Arch Pharm Res Vol 26, No 10, 861-867, 2003

Archives of Pharmacal Research
http://apr.psk.or.kr

Antitumor Activity of the Korean Mistletoe Lectin is Attributed to Activation of Macrophages and NK Cells

Taek Joon Yoon, Yung Choon Yoo[1], Tae Bong Kang, Seong Kyu Song, Kyung Bok Lee[2], Erk Her[3], Kyung Sik Song[4], and Jong Bae Kim

Institute for Biomedical Research, Han Dong University, Namsong-Ri 3, Buk-Ku, Pohang, Korea, [1]Department of Microbiology, [2]Department of Biochemistry, College of Medicine, Konyang University, Nonsan City, Chungnam, Korea, [3]Department of Immunology, College of Medicine, Kon-Kuk University, Chung-Ju 380-701, Korea, and [4]School of Applied Biology and Chemistry, College of Agriculture and Life Science, Kyungpook National University, Daegu 702-701, Korea

(Received June 9, 2003)

Inhibitory effect of the lectins (KML-C) isolated from Korean mistletoe (KM; *Viscum album coloratum*) on tumor metastases produced by murine tumor cells (B16-BL6 melanoma, colon 26-M3.1 carcinoma and L5178Y-ML25 lymphoma cells) was investigated in syngeneic mice. An intravenous (i.v.) administration of KML-C (20-50 ng/mouse) 2 days before tumor inoculation significantly inhibited lung metastases of both B16-BL6 and colon 26-M3.1 cells. The prophylactic effect of 50 ng/mouse of KML-C on lung metastasis was almost the same with that of 100 μg/mouse of KM. Treatment with KML-C 1 day after tumor inoculation induced a significant inhibition of not only the experimental lung metastasis induced by B16-BL6 and colon 26-M3.1 cells but also the liver and spleen metastasis of L5178Y-ML25 cells. Furthermore, multiple administration of KML-C given at 3 day-intervals after tumor inoculation led to a significant reduction of lung metastasis and suppression of the growth of B16-BL6 melanoma cells in a spontaneous metastasis model. In an assay for natural killer (NK) cell activity, i.v. administration of KML-C (50 ng/mouse) significantly augmented NK cytotoxicity against Yac-1 tumor cells 2 days after KML-C treatment. In addition, treatment with KML-C (50 ng/mouse) induced tumoricidal activity of peritoneal macrophages against B16-BL6 and 3LL cells. These results suggest that KML-C has an immunomodulating activity to enhance the host defense system against tumors, and that its prophylactic and therapeutic effect on tumor metastasis is associated with the activation of NK cells and macrophages.

Key words: Korean mistletoe, Lectin, Antitumor activity, NK cell, Macrophages

INTRODUCTION

Mistletoe is a semiparasitic plant growing on deciduous trees all over the world. It has been shown that the extracts of European mistletoe (EM; *Viscum album Loranthaceae*) possess a variety of biological activities such as induction of various cytokines (Mueller *et al.*, 1990b; Hajto *et al.*, 1990; Mannel *et al.*, 1991), enhancement of natural killer (NK) cell activity (Kuttan *et al.*, 1992; Mueller *et al.*, 1989; Mueller *et al.*, 1990a; Hulsen *et al.*, 1989; Hamprecht *et al.*, 1987) and immunoadjuvant activities (Bloksma *et al.*, 1979; Mertzer *et al.*, 1985; Hajto *et al.*, 1986). Moreover, antitumor activities of the EM extract were related to its capability of enhancing the cytotoxic activity of NK cells, lymphokine-activated killer (LAK) cells and macrophages (Kuttan *et al.*,1990; Mueller *et al.*, 1990c; Kuttan *et al.*, 1992). It was reported that the mistletoe preparations exhibited direct cytotoxicity against tumor cells in culture (Gilles *et al.*, 1986). The responsible active components of the EM extract were determined to be lectins: ML-I, -II, and -III (Mertzer *et al.*, 1985; Franz *et al.*, 1986). Among those three lectins, ML-I has been studied most in its antitumor and immunomodulating activities (Franz *et al.*, 1986). The ML-I consists of A and B subchains linked by disulfide bonds (Olsnes *et al.*, 1982), and, recently, the amino acid sequence of the A chain was completely determined (Stiefel *et al.*, 1996).

