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Antitumor Activities of Extract of *Viscum album* var. *coloratum* Modified with *Viscum album* var. *coloratum* Agglutinin

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Abstract – The mistletoe lectins are major active components in the extract of European mistletoe (*Viscum album* L) that have been widely used in adjuvant chemotherapy of cancer. This study was performed to investigate the antitumor activity of extract of Korean mistletoe (*Viscum album* var. *coloratum*) modified with Korean mistletoe lectin (*Viscum album* var. *coloratum* agglutinin, VCA). Compared with the results of VCA, survival rate was increased and experimental lung metastasis was reduced by treatment of modified extract (VCM). In addition, the treatment of VCM reduced angiogenesis and VCA-induced toxicity measured by a CAM assay. And VCM inhibited proliferation and induced apoptosis *in vitro* in tumor cells originated from tissues which are possible to apply topically without surgery. Taken together, the antitumor activities of VCM-treated group outperformed the activities of the VCA-treated group.
Key words – Mistletoe, Lectin, Modified, Antitumor, Angiogenesis, Apoptosis

Introduction

European mistletoe (*Viscum album* L.) has been widely used as adjuvant cancer therapy (Büssing, *et al.*, 1999, 2000). Recent studies have confirmed that the antitumor activity of the mistletoe extract is mediated by direct cytotoxicity of the extract to tumor cells and/or through a potentiation of immune response by the host (Lyu *et al.*, 2002). Several evidences extrapolated from *in vitro* and *in vivo* studies suggest that mistletoe lectins are strong candidates for tumor targeting therapy. However, there are increasing evidences that not only lectins, but also other substances such as viscotoxins and flavonoids in mistletoe extracts exert antitumor activites (Lyu *et al.*, 2000). The European mistletoe lectins (*Viscum album*, L. agglutinins, VAA-I, II, III) are D-galactose- and/or *N*-acetyl-D-galactosamine-specific (Büssing, *et al.*, 1999, 2000). A galactose- and N-acetyl-D-galactosamine-specific lectin (*Viscum album* L. *coloratum* agglutinin, VCA) was also isolated from Korean mistletoe (*Viscum album* L. *coloratum*), a subspecies of European mistletoe (Park *et al*, 1999a, 1999b, 2001, Lyu *et al.*, 2002).

Metastasis, a multistep process involving numerous tumor cell-host cell and cell-matrix associations are the most clinically and enigmatic aspect of tumor behavior. Although advances have been made in conventional tumor therapies and surgical techniques, most deaths caused by cancer still result from metastasis (Yokoda, 2000). Tumor growth and metastasis require persistent angiogenesis. In the absence of new vasculature, tumor cells become necrotic (Brem *et al.*, 1976) or apoptotic (Parangi *et al.*, 1996). Angiostatin (O'Reilly *et al.*, 1994), interleukin 12 (Sunamura *et al.*, 2000), and prolactin fragment have been revealed as endogenous angiogenesis inhibitors. Angiogenesis inhibitors seem most promises when used in conjunction with therapeutic agents aimed at invasive metastatic tumors and of effective adjuvant therapies for use following resection of primary tumor (Hanahan *et al.*, 1996). Mistletoe extract and lectin have been shown to inhibit metastasis of melanoma cells (Yoon *et al.*, 1998, Park *et al.*, 2001) and angiogenesis (Yoon *et al.*, 1995, Park *et al.*, 2001).

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). There are several reports showing that mistletoe lectin induced apoptosis in cancer cells. Mistletoe lectin induced was associated with the mitochondrial release of cytochrome c, and caspase-activation through p53- and p21-independent pathways (Bantel *et al.*, 1999, Yoon *et al.*, 1999, Lyu *et al.*, 2001, 2002). Clinical studies with mistletoe therapy have shown longer survival times, better quality of life or tumor regression (Büssing, *et al.*, 1999, 2000). Intrapleural instillation of mistletoe extracts is reported to result in pleurodesis in cancer patients with malignant pleural effusions (Stumpf and Büssing, 1997). In addition, intratumoral

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application of mistletoe extracts was more effective in reduction of tumor volume (Büssing, 1996). Nevertheless, mistletoe preparation has been used as adjuvant cancer therapy because of its low efficacy compared with conventional cancer chemotherapy (Büssing, *et al.*, 1999, 2000). In the present study, Korean mistletoe extract (VCE) was modified with VCA to improve treatment modalities of cancer. We investigated the antitumor and antimetastatic activities of modified preparation (VCM), and assessed antiangiogenic activity in the chick chorioallantoic membrane (CAM) assay. We also investigated cytotoxicity and patterns of apoptosis of VCM in different tumor-cell lines originated from tissues which are possible to apply topically without surgery.

