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Anti-cancer Activities of Ginseng Extract Fermented with *Phellinus linteus*

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In the present study, the anti-cancer effects of ginseng fermented with *Phellinus linteus* (GFPL) extract were examined through *in vitro* and *in vivo* assays. GFPL was produced by co-cultivating ginseng and *Phellinus linteus* together. Ginsenoside Rg3, Rh1 and Rh2 are important mediators of anti-angiogenesis and their levels in GFPL were enriched 24, 19 and 16 times, respectively, more than that of ginseng itself through the fermentation. GFPL exhibited distinct anti-cancer effects, including growth inhibition of the human lung carcinoma cell line A549, and promotion of immune activation by stimulating nitric oxide (NO) production in Raw 264.7 cells. Further evidence supporting anti-cancer effects of GFPL was its significant prolongment of the survival of B16F10 cancer cell-implanted mice. These results suggest that the GFPL may be a candidate for cancer prevention and treatment through immune activation and anti-angiogenic effects by enriching Rg3, Rh1 and Rh2.

KEYWORDS : Antitumor activity, Ginsenoside Rg3, HUVEC, *Phellinus linteus*

Ginseng has been a popular herbal medicine for the treatment of weakness and fatigue for thousands of years in Asia. Although long recognized, only recently have clinical studies demonstrated solid scientific evidence that ginseng may improve psychological function, immunity and conditions associated with diabetes (Kiefer and Pantuso, 2003). The main active components of ginseng are ginsenosides (saponins), which have been shown to have a variety of beneficial effects (Liu and Zhang, 1995; Kim *et al*., 1998). Until now, 38 ginsenosides have been isolated from ginseng roots, with five major ginsenosides (ginsenosides Rb1, Rb2, Rc, Re and Rg1) constituting more than 80% of the total available (Kim *et al*., 1987).

Many studies have recently focused on the pharmacological activities of the minor ginsenosides, such as Rg3, Rh1 and Rh2, as their potency and range of activity are recognized to be superior to those of their major counterparts. These minor ginsenosides have been shown to both suppress the increase and actually cause the disintegration of cancer cells as well as enhance immunity in counteracting tumors. Therefore, many studies have aimed to convert major ginsenosides to the more active and therefore therapeutic, minor ginsenosides. The sugar chains of the ginsenosides were found to be crucial for their biological activity, so therefore modification of the sugar chains may markedly change their biological activity and possibly provide a method of conversion to the minor form (Kudo *et al*., 1998; Popovich and Kitts, 2002).

Among the minor ginsenosides, Rg3 has been reported *in vitro* and *in vivo* to exhibit anti-carcinogenic and anti-metastatic effects. These include the inhibition of the growth of LNCaP prostate carcinoma cells, metastasis of B16-BL6 melanoma and colon 26-M3.1 carcinoma, invasion of human lung carcinoma (OC10) and pancreatic adenocarcinoma (PSN-1) cells (Yun *et al*., 2001). Patrick *et al.* have also demonstrated the angiogenic inhibition effects of ginsenoside Rg3 (Patrick *et al*., 2006).

Angiogenesis is a multi-step process, tightly regulated by the balance between counteracting angiogenic stimulators and inhibitors (Folkman, 1995), which includes degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs), endothelial cell proliferation, migration and capillary tube formation. Angiogenesis is a prominent pathological feature of many diseases such as rheumatoid arthritis and psoriasis (Tonnesen *et al*., 2000). A more prominent example concerning public health is the obligatory role angiogenesis has in the survival, growth, and metastasis of tumors beyond the size limit (1~2 mm diameter) imposed by adequate supply of nutrients, and oxygen (Folkman, 1974).

Phellinus linteus has been used as a traditional medicinal mushroom in northeast Asia for the treatment of various diseases, including gastroenteric disorder, lymphatic diseases and cancer. In support of this, we have previously demonstrated the anti-cancer and anti-proliferative effects of Mycelial Extract of *Phellinus linteus* (PLME) on the growth of human lung carcinoma cell line A549. Furthermore, PLME increased the production of NO in macrophage cells (Raw 264.7) in a dose-dependent manner. Therefore, PLME has been demonstrated to activate host innate and adaptive immune systems by releasing mediators with cytotoxic activity (Lee *et al*., 2006).

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Although microorganisms have recently been utilized to produce transform ginsenosides, investigations of the transformation pathway have been limited to only a few microorganism species. In addition, most of the microorganisms used for the transformation of ginsenosides in previous studies were inedible. To overcome this problem, we used *Phellinus linteus* that has been safely used in food and drugs.