Correspondence to: Jong Bae Kim, Institute for Biomedical Research, Han Dong University, Namsong-Ri 3, Buk-Ku, Pohang, Korea
Tel: 82-54-260-1350, Fax: 82-54-261-6705
E-mail: jbkim@handong.edu

On the other hand, Korean mistletoe (KM; *Viscum album Coloratum*), a different subspecies of *Viscum album* from EM, was shown to be more cytotoxic to tumor cells than EM in *in vitro* experiments using L1210 murine leukemia cells (Khwaja *et al.*, 1980). We also found that the water extract of KM (KM-110) inhibits tumor metastasis by suppressing tumor growth and tumor-induced angiogenesis (Yoon *et al.*, 1995), and enhances natural killer cell activity in mice (Yoon *et al.*, 1998). In a series of studies on the biological activities of KM and its components, we isolated the lectins (KML-C) from the KM extract and determined the difference of chemical properties between the lectins of KM and EM (Yoon *et al.*, 1999). Our previous study revealed that KML-C was cytotoxic to various tumor cells, and its cytotoxicity was associated with induction of apoptotic cell death. Recently, in addition, we demonstrated that KML-C acted as an immnoadjuvant to enhance humoral and cellular immune responses specific to foreign antigens (Yoon *et al.*, 2001). Even though the lectin fraction of KM-110 was proven as one of the active components of KM responsible for suppressing tumors (Yoon *et al.*, 1999), the underlying mechanisms involved in its antitumor activity *in vivo* have not been fully understood yet.

In this study, we investigated antitumor activity of KML-C with respect to the prophylactic and therapeutic inhibition of tumor metastasis using experimental metastasis models in syngeneic mice, and partly analyzed the mechanism attributed to its antimetastatic effect in the aspect of enhancement of host defense system against tumors through activation of NK cells and macrophages.

MATERIALS AND METHODS

Animals

Seven-to-eight weak old female specific pathogen-free Balb/c, C57BL/6 and CDF1 mice were purchased from the Shizuoka Laboratory Animal Center, Hamamatsu, Japan, and were maintained at the Laboratory of Animal Experiments, the Institute of Biomedical Research, Handong University, Korea.

Cells cultures

B16-BL6 melanoma (Yoo *et al.*, 1997), a highly metastatic line of colon26 carcinoma (colon26-M3.1) (Yoon *et al.*, 1995) and 3LL lung carcinoma cells were maintained as monolayer cultures in Eagle's minimal essential medium (EMEM) supplemented with 7.5% fetal calf serum, vitamin solution, sodium pyruvate, nonessential amino acids and L-glutamine. Liver metastatic L5178Y-ML25 T lymphoma cells (partially metastasizing to the spleen) were maintained in RPMI-1640 supplemented with 5% FBS and L-glutamine [24].

Preparation of Korean mistletoe extract (KM-110) and Lectin (KML-C)

An aqueous extract (KM-110) and lectin (KML-C) from KM were prepared according to the method as previously described (Yoon *et al.*, 1999). Briefly, the chopped leaves of Korean mistletoe plants were grinded in approximately 10 volumes of distilled water by homogenizing for 30 sec and left being stirred overnight at 4°C. After centrifugation at 15,000×g for 30 min, the supernatants were filtered by the stages with 60, 20, 7.2, 0.45 and 0.2 mm pore size and lyophilized. The protein fraction obtained from KM-110 was applied to a Separose 4B column which had been hydrolyzed for 2.5 h with 0.2 M HCl. The absorbed material was eluted with a lactose-containing buffer (0.1 M lactose and 10 mM sodium phosphate in 0.14 M NaCl, pH 7.3). The fraction containing lectins was pooled, dialysed against water and freeze-dried. An appropriate amount of each of KM-110 and KML-C was dissolved with PBS and stored at 4°C until use.

Experimental lung metastasis or liver and spleen metastasis

In experimental lung metastasis, Balb/c or C57BL/6 mice were inoculated i.v. with colon26-M3.1 carcinoma or B16-BL6 melanoma cells (2.5×10^4/mouse), respectively, and given various doses of KM-110 or KML-C on the indicated days before or after tumor inoculation. The mice were sacrificed 14 days after tumor inoculation. The number of lung tumor colonies was counted using a dissecting microscope after fixing the lung samples in Bouins solution (Yoon *et al.*, 1995). An assay for liver and spleen metastasis was conducted by i.v. inoculation of L5178Y-ML25 lymphoma cells (4×10^4/mouse) into CDF1 mice as described previously (Yoo *et al.*, 1995). The weights of the liver and the spleen were recorded 14 days after tumor inoculation to evaluate tumor metastasis.

Spontaneous lung metastasis

C57BL/6 mice were inoculated s.c. with B16-BL6 melanoma cells (5×10^5/mouse) into the right hind footpad, and administered i.v. with KML-C on the indicated days before or after the primary tumor inoculation (Yoon *et al.*, 1995). The primary tumors were surgically removed by amputation 21 days after tumor inoculation. Tumor volume at the time of tumor amputation was calculated by the following formula; $(L\times W^2)/2$, L; long axes, W; width. The mice were sacrificed 35 days after tumor inoculation for the evaluation.