Experimental

Preparation of VCE, VCA and VCM – Water extract of mistletoe (VCE) was prepared as reported previously (Lyu *et al.*, 2000). The concentration (mg/ml) of water extract of fresh plant was expressed as the amount of the fresh plant to prepare 1 ml of solution. VCA was purified from the Korean mistletoe by affinity chromatography on asialofetuin-Sepharose 4B as described previously (Lyu *et al.*, 2000). Water extract modified with VCA (VCM) was prepared by adding 1 ml of VCA (1 mg/ml) to 9 ml of VCE (1 mg/ml). The concentration (ng/ml) of VCM was expressed as the amount of VCA.

ELLA (Enzyme-linked lectin assay) – For ELLA, asialofetuin-1 (100 μl/well of 0.1 mg asialofetuin-1/ml in PBS) in a Nunclon microtiter plate (Nunc Immuno Plate Maxisorb, Nunc. Rosklide, Denmark) was incubated overnight, and washed with PBS-T. Blocking solution (200 μl/well of 1% BSA in PBS) was added, incubated, and washed. Then samples at varied concentrations (100 μl/well of antigen in PBS) were added and incubated. The plate was washed with PBS-Tween 20. Primary antibody (a rabbit polyclonal anti-VAA antibody) solution was added, incubated, and washed. Secondary antibody (rabbit-antimouse-IgG-conjugated peroxidase) solution (100 μl/well) was added, incubated, and washed. Each washing process above repeated three times. Hydroperoxidase substrate ABTS (2,2'-azinobis [3-ethylbenzotiazoline-6-sulfonic acid]-diammonium salt (100 μl/well) solution was added and incubated for 20 min at RT in dark. The development was stopped with 100 μl/well of 1% sodium dodecyl sulfate. Absorbance was measured at 405 nm. Each assay was done in triplicate

Cell culture – The following cancer cell lines were obtained from Korean cell line bank (KCLB, Seoul, Korea): a highly metastatic murine melanoma, B16-BL6; mouth carcinoma, KB; salivary gland carcinoma A253; pharynx carcinoma, FaDu; cervix and uterine carcinoma, SNU-17 and SNU-778, and bladder carcinoma, HT-1197 and 253J. The cells were maintained as monolayer cultures in Eagles minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS) and penicillin/streptomycin. The cells were grown in a humidified incubator in 5% CO_2 at 37°C.

Survival analysis and experimental lung metastasis – Female, syngeneic C57BL/6 mice (7-8 weeks old) were used for the survival rate and antimetastatic studies. For survival analysis, B16-BL6 melanoma cells were detached from culture by application of EDTA, washed, and resuspended in DMEM with 10% serum to make a suspension containing 1×10^6 cells/ml. B16-BL6 melanoma cells (1×10^5 cells in a volume of suspension injected subcutaneously) were inoculated into mice. Two weeks after inoculation of B16-BL6 melanoma cells, sample was injected intraperitoneally into 10 mice per group three times a week while the control group received PBS (phosphate buffered saline) only. Experimental lung metastasis of B16-BL6 melanoma cells was assessed by intravenous inoculation of 4×10^4/mouse tumor cells into mice. Sample was administered intravenously into 10 mice per group 2 days before, and 1 day after inoculation of B16-BL6 melanoma cells. The mice were killed 14 days after tumor inoculation and their lungs were fixed with Bouin's solution. Lung tumor colonies were counted under a dissecting microscope. All animals were treated according to the Laboratory Animal Control Guidelines, which basically conform to those of the National Institutes of Health-American Association of Laboratory Animal Control.

