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Anti-metastatic activity of *Acanthopanax senticosus* extract and its possible immunological mechanism of action

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Abstract

Antitumor and immunomodulatory activities of an aqueous extract (GF100) of *Acanthopanax senticosus* were examined. In experimental lung metastasis of colon26-M3.1 carcinoma cells, intravenous (i.v.) administration of GF100 2 days before tumor inoculation significantly inhibited lung metastasis in a dose-dependant manner. The i.v. administration of GF100 also exhibited the therapeutic effect on tumor metastasis of colon26-M3.1 cells, when it was injected 1 day after tumor inoculation. In an in vitro cytotoxicity analysis, GF100 at the concentration up to 1000 μg/ml did not affect the growth of colon26-M3.1 cells. In contrast, GF100 enhanced the responsiveness to a mitogen, concanavalin A (ConA), of splenocytes in a dose-dependent manner. Peritoneal macrophage stimulated with GF100 produced various cytokines such as IL-1β, TNF-α, IL-12 and IFN-γ in an in vitro experiment. The macrophages obtained from the mice which were injected with GF100 (500 μg) 3 days before the assay showed significantly higher tumoricidal activity against tumor cells than that of the untreated macrophages. In addition, the i.v. administration of GF100 significantly augmented NK cytotoxicity to Yac-1 cells. The depletion of NK cells by injection of rabbit anti-asialo GM1 serum completely abolished the inhibitory effect of GF100 on lung metastasis of colon26-M3.1 cells. These data suggest that GF100 has antitumor activity to inhibit tumor metastasis prophylactically as well as therapeutically, and its antitumor effect is associated with activation of macrophages and NK cells.

Keywords: *Acanthopanax senticosus*; Tumor metastasis; Immunomodulation; Macrophage; NK cell

1. Introduction

Acanthopanax senticosus, also called the "Siberian Ginseng" or "*Eleutherococcus senticosus*", has been well known to be prophylactic for various diseases such as chronic bronchitis, hypertension and ischemia (Yi et al., 2001). *Acanthopanax senticosus* is also known to be effective for reducing many kinds of stress (Gaffney et al., 2001) or fatigue (Dowling et al., 1996). Today this oriental herb is called 'adatogen' in the US (Davydov and Krikorian, 2000). Recently, Davydov and Krikorian (2000) reported that the herb included various compounds such as acanthosides, eleutherosides, senticoside, triterpenic saponin, flavon, vitamins and minerals, and they are related to its diverse biological activities. Interestingly, many investigators demonstrated two contrary views on the immunomodulatory effect of *Acanthopanax senticosus*: the stimulation (Schmeda-Hirschmann et al., 2001; Schmolz et al., 2001) and the suppression (Jeong et al., 2001; Yi et al., 2001; Umeyama et al., 1992) of immune responses. In fact, the stem of the herb has been clinically used for treatment of allergy in Korea. However, the precise mechanisms related to its immunomodulatory activities are still unclear.

It is well known that most death caused by cancer are not due to primary tumor but the dissemination of tumor cells to secondary sites by a series events known to collectively as the metastatic cascade (Fidler, 1991; Liotta et al., 1991). Many experimental studies and clinical trials showed that natural immunity played an important role in blocking of metastasis from primary tumors (Schantz

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et al., 1987). Among various immune-related cells, NK cells and macrophages were thought to be the relevant effectors responsible for the natural immunity against tumors (Barlozzari et al., 1985; Andreesen et al., 1990). Thus, it is possible that the functional activation of NK cells and macrophages elicit the suppression of tumor growth as well as the inhibition of tumor metastasis. Indeed, it was reported that activation of NK cells and macrophages by plant extracts could control the growth of tumor cells and their metastasis (Yoon et al., 1998; Saiki, 2000).

In this study, we investigated the anti-tumor activity of the aqueous extract (GF100) of *Acanthopanax senticosus* in respect to the prophylactic and therapeutic inhibition of lung metastasis of colon26-M3.1 carcinoma cells using experimental metastasis model in syngeneic mice, and analyzed the involvement of activation of NK cells and macrophages in its antimetastatic effect against tumors.