In vitro assay of macrophage-mediated cytotoxicity

Macrophage-mediated cytotoxicity was assessed by a radioactive release assay as described elsewhere (Yoo *et al.*, 1994). Briefly, [^{125}I]-IdURd-labeled target cells (B16-BL6 cells) were added to the macrophage monolayer in a

96-well plate to obtain a macrophage:target cell ratio of 20:1, and the culture was incubated for 2 days. After incubation, the plate was centrifuged for 10 min at 900×g. The supernatants (100 μL) from each well was absorbed on cotton swabs and monitored for radioactivity using a gamma counter. The percentage of macrophage cytotoxicity generated was calculated from the radioactivity (count min^{-1}) according to the following formula: Cytotoxicity (%) = [(experimental release – spontaneous release)/(maximum release spontaneous release)]×100.

Assay of NK-mediated tumor cytotoxicity

An NK-mediated assay was carried out by the radioactive ^{51}Cr-release assay as described previously (Yoo *et al.*, 1997). Three Balb/c mice per group were administered i.v. with the indicated doses of KML-C, and their splenocytes were harvested 2 days after KML-C treatment. Single cell suspensions of the splenocytes were added to ^{51}Cr-labeled Yac-1 cells (1×10^4/well) to obtain effector-to-target cell ratio (E/T ratio) of 100:1, 50:1 or 25:1 in a U-bottomed 96 well plate, and the cultures were incubated for 6 h. After incubation, the plate was centrifuged for 10 min at 900 g. The supernatants (100 μL) of each well were absorbed onto cotton swabs, and their radioactivity was monitored using a gamma counter. NK cell cytotoxicity was calculated from the radioactivity (count min^{-1}) according to the following formula: Cytotoxicity (%) = [(experimental release – spontaneous release)/(maximum release spontaneous release)]×100.

Depletion of NK cells or macrophages *in vivo*

Depletion of NK cells and macrophages *in vivo* was performed as the methods described previously (Yoon *et al.*, 1998; Hayakawa *et al.*, 1998) with some modifications. To deplete NK cells, mice were injected intraperitoneally (i.p.) twice with 500 μL/mouse of 50-fold diluted rabbit anti-asialo GM1 serum (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) 1 and 3 days before tumor inoculation. Depletion of macrophages was carried out by i.p. injection of carrageenan (1.2 mg/mouse) twice 1 and 3 days after tumor inoculation.

Statistical analysis

The statistical significance of differences between the groups was determined by applying the Student's two-tailed *t*-test.

RESULTS

Inhibitory effect of KML-C on experimental tumor metastasis

To investigate the inhibitory effect of KML-C on tumor metastasis, we first examined the prophylactic activity of KML-C against experimental lung metastasis produced by B16-BL6 melanoma and colon 26-M3.1 carcinoma cells. As shown in Table I, i.v. administration of KML-C 2 day before tumor inoculation significantly reduced lung metastasis of both highly metastatic tumor cells, and KML-C treatment was still active as low a dose as 5 ng/mouse. The antimetastatic effect of KML-C at the dose of 50 ng/mouse was almost equivalent to that produced by 100 μg/mouse of KM-110. Furthermore, KML-C treatment 1 day after tumor inoculation induced a significant inhibition of experimental lung metastasis of B16-BL6 and colon 26-M3.1 cells in a dose dependent manner (Table II). The therapeutic effect of KML-C on tumor metastasis was also found in liver and spleen metastasis produced by L5178Y-

Table I. Prophylactic effect of KML-C on lung metastasis produced by i.v. inoculation of colon 26-M3.1 carcinoma or B16-BL6 melanoma cells

Treatment	Dose (mg)	Number of lung metastasis (% inhibition)			
		colon 26-M3.1		B16-BL6	
		Mean±SD	Range	Mean±SD	Range
Untreated (PBS)		55 ± 17	(39-80)	66 ± 13	(52-80)
KM-110	100	12 ± 5 (78.2) [a]	(6-19)	37 ± 11 (43.8) [a]	(26-49)
KML-C	0.05	12 ± 8 (78.2) [a]	(0-22)	36 ± 8 (45.5) [b]	(28-45)
	0.02	28 ± 11 (49.1) [b]	(17-41)	42 ± 6 (36.4) [b]	(35-51)
	0.005	50 ± 14 (9.1)	(30-74)	55 ± 10 (16.7)	(46-71)

Groups of five C57BL/6 or Balb/c mice were inoculated i.v. with 4×10^4 B16-BL6 melanoma or 2.5×10^4 colon 26-M3.1 carcinoma cells, respectively. All mice were administered i.v. with the indicated doses of KM-110 or KML-C 2 days before tumor inoculation, and sacrificed 14 days after tumor inoculation for evaluation.

