspase-8/FLICE independently of death receptor signaling, breakdown of mitochondrial transmembrane potential, as a consequence, activation of caspase-3 cascade (Bantel *et al.*, 1999) and by promoting down-regulation of the nuclear p53 and Bcl-2 protein and telomeric associations (Büssing, 1998). We also demonstrated that VCA induced apoptosis by down-regulation of Bcl-2 and telomerase activity and by up-regulation of Bax through p53- and p21-independent pathway (Lyu *et al.*, 2002). Mistletoe lectin activates a variety of intracellular signaling pathways leading to amplification of tumor necrosis

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factor- α (Pae *et al.*, 2000), rises in cytosolic calcium concentration (Walzel *et al.*, 2000), or increases intracellular expression of interleukin-4 (Stein *et al.*, 2000). Some of these signaling actions correlate with activation of mitogen-activated protein (MAP) kinase cascades including ERK, JNK and p38 MAPK (Kim *et al.*, 2000; Pae *et al.*, 2001).

Serine/threonine kinase Akt/protein kinase B (PKB), an important target of PI3K, is involved in the cell survival mediated by many growth factors. PI3K-dependent activation of Akt occurs through phosphatidylinositol 3,4,5-trisphosphate-dependent kinase-1-mediated phosphorylation of threonine 308, which leads to the autophosphorylation of serine 473 (Yao and Cooper, 1995; Kennedy *et al.*, 1997; Bellacosa *et al.*, 1998). Akt signaling pathway inhibits apoptosis in a variety of cell types. Akt mediates anti-apoptotic functions by phosphorylating apoptosis regulatory molecules including BAD, caspase-9, I κ B kinase- α (IKK- α), and the forkhead transcriptional factor (FKHR) (Zha *et al.*, 1996; Datta *et al.*, 1997; Cardone *et al.*, 1998). Protein phosphatase 2A (PP2A) is a multimeric serine/threonine phosphatase and affects diverse cellular function through reversible protein phosphorylation of several protein kinases (Zhang *et al.*, 1999). It has been reported that protein phosphorylation reversibly regulates the function of telomerase and that PP2A is a telomerase inhibitory factor in human breast cancer cells (Herbert *et al.*, 1999).

Telomerase plays a key role in maintenance of chromosomal stability and cell proliferation (Datta *et al.*, 1999). Thus, telomerase becomes a potential target for anticancer therapeutic application based on the strong correlation between telomerase activity and cell immortality. It has been found that inhibition of telomerase activity in human cancer cells leads to progressive telomere shortening and apoptotic cell death (Counter *et al.*, 1992; Kondo *et al.*, 1998; Kang *et al.*, 1999; Breitschopf *et al.*, 2001). In addition, recent studies have demonstrated that Akt plays an important role in the regulation of telomerase activity through phosphorylation of TERT subunit, which is the most important component responsible for the enzymatic activity of telomerase (Cardone *et al.*, 1998; Brunet *et al.*, 1999).

Although several studies have shown the mechanisms of mistletoe lectin involved in the induction of apoptosis, the effect of mistletoe lectin on the regulation of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway has not been reported yet. In this study, we demonstrate that VCA induces dephosphorylation of Akt in A253 cells and that this could be a potential mechanism of telomerase inhibition and apoptotic cell death.

MATERIALS AND METHODS

Materials

We previously isolated mistletoe lectin from the extracts

of *Viscum album* L. *coloratum* (VCA) and characterized its biochemical properties (Lyu *et al.*, 2000). 3-(4,5-dimethylthiazol-2-yl) 5-diphenyltetrazolium bromide (MTT) and okadaic acid, LY294002, and wortmannin were purchased from Sigma Chemicals (St. Louis, MO, USA). zVAD-fmk and acetyl-Asp-Glu-Val-Asp-aminomethyl-coumarin (Ac-DEVD-AMC) were obtained from Calbiochem-Novabiochem (La Jolla, CA, USA). Anti-phospho-Akt (Ser-473) and anti-Akt antibodies were purchased from New England Biolabs (Beverly, MA). Anti-caspase-3 and anti-PARP antibodies were the product of Santa Cruz Biotechnology (Santa Cruz, CA). If not stated otherwise, all other compounds were used from Sigma.

Cell culture

Human A253 cancer cell line was obtained from Korea Cell Line Bank (KCLB, Seoul, Korea) and maintained in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL), streptomycin (100 μ g/mL; Sigma), and penicillin (100 U/mL; Sigma) at 37°C in a humidified atmosphere containing 5% CO₂. Cells (1 \times 10⁵ cells per mL) were cultured for indicated time periods. Cell counts were determined by trypan blue dye exclusion.

MTT assay

The MTT assay is a colorimetric assay based on the ability of viable cells to reduce a soluble MTT to blue formazan crystals. Cells were seeded onto 96-well plates, grown for 24 h treated with VCA. After treatment, MTT dye (5 mg/mL; Sigma) was added and the plates were incubated for 4 h at 37°C. Optical density was determined by dissolving the formazan crystals with DMSO, and the absorbance was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Fluorescence microscopy

Morphological assessment of the cells was made on cytospin slide preparations stained with DNA-binding fluorescent dye. Cells (1 \times 10⁵ cells per mL) were seeded to 60-mm dishes and treated with 10 ng/mL VCA for 24 h. Cells were washed three times with PBS, fixed with 4% formaldehyde and then treated 1 mg/mL Hoechst 33258 (Molecular Probes, Eugene, OR, USA) at room temperature for 10 min. Nuclei condensation and fragmentation were visualized using a Zeiss Axiophoto photomicroscope (Carl Zeiss, Jena, Germany).

