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Anti-Metastatic Activity of Glycoprotein Fractionated from *Acanthopanax senticosus*, Involvement of NK-cell and Macrophage Activation

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Previously, we reported that water-extracted *Acanthopanax senticosus* exhibited anti-metastatic activity by stimulating the immune system. In this study, we fractionated glycoproteins (EN-SP) from the soluble protein layer (GF-AS) of *A. senticosus* and determined their basic chemical properties. We also investigated the anti-tumor and immunostimulating activities of the fractionated glycoprotein, EN-SP. We found that intravenous (i.v.) administration of GF-AS dramatically inhibited metastasis of colon26-M3.1 carcinoma cells to the lung in a dose-dependent manner. *In vitro* analysis showed GF-AS to enhance the proliferation of splenocytes. GF-AS also stimulated peritoneal macrophage, which was followed by the production of various cytokines such as IL-1β, TNF-α, IL-12 and IFN-γ. Furthermore, the production of these cytokines was partially blocked when peritoneal macrophage was cultured with the polyclonal antibodies against GF-AS. The depletion of NK cells by rabbit anti-asialo GM1 serum partly abolished the inhibitory effect of GF-AS on lung metastasis of colon26-M3.1 cells. Using gel filtration, EN-SP, an active glycoprotein fraction, is isolated from GF-AS. While both GF-AS and EN-SP stimulated the proliferatation of splenocytes of normal mice, EN-SP showed higher anti-metastatic activity and more potently stimulated the proliferation of splenocytes compared to GF-AS. These results suggest the use of EN-SP, the fractionated glycoprotein from *A. senticosus*, can be used as a therapeutical reagent to prevent or inhibit tumor metastasis.

Key words: *Acanthopanax senticosus*, Tumor metastasis, Cytokines, Macrophage, NK cell

INTRODUCTION

Acanthopanax senticosus (*A. senticosus*), called the 'Siberian Ginseng', is known to be prophylactic to various diseases such as chronic bronchitis, hypertension, and ischemia (Yi *et al.*, 2002). *A. senticosus* is also known to be effective in reducing many kinds of stress (Gaffney *et al.*, 2001) or fatigue (Dowling *et al.*, 1996) hence it is called an 'adatogen'. Various compounds such as acanthosides, eleutherosides, senticoside, triterpenic saponin, flavon, vitamins and minerals are related to diverse biological activities (Lee *et al.*, 2003; Davydov and Krikorian, 2000). There are two contradictory views on the immunomodulatory effect of *A. 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, our data also showed crude extracts from the herbs stem to have different activities based on the extraction methods (Yoon *et al.*, 2002, 2003). These results prompted us to purify the constituents from *A. senticosus* in order to modulate immune activities.

Most deaths caused by cancer are not due to primary tumor, but the dissemination of tumor cells to secondary sites, which is called "metastasis" (Fidler, 1991; Liotta *et al.*, 1991). Many experimental studies and clinical trials showed that natural immunity plays important roles in

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blocking the metastasis of primary tumors (Schantz *et al.*, 1987). Among various immune-related cells, NK cells, and macrophages are believed to be relevant effectors for the natural immunity against tumors (Barlozzari *et al.*, 1985; Andreesen *et al.*, 1990). These results suggest that the functional activation of NK cells and macrophages suppress tumor growth as well as metastasis. We hypothesized that various plant extracts, including the extract of *A. senticosus*, regulate the growth of tumor cells and their metastasis by activating NK cells and macrophages (Yoon *et al.*, 1998; Saiki, 2000).

In this study, we fractionated the crude proteins (GF-AS) from aqueous extracts of *A. senticosus* and identified EN-SP glycoproteins. To characterize its anti-tumor activity, EN-SP was applied to an experimental tumor metastasis model system. Colon26-M3.1 carcinoma or B16-BL6 melanoma cells were transplanted to syngeneic mice, and the anti-metastatic effect of the fractionated EN-SP against the tumors and its role in the activation of NK cells and macrophages were analyzed.

Our results strongly suggest EN-SP to have immunostimulatory activity both under *in vivo* and *in vitro* conditions.