Choriallantoic membrane (CAM) assay for angiogenesis – The CAM assay was carried out by the method reported previously (Oikawa *et al.*, 1998, Park *et al.*, 2001). The fertilized chicken eggs (Pulmuwon, Seoul, Korea) were kept in a humidified incubator (JS-IN-180, Johnsam Co., Seoul, Korea) at 37°C. After 3.5-day incubation, 3 ml of albumin was aspirated from the eggs with a 22-gauge hypodermic needle through the small hole drilled at the narrow end of the eggs, allowing the small CAM and yolk sac to drop away from the shell membrane. The shell covering the air sac was punched out and removed by forceps, and the shell membrane on the floor of the air sac was peeled away. At 4.5-days, one μl of sample at the given concentrations was loaded on a Thermanox coverslip (Nunc, Naperville, IL, USA) surface, and the coverslip was placed on the CAM. After incubation of the chick embryo for two days, an appropriate volume of a 10% fat emulsion (Intralipose 10%, Korea Green Cross, Seoul, Korea) was injected

using a 22-gauge needle into the embryo chorioallantois. The eggs were observed on day 6.5 under a microscope. Twenty eggs were used in each concentration and the percentage of relative activity was calculated from the percent of avascular eggs per total number of eggs tested.

Cytotoxic assay – The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, St. Louis, MO, USA) colorimetric assay was used for testing cell survival. The cell suspension was aliquoted into 96-well microtiter plates (one hundred microliter of cell suspension containing 1×10^5 cells/ml in each well). One hundred microliter of indicated concentration of sample solution was added to the cell suspension, and the plate was incubated for 48 h. At the end of incubation, the MTT solution was added and the plate was incubated for a further 4 h followed by detection using an ELISA plate reader at 570 nm. The inhibitory concentration values, i.e., drug concentration required to inhibit viability by 50% (IC_{50} values) were determined directly from sample concentration resulting in 50% inhibition of MTT dye formation, compared to untreated controls, with each assay was done in triplicate. Cell growth was determined by trypan blue exclusion method at each time-point. The cells treated with sample were washed twice with PBS. Cells were stained with 0.4% trypan blue solution and the cells that excluded the dye were counted. Cell growth was determined by percentage of the control cells.

DNA fragmentation analysis – For DNA fragmentation analysis, cells were harvested and washed twice with PBS after treatment of cells with the indicated concentrations of sample. Cells were suspended in lysis buffer containing 0.1 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), in the presence of 10% SDS and proteinase K (20 mg/ml), and the resuspended cells were incubated in a shaking incubator at 56°C for 2 h. DNA was then extracted with 5 M sodium chloride solution, precipitated in ethanol, centrifuged at 12,000×g for 30 min, and resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). After quantitation of DNA, 2.5 μg of DNA per lane was electrophoresed on a 1.5% agarose gel for 30 min at 100V and the gel was visualized with ethidium bromide.

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

Determination of VCA in water extract of mistletoe by ELLA – The asialofetuin is asialo-triantenary glycopeptide that contains three terminal galactose residues. We previously reported that the VCA was isolated efficiently by asialofetuin-Sepharose 4B and that the higher recognition of VCA in ELLA may come from higher affinity of VCA to asialofetuin (Lyu *et al.*, 2000). In the ELLA based on lectin affinity to sugars, asialofetuin is used as coating antigen (Lyu *et al.*, 2000). Accordingly, ELLA only determines sugar binding lectin. In the present study, the concentration of VCA in water extract of mistletoe was determined by ELLA using purified VCA as standard. A dose-dependent curve was obtained up to 100 ng/ml of VCA. The concentration of VCA in 1 mg/ml of water extract was 30 ng/ml. As described in materials and methods, water extract modified with VCA (VCM) was prepared by adding 1 ml of VCA (1 mg/ml) to 9 ml of VCE (1 mg/ml). Consequently the concentration of VCA in VCM is calculated at 1,030 ng/ml.

Survival analysis and inhibition of tumor metastasis – Two weeks after inoculation of B16-BL6 melanoma cells into mice, VCM was injected intraperitoneally into mice three times a week. To analyze the antitumor potential of VCM the survival rate of the animals was used as the principal outcome measure. We previously reported that tumor formation was not blocked by treatment of VCA, but the survival of mice was slightly prolonged (Park *et al.*, 2001). In the present study, we performed the survival analysis of treatment of VCM and compared with the results of treatment of VCA. Similarly to the results of treatment of VCA, tumor formation was not blocked by treatment of VCM. However, we found that the survival of mice was prolonged by VCM-treatment. On day 27 after inoculation of tumor cell, 50% in the VCA-treated group and 70% in the VCM-treated group were alive, while all animals in the