Cell cycle analysis

After exposure to VCA, cells were harvested, washed twice in PBS and fixed with cold 70% ethanol at 4°C for 4 h. Fixed cells were incubated with in phosphate-citrate buffer (0.2 M Na₂HPO₄ and 0.1 M citrate) at room tem-

perature for 30 min. Following treatment with 100 µg/mL RNAase (Sigma) at 37°C for 30 min, the cells were stained with 50 µg/mL propidium iodide (PI; Sigma) at 37°C for 30 min in the dark. The numbers of cells in different cell cycle phases and in apoptosis were determined by FACScan flow cytometer (Becton Dickinson, Bedford, MA, USA). Twenty thousand cells were analyzed in each sample.

Fluorimetric assay of caspase-3 activity

To measure the activity of caspase-3 activity, the cells were harvested, washed twice with ice-cold PBS and then lysed in a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM DTT, 2 mM EDTA and 0.1% CHAPS. The amount of protein was determined for each lysate using the protein assay kit (Bio-Rad). Fifty to twenty µL of each of the cell lysates, which had the same protein concentrations, was incubated in a final volume of 100 µL of a reaction buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM DTT, 10% glycerol and 100 mM Ac-DEVD-AMC (Calbiochem-Novabiochem) at 37°C for 1 h. Fluorescence intensity was quantified using the Luminescence spectrometer LS 55 (Perkin Elmer) at excitation and emission wavelengths of 380 and 460 nm respectively. Enzyme activity is expressed as fluorescent units (FU) per mg of protein per min.

Western blot analysis

Cells cultured in 100-mm dishes were washed twice with PBS and lysed in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, 1% NP-40, 0.5 mM phenylmethylsulfonyl fluoride and proteinase inhibitor cocktail (Sigma)]. After incubation for 30 min on ice, cell lysates were centrifuged at 14,000 rpm, 4°C, for 20 min. Protein concentrations were determined using the protein assay kit (Bio-Rad). Aliquots of cell lysate containing 50 mg of total proteins were separated on 10 or 12% SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking with 5% skim milk in the Tris-buffered saline (TBS-T) (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20), the membranes were incubated overnight at 4°C with anti-phospho-Akt, anti-Akt, anti-caspase-3, and anti-PARP antibodies and further incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Buckinghamshire, England). The membranes were visualized by enhanced chemiluminescence detection kit (Amersham-Pharmacia).

Telomerase assay

The telomerase activity in cell extracts was measured by the PCR-based telomeric repeat amplification protocol (TRAP) (Kim *et al.*, 1994) using the TRAP_{EZE} telomerase detection kit (Intergen, Purchase, NY, USA) according to

the manufacturer's instructions. Briefly, the cells were washed twice in phosphate-buffered saline (PBS) and lysed in CHAPS lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 1 mM EGTA, 0.1 mM benzamidine, 5 mM β-mercaptoethanol, 0.5% CHAPS and 10% glycerol) for 30 min on ice and centrifuged at 12,000 rpm for 30 min at 4°C. Supernatants were collected and the protein concentration was measured using protein assay kit (Bio-Rad, Hercules, CA, USA). When performing the TRAP assay, 1 µg of protein from each cell extracts were analyzed in the TRAP reaction buffer (20 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 63 mM KCl, 0.05% Tween-20, 1 mM EGTA, 0.01% bovine serum albumin, 25 mM each dNTPs, TS primer, RP primer, 2 units of Taq DNA polymerase, and distilled water). The reaction mixture was incubated at room temperature for 30 min to allow telomerase to extend TS primer and heated to 95°C for 2 min to inactivate telomerase activity. The extended telomerase products were then amplified by PCR (94°C for 30 s, 60°C for 30 s and 72°C for 90 s) for 30 cycles with GeneAmp Thermal Cycler (Perkin-Elmer, Foster, USA). The PCR products were separated on 12% polyacrylamide gel and stained with SYBR Green I (BMA, Rockland, ME, USA) and photographed using an ultraviolet transilluminator (Bio-Rad).

Reverse transcription (RT)-PCR

The expression of hTERT and hTR mRNA was analyzed by RT-PCR amplification as previously described (Choi *et al.*, 2000). Briefly, total RNA was isolated from the cells using RNAzol B extraction kit (Tel-Test, Friendswood, TX, USA) according to the manufacturer's protocol and cDNA was synthesized from 2 µg of RNA using the RNA PCR kit (Perkin Elmer). Two-microliter aliquots of cDNA were amplified by PCR in 50 µL of 1× buffer (10 mM Tris-HCl, pH 8.3, 2.5 mM MgCl₂ and 50 mM KCl) containing 1 mM each dNTPs, 2.5 units of Taq DNA polymerase (Takara) and 0.2 µM each specific primer. The specific primers were used: 5'-TGA GTG TGT ACG TCG TCG AG-3' (sense) and 5'-GAA CGT TCT GGC TCC CAC GA-3' (antisense) for hTERT; 5'-TCT AAC CCT AAC TGA GAA GGG CGT AG-3' (sense) and 5'-GTT TGC TCT AGA ATC AAC GGT GGA AG-3' (antisense) for hTERC. The reaction sequence was an initial incubation step at 94°C for 5 min, followed by 31 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 90 s for hTERT, hTERC, and GAPDH. The PCR products were separated on 8% polyacrylamide gel visualized by SYBR green I (BMA) and photographed using an ultraviolet transilluminator (Bio-Rad).