2. Materials and methods

2.1. Preparation of Acanthopanax senticosus extract

The barks of *Acanthopanax senticosus* originated from China were purchased from a herbal medicine company (Daehyo Pharm., Korea). The barks were mixed with 20 volumes of distilled water, homogenized for 1 min and then stirred at 4 °C overnight. After centrifuge at 9000 rpm for 20 min, the supernatant was filtered with 0.2 μm pore sized filters and lyophilized. Finally, 5.5 g of the dried extract was obtained from 100 g of the barks of *Acanthopanax senticosus*. An appropriate amount of obtained brown powder (GF100) was dissolved with PBS and stored at 4 °C until use. Protein content of GF100 was determined by using of commercial Bio-Rad protein assay kit and the carbohydrate content was measured as described previously (Dubois et al., 1956). The portion of polysaccharide content of GF100 was 35%.

2.2. Mice and cell cultures

Specific pathogen-free female Balb/c (6–7-weeks-old), were purchased from Dae-Han Biolink Ltd., Korea. The mice were maintained in a clean rack in the laboratory of Department of Food and Biotechnology at Kyonggi University. Water and diet of pellets were supplied ad libitum. A lung metastatic subline of a highly metastatic cell line of colon 26 carcinoma, colon 26-M3.1, was maintained as mono-layer cultures in Eagle's MEM supplemented with 7.5% FBS, vitamin solution, sodium pyruvate, non-essential amino acids and L-glutamine as described previously (Yoo et al., 1994). Yac-1 cells, a NK-sensitive mouse lymphoma cell line, sarcoma-180 cells and murine lymphocytes were maintained in RPMI-1640 supplemented with 7.5% FBS and L-glutamine.

2.3. Cytotoxicity and mitogenic response assay

For mitogenic response assay, splenic lymphocytes (5×10^5 per well) from Balb/c mice were co-cultured with or without the indicated doses of GF100 in the presence of ConA (1 μg/ml) in 96-well culture plates for 72 h. In cytotoxicity test, various doses of GF100 in culture medium were added into each well of the culture plate of colon26-M3.1 cells (5×10^5 per well), and incubated for 48 h. Cytotoxicity to tumor cells and lymphocyte proliferation were assayed by a MTT-based colorimetric assay. The cultures were incubated with 0.5 μg/ml of MTT solution for the last 6-h incubation. Then, the supernatants of the cultures were mixed with 100 μl of dimethyl sulfoxide (DMSO). Absorbance of each well was monitored at 570 nm using a microtitration plate reader (Molecular Device Co., USA).

2.4. Experimental lung metastasis

Experimental lung metastasis was assessed by i.v. inoculation of colon26-M3.1 carcinoma cells (3×10^4 per mouse) in syngeneic Balb/c mice (Yoo et al., 1994). Treatment with various doses of GF100 was carried out 2 days before or 1 day after i.v. inoculation of colon26-M3.1 cells. The mice were sacrificed 14 days after tumor inoculation and their lungs were fixed in a Bouin's solution. Lung tumor colonies were counted under a dissecting microscope.

2.5. Cytokine assay

Peritoneal macrophages were harvested from thioglycollate-treated mice as described previously (Saiki et al., 1988). The cells (1×10^6/1 ml/well) suspended in complete RPMI medium were plated into 24-well culture plates. After 2-h incubation, non-adherent cells were removed by wash with PBS, the adherent macrophages were co-incubated with the indicated doses of GF100 for 24 h. The concentration of various cytokines (IL-1β, TNF-α, IL-12 and IFN-γ) in the culture supernatants was determined by ELISA kits (Pharmingen Co., USA) according to the manufacturer's protocol.

2.6. Assay of macrophage-mediated cytotoxicity against tumor cells

Macrophage-mediated cytotoxicity was assayed as described previously (Saiki et al., 1988) with some modifications. In brief, sarcoma 180 cells (1×10^4) were added to the macrophage monolayers harvested from the mice given intraperitoneal (i.p.) administration of GF100 (500 μg) 3 days before the assay in 96-well plates to obtain various macrophage-to-target cell ratios. The cultures were incubated at 37 °C for 18 h. After centrifugation, the culture supernatants were admixed with LDH solution (Promega Co., USA), and the absorbance value of each well was measured

at 620 nm. The percentage of macrophage cytotoxicity was calculated from the following formula:

$$\text{Percentage inhibition} = \left[1 - \frac{\text{OD value of experimental group} - \text{OD value of spontaneous group}}{\text{OD value of untreated group} - \text{OD value of spontaneous group}}\right] \times 100$$