MATERIALS AND METHODS

Preparation of glycoprotein fractions from *Acanthopanax senticosus* extract

The barks of *A. senticosus* from China were purchased from an herbal medicine company (Daehyo Pharm., Korea). The chopped stem bark of *A. senticosus* (100 g) were placed in distilled water (1000 mL) and stirred overnight at 4°C. After centrifugation at 7000 rpm for 30 min, the supernatant was adjusted with 70% saturated ammonium sulfate and slightly stirred overnight at 4°C. After centrifugation, the precipitate was collected and then dissolved minimally in PBS. The extraction (GF-AS) was filtered in stages using filters with 7.2, 0.45, and 0.2 μm pores (ADVENTIC MFS, Inc. USA) and kept at 4°C. To purify the extract for further identification of active molecules, GF-AS was applied to the sephadex-G50 gel filtration (2.5×90 cm, FPLC system of Pharmacia Biotech. Sweden) and the column was eluted at a speed of 1 mL/min with PBS. Each fraction was measured for the content of proteins (Bio-Rad, USA) and the mitogenic effect on splenocytes from normal mice was assayed.

Mice and cell cultures

Specific pathogen-free female BALB/c and C57BL/6, 6 weeks old, were purchased from Dae Han Biolink Ltd. (Korea). The mice were maintained in a clean rack in the laboratory of the Department of Food and Biotechnology at Kyonggi University. Water and a 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, and B16-BL6 melanoma were 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. Splenocytes from the mice were maintained in RPMI-1640 supplemented with 7.5% FBS and L-glutamine.

Electrophoresis and western blotting

Electrophoresis in the presence of sodium dodecyl sulfate was carried out in 10% polyacrylamide gel, as previously described (Laemmli. 1970). It was performed at a constant voltage of 100 V until the marker dye reached the bottom of the gel. After electrophoresis, the antigens were transferred to PVDF membranes as previously described (Towbin *et al.*, 1979). Anti-serum was probed at a dilution of 1:1000. The peroxidase-conjugated mouse IgG (HRP-conjugates, Pierce, USA) were diluted 1:5000. The blots were incubated for 1 h at 20°C. HRP-conjugates were visualized by enhanced chemiluminescence (ECL) using the manufacture's protocol (Amersham, Buckinghamshir, UK). To obtain anti-GF-AS antibody for blotting, BALB/c mice were immunized subcutaneously (s.c.) with Freund's complete adjuvant (FCA) and 5 μg/mL of GF-AS. The first injection was followed by booster injections at two week intervals. Ten days after secondary immunization, the mice were sacrificed and serum was collected.

Chemical analysis

The protein concentration was determined using the Bio-Rad assay kit with bovine serum albumin (BSA) as a standard, according to the manufacture's protocol. The total neutral sugar content of the EN-SP was measured colorimetrically by the phenol-sulfuric acid method (Dubois *et al.*, 1956) using glucose as a standard.

Mitogenic response assay

For mitogenic response assay, splenic lymphocytes (5×10^5/well) from BALB/c mice were co-cultured with or without the indicated doses of EN-SP in 96-well culture plates for 72 h. Lymphocyte proliferation was assayed by MTT-based colorimetric assay. The cultures were incubated with 0.5 mg/mL MTT solution for the last 6 h of incubation. Then, the supernatants of the cultures were mixed with 100 mL dimethyl sulfoxide (DMSO). Absorbance of each well was monitored at 570 nm using a microtiter plate reader (Molecular Device Co. USA).

Experimental lung metastasis

Experimental lung metastasis of Colon26-M3.1 and B16-BL6 cells were assessed by intravenous (i.v.) inoculation of tumor cells into BALB/c and C57BL/6 mice, respectively

(Yoo *et al.*, 1994). In the lung metastasis experiment, mice were given i.v. administrations of GF-AS (0.5~50 mg/20 g) or EN-SP (0.2~5 mg/20 g) 3 days after i.v. injections of 2.7×10^4 colon26-M3.1/mouse and 5×10^4 B16-BL6 cells/mouse. The mice were sacrificed 14 days after tumor inoculation, and their lungs were fixed in Bouin's solution. Lung tumor colonies were counted microscopically.

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-1640 medium were plated into 24-well culture plates. After 2 h of incubation, non-adherent cells were removed by washing with PBS, and the adherent macrophages were co-incubated with the indicated doses of GF-AS or EN-SP for 24 h. The concentrations of various cytokines (IL-1β, TNF-α, IL-12, and IFN-γ) in the culture supernatants were determined by ELISA kits (Pharmingen Co. USA) according to the manufacturer's recommendations.

Depletion of NK cells in mouse

Depletion of NK cells *in vivo* was performed according to the method described previously (Kasai *et al.*, 1970). Mice were injected intraperitoneally (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.