RESULTS

VCA induces apoptotic cell death

To measure the IC₅₀ of VCA in A253 cells, the cells

were treated for 24 h with VCA at the indicated doses. The IC_{50} of VCA was 10 ± 2 ng/mL (Fig. 1A) which is similar to the results for other cells reported previously (Lyu *et al.*, 2001, 2002). The cell growth was measured by counting the cells treated with 10 ng of VCA at each time point. The viability began to decrease at 6 h after treatment and 70% cells died in 24 h (Fig. 1B). Next, the levels of apoptosis following VCA treatment were determined in A253 cells using nuclear morphology and cell cycle distribution. When A253 cells were treated with VCA, they became rounded, showed retraction of cellular processes and eventually detached from the culture plate (Fig. 2A). The morphological changes in A253 cells suggest that they were undergoing cell death. To examine whether VCA-induced A253 cell death has features indicative of apoptosis, we explored evidence of nuclear fragmentation by staining the cells with a DNA specific fluorescent dye Hoechst 33258. Fig. 2B shows the nuclei of some VCA-treated cells were exhibited prominent fragmentation

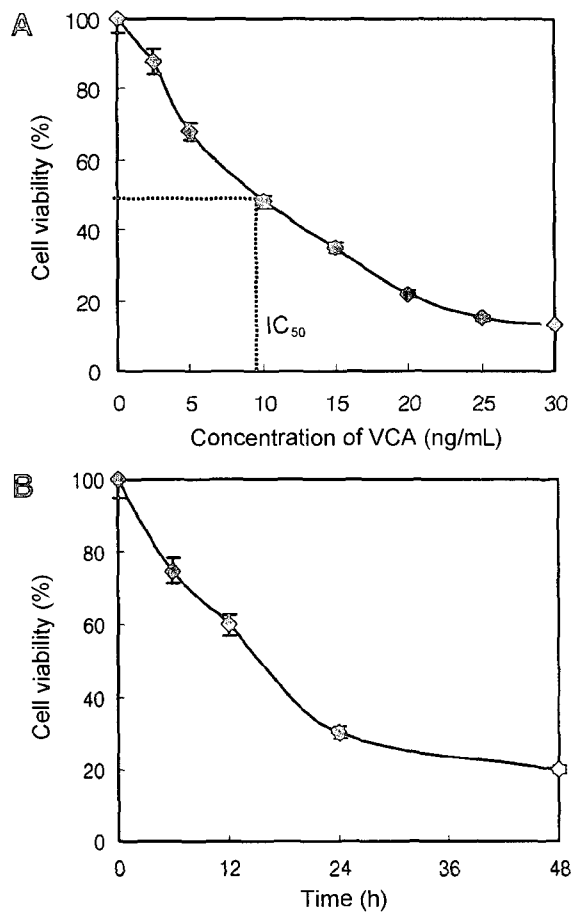


Fig. 1. Dose- (A) and time- (B) dependent cytotoxicity of VCA in A253 cells. The cells were treated for 24 h with VCA at the indicated doses (A) and treated with 10 ng of VCA at each time point (B). Cell viability was determined by MTT reduction assay. Values represent as mean \pm standard deviations from three independent experiments.

forms. In order to quantify the induction of apoptosis by VCA treatment, cells were labeled with PI and analyzed by FACScan flow cytometer. Although numbers of hypodiploid peak were increased 24 and 48 h after VCA treatment without the arrest of growth in any phase of the cell cycle, a characteristic hypodiploid (sub-G₁) peak was not observed following treatment of A253 cells with VCA (Fig. 2C).

Activation of the caspase-3 is involved in apoptotic cell death

We next examined whether caspases, the critical regulator of apoptotic cell death, are involved in the regulation of the apoptotic cell death induced by VCA. When the

