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Korean Mistletoe Lectin-induced Apoptosis in Hepatocarcinoma Cells is Associated with Inhibition of Telomerase *via* Mitochondrial Controlled Pathway Independent of p53

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The extract of European mistletoe (*Viscum album*, L) has been used in adjuvant chemotherapy of cancer and mistletoe lectins are considered to be major active components. The present work was performed to investigate the effects of Korean mistletoe lectin (*Viscum album* L. *coloratum agglutinin*, VCA) on proliferation and apoptosis of human hepatoma cells as well as the underlying mechamisms for these effects. We showed that VCA induced apoptosis in both SK-Hep-1 (*p53*-positive) and Hep 3B (*p53*-negative) cells through p53- and p21-independent pathways. VCA induced apoptosis by down-regulation of Bcl-2 and by up-regulation of Bax functioning upstream of caspase-3 in both cell lines. In addition, we observed down-regulation of telomerase activity in both VCA-treated cells. Our results provide direct evidence of the anti-tumor potential of this biological response which comes from inhibition of telomerase and consequent inducing apoptosis. VCA-induced apoptosis is regulated by mitochondrial controlled pathway independently of p53. These findings are important for the therapy with preparation of mistletoe because they show that telomerase-dependent mechanism can be targeted by VCA in human hepatocarcinoma. Taken together, our results suggest that the VCA, considered as a telomerase-inhibitor, can be envisaged as a candidate for enhancing sensitivity of conventional anticancer drugs.

Key words: Korean mistletoe, Lectin, Telomerase, Apoptosis, p53, p21, Hepatocarcinoma, Bax, Bcl-2

INTRODUCTION

The aqueous extract of European mistletoe (*Viscum album*, L.) has been used in conventional cancer therapy for decades, mainly in Europe (Büssing *et al.*, 2000, Lyu *et al.*, 2000). Among several components in mistletoe, lectins are considered to be major active components. The European mistletoe lectins (*Viscum album*, L. agglutinins, VAA-I, II, III) with Mr between 55 and 63 kDa are D-galactose- and/or *N*-acetyl-D-galactosamine-specific (Jung *et al.*, 1990, Büssing, *et al.*, 1999, 2000). The cloning of three VAAs has recently revealed the presence of one gene only, and they can be distinguished from each other by the degree of glycosylation that is dependent on post-translational processing in the plant (Eck *et al.*, 1999a, 1999b). A galactose- and N-acetyl-D-galactosamine-specific lectin (*Viscum album* L. *coloratum* agglutinin, VCA) was isolated from Korean mistletoe (*Viscum album* L. *coloratum*) and proved to possess anticancer activity (Park *et al.*, 1998, 1999a,b, Lyu *et al.*, 2000). The sequence of N-terminal amino acid and gene of VCA were different from VAAs (Yoon *et al.*, 1999, Park *et al.*, 2001). The antitumor effect of mistletoe lectins is thought to induce the death of tumor cells *via* binding of B-chain to the cell surface and inhibition of protein synthesis by A-chain (Büssing, *et al.*, 1999, Lyu *et al.*, 2001).

It is now well established that many types of cancer have been associated with reduced apoptosis (Schwartz and Osborn, 1993, Kerr *et al.*, 1994, Arends *et al.*, 1994, Green and Martins 1995). *p53* mutations occur in the majority of human tumors and are often associated with advanced tumor stage and poor patient prognosis (Wallace and Lowe, 1999). In many cases, *p53* loss is associated with reduced apoptosis *in situ* (Lowe and Lin,

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2000) and is upregulated in response to DNA damage (Tereda *et al.*, 1991, Kuirbitz *et al.*, 1992). However, p53 is not strictly required for drug-induced cell death; indeed, at sufficient doses virtually all anticancer-agents induce apoptosis independently of p53. In fact, the contribution of p53 to drug-induced apoptosis is determined by a variety of factors including agent, dose, tissue and mutational background of the tumor (Yonish-Rouach, 1991, Lowe and Lin, 2000). p21 has been identified as a major cell cycle protein acting as a cyclin-dependent kinase (cdk) inhibitor and a downstream mediator of the tumor suppressor p53 in regulating cell cycle progression (El-Deiry *et al.*, 1994, Jiang *et al.*, 1994, Kim *et al.*, 1998, 2000). Direct induction of p21 by wild-type p53 contributes to growth arrest by inhibiting cdk activity. Recent reports examining the effect of DNA damaging agent on the apoptosis of the cells containing mutant or null p53 suggested that marked up-regulation of p21 gene expression is often coupled with induction of p53-independent apoptosis (Kano *et al.*, 1997, Kim *et al.*, 1998, 2000).