2.7. Assay of NK-mediated tumor cytotoxicity

NK-mediated cytotoxicity was determined by the radioactive ^{51}Cr-release assay as described previously (Yoon et al., 1998). Balb/c mice were administered i.v. with GF100 (500 μg), and their splenocytes were harvested 3 days after the GF100 treatment. The splenocytes (100 μl/well) were added to ^{51}Cr-labeled Yac-1 cells (1×10^4/100 μl/well) to obtain effector-to-target cell ratios (E/T ratio) of 100:1, 50:1, 25:1 or 12.5:1 in U-bottomed 96 well plates, and the cultures were incubated for 6 h. After the incubation, the culture supernatants (100 μl/well) were absorbed onto a cotton swab and monitored for radioactivity using a gamma counter. The percentage of cytotoxicity generated by NK cells was calculated from the radioactivity (counts/min) according to the following formula:

$$\text{Cytotoxicity (\%)} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

2.8. Depletion of NK cells in vivo

Depletion of NK cells in vivo was performed according to the method described previously (Yoon et al., 1998). Mice were injected i.p. with 500 μl/mouse of 50-fold diluted rabbit anti-asialo GM1 serum (Wako Pure Chemicals Industries, Ltd., Japan) 1 and 3 days before tumor inoculation.

2.9. Statistical analysis

The statistically significant difference between the groups was calculated by applying the Student's two-tailed *t*-test.

3. Results

3.1. Inhibitory effect of GF100 on experimental lung metastasis

In order to investigate if GF100 has antitumor activity to inhibit tumor metastasis, we examined the prophylactic effect of GF100 on the experimental lung metastasis produced by colon26-M3.1 cells. Table 1 shows that i.v. administration of GF-100 (100–500 μg/mouse) 2 days before tumor inoculation significantly inhibited lung metastasis of colon26-M3.1 cells in a dose-dependent manner. However, the smaller amount of GF100 (20 μg/mouse) had no effect. Similarly, treatment with 100–500 μg/mouse of GF100 1 day after tumor inoculation significantly reduced lung metastasis of colon26-M3.1 cells, whereas 20 μg/mouse of GF100 did not (Table 2). This suggests that GF100 is able to induce prophylactic as well as therapeutic effect against lung metastasis produced by colon26-M3.1 tumor cells.

Table 1
Prophylactic effect of GF100 on lung metastasis produced by i.v. inoculation of colon26-M3.1 carcinoma cells

Treatment	Dose (μg/head)	Number of lung metastasis	
		Mean ± SD (% inhibition)	Range
Untreated	–	131 ± 14	117–148
GF100	500	9 ± 15 (93.1)[b]	3–20
	100	55 ± 22 (58.0)[a]	26–85
	20	100 ± 27	69–129

Groups of five Balb/c mice were administered i.v. with the indicated doses of GF100 two days before i.v. inoculation of 2.5×10^4 colon26-M3.1 cells. Mice were sacrificed 14 days after tumor inoculation for evaluation.
[a] $p < 0.01$.
[b] $p < 0.001$, compared with untreated control (by Student's two-tailed *t*-test).

3.2. Cytotoxic effect of GF100 on tumor cells and spleen cells

Cytotoxic effect of GM100 on tumor cells and murine splenocytes was examined in an in vitro experiment. As shown in Fig. 1A, GF100 at the concentration up to 1000 μg/ml did not affect the growth of colon26-M3.1 tumor cells. In contrast, treatment of ConA-stimulated splenocytes with GF100 enhanced the proliferation of the cells in a dose-dependent manner (Fig. 1B), showing the maximal activity at the concentration of 500 μg/ml. These results indicate that the inhibitory activity of GF100 on tumor metastasis is not due to its cytotoxic effect on tumor cells, and GF100 may up-regulate the function of immune-related cells.

Table 2
Therapeutic effect of GF100 on lung metastasis produced by i.v. inoculation of colon26-M3.1 carcinoma cells

Treatment	Dose (μg/head)	Number of lung metastasis	
		Mean ± SD (% inhibition)	Range
Untreated	–	80 ± 12	64–93
GF100	500	51 ± 6 (36.8)[a]	48–59
	100	58 ± 5 (27.9)[a]	50–62
	20	69 ± 14	60–85

Groups of five Balb/c mice were inoculated i.v. with 2.5×10^4 colon26-M3.1 cells, and administered i.v. with the indicated doses of GF100 one day after tumor inoculation. Mice were sacrificed 14 days after tumor inoculation for evaluation.
[a] $p < 0.01$, compared with untreated control (by Student's two-tailed *t*-test).