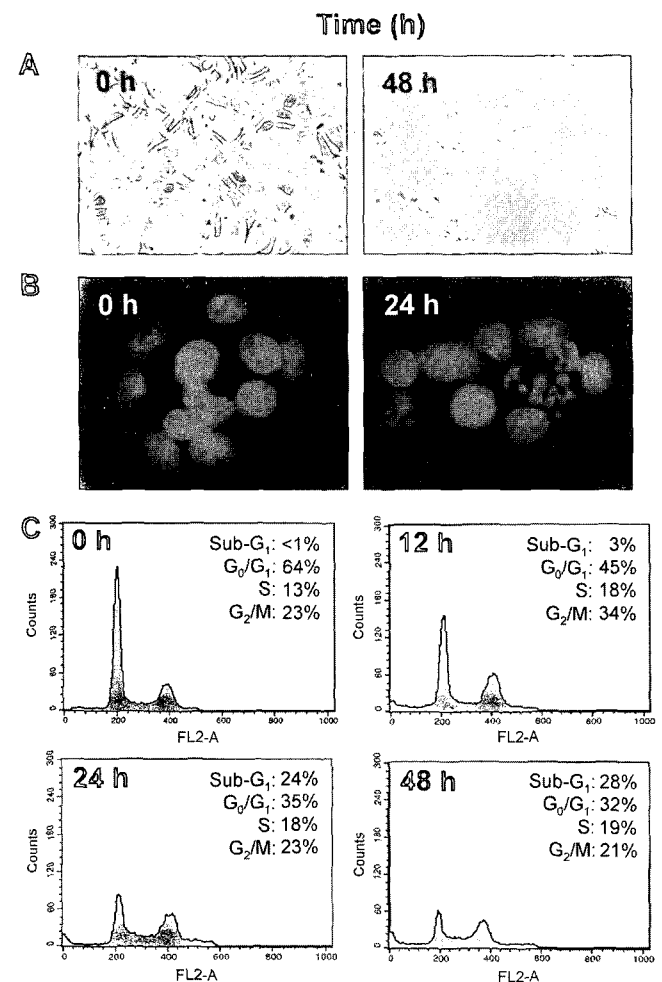


Fig. 2. VCA induces apoptosis in A253 cells. (A) Morphological changes of A253 cells after treatment with 10 ng/mL VCA for 48 h. Magnification, $\times 100$. (B) Fluorescence micrographs of A253 cells. Cells were treated with 10 ng/mL VCA for 24 h. The cells were stained with Hoechst 33258 and examined by fluorescence microscope. Magnification, $\times 400$. (C) Cell cycle analysis of A253 cells. Cells were treated with 10 ng/mL VCA for 24 h. The PI-stained cells were analyzed for DNA contents by flow cytometry.

activity of caspase-3 recognizing and cleaving after the DEVD sequence was monitored *in vitro* using a synthetic fluorogenic substrate, significant increase of DEVD-cleaving activity was observed in A253 cells as a result of VCA treatment in contrast to the untreated control (Fig. 3).

In addition, the DEVD-cleaving activity was significantly inhibited by caspase inhibitor, zVAD-fmk (Fig. 3). The time course of cleavage in the pro-forms of caspase-3 and its substrate, poly (ADP-ribose) polymerase (PARP) were examined by Western blot analysis. As shown in Fig. 3B, the activation of caspase-3 was with an increase in pro-caspase-3 cleavage of procaspase-3 (32-kDa). The 32-kDa levels of caspase-3 decreased time-dependently in cells treated with 10 ng/mL VCA. We also found significant processing of PARP, a major substrate for active form of caspase-3.

Effects of VCA on the telomerase activity and the expression of telomerase components

To examine the effects of VCA on telomerase activity in A253 cells were cultured in the absence or presence of 10 ng/mL VCA, we measured telomerase activity by PCR-based TRAP assay (Kim *et al.*, 1994). As shown in Fig. 4A, VCA induced a time-dependent decrease in telomerase activity compared with untreated control. Telomerase

activity was slightly decreased after VCA treatment for 6 h, and the treatment of VCA for 24 and 48 h resulted in a significant inhibition of telomerase activity (about 80 to 90%).

It has been reported that expression of human telomerase reverse transcriptase (hTERT) catalytic subunit, which modulates telomerase activity, is regulated at both the transcriptional level and *via* phosphorylation by Akt kinase (Akiyama *et al.*, 2003). Moreover, nuclear localization of hTERT is required to promote elongation of telomere sequences (Akiyama *et al.*, 2003). To investigate possible effect of VCA on the transcription of gene coding for telomerase, the effects of VCA on levels of hTERT and hTERC mRNA were examined by RT-PCR. Three independent experiments showed that VCA reduced levels of hTERT mRNA at 12 h and bands were mostly disappeared at 48 h. In contrast, no significant change was observed in hTERC mRNA. GAPDH was used for the efficiency of the cDNA synthesis from each sample (Fig. 4B).

Effect of VCA on the phosphorylation of Akt

We next investigated whether inhibition of telomerase activity and induction of apoptosis resulted from decreased PI3K/Akt survival signaling pathways. Since the PI3K/Akt