An early event in the process is mitochondrial release of cytochrome *c*, which upon entry into the cytosol forms a complex with Apaf and caspase-9. This complex processes and activates the caspases, which can trigger a cascade by processing and activating other caspases (Finucane *et al.*, 1999, Kaufmann and Earnshaw, 2000). Bax is a pro-apoptotic Bcl-2-family protein that resides in the cytosol and is translocated to mitochondria upon induction of apoptosis. Bax can directly induce cytochrome c release from mitochondria and caspase activation. In contrast, Bcl-2 prevents the release of cytochrome c in cells undergoing apoptosis (Antonsson and Martinou, 2000). Bcl-2 itself was shown to be down-regulated by the tumor suppressor molecule p53 (Hoffman and Reed 1994, Miyashita *et al.*, 1995). To date, at least 15 Bcl-2 family member proteins have been identified in mammalian cells including proteins that promote or prevent apoptosis (Kim *et al.*, 1999).

Human telomerase, a cellular reverse transcriptase (hTERT), is a nuclear ribonucleoprotein enzyme complex that catalyzes the synthesis and extension of telomeric DNA. This enzyme is specifically activated in most malignant tumors but is usually inactive in normal somatic cells, suggesting that telomerase plays an important role in cellular immortalization and tumorigenesis. The correlation between telomerase activity and human tumors has led to the hypothesis that tumor growth requires reactivation of telomerase. Therefore, telomerase is a challenging target for anticancer-drug development (Hodes, 2001, Lee *et al.*, 2001, Pendino *et al.*, 2001). In addition, several proto-oncogenes and tumor suppressor genes have been implicated in the regulation of telomerase activity, both directly and indirectly; these include Bcl-2, p21, Rb, p53, PKC, and Akt/PKB (Liu, 1999).

There are several reports showing that mistletoe lectins induce apoptosis (Büssing, *et al.*, 1996, 1999, Bantel *et al.*, 1999, Yoon *et al.*, 1999, Lyu *et al.* 2001). Mistletoe lectins induced-apoptosis was associated with the mitochondrial release of cytochrome c, and caspase-activation by mitochondrial controlled pathway (Bantel *et al.*, 1999 Lyu *et al.*, 2001). In addition, VAAs induce apoptosis by promoting down-regulation of the nuclear p53 and Bcl-2 protein and telomeric associations in human lymphocytes (Büssing, *et al.*, 1999). However, further studies on the regulatory mechanism of apoptosis by mistletoe lectins have not been reported yet. In the present study to clarify the mechanism of mistletoe lectins focused on VCA, we investigated the possible role of p53, p21, caspase-3, Bax, Bcl-2, and telomerase in undergoing apoptosis. We employed two human hepatocarcinoma cell lines that differed in their status of *p53*; SK-Hep-1 cells expressing wild-type *p53* and *p53* null Hep 3B cells.

MATERIALS AND METHODS

Cell culture and cytotoxic assay

SK-Hep-1 and Hep3B human hepatocarcinoma cell lines were obtained from Korea Cell Line Bank (KCLB, Seoul, Korea) and maintained in RPMI 1640 medium (GIBCO BRL, Grand Island, MD, USA) containing 10% fetal bovine serum (GIBCO BRL), 100 U/ml penicillin (GIBCO BRL), and 100 μg/ml streptomycin (GIBCO BRL) at 37°C in a humidified atmosphere composed of 95% air and 5% CO_2.

VCA was isolated and identified as reported previously (Lyu *et al.*, 2000). Cells (1×10^5 cells/ml) were plated, maintained and treated with varied concentrations of VCA for indicated time periods. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, St. Louis, MO, USA) assay colorimetric dye reduction method (Freshney, 2000). The cyto-toxicity was measured as IC_{50} values (inhibitory concen-tration values, i.e. drug concentration required to inhibit viability by 50%) and each assay was done in triplicate.

Nuclear staining and DNA fragmentation assay

Cells were exposed to 10 ng/ml VCA for 24 h and nuclei were stained with 5 μg/ml Hoechst 33258 for 30 min. Stained nuclei were observed and photographed with an Olympus B × 50 fluorescence microscope.