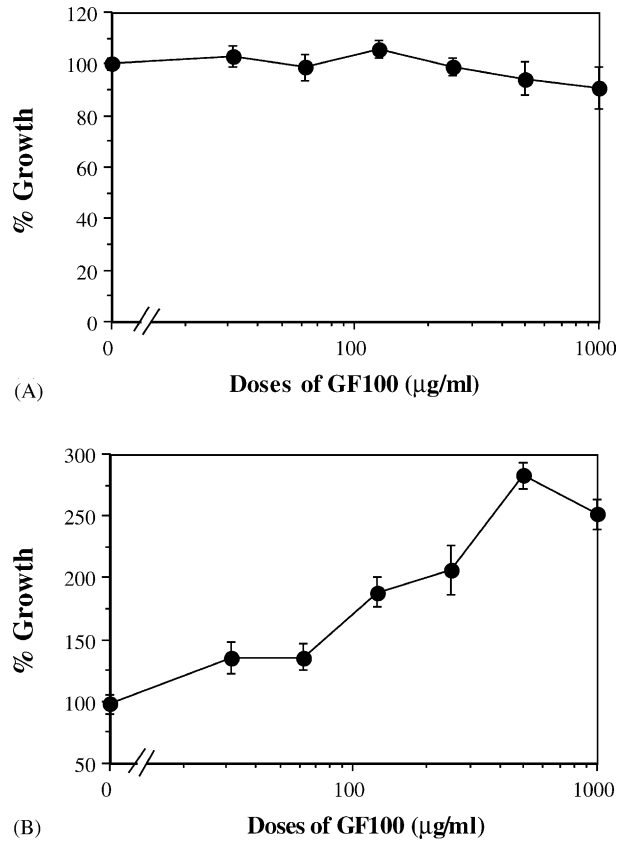

Fig. 1. Effect of GF100 on the growth of tumor cells and mitogen-stimulated splenocytes: colon26-M3.1 cells (A) and the splenocytes (5×10^5 per well) (B) were co-incubated with the indicated doses of GF110 for 72 or 48 h, respectively. The splenocytes were incubated in the presence of ConA (1 μg/ml). The proliferation of these cells was measured by a MTT-based colorimetric assay.

3.3. Effect of GF100 on activation of macrophages

It is well known that activated macrophages release various cytokines (Saiki et al., 1988), and these cytokines play a role in modulating immune responses. Macrophages also act as a killer against tumor cells when these cells are potentially activated by some stimuli (Andreesen et al., 1990). The ability of GF100 to activate macrophages was investigated in aspect of the induction of cytokine production and tumoricidal activity.

As described in Table 3, treatment of peritoneal macrophages with GF100 in an in vitro experiment induced various cytokines such as IL-1β, TNF-α, IL-12 and IFN-γ in a dose-dependent manner. In addition, peritoneal macrophages obtained from GF100-treated mice displayed a higher cytolytic activity against tumor cells than those of the untreated mice (Fig. 2). This suggests that GF100 can activate macrophages, and its ability to induce cytokines from macrophages and enhance macrophage-mediated cytotoxicity against tumor cells may be related to its antitumor activity.

Table 3
Effect of GF100 on induction of cytokines from macrophages

GF100 treatment (μg/ml)	The level of cytokines (pg/ml)			
	TNF-α	IL-12	IL-1β	IFN-γ
0	16 ± 5	10 ± 2	3 ± 1	11 ± 5
0.16	27 ± 5	22 ± 7	7 ± 1	21 ± 2
0.8	58 ± 6	132 ± 3	25 ± 2	88 ± 5
4	160 ± 25	289 ± 25	55 ± 9	202 ± 9
20	220 ± 32	314 ± 16	62 ± 9	157 ± 22
100	540 ± 44	197 ± 22	66 ± 4	71 ± 10

Peritoneal macrophages (1×10^6/1 ml/well) were treated with the indicated doses of GF100 in 24-well plate for 24 hr. The level of each cytokine in the supernatants of the cultures was determined by ELISA kits. The level of cytokines in the group treated with LPS (5 μg/ml) was as follows. TNF-α; 3470 ± 258, IL-12; 607 ± 42, IL-1β; 127 ± 9, IFN-γ; 1012 ± 59.