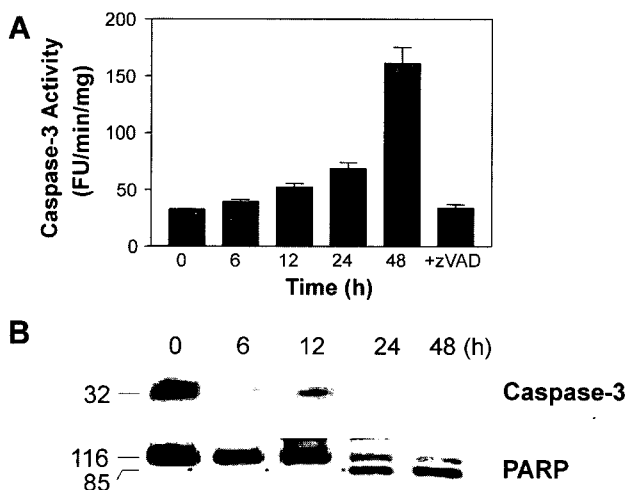


Fig. 3. Effects of VCA on caspase-3 activity and expression of caspase-3 and PARP in A253 cells. (A) Caspase-3 was determined using fluorogenic peptide substrate Ac-DEVD-AMC. Cells were incubated in the absence or presence of 10 ng/mL VCA. The reactions were analyzed with a luminescence spectrometer using a 380-nm excitation and a 460-nm emission wavelength. Values represent as mean \pm standard deviations from three independent experiments. (B) Specific cleavage of caspase-3 and PARP was observed 6 h (caspase-3) and 24 h (PARP) after treatment with VCA. Protein extracts were harvested, separated by SDS-PAGE and subjected to Western blot analysis using either total Akt and phospho-Akt antibodies. The proteins are indicated by sizes of molecular weight markers are shown on the left. The figure is representative of three independent experiments.

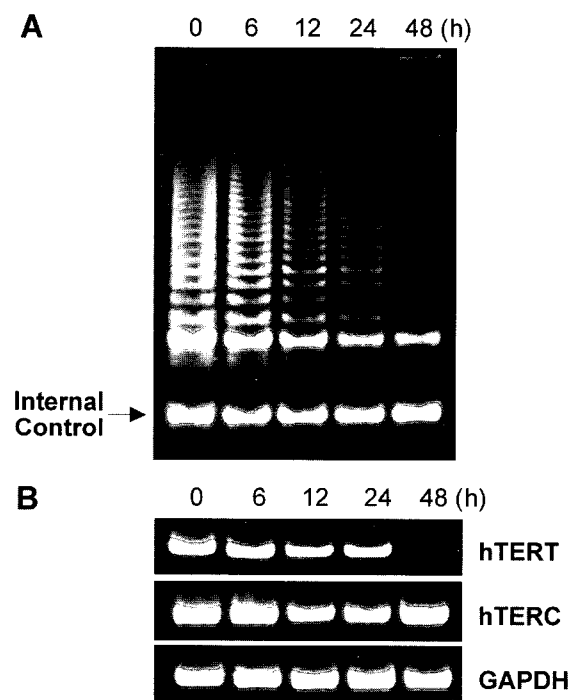


Fig. 4. Time-course experiments showing decreased telomerase activity and hTERT mRNA expression in A253 cancer cells following by treatment 10 ng/mL VCA. Representative result from (A) TRAP assay for telomerase activity and (B) RT-PCR for mRNA levels of telomerase components, hTERT, hTERC, and GAPDH (as a control).

pathway protects cells from apoptosis caused by diverse stress stimuli (Kennedy *et al.*, 1997; Bellacosa *et al.*, 1998), we investigated the changes of levels of phosphorylated Akt in A253 cells. The levels of phosphorylated Akt decreased in a time-dependent manner following VCA treatment (Fig. 5). Dephosphorylation of Akt was demonstrated within 12 h after VCA treatment with no change of total Akt protein.

Effects of PI3K inhibitors on the VCA-induced dephosphorylation of Akt and inhibition of telomerase activity

To investigate whether cotreatment of A253 cells with

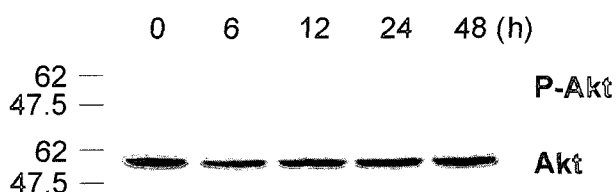


Fig. 5. VCA induces a time-dependent decrease in Akt phosphorylation in A253 cells. Cells were incubated for 24 h in 10% serum condition following by treatment 10 ng/mL VCA. Protein extracts were harvested, separated by SDS-PAGE and subjected to Western blot analysis using either total Akt and phospho-Akt antibodies.

VCA and PI3K inhibitors would have an enhanced effect over either compound alone on the dephosphorylation of Akt, A253 cells were treated with PI3K inhibitors, wortmannin (200 nM) or LY294002 (10 μM), and VCA (10 ng/mL) for 48 h. As shown in Fig. 6A, the Akt phosphorylation was decreased following treatment with both PI3K inhibitors. However, combined effects of VCA and PI3K inhibitors on the dephosphorylation of Akt were not observed possibly due to high concentration of VCA or prolonged treatment of VCA. We also observed that the VCA-induced telomerase inhibition in the absence or presence of PI3-kinase inhibitors. When these inhibitors were given without VCA, they did not show any significant effects on the telomerase activity. The pretreatment of LY294002 enhanced significantly the inhibition of telomerase activity by VCA. On the other hand, the pretreatment of wortmannin slightly increased telomerase activity (Fig. 6B).