[a]$p<0.001$; [b]$p<0.01$, compared with the untreated group (by Student's two-tailed *t*-test)

Table II. Therapeutic effect of KML-C on lung metastasis produced by i.v. inoculation of colon 26-M3.1 or B16-BL6 tumor cells

Treatment	Dose (mg)	Number of lung metastasis (% inhibition)			
		colon 26-M3.1		B16-BL6	
		Mean ± SD	Range	Mean ± SD	Range
Untreated (PBS)		100 ± 13	(85-117)	89 ± 16	(53-105)
KM-110	100	60 ± 13 (40) [a]	(42-74)	48 ± 12 (46.1) [a]	(36-60)
	0.05	64 ± 16 (36) [a]	(47-81)	52 ± 8 (41.6) [a]	(44-60)
KML-C	0.02	74 ± 21 (26) [b]	(53-93)	58 ± 11 (34.8) [b]	(47-69)
	0.005	98 ± 11	(87-109)	74 ± 19	(55-94)

Groups of five C57BL/6 or Balb/c mice were inoculated i.v. with 4×10^4 (B16-BL6) or 2.5×10^4 (colon 26-M3.1) tumor cells, respectively. All mice were administered i.v. with the indicated doses of KM-110 or KML-C 1 day after tumor inoculation, and sacrificed 14 days after tumor inoculation for evaluation.

[a]$p<0.01$; [b]$p<0.05$, compared with the untreated group (by Student's two-tailed *t*-test)

ML25 lymphoma cells (Table III). These results suggest that KML-C prophylactically as well as therapeutically inhibit the tumor metastasis of both lung metastatic tumor cell lines (B16-BL6 and colon 26-M3.1), and reduce tumor metastasis of hematogenous tumor cells (L5178Y-ML25).

Inhibition of spontaneous lung metastasis by KML-C

The inhibitory effect of KML-C on a spontaneous tumor metastasis was investigated in a spontaneous lung metastasis model in which KML-C (50 ng) was administered 5 times at an interval of 3 days beginning with the day 5 after tumor inoculation. As shown in Table IV, the multiple administration of KML-C before the amputation of the primary tumors resulted in a significant inhibition of spontaneous lung metastasis. In addition, the multiple administration of KML-C apparently suppressed the growth of the primary tumors 21 days after tumor inoculation. The multiple administration of KML-C after tumor amputation was also therapeutically active.

Table III. Therapeutic effect of KML-C on liver and spleen metastasis produced by L5178Y-ML25 lymphoma cells

Treatment	Dose (μg)	Mean weight (g) ± SD, (% inhibition)	
		Liver	Spleen
Normal		1.07 ± 0.1	0.09 ± 0.02
Untreated (PBS)		3.54 ± 0.48	0.21 ± 0.05
KM-110	100	1.59 ± 0.22 (56.5) [a]	0.14 ± 0.02 (33.3) [a]
KML-C	0.05	1.83 ± 0.48 (48.3) [a]	0.15 ± 0.04 (28.6) [a]
	0.02	2.12 ± 0.35 (40.1) [a]	0.17 ± 0.06 (19.1)
	0.005	3.21 ± 1.31	0.22 ± 0.04

Groups of five CDF1 mice were inoculated i.v. with 5×10^4 L5178Y-ML25 lymphoma cells, and given i.v. injection of the indicated doses of KM-110 or KML-C 1 day after tumor inoculation. Mice were sacrificed 14 days after tumor inoculation for evaluation.
[a]$p<0.01$; [b]$p<0.05$, compared with each vehicle control (by Student's two-tailed *t*-test)

Table IV. Inhibitory effect of KML-C on tumor growth and spontaneous lung metastasis of B16-BL6 melanoma cells

Treatment (on day)	Primary tumor volume (mm^3)		No. of lung metastasis (% inhibition)	
	Mean ± SD (% inhibition)		Mean ± SD	Range
Untreated	980 ± 253		56 ± 13 (33-69)	
5,8,11,14,17	495 ± 149	(49.2) [a]	24 ± 9 (57.1) [a]	(15-34)
22,25,28,31,34			29 ± 13 (48.2) [a]	(16-42)

Groups of five C57BL/6 or Balb/c mice were administered i.v. with KML-C (50 ng) on the indicated days after footpad inoculation of B16-BL6 cells ($5X10^5$/site). The primary tumors were amputated surgically 21 days after tumor inoculation. Mice were sacrificed 14 days after tumor inoculation for evaluation.
[a]$p<0.01$, compared with the untreated group (by Student's two-tailed *t*-test)

Activation of NK cell activity by KML-C

The ability of KML-C to enhance NK activity was estimated by its cytotoxic activity against Yac-1 NK-sensitive cells in 6 h ^{51}Cr-release assay 2 days after KML-C treat0 ment. As seen in Fig. 1, the splenocytes of the mice treated with KML-C (550 ng/mouse) showed a higher NK activity than that of the control mice 2 days after KML-C treatment. This increased activity was E/T ratio-dependent, and maintained even at a low dose of 20 ng/mouse.