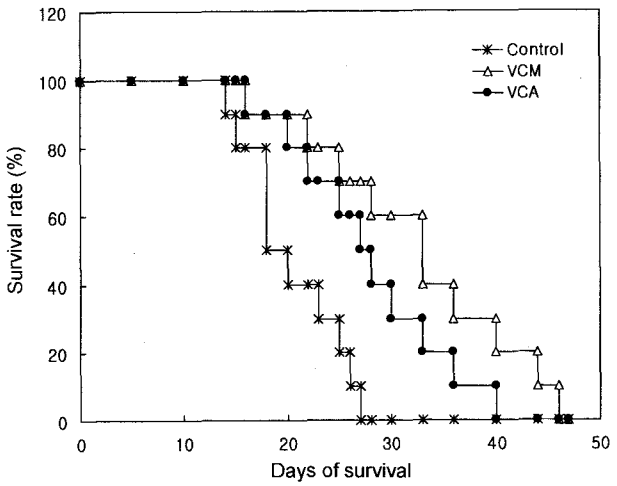

Fig. 1. Survival rate of C57BL6 mice harboring the B16-BL6 melanoma cells. The mice were inoculated with B16-BL6 melanoma cells and injected with VCA (10.0 ng/ml) or VCM (10.3 ng/ml) two weeks after inoculation. To analyze antitumor potential of the samples, the survival of the animals was taken as the principal outcome.

Table 1. Effect of VCM[a] on the tumor-metastasis produced by B16-BL6 melanoma cells. The mice were injected with samples two days before and 1 day after inoculation of melanoma cells and killed 14 days after tumor inoculation. The concentration (ng/ml) of VCES was expressed as the amount of VCA ($p<0.01$, compared with the untreated group by ANOVA test)

Dose	No. of lung metastasis (Inhibition %) (ng/mouse)		
	No.	Mean ± Std.	Std. Error
Control	10	65.90 ± 5.51	1.74
10.3	10	21.10 ± 3.52	1.33
51.5	10	30.50 ± 6.43	1.47
Total	50	41.80 ± 15.08	2.54

[a]VCM: Water extract modified with VCA prepared by adding 1 ml of VCA (1 mg/ml) to 9 ml of VCE (1 mg/ml). The concentration (ng/ml) of VCM was expressed as the amount of VCA.

Table 2. Effects of VCM on the angiogenic activity by CAM assay. Each assay employed about 20 eggs per sample. % Relative activity = (No. of positive eggs / No. of surviving eggs) ×100

Conc. of VCM (ng/ml)	No. of survived eggs	No. of positive eggs	% Relative activity
1.6	19	9	47
3.2	18	10	56
6.5	17	12	71
13.0	19	15	79
26.0	19	16	84
52.0	18	18	100
103.0	16	16	100
206.0	15	15	100
PBS	20	0	0
Retinoic Acid	20	14	70

control group had died. On day 40, all animals in the VCA-treated group had died, while 20% in the VCM-treated group were still alive, and all animals died within 46 days after tumor cell inoculation (Fig. 1).

The antimetastatic effect of VCM was examined in experimental lung metastasis of B16-BL6 cells. VCM was administered into mice 2 days before and 1 day after inoculation of B16-BL6 melanoma cells. VCM inhibited more effectively than the results of VCA reported previously (Park *et al.*, 2001). Each group administered with VCM inhibited distinctly lung metastasis and the group treated with 10.3 ng showed the highest inhibition of metastasis (Table 1).

Antiangiogenic effects of VCM – Next, we investigated the antiangiogenic activity of VCM using a CAM assay. Fig. 2 shows photographs of representative results showing the antiangiogenic acitivity of VCM at the indicated concentrations. VCM appeared to inhibit angiogenesis, so an avascular zone as was seen in the positive control, retinoic acid whereas PBS alone, as a negative control, had no effect. VCM inhibited angiogenesis in 44% of the surviving eggs at 0.8 ng/egg and inhibition reached 100% at 52 ng/egg (Table 2). The number of survived eggs was slightly decreased at higher concentrations of VCM.