Statistical analysis

Statistically significant differences between the groups were calculated by applying the Student's two-tailed *t*-test.

RESULTS

Inhibitory effect of GF-AS on experimental lung metastasis

In order to investigate whether GF-AS inhibited tumor metastasis, we examined the prophylactic effect of GF-AS on the experimental lung metastasis, which was induced by colon26-M3.1 cells. Table I shows that i.v. administration of GF-AS (50 μg/20 g) and crude extract (500 μg/20 g) 2 days before tumor inoculation similarly inhibited lung metastasis of colon26-M3.1 cells. The anti-metastatic activity of GF-AS (50~0.5 μg/20 g) was shown in a dose-dependent manner.

This suggests that *A. senticosus* is able to induce a prophylactic effect against lung metastasis induced by colon26-M3.1 tumor cells, and GF-AS, the protein part of *A. senticosus*, is one of the components related with this anti-tumor activity.

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

Treatment	Doses (μg/head)	Number of lung metastasis	
		Mean ± SD (Inhibition %)	Range
Untreated	–	163.6 ± 33.4	137 - 221
Crude Ext.	500	20.1 ± 16.7 (87.7)[a]	6 - 42
GF-AS	50	12.4 ± 4.9 (92.4)[a]	5 - 17
	5	69.0 ± 7.0 (57.8)[a]	64 - 74
	0.5	140.3 ± 48.6	94 - 191

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

Cytokine production from macrophages

Activated macrophages release various cytokines (Saiki *et al.*, 1988), and these cytokines play a role in modulating immune responses. The ability of GF-AS to activate macrophages was investigated by observing cytokine production. As presented in Fig. 1, treatment of peritoneal macrophages with GF-AS in an *in vitro* experiment induced various cytokines such as IL-1β, TNF-α, IL-12 and IFN-γ. This suggests that GF-AS can activate macrophages, and its ability to induce cytokines from macrophages may enhance macrophage-mediated cytotoxicity against tumor cells (Kasai *et al.*, 1981; Yoon *et al.* 1998). In addition, anti-GF-AS Ab partially inhibited such cytokine production, suggesting that other proteins or another constituents from *A. senticosus* may also take part in activating macrophages.

Effect of GF-AS on NK cell activity

Since NK cells are known to be important in suppressing tumor growth and metastasis (Kasai *et al.*, 1981), we addressed whether the increase of NK cell activity by GF-AS corresponded to its anti-tumor activity. Our previous data showed that the administration of crude extracts from *A. senticosus* increased the activity of cytotoxic NK cell against Yac-1 cells (Yoon *et al.*, 2003). To estimate whether the anti-metastatic activity of GF-AS was related to NK cell activation, we examined anti-aqsialo GM1 Ab treated NK cell deleted mouse. As seen in Fig. 2, the depletion of NK cells by anti-asalo-GM1 antibody partially abolished the anti-tumor effect of GF-AS on lung metastasis of colon26-M3.1 cells. This indicates that the inhibitory effect of GF-AS on tumor metastasis was partly *via* NK cell activation.

Immunostimulating sub-fractions of GF-AS

In order to determine the molecular size of GF-AS, the crude proteins from *A. senticosus* were applied to Sephadex G50. The elution patterns suggested GF-AS to