The attached cells were collected and resuspended in 400 ml of ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM EDTA and 0.3% Triton X-100, incubated on ice for 30 min and centrifuged. Proteinase K (200 μg/ml, GIBCO BRL) was added to the supernatant and

incubated at 50°C for 2 h, followed by the addition of 100 mg/ml RNase A (Sigma) and further incubation at 37°C for 2h. Fragmented DNA was extracted with phenol/chloroform and precipitated at -20°C with ethanol/sodium acetate. The DNA sample was separated by electrophoresis on a 1.5% agarose gel containing 0.1 μg/ml ethidium bromide.

Flow cytomeric analysis

The cells were harvested and fixed in cold 70% ethanol for 24 h. After extensive washing with phosphate-buffered saline (PBS), the samples were incubated for 30 min at room temperature with the DNA staining solution containing 50 μg/ml propidium iodide (Sigma) and 100 mg/ml RNAase (Sigma) and measured on a FACScan flow cytometer (Becton Dickinson, NJ, USA). The DNA histograms were analyzed for evaluation of apoptosis and the percentage of cells in the various phases of the cell cycle.

Protein preparation and Western blot analysis

Cells were washed twice with cold PBS on ice and lysed in buffer (50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, and proteinase inhibitors) for 30 min on ice. The protein concentration in extracts was determined using BCA reagent (Pierce, Rockford, IL, USA). The cell lysate was mixed with sample buffer and boiled for 5 min. Cellular extracts equivalent to 100 μg protein from each sample were resolved on a 8~12% SDS-PAGE and were transferred to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). The membrane was incubated with a specific human monoclonal antibody (Santa Cruz, Santa Cruz, Calif, USA) overnight at 4°C and was developed by enhanced chemiluminescence regents (Pierce, Rockford, IL, USA). Protein concentration was analyzed by image analyzer (Vilber Lourmat, Marne-La-Vallee Cedex, France).

Telomerase assay

Telomerase activity was assayed by telomere repeat amplification protocol (TRAP) using $TRAP_{EZE}$ telomerase detection kit (Oncor, Gaithersburg, MD, USA) following provided procedures. Briefly cell lysate was prepared from 10^4-10^5 cells and telomerase product in the cell lysate was amplified by PCR (at 94°C for 30 sec., at 72°C for 1 min, and repeated 27 cycles) in the presence of pg of the internal TRAP assay standard. Signals obtained by TRAP assay were analyzed by image analyzer (Vilber Lourmat, Marne-La-Vallee Cedex, France).

Statiscal analysis

All data are expressed as mean ± SD of the three independent experiments. A one-way ANOVA was used for multiple comparisons (SPSS program, ver 10.0).

RESULTS

p53 and p21 are downregulated in VCA-induced apoptosis

To determine whether p53 is involved in VCA-induced apoptosis, we first investigated growth inhibition of SK-Hep-1 (*p53*-positive) and Hep 3B (*p53*-negative) human hepatocarcinoma cells by VCA-treatment. Fig. 1 shows dose- and time- dependent growth inhibition of cells. The response of the two cell lines to VCA appeared to be independent of p53 status, because cells with either wild-type or mutant *p53* were affected similarly by VCA. During these experiments, we consistently observed that a significant number of Hep3B cells started rounding-up and changed their morphology between 12~24 h after VCA treatment. To test whether the observed growth inhibition of cells by VCA was related to the induction of apoptosis, we examined the effect of VCA (10 ng/ml) on SK-Hep-1 and Hep 3B cells. Morphological findings of apoptosis such as cell shrinkage, chromatin condensation and nuclear