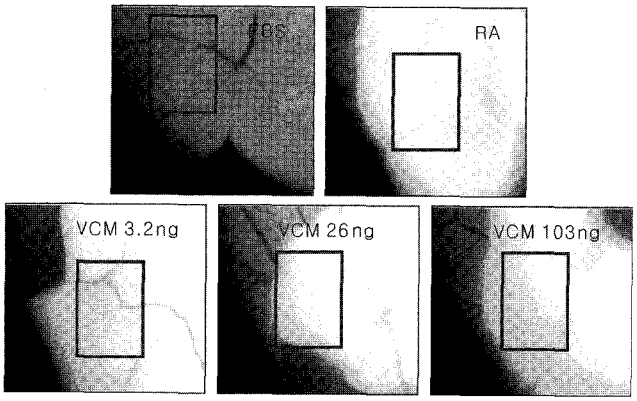

Fig. 2. Inhibition of angiogenesis by the VCM by CAM assay. After 4.5 days of development, a thermanox-coverslip (red-box) containing indicated concentration of VCM, RA (1μg, retinoic acid as positive control), or PBS (negative control) was placed at the vascular membrane of the CAM. After 2 days the membranes were evaluated and photographed (original magnification × 14).

VCM induces cell death in tumor cells *via* apoptosis *in vitro* – Table 3 shows the effect of VCA and VCM on the proliferation of a variety of tumor cell lines *in vitro* as measured by the MTT assay. The tumor-cell lines were originated from tissues which are possible to apply topically without surgery. Both samples were highly cytotoxic against tumor cells and caused time- and dose-dependent reduction of cell growth after incubation with cells (data not shown). No significant difference of IC_{50} was observed between treatment of VCA and VCM, but the IC_{50} of VCA and VCM on B16-BL6 was higher than those of other tumor cell lines. To determine the mode of cell death, tumor cells

Table 3. Cytotocitiy of VCA and VCM against different tumor cells. Cells were treated for 48 h with VCA and VCM. Growth inhibition was determined by the MTT assay. The activity was compared to the control of the same cell line and results are expressed as a percentage of the controls. Each assay was done in triplicate and the results are shown an average

Cell lines	Tissue	IC_{50}[a] (ng/ml)	
		VCA	VCM
Murine cell lines			
B16-BL6	Melanoma	25±6	20±5
Human cell lines			
KB	Mouth carcinoma	4±3	4±1
A253	Salivary gland carcinoma	3±2	3±2
FaDu	Pharynx carcinoma	2±1	4±3
SNU-17	Cervix and uterine carcinoma	7±3	5±3
SNU-778	Cervix and uterine carcinoma	8±5	10±2
HT-1197	Bladder carcinoma	10±4	9±3
253J	Bladder carcinoma	7±5	7±3

[a]IC_{50}: The inhibitory concentration values, i.e., drug concentration required to inhibit viability by 50%.

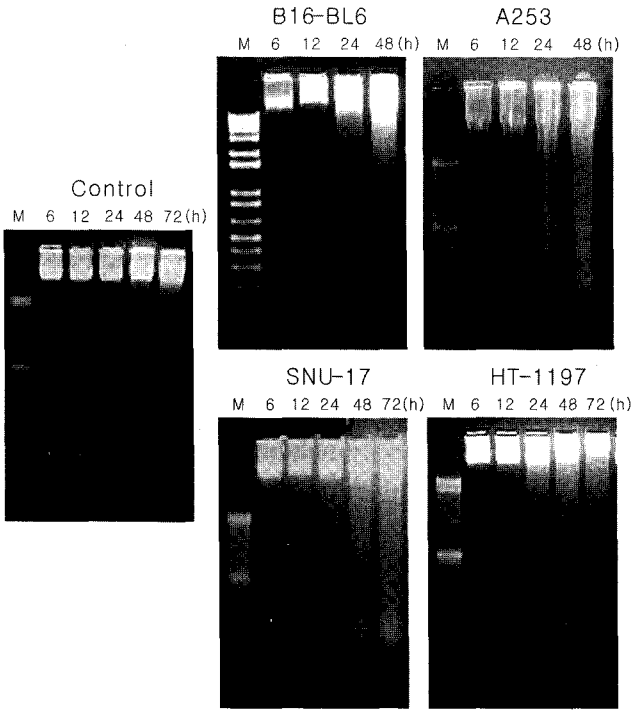

Fig. 3. DNA ladder formation of tumor cells after treatment with VCM. Cells for DNA fragmentation analysis were treated with 10.3 ng/ml of VCM for indicated time periods. DNA was extracted and analyzed on 1.5% agarose gel. Lane M was standard DNA marker (bp).

were treated with 10.3 ng/ml of VCM and agarose gel electrophoresis was performed. The VCM-treated chromosomal DNA showed a ladder-like pattern of DNA fragments (Fig. 3). The apoptosis-inducing activities of VCM in A253, SNU-17, and HT-1197 were dose- (data not shown) and time-dependent, being observed at 72 h. However, induction of apoptosis was not observed up to 48 h in B16-BL6 cells.