Induction of macrophage-mediated cytotoxicity against tumor cells by KML-C

It is well known that macrophages play important roles in non-specific immune response against tumors, and activated macrophages can eliminate neoplastic cells. We examined whether KML-C was able to elicit macrophages having tumoricidal activity. As depicted in Fig. 2, treatment with various doses of KML-C 2 days before assay showed a higher macrophage-mediated cytotoxic activity against B16-BL6 tumor cells than the untreated control. The tumoricidal macrophages activated by KML-C also effectively killed another tumor cells (3LL lung carcinoma).

Effect of the depletion of NK cell and macrophages on KML-C-induced antitumor activity

To address the contribution of NK cells and macrophages to the inhibitory effect of KML-C on tumor metastasis *in vivo*, KML-C (50 ng/mouse) was administered into NK-depleted or macrophage-depleted mice 2 days before

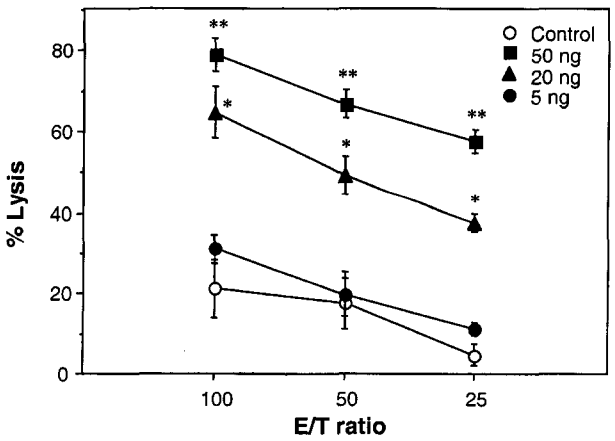

Fig. 1. Effect of KML-C on enhancement of NK cell activity. Three Balb/c mice per group were administered i.v. with KM-110 (100 μg/mouse) or KML-C (50 ng/mouse). NK activity was determined by a 6 hr-incubation assay using splenocytes (effector) and Yac-1 cells (target) 2 days after KML-C treatment as described in Materials and Methods. *$p<0.01$; **$p<0,001$, compared with the untreated group (by Student's two tailed *t*-test)

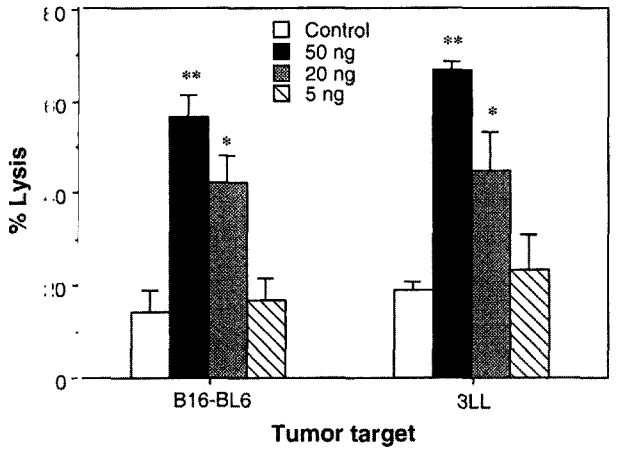

Fig. 2 Effect of KML-C on induction of tumoricidal macrophages. C57BL/6 mice were administrated intraperitoneally with KML-1 (50 ng/mouse), and the peritoneal macrophages were collected 2 days after treatment. ^{125}I-labeled B16-BL6 or 3LL tumor cells were plated in a 96-well culture plate at 1×10^4/well with macrophages and incubated for 48 hr. Then the radio activity was measured by a g-ray counter. *p<0.05; **p<0.01, compared with the untreated group (by Student's two tailed *t*-test).

tumor inoculation. Both NK- and macrophage-depletion by anti-asialo GM1 serum or carragenan, respectively, resulted in a marked increase of experimental lung metastasis as compared with that of the untreated control (Table V). Treatment with KML-C reduced experimental lung metastasis in NK-depleted as well as macrophage-depleted mice although its inhibitory effect was not significant.