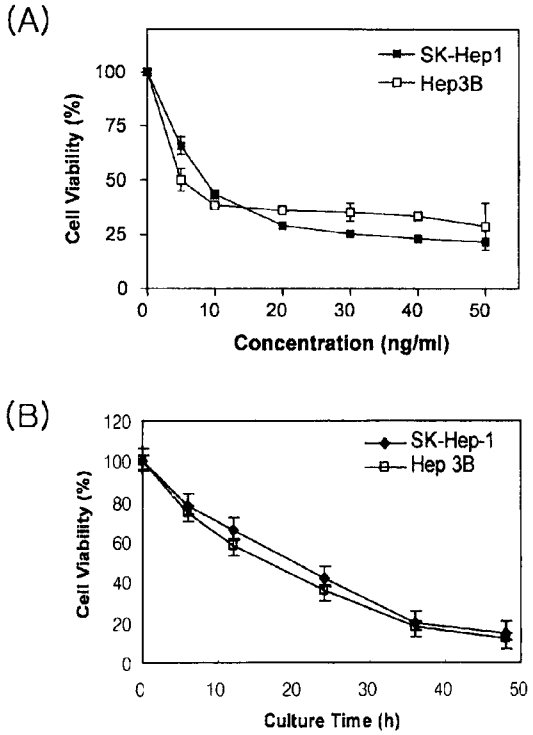

Fig. 1. Dose- (A) and time- (B) dependent viability of SK-Hep-1 and Hep 3B cells treated with VCA. (A) Cells were treated with VCA for 48 h. (B) Cells were treated with 10 ng/ml for indicated indicated time period. Cell viability was determined by the MTT assay. Values from each treatment were expressed as a percent relative to the control. Each point represents the mean ± SD of the three independent experiments.

segmentation were observed in both cells (Fig. 2A). And undergoing apoptosis of both cells was observed by nucleosomal DNA fragmentation, which is the biochemical hallmark of apoptosis. The amount of nucleosomal DNA fragments gradually increased with the time of exposure to VCA (Fig. 2B). Since DNA ladder pattern may also be associated with necrosis, we performed flow cytometric analysis of VCA-treated cells to verify that VCA-induced cell death occurred *via* an apoptotic mechanism. Both cells were exposed to VCA (10 ng/ml) for indicated periods of time, and cell cycle progression was examined. As shown in Fig. 3, apoptotic peaks were detected following

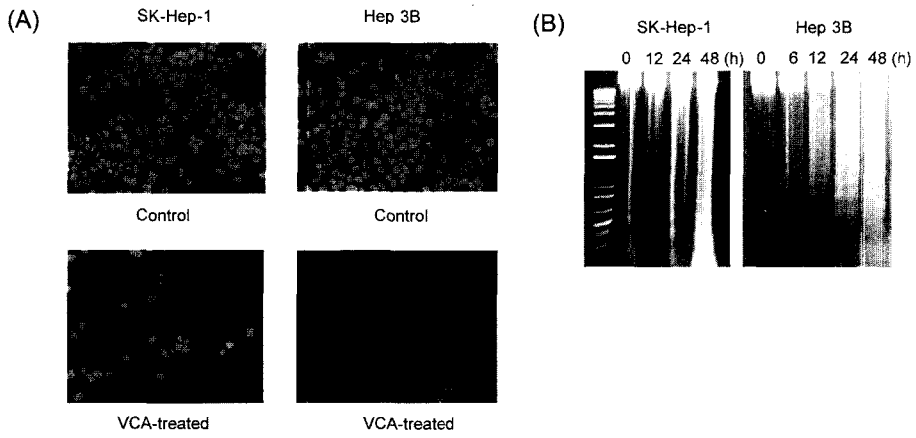

Fig. 2. (A) Morphological changes of SK-Hep-1 and Hep 3B cells by treatment of VCA. Cells were exposed to 10 ng/ml VCA for 24 h and were stained nuclei with 5 μg/ml Hoechst 33258 for 30 min. Stained nuclei were observed and photographed with an Olympus B × 50 fluorescence microscope. The controls are the data of cells in the absence of VCA. (B) DNA fragmentation induced by VCA in SK-HEP-1 and Hep3B cells. Cells were incubated in absence or presence of VCA (10 ng/ml). Total genomic DNA was extracted and was resolved on 1.5% agarose gel. DNA fragmentation was stained with ethidium bro-mide.