Discussion

Clinical studies with mistletoe therapy have shown tumor regression and even more effective reduction of tumor size by intratumoral application (Büssing, 1996). Neverthless, mistletoe preparation has been used as adjuvant cancer therapy because of its lower efficacy than conventional cancer therapy (Büssing, *et al.*, 1999, 2000). Although mistletoe lectin is known to be main active antitumor-components, other substances in the extracts are also known to have antitumor- activities (Lyu *et al.*, 2000). In addition, application of purified lectin is known to be highly toxic *in vivo* (Büssing, 2000). Consequently, aqueous extract of mistletoe has been used clinically.

It has been reported that mistletoe extract showed immunostimulatory properties at low concentrations and cyotstatic/cytotoxic properties at higher concentrations (Büssing, 1996). We also previously reported that both analysis of survival and metastasis showed that low dose group outperformed the high dose group (Park *et al.*, 2001). On the other hand, higher doses produced more DNA laddering, with no evidence of apoptosis seen at the lower doses. The present study was designed to develop mistletoe preparations to improve for the use of topical application without surgery for external cancer-patients such as melanoma and mouth carcinoma.

We prepared water extract of mistletoe modified with Korean mistletoe lectin (VCA) and examined whether the modified preparation (VCM) blocked the growth of pre-inoculated tumors *in vivo*. To analyze anti-tumor potential of VCM, the length of survival of mice was taken as the principal outcome measure. We found that the length of survival was increased by VCM-treatment compared with the previous results of VCA-treatment (Park *et al.*, 2001). Next, the antimetastatic effect of VCM was examined in experimental lung metastasis of B16-BL6 cells, resulting that the VCM improved inhibition of tumor metastasis compared with the results of VCA-treatment (Park *et al.*, 2001). Each group administered with VCM inhibited distinctly experimental lung metastasis and the group treated with 10.3 ng of VCM showed the highest inhibition of metastasis. Considering that prophylactic effect of Korean mistletoe extract on tumor metastasis is mediated by enhancement of NK cell activity (Yoon, 1998), these results support that VCM may also augment more effectively the host defense system against tumors at lower concentrations.

It is well known that most deaths caused by cancer are not the result of primary tumor growth but, rather, are due to the dissemination of tumor cells to secondary sites by a series of events known collectively as the metastatic cascade (Filder, 1991). We investigated that VCM could inhibit metastasis by another mechanism such as antiangiogenesis. We previously reported that VCA inhibited angiogenesis up to 100% in 55% of the surviving eggs at 50 ng/egg. In the present study, the antiangiogenic activity of VCM was investigated using a CAM assay showing improved antiangiogenic effects. Treatment of VCM (52 ng/egg) inhibited angiogenesis up to 100% and the number of survived eggs was increased to 90%, indicating the reduction of VCA-induced toxicity by modification.

Next, to investigate the cytotoxic activities and pattern of apoptosis of VCA and VCM *in vitro*, we selected different cell lines of melanoma, mouth carcinoma, salivary gland carcinoma, pharynx carcinoma, cervix and uterine carcinoma, and bladder carcinoma. The selected cell lines

are originated from tissues which are possible to apply topically without surgery. The addition of VCM to tumor cells resulted in growth suppression, DNA fragmentation inducing apoptosis. These results are also consistent with our previous results where VCA did not induce cell cycle arrest, but induced apoptosis (Lyu *et al.*, 2001).

Taken together, both survival and metastasis analysis showed that the antitumor activities of VCM-treated group outperformed the activities of the VCA-treated group. The enhancing antiumor-activity and reduced toxicity of VCM may come from synergistic effect of lectin and other substances such as viscotoxins and flavonoids in mistletoe extracts (Lyu *et al.*, 2000). Our results suggest that the VCM can be visualized as a new candidate of mistletoe preparation for enhancing efficacy and reducing toxicity specifically for the use of topical application for cancer patients.

Acknowledgements

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