Table V. Effect of anti-asialo GM1 serum and carragenan on KML-C-induced inhibition of lung metastasis of colon 26-M3.1 carcinoma cells

	Number of lung metastasis (% inhibition)	
	Mean ± SD	Range
Untreated (PBS)	122 ± 20	(107-214)
KML-C	42 ± 7 (65.6) [a]	(35-50)
a-asialo GM1	354 ± 38	(306-394)
a-asialo GM1 + KML-C	305 ± 41	(265-347)
Carragenan	174 ± 35	(138-209)
Carragenan + KML-C	128 ± 37	(91-165)

Groups of five Balb/c mice were inoculated i.v. with 2.5×10^4 colon 26-M3.1 carcinoma cells. To delete NK cells and macrophages in vivo, mice were injected i.p. with 50-fold diluted rabbit anti-asialo GM1 serum (500 ml/mouse) or carragenan (1.2 mg/mouse)1 and 3 days before tumor inoculation, respectively. In KML-C-treated group, mice were administered i.v. with KML-C (50 ng/mouse) 2 days before tumor inoculation. All mice were sacrificed 14 days after tumor inoculation for evaluation.

[a]p<0.001, compared with the untreated group (by Students two-tailed *t*-test)

DISCUSSION

The lectins purified from EM were shown to possess a variety of immunostimulating actions and antitumor activities (Bloksma *et al.*, 1979; Kuttan *et al.*, 1990; Ribereau-Gayo *et al.*, 1986). It was also documented that the water extract (KM-110) of Korean mistletoe (KM), a different subspecies of *Viscum album* from EM, stimulated macrophages to produce IL-1 and TNF-α *in vitro* system (Yoon *et al.*, 1994), and activated NK cells to enhance its cytolytic activity against tumor cells (Yoon *et al.*, 1998). In addition, the protein fractions isolated from KM-110 by 80% ammonium sulfate precipitation induced TNF-a from macrophages in mice (data not shown). These data strongly suggest that the active materials of KM-110 responsible for nonspecific potentiation of immune-related cells are mainly contained in its protein part.

Recently, we isolated KM-derived lectins (KML-C) specific to *N*-acetylgalactosamine and galactose, and found its two different biological functions such as apoptosis-inducing activity in tumor cells (Yoon *et al.*, 1999) and immunoadjuvant activity to augment cellular and humoral immune responses against protein antigens (Yoon *et al.*, 2001). These findings let us suppose the possibility that KML-C is able to induce antitumor activity through either of apoptotic cell death of tumor cells or immunomodulating activity, or through both mechanisms. As expected, i.v. administration of KML-C 2 days before tumor inoculation significantly inhibited lung metastasis caused by two different murine tumor cells, B16-BL6 and colon 26-M3.1 cells in a dose-dependent manner (Table I). Moreover, the administration of KML-C 1 day after tumor inoculation resulted in a significant inhibition of experimental lung metastasis of both tumor cells (Table II). The therapeutic effect of KML-C was also found in liver and spleen metastasis produced by L5178Y-ML25 lymphoma cells (Table III), and in spontaneous lung metastasis of B16-BL6 cells (Table IV). Considering the prophylactic inhibition of tumor metastasis by KML-C in a lung metastasis model in which KML-C was administered 2 days before tumor inoculation (Table I), it seemed that the antitumor activity of KML-C administered prior to tumor inoculation was due to immunological pathway rather than apoptotic death of tumor cells.

Numerous experimental and clinical studies have shown that natural immunity plays an important role in both immuno-surveillance and blockade of metastasis from the primary tumors (Schantz *et al.*, 1987; Pollack *et al.*, 1982). In addition, the relevant effectors responsible for natural immunity against tumors have been identified as NK cells (Barlozzari *et al.*, 1985), LAK cells (Grimm *et al.*, 1982) and macrophages (Andreesen *et al.*, 1990). Since it is possible that the functional stimulation of these immune-

related cells results in suppression of tumors, the activation of NK cells and macrophages by biological response modifiers has been thought to be an useful tool to suppress tumor growth and tumor metastasis (Barlozzari *et al.*, 1985; Andreesen *et al.*, 1990). Actually, many investigators demonstrated that the activation of NK cells and macrophages by immuno-stimulants led to reduction of metastatic colonization of tumors (Herberman *et al.*, 1984; Hanna *et al.*,1985). Thus, we analyzed the mechanism of the prophylactic effect of KML-C on tumor metastasis in the point of NK cell and macrophage activation.

When mice were treated with KML-C, NK cell activity and macrophage-mediated cytotoxicity against tumor cells are apparently raised 2 days after treatment (Fig .1 and Fig. 2), indicating that NK cells and macrophages activated by KML-C are responsible for KML-C-induced prophylactic effect on tumor metastasis. However, although it seemed that the both immune-related cells are associated with KML-C-induced antitumor activity, the depletion of either of NK cells or macrophages elicited just a slight, not significant, reduction of lung metastasis (Table V). These results suggest that co-activation of the both NK cells and macrophages is required for full antitumor activity induced by KML-C; either of these immune-related cells alone is limited in its antitumor activity.