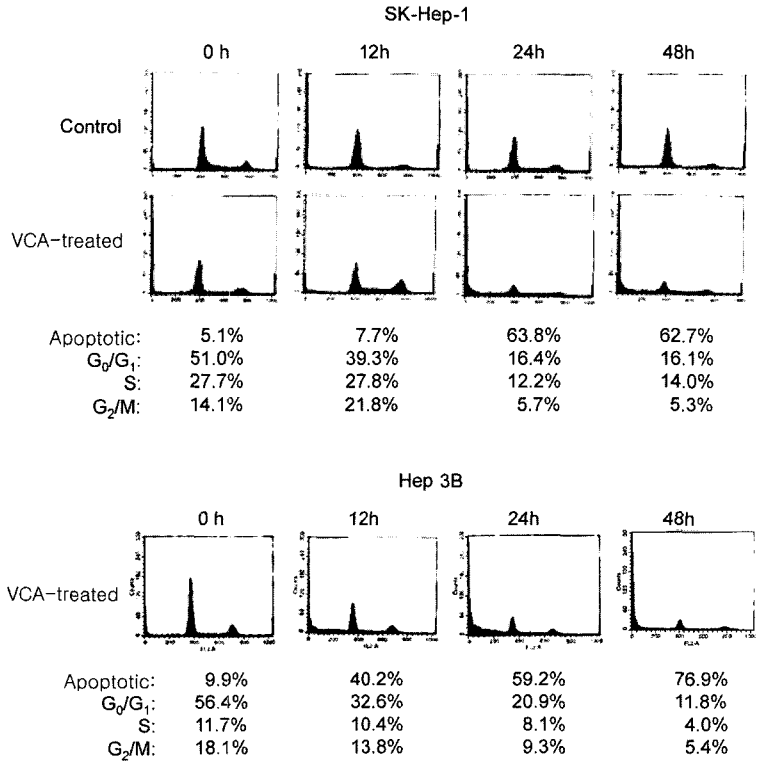

Fig. 3. Flow cytometry analysis of SK-Hep-1 and Hep 3B cells. Cells were maintained in presence of 10 ng/ml lectin for 4 h, fixed, stained with propidium iodide, and analyzed on a FACScan flow cytometer (Becton Dickinson) for relative DNA content. Apoptotic cells were calculated by measuring the area of a 'sub-G1' peak. The controls are the data of cells in the absence of VCA.

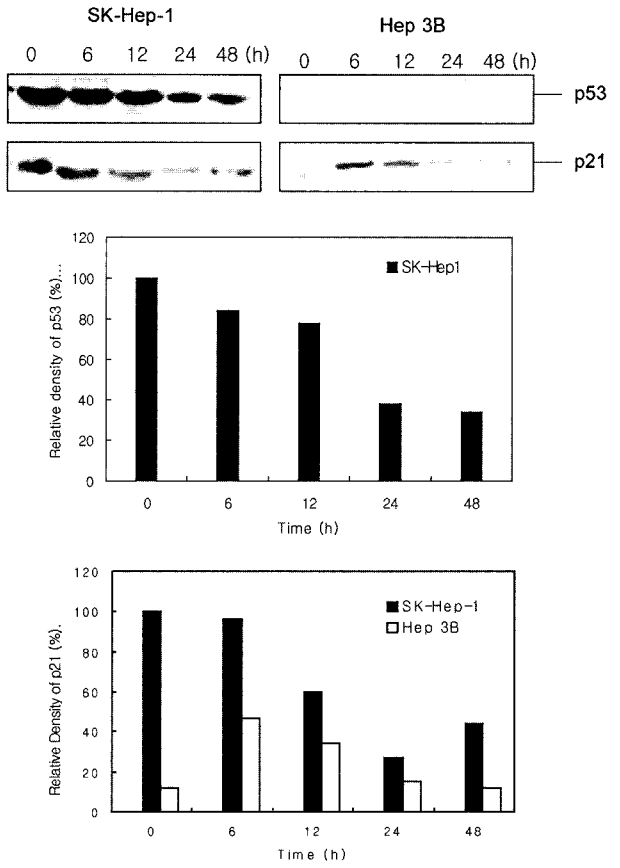

Fig. 4. Effect of VCA on the expression level of p53 and p21 in SK-Hep-1 and Hep 3B cells. Cells were treated with 10 ng/ml of VCA for indicated time-periods. The expression of each protein was assayed by Western blot analysis using anti-human p53- and p21-monoclonal antibody. The detection was performed by the ECL method.