Effects of protein phosphatase inhibitor on the VCA-induced dephosphorylation of Akt and on the VCA-induced inhibition of telomerase activity

To investigate the role of protein phosphatase in VCA-induced dephosphorylation of Akt and inhibition of telomerase, A253 cells were pretreated with PP2A inhibitor, okadaic

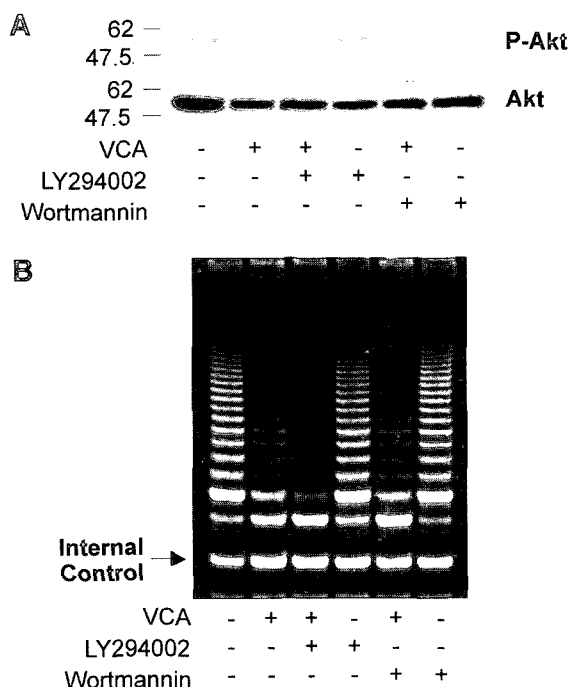


Fig. 6. Effects of PI3K inhibitors on the VCA-induced dephosphorylation of Akt (A) and on the VCA-induced inhibition of telomerase activity (B). (A) Levels of Akt phosphorylation were determined by Western blot analysis using either total Akt and phospho-Akt antibodies. (B) Telomerase activity assessed by the TRAP assay. The figure is representative of three independent experiments.

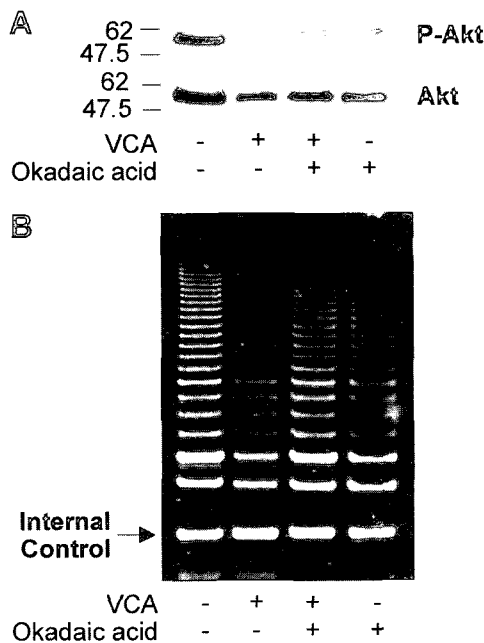


Fig. 7. Effects of protein phosphatase inhibitor on the VCA-induced dephosphorylation of Akt (A) and on the VCA-induced inhibition of telomerase activity (B). A253 cells were pretreated with okadaic acid and 10 ng/mL VCA or treated with either VCA or okadaic acid alone for 48 h. (A) Levels of Akt phosphorylation were determined by Western blot analysis using either total Akt and phospho-Akt antibodies. (B) Telomerase activity assessed by the TRAP assay. The figure is representative of three independent experiments.

acid (1.25 nM) for 1 h, and then treated with VCA (10 ng/mL). As shown in Fig. 7A, the treatment of A253 cells with okadaic acid alone decreased the phosphorylation of Akt and telomerase activity. However, the VCA-induced dephosphorylation of Akt was not affected by treatment of okadaic acid. In addition, pretreatment of okadaic acid followed by the addition of VCA increased telomerase activity.

DISCUSSION

Apoptosis is a multistep process and the multiple actions of Akt/PKB would be implicated both in the upstream induction phase of apoptosis and in the downstream executive stage, as the direct targets for caspases (Stein *et al.*, 2000; Walzel *et al.*, 2001; Pae *et al.*, 2001). Akt mediates antiapoptotic functions by phosphorylating apoptosis regulatory molecules (Kennedy *et al.*, 1997; Bellacosa *et al.*, 1998) and the inhibition of telomerase activity in human cancer cells leads to progressive telomere shortening and apoptotic cell death (Counter *et al.*, 1992; Kang, *et al.*, 1999; Breitschopf, *et al.*, 2001). Reactivation of telomerase and maintenance of telomere stability increase cellular resistance to apoptosis caused by anticancer agents (Breitschopf *et al.*, 2001), which suggested a protective function of telomerase activity against apoptotic cell death.

We previously observed the growth inhibition, morphological changes of apoptotic nuclei, and DNA fragmentation in VCA-treated HL-60 cells (Lyu *et al.*, 2001). In addition, we demonstrated the VCA-induced apoptosis by down-regulation of Bcl-2 and telomerase activity and by up-regulation of Bax through p53- and p21-independent pathway in hepatoma cells (Lyu *et al.*, 2002). In the present study, we observed the induction of apoptotic cell death through activation of caspase-3 and the inhibition of telomerase activity through transcriptional down-regulation of hTERT in the VCA-treated A253 cells. We also observed the inhibition of telomerase activity and induction of apoptosis resulted from decreased phosphorylation of Akt survival signaling pathways. In the presence of VCA, the extent of Akt/PKB phosphorylation was reduced greatly in A253 cells.