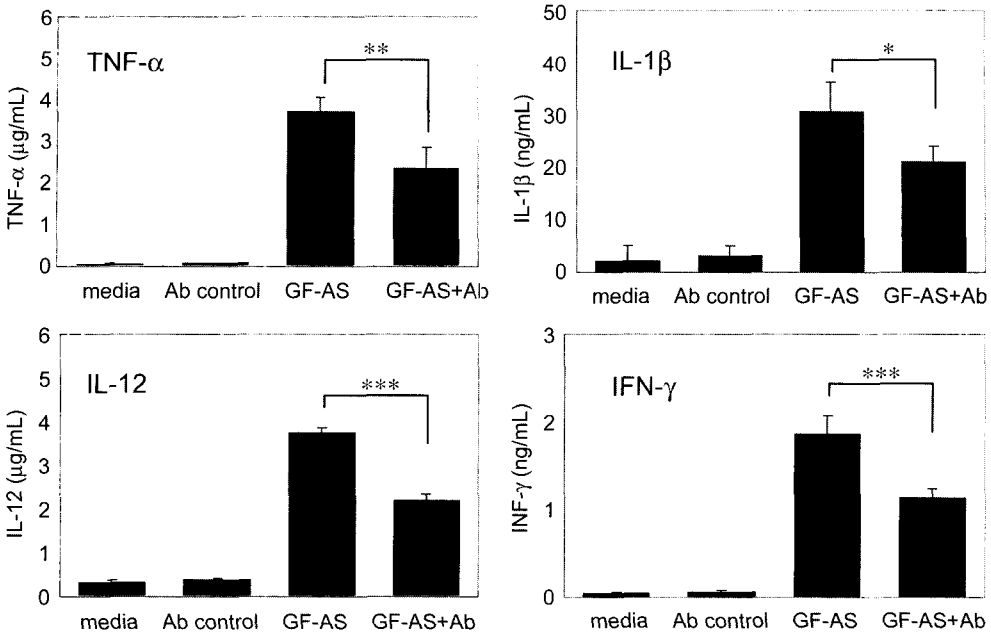

Fig. 1. Effects of GF-AS on the production of cytokines by peritoneal exudate macrophages (PEMs). Peritoneal macrophages were harvested from 3% thioglycollate-treated mice. The PEMs suspended in culture medium were plated into 24-well culture plates and the non-adherent cells were removed after 2 h incubation. The PEMs were co-incubated with the 1 mg/mL of GF-AS for 24 h. The anti-GF-AS antibody was treated with GF-AS at 37°C for 30 min before adding to PEMs. The concentrations of each cytokines in the cultured supernatants were determined by ELISA method. *p<0.05, **p<0.01, ***p<0.001 as compared to each control by Student's two-tailed t-test.

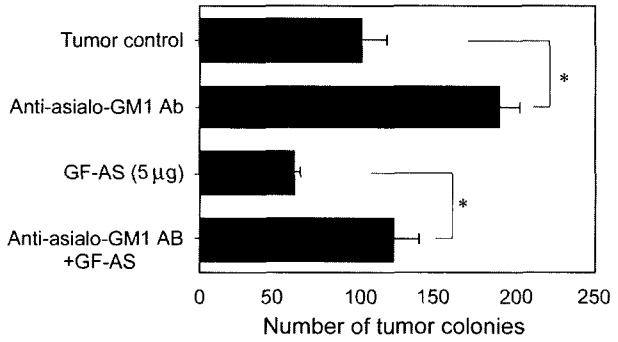

Fig. 2. Effect of NK cell depletion on GF-AS-induced inhibition of lung metastasis. To deplete NK cells *in vivo*, rabbit anti-asialo-GM1 serum was injected into mice 1 and 3 days before inoculation of colon26-M3.1 cells. Mice were administered i.v. with GF-AS (5 μg) for 2 days before tumor inoculation. 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)

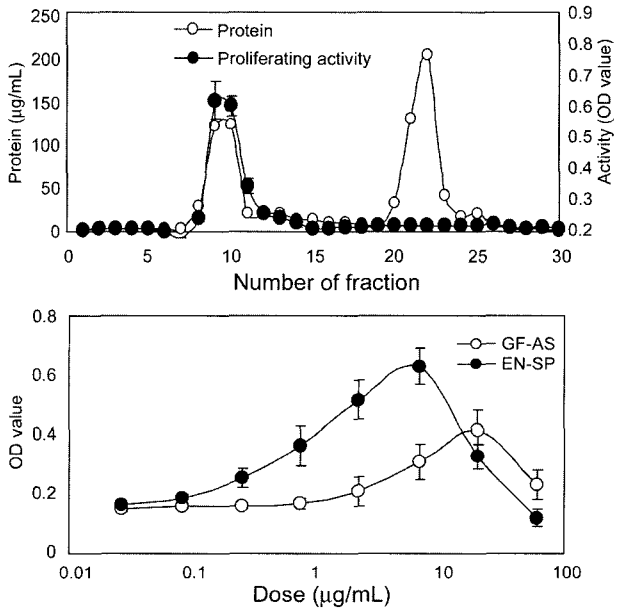

Fig. 3. Profiles of gel chromatography of GF-AS. Gel chromatography of GF-AS was carried out on a 2.5×90 cm sephadex-G50 column with PBS as an eluent. In a typical experiment, 1 mg/mL of GF-AS dissolved in PBS were applied to the column and eluted with a flow rate of 1 mL/min. To determined the elution pattern, each fraction was measured the protein concentration and assayed the proliferating activity of normal splenocytes (A). To compare the proliferating activity GF-AS and EN-SP, the indicated concentrations of each samples were added to splenocytes and incubated for 72 h. The proliferation of these cells was measured by MTT-based colorimetric assay (B).

be composed of two main proteins (Fig. 3A). We screened each fraction for immunostimulating activity and found that the first peak induced a mitogenic response to spenocytes. This suggested that the first peak was an active fraction to stimulate immune-related cells. While the first peak (called it EN-SP) had affinity to anti-GF-AS, the second peak did not (data not shown). To clarify the biological properties of EN-SP, the mitogenic response to murine splenocytes was examined in an *in vitro* experiment.