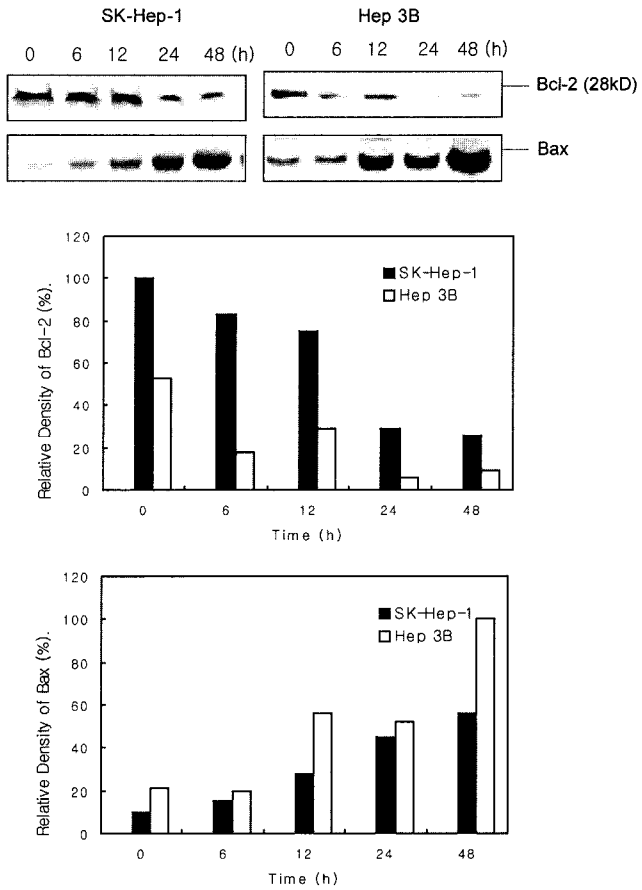

Fig. 5. Effect of VCA on the expression level of Bcl-2 and Bax in SK-Hep-1 and Hep 3B cells. Cells were treated with 10 ng/ml of VCA for various periods of time. The expression of each protein was assayed by Western blot analysis using anti-human Bcl-2- and Bax-monoclonal antibody. The detection was performed by the ECL method.

VCA exposure in both cells. The increase of apoptotic cells appeared 12 h after VCA-treatment in Hep 3B cells, whereas 24 h in SK-Hep-1 cells. VCA-induced apoptosis in *p53*-negative cells was more sensitive than that in *p53*-positive cells.

p21 has been known to induce G1 arrest through p53-dependent or p53-independent manner (Kim *et al.*, 2000). Although VCA was observed to induce apoptosis through p53-independent pathway, *p21* expression may be linked to VCA-induced apoptosis. To confirm the possible involvement of p21 in VCA-induced apoptosis, the *p53*-positive and *p-53*-negative cells were treated with 10 ng/ml of VCA at each time-point and the level of protein was assayed by Western blot analysis. As demonstrated in Fig. 4, the expression of *p53* was observed distinctly in SK-Hep-1 cells but not observed in Hep 3B cells. The protein level of p53 was decreased in SK-Hep-1 cells after treatment of VCA for 24 h. The relative abundance of p21 in SK-Hep-1 cells was detected and decreased significantly 12 h after VCA-treatment, while p21 in Hep 3B cells was appeared and decreased gradually by VCA-treatment.

VCA induced apoptosis through activation of Bax and inhibition of Bcl-2

Bax, an accelerator of apoptosis, and Bcl-2, a suppressor of apoptosis, have been known to regulate many types of apoptosis (Lowe and Lin, 2000). Bcl-2 prevents various types of mammalian cells from apoptosis by preventing activation of caspases, indicating that Bcl-2 family functions upstream of caspases (Kim *et al.*, 1999). In addition, Bcl-2 is down-regulated by the tumor suppressor molecule p53 (Miyashita *et al.*, 1995). To elucidate the downstream events of p53 and p21 in VCA-induced apoptosis, the expression levels of Bcl-2 and Bax were measured in SK-Hep-1 and Hep 3B cells. In VCA-treated SK-Hep-1 cells, Bcl-2 was detected and the protein level decreased in time-dependent manner, while indistinct level of Bcl-2 was detected in Hep 3B cells at each time-point (Fig. 5).