Here we demonstrated that KML-C inhibited tumor metastasis prophylactically as well as therapeutically, and its antitumor activity was partly associated with activation of NK cells and macrophages. The present study suggests that the KML-C is one of potent molecules responsible for antitumor activity of Korean mistletoe.

ACKNOELEDGEMENT

This research was supported by a grant (PF002201) from Plant Diversity Research Center of 21st Century Frontier Research Program funded by Ministry of Science and Technology of Korean government.

REFERENCES

Andreesen, R., Scheibenbogen, C., Brugger, W., Krause, S., Meerpohl, H. G., Leser, H. G., Engler, H., and Lohr, G. W., Adoptive transfer of tumor cytotoxic macrophages generated *in vitro* from curculating monocytes: a new approach to cancer immunotherapy. *Cancer Res.*, 50, 7450-7456 (1990).

Barlozzari, T. J., Leonhardt, R. H., Wiltrout, R. B., Herberman, R. B., and Reynolds, C. W., Direct evidence for the role of LGL in the reduction of experimental metastasis. *J. Immunol.*, 134, 2783-2789 (1985).

Bloksma, N., Dijk, H. V., Korst, P., and Willers, J. M., Cellular and humoral adjuvant activity of Mistletoe extract. *Immunobiol.*, 156, 309-319 (1979).

Franz, H., Mistletoe lectins and their A & B chains. *Oncology*, 43:suppl. 1, 23-28 (1986).

Gilles, R. G., Jung, M. L., Dominique, D. S., and Beck, J. P., Comparison of the effects of fermented and unfermented mistletoe preparations on cultured tumor cells. *Oncology*, 43:suppl 1, 35-41 (1986).

Grimm, E. A., Mazumder, A., Zhang, H. Z., and Rosenberg, S. A., Lymphokine-activated killer cell phenomenon: Lysis of natural killer resistant fresh solid tumor cells by interleikin-2 activated autologous human peripheral blood lymphocytes. *J. Exp. Med.*, 155, 1823-1841 (1982).

Hajto, T., Immunomodulatory effect of Iscador: a Viscum album preparation. *Oncology* 43:suppl 1, 51-61 (1986).

Hajto, T., Hostanska, K., Frei, K., Rordorf, C. and Gabius, H. J., Increased secretion of tumour necrosis factor-α, interleukin 1, and interleukin 6 by human mononuclearcells exposed to β-galactoside lectin from clinically applied mistletoe extract. *Cancer Res.*, 50, 3322-3326 (1990).

Hamprecht, K., Handgretinger, R., Voetsch, W., and Anderer, F. A., Mediation of human NK-activity by component in extracts of Viscum album. *Int. J. Immunopharmacol.*, 9, 199-209 (1987).

Hanna, N., The role of natural killer cells in control of tumor growth and metastasis. *Biochim. Biophys. Acta*, 780, 2132-2630 (1985).

Hayakawa, Y., Fujii, H., Hase, K., Ohnishi, Y., Sakukawa, R., Kadota, S., Namba, T., and Saiki, I., Anti-metastatic and immunomodulating properties of the water extract from Celosia argentea seeds. *Bio. Pharm. Bull.*, 21, 1154-1159 (1998).

Herberman, R. B., Possible role of natural killer cells and other effector cells in immune surveillance against cancer. *J. Invest. Dermatol.*, 83:suppl 1, 137-140 (1984).

Hulsen, H., Kron, R., and Mechelke, F., Influence of Viscum album preparations on the natural killer cell-mediated cytotoxicity of peripheral blood. *Naturwissenschaften*, 76, 530-531 (1989).

Kuttan, G., Vasudevan, D. M., and Kuttan, R., Effect of a preparation from Viscum album an on tumor development *in vitro* and in mice. *J. Ethnopharmacology*, 29, 35-41 (1990).

Kuttan, G. and Kuttan, R., Immunological mechanism of action of the tumor reducing peptide from mistle extract (NSC 635089) cellular proliferation. *Cancer Letters*, 66, 123-130 (1992a).

Kuttan, G. and Kuttan, R., Immunomodulatory activity of a peptide isolated from Viscum album extract (NSC 635089). *Immunological Investigations*, 21, 285-296 (1992b).

Khwaja, T. A., Varven, J. C., Pentescost, S., and Pande, H., Isolation of biologically active alkaloids from Korean mistletoe Viscum album, coloratum. *Experientia*, 36, 599-600 (1980).

Mannel, D. N., Becker, H., Gundt, A., Kist, A., and Franz, H., Induction of tumour necrosis factor expression by a lectin from Viscum album. *Cancer Immunol. Immunother.*, 33, 177-182 (1991).