As shown in Fig. 3B, treatment with EN-SP resulted in significant increase of cell proliferation without mitogenic *stimuli*, showing maximal activity at the concentration of 7 μg/mL. These results indicate that EN-SP may up-regulate the function of immune-related cells.

Patterns of electrophoresis and western blotting of EN-SP

As shown in Fig. 4, gel electrophoresis analysis of EN-SP in the presence of 2-mercaptoethanol revealed that the molecular size of EN-SP was about 30.5 kDa. Western blotting results also revealed that anti-GF-AS Ab reacted with EN-SP. These results suggest that anti-GF-AS was elicited by EN-SP molecules of GF-AS.

Protein and carbohydrate analysis of EN-SP

The protein concentrations of GF-AS solution and EN-SP solution were 384.5 μg/mL and 436.7 μg/mL and the neutral sugar concentrations were 222.4 μg/mL and 2592.1 μg/mL, respectively. According to these results, the amount of total protein and carbohydrate of GF-AS and EN-SP were approximately 63.4 and 16.8, and 36.6 and 83.2%, respectively. These results indicated that EN-SP, active fraction in GF-AS, was mainly composed of carbohydrates (Table II).

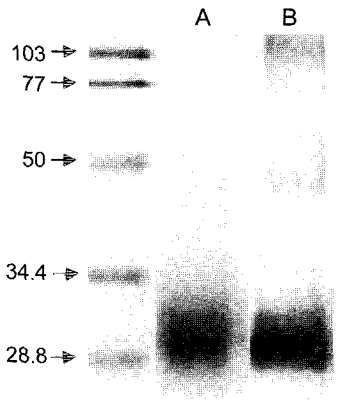

Fig. 4. Patterns of electrophoresis (A) and western blotting (B) of EN-SP. SDS-PAGE of 20 mg of EN-SP was performed and the proteins was separated, and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane as described in Materials and Methods. The detected protein bands were visualized by enhanced chemiluminescence method.

Table II. Approximate contents of total proteins and carbohydrate in GF-AS and EN-SP

Group	total protein (%)	neutral sugar (%)
GF-AS	63.4	36.6
EN-SP	16.8	83.2

Protein concentration was determined using the Bio-Rad assay kit with bovine serum albumin (BSA) as a standard. The total neutral sugar content of the EN-SP was measured colorimetrically by the phenol-sulfuric acid method using glucose as a standard.

Inhibitory effect of EN-SP on experimental lung metastasis

As shown in Table III, administration of EN-SP induces the inhibition of tumor metastasis by colon26-M3.1 lung carcinoma and B16-BL6 melanoma cells. The optimal dosage of EN-SP for blocking tumor metastasis was in the range 5 to 1 μg/mouse, which suggests that the anti-tumor activity of EN-SP was stronger than that of GF-AS (5 to 50 μg/20 g).

This suggests that EN-SP is one of the active constituents of *A. senticosus* that induces the inhibitory effect against these two types of tumors and the glycoproteins are major components in stimulating the immune system and inhibiting tumor metastasis.

DISCUSSION

Biological response modifiers (BRMs) have been used as useful tools to suppress tumor growth and inhibit tumor metastasis (Suto T *et al.*, 1994; Yoo *et al.*, 1994). In fact, various BRMs, such as oriental medicines, cytokines, and synthetic adjuvants, have shown biological activity that

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

Treatment	Doses (μg/head)	Number of lung metastasis			
		Colon26-M3.1 lung carcinoma		B16-BL6 melanoma	
		Mean ± SD (Inhibition %)	Range	Mean ± SD (Inhibition %)	Range
Untreated	–	102.2 ± 16.5	87 - 121	65.0 ± 8.3	55 - 77
GF-AS	5	35.8 ± 8.4 (65.0)[a]	26 - 45	36.4 ± 9.5 (44.0)[a]	28 - 48
EN-SP	5	29.2 ± 3.4 (71.4)[a]	25 - 34	25.3 ± 4.2 (61.1)[a]	20 - 32
	1	59.6 ± 4.6 (41.7)[a]	52 - 64	N.T	
	0.2	94.2 ± 8.5	81 - 103	N.T.	