To evaluate the functional significance of PI3-kinase activation, we further observed the effect of inhibitors of PI3-kinase on the phosphorylation of Akt/PKB and telomerase activity. The Akt phosphorylation was decreased following treatment with both PI3K inhibitors. However, the combined effects of VCA and PI3K inhibitors on the dephosphorylation of Akt were not observed possibly due to high concentration of VCA or prolonged treatment of VCA. The pretreatment of LY294002 enhanced significantly inhibition of telomerase activity by VCA, while the pretreatment of wortmannin slightly increased telomerase

activity showing competition with VCA in inhibition of telomerase activity. These results suggest that VCA may act by a mechanism from that of PI3K inhibitor, LY294002, by enhancing dephosphorylation of Akt or by inhibition of telomerase activity.

The protein phosphatases also play a role in apoptosis and the inhibitors of protein phosphatase could inhibit apoptosis (Song, *et al.*, 1993; Gjertsen and Doskeland, 1995). The activated Akt/PKB kinase would become available to phosphorylate its downstream targets until inactivated by protein phosphatase (Kim *et al.*, 1994; Meier *et al.*, 1997). We observed that the PP2A inhibitor restored the reduced phosphorylation of Akt/PKB caused by the VCA and that the activated protein phosphatase could affect the change in the phosphorylation state of the Akt/PKB.

Taken together, together with our previous studies, we suggest that VCA triggers molecular changes resulting in the inhibition of cell growth and the induction of apoptotic cell death of cancer cells. The anti-tumor potential of this biological response comes from inhibition of telomerase and consequent inducing apoptosis. In addition, the induction of apoptosis results from dephosphorylation of Akt in survival signaling pathways. In conclusion, our findings provide the first evidence that VCA modulates Akt/PKB signaling pathways in A253 cells and that this could be a potential mechanism of telomerase inhibition and apoptotic cell death suggesting that VCA may be useful as chemotherapeutic agent for cancer cells.

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REFERENCES

- Akiyama M., Hideshima T., Hayashi T., Tai Y. T., Mitsiades C. S., Mitsiades N., Chauhan D., Richardson P., Munshi N. C., and Anderson K. C., Nuclear factor-kappaB p65 mediates tumor necrosis factor alpha-induced nuclear translocation of telomerase reverse transcriptase protein. *Cancer Res.*, 63, 18-21 (2003).
- Bantel, H., Engels, I. H., Voelter, W., Schulze-Osthoff, K., and Wesselborg, S., Mistletoe lectin activates caspase-8/FLICE independently of death receptor signaling and enhances anticancer drug-induced apoptosis. *Cancer Res.*, 59, 2083-2090 (1999).
- Bellacosa, A, Chan, T. O., Ahmed, N. N., Datta, K., Malsrom, S., Stokoe, D., McCormick, F., Feng, J., and Tschlis, P., Akt activation by growth factors is a multiple-step process: The role of the PH domain. *Oncogene*, 17, 313-325 (1998).
- Breitschopf, K., Zeiher, A. M., and Dimmeler, S., Pro-atherogenic