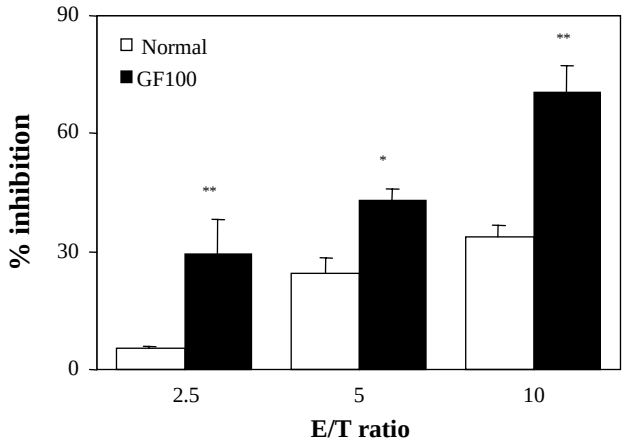

Fig. 2. Effect of GF100 on macrophage-mediated cytotoxicity against tumor cells. Peritoneal macrophages were co-incubated with sarcoma 180 cells for 18 h. After incubation, LDH solution was added to the cultures, and the absorbance value of each well was measured at 620 nm. E/T ratio means the ratio of effector cells (splenocytes) to target cells (Yac-1 cells). $^*P < 0.01$, $^{**}P < 0.001$, compared with the untreated group (by Student's two-tailed *t*-test).

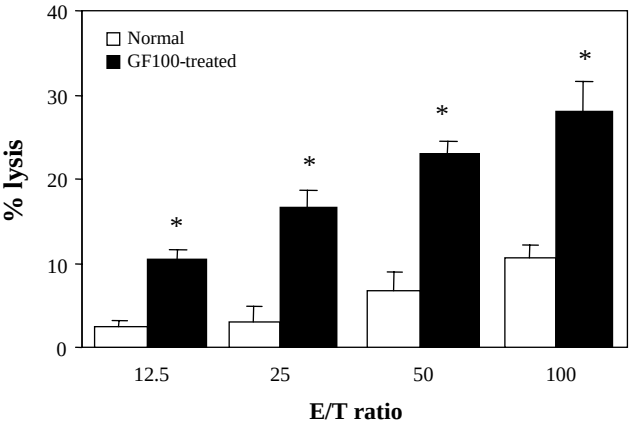

Fig. 3. Effect of GF100 on the enhancement of NK cell activity. NK activity was determined by ^{51}Cr-release assay as described in Materials and Methods. $^*P < 0.001$, compared with the untreated group (by Student's two-tailed *t*-test).

3.4. Effect of GF100 on NK cell activity

The effect of GF100 on NK cell activity was estimated by the cytotoxic activity against Yac-1 cells by ^{51}Cr-release assay. As seen in Fig. 3, the splenocytes obtained from mice administered i.v. with GF100 2 days before the assay showed a higher cytotoxicity than those of the untreated mice in a E/T ratio-dependent manner. Since NK cells are known to be an important effector to suppress tumor growth and metastasis, we addressed if the activity of GF100 to enhance NK activity has relation to its antitumor activity. The deletion of NK cells by treatment with anti-asalo-GM1 antibody completely abolished the antitumor effect of GF100 on lung metastasis of colon26-M3.1 cells, indicating that the inhibitory effect of GF100 on tumor metastasis was mediated by NK cell activation.

4. Discussion and conclusions

Biological response modifier (BRM) has been thought to be a useful tool to suppress tumor growth and inhibit tumor metastasis (Suto et al., 1994; Yoo et al., 1994). In fact, various BRMs such as natural products involving oriental medicines, cytokines, synthetic adjuvants having biological activity to enhance host defense system have been applied to cancer immunotherapy (Suto et al., 1994; Schmeda-Hirschmann et al., 2001; Kang et al., 2002; Saiki, 2000). In many cases, BRMs activate immune-related cells such as NK cells, lymphokine-activated killer (LAK) cells and macrophages to control the growth of the cancer and its metastasis (Yoon et al., 1998; Sakamaki et al., 1992; Habu et al., 1981). Thus, the traditionally used natural resources to stimulate immune system may be important candidates for cancer therapy (Saiki, 2000; Ohnishi et al., 1998).

Acanthopanax senticosus is a well-known typical oriental herb consisting of various constituents having diverse pharmacological effects (Deyama et al., 2001). Recently, Hibasami et al. (2000) reported that the extract of the stem bark of *Acanthopanax senticosus* HARMS originated from Japan induced apoptosis in human stomach cancer cells, and one of its components, sesamin, was responsible for its apoptosis-inducing activity. Although a variety of biological activities of *Acanthopanax senticosus* have been reported, it is yet unclear that this herb can induce antitumor activity enough to inhibit the metastasis of tumor cells.