Groups of five BALB/c or C57/BL6 mice were administered i.v. with the indicated doses of GF-AS or EN-SP two days before i.v. inoculation of 2.5×10^4 colon26-M3.1or B16-BL6 cells. Mice were sacrificed 14 days after tumor inoculation for evaluation. [a]$p<0.001$, compared with untreated control (by Student's two-tailed *t*-test). N.T. is not treated.

enhanced the host defense system and have been applied for cancer immunotherapy (Suto *et al.*, 1994; Schmeda-Hirschmann *et al.*, 2001; 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 cancer and its metastasis (Kasai *et al.*, 1981). Thus, traditionallyused natural resources for stimulating the immune system have emerged as important molecules for cancer therapy (Saiki 2000; Ohnishi *et al.*, 1998).

A. senticosus is a well-known typical oriental herb, which consists of various constituents having diverse pharmacological effects (Deyama *et al.*, 2001; Davydov *et al.*, 2000). Although a variety of biological activities of *A. senticosus* have been reported (Hibasami *et al.*, 2000; Davydov *et al.*, 2000), it is unclear which component induces anti-tumor activity thereby inhibiting the metastasis of tumor cells and how it is exerted.

This study demonstrated that glycoprotein (EN-SP) from *A. senticosus* inhibits lung metastasis, when colon26-M3.1 carcinoma or B16-BL6 melanoma cells were transplanted to syngenic mice. Its anti-tumor activity was associated with the activation of macrophages and NK cells.

As shown in Table I and III, treatment with GF-AS or EN-SP caused prophylactic effects on lung metastasis. Activated macrophages and NK cells are known to be relevant effectors responsible for natural immunity against tumors (Barlozzari *et al.*, 1985). In experiments for immunomodulatory activity test, GF-AS enhanced the proliferation of splenocytes (Fig. 3) and activated macrophages (Fig. 1). In addition, GF-AS induced the secretion of various cytokines, such as IL-1β, TNF-α, IL-12, and IFN-γ, from macrophages. The immunostimulating activities of GF-AS were partially blocked by adding the antibody against GF-AS, suggesting that yet unknown additional proteins may be involved in inducing these cytokines (Fig. 1). 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). IL-12 is secreted from phagocytic antigen-presenting cells, such as macrophages and dendritic cells, in early stages of immune response. IL-12, a so-called multi-functional cytokine, has been studied as one of the most essential cytokines to elicit tumor immunity (Ogawa *et al.*, 1998; Lasek *et al.*, 1997). Animal models have demonstrated that IL-12 has potent anti-tumor growth and metastatic activity, and its effects are most likely through IFN-γ (Lasek *et al.*, 1997; Liu *et al.*, 2002). In fact, treatment with GF-AS enhanced NK cell activity and its anti-tumor activity, which were partially abolished by depletion of NK cells (Fig. 2). Thus, our study suggests that GF-AS, including the fractionated glycoprotein, EN-SP, potentiate natural immunity of the host and/or inhibit tumor metastasis through the activation of macrophages and NK cells followed by production of various cytokines.

In order to obtain biochemical/functional profiles of this active component(s) of GF-AS, we fractionated GF-AS extracts by gel filtration. The analysis revealed that EN-SP glycoprotein was a major component related to boosting NK cell and macrophage activation, thus inhibiting tumor metastasis *in vivo* (Table III). Similar results reported that immunostimulating activities were directly proportional to the content of EN-SP (Ha *et al.*, 2003). The chemical analysis of EN-SP revealed EN-SP protein to consist of apporox. 16.8% of protein and approx. 83.2% carbohydrate (Table II).

The present study demonstrated that glycoproteins EN-SP fractionated from GF-AS in aqueous extract of *A. senticosus* significantly inhibited lung metastasis in an *in vivo* model system, in which colon26-M3.1 tumor cells and B16-BL6 melanoma cells were transplanted to syngenic mice. Our results also suggest that the anti-tumor effect of EN-SP is partially due to the activation of NK cells and macrophages. We are currently carrying out further studies to elucidate the biological mechanisms of EN-SP, related to its anti-tumor activity.

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