Mertzer, G., Franz, H., Kindt, A., Fahlbusch, B., and Sus, J.,

The *in vitro* activity of lectin 1 from mistletoe (ML 1) and its isolated A and B chains in functions of macrophages and polymorphonuclear cells. *Immunobiol.*, 169, 461-471 (1985).

Mueller, E. A., Hamprecht, K., and Anderer, F. A., Biological characterization of a component in extract of Viscum album enhancing human NK cytotoxicity. *Immunopharmacology*, 17, 11-18 (1989).

Mueller, E. A. and Anderer, F. A., Chemical specificity of effector cell/tumor cell bridging by a Viscum album rhamnogalacturonan enhancing cytotoxicity of human NK cells. *Immunopharmacology*, 19, 69-77 (1990a).

Mueller, E. A. and Anderer, F. A., Viscum album oligosaccharide activating human natural cytotoxicity is an interferon g inducer. *Cancer Immunol. Immunother.*, 32, 221-227 (1990b).

Mueller, E. A. and Anderer, F. A., Synergistic action of a plant rhamno-galacturonan enhancing antitumor cytotoxicity of human natural killer and lymphokine-activated killer cells: chemical specificity of target cell recognition. *Cancer Res.*, 50, 3646-3651 (1990c).

Olsnes, S., Stirpe, F., Sandvig, K., and Pihl, A., Isolation and characterization of viscumin, a toxic lectin from *Viscum album* L. (mistletoe). *J. Biol. Chem.*, 257, 13263-13270 (1982).

Pollack, S. B. and Hallenbeck, L. A., *In vivo* induction of NK activity with anti-NK1 serum: direct evaluation of NK cells in tumor clearance. *Int. J. Cancer*, 29, 203-207 (1982).

Ribereau-Gayon, G., Jung, M. L., Di Scala, D., and Beck, J. P., Comparison of the effects of fermented and unfermented mistletoe preparations on cultured tumor cells. *Oncology*, 43: suppl.1, 35-41 (1986).

Schartz, S. P., Brown, B. W., Lira, E., Taylor, D. L., and Beddingfield, N., Evidence for the role of natural immunity in the control of metastatic spread of head and neck cancer. *Cancer Immnunol. Immunother.*, 25, 141-148 (1987).

Stiefel, T. and Voelter, W., Complete amino acid sequence of the A chain of mistletoe lectin I. *FEBS Lett.*, 399, 153-157 (1996).

Yoo, Y. C., Saiki, I., Sato, K., and Azuma, I., MDP-Lys(L18), a lipophilic derivative of muramyl dipeptide, inhibits the metastasis of hematogenous and non-hematogenous tumors in mice. *Vaccine*, 12, 175-180 (1994).

Yoo, Y. C., Watanabe, S., Watanabe, R., Hata, K., Shimazaki, K., and Azuma, I., Bovine lactoferrin and lactoferricin, a peptide derived from bovine lactoferrin, inhibit tumor metastasis in mice. *Jpn. J. Cancer Res.*, 88, 184-190 (1997).

Yoon, T. J., Yoo, Y. C., Hong, E. K., Cho, Y. H., Lee, S. W., Azuma, I., Yoo, B. I., and Kim, J. B., Effect of Korean mistletoe extract on the induction of IL-1 and TNF-a from mouse macrophages. *Kor. J. Pharmacognosy*, 25, 132-139 (1994).

Yoon, T. J., Yoo, Y. C., Choi, O. B., Do, M. S., Kang, T. B., Lee, S. W., Azuma, I., and Kim, J. B., Inhibitory effect of Korean mistletoe (*Viscum album coloratum*) extract on tumor angiogenesis and metastasis of haematogenous and non-haematogenous tumour cells in mice. *Cancer Lett.*, 97, 83-91 (1995).

Yoon, T. J., Yoo, Y. C., Kang, T. B., Baek, Y. J., Huh, C. S., Song, S. K., Lee, K. H., Azuma, I., and Kim, J. B., Prophylactic effect of Korean mistletoe (*Viscum album coloratum*) extract on tumor metastasis is mediated by enhancement of NK cell activity. *Int. J. Immunopharmacol.*, 20, 163-172 (1998).

Yoon, T. J., Yoo, Y. C., Kang, T. B., Shimazaki, K., Song, S. K., Lee, K. H., Kim, S. H., Park, C. H., Azuma, I. and Kim, J. B., Lectins isolated from Korean mistletoe (*Viscum album coloratum*) induce apoptosis in tumor cells. *Cancer Lett.*, 136, 33-40 (1999).

Yoon, T. J., Yoo, Y. C., Kang, T. B, Her, E., Kim, S. H., Kim, K., Azuma, I., and Kim, J. B., Cellular and humoral adjuvant activity of lectins isolated from Korean mistletoe (*Viscum album coloratum*). *Int. Immunopharmacol.*, 1, 881-889 (2001).