This study demonstrated that the water extract (GF100) from *Acanthopanax senticosus* inhibited the experimental lung metastasis produced by colon26-M3.1 carcinoma cells, and its antitumor activity was associated with activation of macrophages and NK cells. As shown in Tables 1 and 2, treatment with GF100 resulted in the prophylactic as well as therapeutic effect on lung metastasis produced by colon26-M3.1 carcinoma cells. Since *Acanthopanax senticosus* HARMS extract induced apoptosis in tumor cells (Deyama et al., 2001), we examined the cytotoxic effect of GF100 on colon26-M3.1 cells used in the experimental lung metastasis model. Unexpectedly, GF100 at the concentration up to 1000 μg/ml did not induce cell death in colon26-M3.1 cells (Fig. 1A). This suggests that GF100 is not cytotoxic, and its antitumor activity does not result from apoptotic death of tumor cells. The difference in cytotoxic activity between GF100 and *Acanthopanax senticosus* HARMS is not clear. It is possible that the cytotoxic activity of *Acanthopanax senticosus* varies with its growing district and the kind of tumor cells used in experiments.

Activated macrophages and NK cells are relevant effectors responsible for natural immunity against tumors (Barlozzari et al., 1985). In experiments for immuno-modulatory activity test, GF100 was shown to enhance the proliferation of mitogen-stimulated splenocytes (Fig. 1B) and activate macrophages (Table 3 and Fig. 2). It is of particular significance that GF100 induced the secretion of various cytokines such as IL-1β, TNF-α, IL-12 and IFN-γ from macrophages.

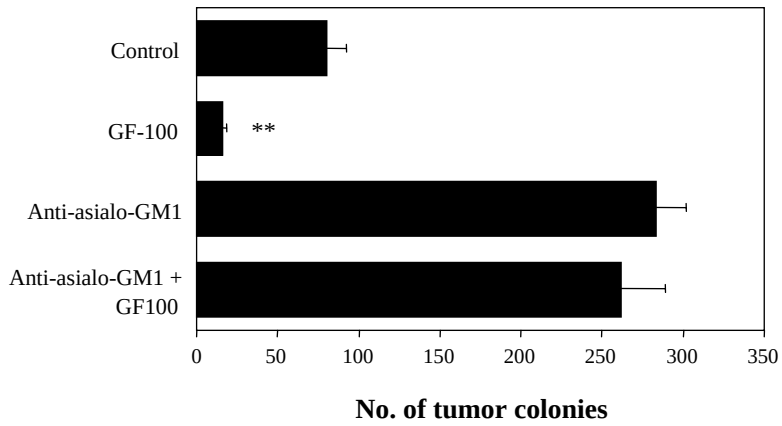

Fig. 4. Effect of NK cell depletion on GF100-induced inhibition of lung metastasis. To deplete NK cells in vivo, rabbit anti-asialo GM1 serum was injected into mice 1 and 3 days before inoculation of colon26-M3.1 cells. Mice were administered i.v. with GF100 (500 μg) 2 day before tumor inoculation. Mice were sacrificed 14 days after tumor inoculation for evaluation. $^{*}P < 0.001$, compared with the untreated group (by Student's two tailed *t*-test).

Inflammatory cytokines like IL-1β and TNF-α secreted from macrophages play a role in activating T cells and rejecting tumor cells (Tanigawa et al., 2000; Baxevanis et al., 2000). IL-12, called multi-functional cytokine, is said to be one of the most essential cytokines to elicit tumor immunity (Ogawa et al., 1998; Lasek et al., 1997). It was also reported that IL-12 and IFN-γ inhibit tumor growth and metastasis by activating NK cells as well as cytolytic T lymphocytes (CTL) (Lasek et al., 1997; Markovic and Murasko, 1991). In fact, treatment with GF100 enhanced NK cell activity (Fig. 3), and its antitumor activity was abolished by depletion of NK cells in vivo (Fig. 4). Thus, the ability of GF100 to induce various cytokines from macrophages may be one of the major mechanisms related to enhancement of natural immunity of the host and inhibition of tumor metastasis by this herb.

The present study demonstrated that the aqueous extract of *Acanthopanax senticosus* significantly inhibited lung metastasis produced by colon26-M3.1 tumor cells prophylactically as well as therapeutically, and that the antitumor effect of this herb is partly due to activation of NK cells and macrophages. Further study to elucidate active molecules and biological mechanisms related to antitumor activity of GF100 is underway in our laboratory.

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