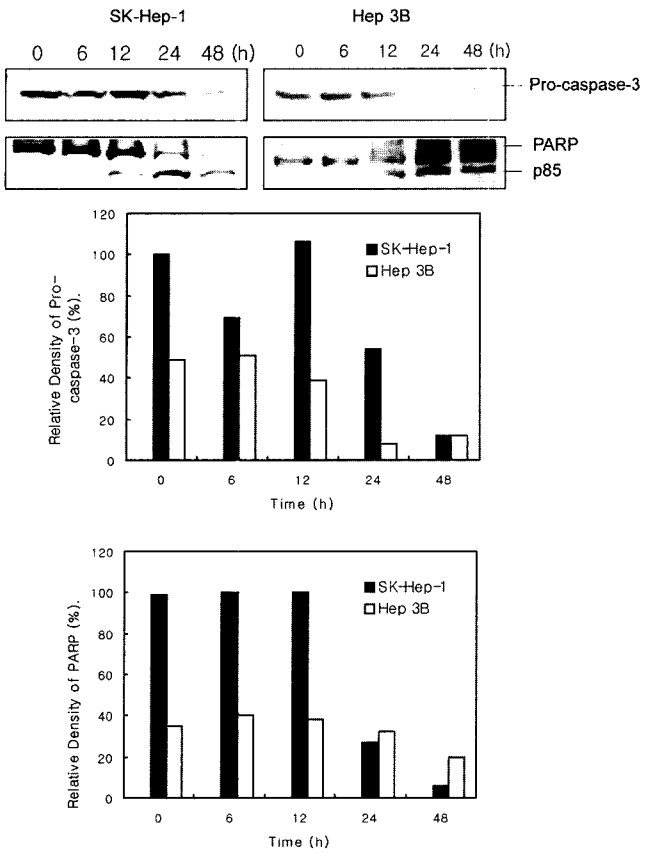

Fig. 6. Effect of VCA on the expression level of caspase-3 and PARP in SK-Hep-1 and Hep 3B cells. Cells were treated with 10 ng/ml of VCA for various periods of time. The expression of each protein was assayed by Western blot analysis using anti-human caspase-3 and PARP-monoclonal antibody. The detection was performed by the ECL method.

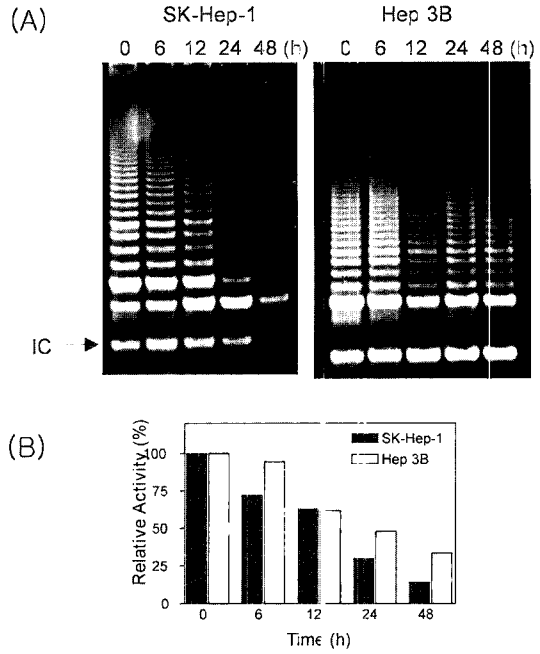

Fig. 7. Inhibitory effect of VCA on the telomerase activity in SK-Hep-1 and Hep 3B cells. Telomerase activity was assayed by telomere repeat amplification protocol (TRAP) using the $TRAP_{EZE}$ telomerase detection kit (Oncor). Telomerase product in the cell lysate was amplified by PCR (A) and signals obtained by TRAP assay were analyzed by image analyzer (B).

In contrast, the expression of Bax protein was dramatically elevated after treatment of VCA in the whole extract of both cells in time-dependent manner, and the elevation was more significant in *p53*- negative cells.

VCA induced apoptosis through activation of caspase-3 proteases in SK-Hep-1 and Hep 3B cells

The observation of VCA-induced apoptotic cell death in *p53*-negative Hep 3B cells has led us to investigate the patterns of caspase-3 in both cell lines. Western blot analysis confirmed that the apoptotic process elicited by the exposure of both cells to VCA was executed through the activation of caspase-3, which plays an important role for several key events during apoptosis (Fig. 6). The cleavage of poly (ADP-ribose) polymerase (PARP) by activated caspase-3 subfamily members is considered to be one of the hallmarks of apoptosis. Proteolysis of the 116 kDa intact form of PARP into 85 kDa and 25 kDa fragments results in the loss of normal PARP function. Thus, to investigate if caspase-3 activation during VCA-induced apoptosis induces the cleavages of PARP, cells were treated with 10 ng/ml of VCA at each time-point. The native and processed PARP forms typically seen in both of apoptotic cells can be observed 12 h after VCA treatment. The relative abundance of the 85 kDa fragment reached a maximum at 24 h. Next, a parallel set of experiments above was done to assess the level of caspase-3 by Western blot analysis. Upon activation, 32 kDa procaspase-3 is cleaved into 17 kDa fragments. The disappearance of 32 kDa procaspase-3 was therefore used to estimate the activation of caspase-3. As shown in Fig. 5, treatment with VCA at the indicated time in both cells resulted in a decrease in the 32 kDa form of procaspase-3 in a time-dependent manner. The patterns of caspase-3 activated by VCA are correlated well with that of VCA-induced PARP cleavage. These results confirmed that the VCA is capable of inducing apoptosis through caspase-3 activation in SK-Hep-1 and Hep 3B cells.