- factors induce telomerase inactivation in endothelial cells through an Akt-dependent mechanism. *FEBS Lett.*, 493, 21-25 (2001).
- Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E., Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell*, 96, 857-868 (1999).
- Büssing, A., Suzar, K., Bergmann, J., Pfüller, U., Schietzel, M., and Schweizer, K., Induction of apoptosis in human lymphocytes treated with *Viscum album* L. is mediated by the mistletoe lectins. *Cancer Lett.*, 99, 59-72 (1996).
- Büssing, A., Multani, A. S., Pathak, S., Pfüller, U., and Schietzel, M., Induction of apoptosis by the *N*-acetylgalactosamine-specific toxic lectin from *Viscum album* L is associated with a decrease of nuclear p53 and Bcl-2 proteins and induction of telomeric associations. *Cancer Lett.*, 130, 57-68 (1998).
- Büssing, A., Biological and pharmacological properties of *Viscum album* L.; From tissue flask to man. In A. Büssing (eds.), Mistletoe, genus *Viscum* & other genera, Medicinal and aromatic plants/Industrial profiles. Harwood Academic Publishers, Netherland, pp 45-60 (2000).
- Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C., Regulation of cell death protease caspase-9 by phosphorylation. *Science*, 282, 318-321 (1998).
- Choi, S. H., Kang, H. K., Im, E. O., Kim, Y. J., Bae, Y. T., Choi, Y. H., Lee, K. H., Chung, H. Y., Chang, H. K., and Kim, N. D., Inhibition of cell growth and telomerase activity of breast cancer cells *in vitro* by retinoic acids. *Int. J. Oncol.*, 17, 971-976 (2000).
- Counter, C. M., Avilion, A. A., LeFeuvre, C. E., Stewart, N. G., Greider, C. W., Harley, C. B., and Bacchetti, S., Telomere shortening associated with chromosome instability in immortal cells which express telomerase activity. *EMBO J.*, 11, 1921-1929 (1992).
- Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E., Akt phosphorylation of Bad couples survival signals to the cell-intrinsic death machinery. *Cell*, 91, 231-241 (1997).
- Datta, S. R., Brunet, A., and Greenberg, M. E., Cellular survival: a play in three Akts. *Genes Dev.*, 13, 2905-2927 (1999).
- Gjertsen, B. T. and Doskeland, S. O., Protein phosphorylation in apoptosis. *Biochim. Biophys. Acta*, 1269, 187-199 (1995).
- Herbert, B. S., Pitts, A. E., Baker, S. I., Hamilton, S. E., Wright, W. E., Shay, J. W., and Corey, D. R., Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. *Proc. Natl. Acad. Sci. USA*, 96, 14276-14281 (1999).
- Kang, S. S., Kwon, T. G., Kwon, D. Y., and Do, S. I., Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. *J. Biol. Chem.*, 274, 13085-13090 (1999).
- Kennedy, S. G., Wagner, A. J., Conzen, S. D., Jordan, J., Belacosa, A., Tsichlis, P. N., and Hay, N., The PI3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. *Genes Dev.*, 11, 701-713 (1997).
- Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L., and Shay, J. W., Specific association of human telomerase activity with immortal cells and cancer. *Science*, 266, 2011-2015 (1994).
- Kim, M. S., So, H. S., Lee, K. M., Park, J. S., Lee, J. H., Moon, S. K., Ryu, D. G., Chung, S. Y., Jung, B. H., Kim, Y. K., Moon, G., and Park, R., Activation of caspase cascades in Korean mistletoe (*Viscum album* var. *coloratum*) lectin-II-induced apoptosis of human myelocytic U937 cells. *Gen. Pharmacol.*, 34, 349-355 (2000).
- Kondo, Y., Kondo, S., Tanaka, Y., Haqqi, T., Barna, B. P., and Cowell, J. K., Inhibition of telomerase increases the susceptibility of human malignant glioblastoma cells to cisplatin-induced apoptosis. *Oncogene*, 16, 2243-2248 (1998).
- Lyu, S. Y., Park, S. M., Choung, B. Y., and Park, W. B., Comparative study of Korean (*Viscum album*, var. *coloratum*) and European mistletoes (*Viscum album*). *Arch. Pharm. Res.*, 23, 592-598 (2000).
- Lyu, S. Y., Park, W. B., Choi, K. H., and Kim, W. H., Involvement of caspase-3 in apoptosis induced by *Viscum album* var. *coloratum* agglutinin in HL-60 cells. *Biosci. Biotechnol. Biochem.*, 65, 534-541 (2001).
- Lyu, S. Y., Choi, S. H., and Park, W. B., Korean mistletoe lectin-induced apoptosis in hepatocarcinoma cells is associated with inhibition of telomerase *via* mitochondrial controlled pathway independent of p53. *Arch. Pharm. Res.*, 25, 93-101 (2002).
- Meier, R., Alessi, D. R., Cron, P., Andjelkovic, M., and Hemings, B. A., Mitogenic activation, phosphorylation, and nuclear translocation of protein kinase B beta. *J. Biol. Chem.*, 272, 30491-30497 (1997).
- Pae, H. O., Seo, W. G., Oh, G. S., Shin, M. K., Lee, H. S., Kim, S. B., and Chung, H. T., Potentiation of tumor necrosis factor- α -induced apoptosis by mistletoe lectin. *Immunopharmacol. Immunotoxicol.*, 22, 697-709 (2000).
- Pae, H. O., Oh, G. S., Kim, N. Y., Shin, M. K., Lee, H. S., Yun, Y. G., Oh, H., Kim, Y. M., and Chung, H. T., Roles of extracellular signal-regulated kinase and p38 mitogen-activated protein kinase in apoptosis of human monocytic leukemia U937 cells by lectin-II isolated from Korean mistletoe. *In vitro Mol. Toxicol.*, 14, 99-106 (2001).
- Ribereau-Gayon, G., Jung, M., Frantz, M., and Anton, R., Modulation of cytotoxicity and enhancement of cytokine release induced by *Viscum album* L. extracts or mistletoe lectins. *Anticancer Drugs*, 8, S3-8 (1997).
- Song, Q. and Lavin, M., Calyculin A, A potent inhibitor of phosphatase-1 and 2A, prevents apoptosis. *Biochem. Biophys. Res. Commun.*, 190, 47-55 (1993).

- Stein, G. M., Pfuller, U., Schietzel, M., and Büssing, A., Intracellular expression of IL-4 and inhibition of IFN-gamma by extracts from European mistletoe is related to induction of apoptosis. *Anticancer Res.*, 20, 2987-2994 (2000).
- Walzel, H., Blach, M., Neels, P., Schulz, U., Wollenhaupt, K., and Brock, J., The B-chain of mistletoe lectin I efficiently stimulates calcium signaling in human Jurkat T-cells. *Immunol. Lett.*, 78, 57-66 (2001).
- Yao, R. and Cooper, G. M., Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by the nerve growth factor. *Science*, 267, 2003-2005 (1995).
- Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J., Serine phosphorylation of death agonist Bad in response to survival factor results in binding to 14-3-3 not Bcl-xL. *Cell*, 87, 619-628 (1996).
- Zhang, X., Mar, V., Zhou, W., Harrington, L., and Robinson, M. O., Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. *Genes Dev.*, 13, 2388-2399 (1999).