VCA inhibited telomerase activity in SK-Hep-1 and Hep 3B cells

Several proto-oncogenes and tumor suppressor genes have been associated in the regulation of telomerase activity, both directly and indirectly (Liu, 1999). Bcl-2 was reported as a direct modulator of telomerase activity, and

correlation between p53 and telomerase activity was reported (Lee *et al.,* 2001). Thus, to investigate whether telomerase activity may be associated in the regulation of VCA-induced apoptosis, *p53*-positive and *p53*-negative cells were treated with 10 ng/ml of VCA at each time-point and telomerase activity was tested by TRAP assay. As demonstrated in Fig. 7, telomerase activity was observed in both cell lines, and the activity was higher in *p53*-positive cells than in *p53*-negative cells. Telomerase activity was reduced in time dependent manner and greatly reduced in SK-Hep-1 cells 24 h after VCA-treatment. On the other hand, different reducing pattern of telomerase activity was observed in *p53*-negative Hep 3B cells. Telomerase activity was slowly reduced by VCA and was still observed in Hep 3B cells 48 h after VCA-treatment.

DISCUSSION

Many types of cancer have been known to be associated with reduced apoptosis and *p53* loss is associated with reduced apoptosis. Bax, an accelerator of apoptosis, and Bcl-2, a suppressor of apoptosis, have been known to regulate many types of apoptosis. In addition, p53 regulates the expression of *p21*, Bax, and Bcl-2 in inducing apoptosis (Kim *et al.,* 1998, 2000). Bcl-2 prevents various types of cells from apoptosis by preventing activation of caspases, indicating that Bcl-2 family functions upstream of caspases (Kim *et al.,* 1999). Bcl-2 is down-regulated by the tumor suppressor molecule p53 (Miyashita *et al.,* 1995), which is up-regulated in response to DNA damage (Kuirbitz *et al.,* 1992, Lowe and Lin, 2000). However, it has been suggested that p53 is not strictly required for drug-induced cell death; all anti-cancer agents at sufficient doses induce apoptosis independently of p53. In fact, the contribution of p53 to drug-induced apoptosis is determined by a variety of factors (Lowe and Lin, 2000). Additionally, it has been reported that several proto-oncogenes and tumor suppressor genes have been implicated in the regulation of telomerase activity, both directly and indirectly (Liu, 1999). And telomere dysfunction was found to be the principle determinant governing sensitivity to anticancer agents (Lee *et al.*, 2001).

Previous studies reported that VAA induced apoptosis by promoting down-regulation of the nuclear p53 and Bcl-2 protein and that the down-regulation might be due to an inhibition of protein synthesis (Büssing *et al.*, 1999). We also reported that VCA induced apoptosis by activation of caspase-3 in HL-60 cells (Lyu *et al.*, 2001). However, further studies have not been reported yet.

In the present work, we observed that VCA induced apoptosis in both SK-Hep-1 (*p53*-positive) and Hep 3B (*p53*-negative) cells through p53- and p21-independent pathways, but the expression of *p21* is independent of *p53*. We also showed that VCA induced apoptosis by down-regulation of Bcl-2 and by up-regulation of Bax functioning upstream of caspase-3 in both cell lines. Hence, the up-regulation of Bax protein provides evidence that the down-regulation of p53 may not be due to an inhibition of protein synthesis, which was suggested previously (Büssing *et al.*, 1999). Furthermore, we observed down-regulation of telomerase activity in both VCA-treated cell lines in different patterns. Telomerase activity in p53-positive cells was greatly reduced 24 h after VCA-treatment, while the activity was gradually reduced in *p53*-negative cells. The results indicate that the p53 affect inhibition of telomerase activity and suggest that the regulation of p53 in VCA-induced apoptsis may be associated with the inhibition of telomerase.

Taken together, our results provide direct evidence of, in human hepatocarcinoma cells, the anti-tumor potential of this biological response which comes from inhibition of telomerase and consequent inducing apoptosis. In addition, VCA-induced apoptosis is regulated by mitochondrial pathway independent of p53. These findings are important for the therapy with preparation of mistletoe because they show that telomerase-dependent mechanism can be targeted by VCA in hepatoma cells. Furthermore, the VCA, considered as a telomerase-inhibitor, can be envisaged as a candidate for enhancing sensitivity of conventional anticancer drugs.

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