This study attempted to verify the anti-tumor effect of ginseng extract fermented with *Phellinus linteus* using our established in vitro and in vivo assays. The up-regulation of nitric oxide in macrophage cells (Raw 264.7) was also measured to see effect on immune system activation by GFPL for possible use in cancer treatment.

Materials and Methods

Materials. A549, L1210, NIH3T3, B16F10 and Raw 264.7 cells were purchased from Korean Cell Line Bank (Seoul, KOREA). Human umbilical vein endothelial cells (HUVEC) and EGM-2 medium kit were purchased from Lonza Inc. (MD, USA). Matrigel and the other growth factors were provided by BD Bioscience (CA, UAS). C57BL/6 and ICR mice were given by DaeHan Biolink Co. (ChungBuk, KOREA). Ginseng extract fermented with *Phellinus linteus* (GFPL) was manufactured by Whanin Pharm. Co. (Seoul, Korea). RPMI1640 and DMEM culture media were obtained by Invitrogen Co. (N.Y., USA). Standard ginsenosides Rg3, Rh1 and Rh2 were obtained from LKT laboratory Inc. (MN, USA). Other reagents and antibiotics were purchased from Sigma Chemical Co. (MO, USA).

Preparation of GFPL. GFPL was produced as previously described by fermentation of *Phellinus linteus* mycelia with dried ginseng with the following modification (Kim *et al*., 2000). Briefly, a WIM broth was used as a medium in a 4 *l* fermenter in which seed cultivations were carried out for 3 days at 27°C and 100 rpm. Four liters of this culture broth were then added to a 70 *l* fermenter containing 36 *l* of fresh WIM medium and 1 kg dried ginseng. After 7 days of cultivation, Mycelia was harvested by filtration of culture broths and was evaporated to 60 brix, mixed with remaining media and then heated for 24 hrs at 60°C. A final dried powder was produced by using 100 mesh size sieve and obtained through an overall process of boiling water extraction, evaporation and lyophilization.

HPLC analysis. The analytical HPLC system consisted of an Alliance 2690-996 (Waters Co., MA, USA) and Xterra RP18 (4.6 × 33 mm) that used gradient conditions with an acetonitrile-water flow rate of 1.2 *ml*/min and with 203 nm UV detection. The injection volume was 20 μl.

Cell culture. A549, L1210, NIH3T3, B16F10 and Raw 264.7 cells were incubated as previously described (Lee *et al*., 2006). HUVEC were cultured in culture flasks containing EGM-2 medium. The cells were grown at 5% CO_2 at 37°C in humidified air using an incubator. All experiments were conducted with HUVEC from passages 3 to 5.

Cell viability assay. Cell viability was assayed by MTT colorimetry (Duan *et al*., 2005). Briefly, 3-[4,5-dimethylthiazol-zyl]-2,5-diphenyltetrazolium bromide (MTT) 0.5 mg/ml was added to every well and incubated for 4 hr, after which the media was removed and replaced with dimethyl sulphoxide (DMSO). Optical density (OD) was measured by Spectra MAX 190 Reader (MDS Inc., CA, USA) at 570 nm after reduced MTT was dissolved in DMSO for 30 min.

Nitric oxide assay. Stable nitrite is the end product of NO generation by activated macrophages and its amount was determined by colorimetric assay in which nitrite concentration was determined by extrapolation from a sodium nitrite standard curve. Culture supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% H_3PO_4) and were incubated at room temperature for 15 min. The absorbance at 540 nm was read on Spectra MAX 190 Reader.

***In vitro* proliferation assay.** HUVEC were grown to about 80% capacity of the flask then were digested and seeded to 24-well plates (2×10^4 cells/*ml*) for 24 hrs. Cells were then exposed to different doses of GFPL in EGM-2 culture media for 48 hrs after which they were trypsinized. The trypan blue exclusion method was used to distinguish viable cells and results were expressed as number of cells per culture.

***In vitro* tube formation assay.** Tube formation assays were performed on 24-well plates coated with 500 μl of Matrigel (diluted in PBS, 1 : 3, v/v) per well and polymerized at 37°C for 1 hr. HUVEC were plated at a density of 4×10^4 cells/well with GFPL added to the culture medium. After 24 hrs, five fields were randomly selected from each culture and images of tube formation were captured and analyzed by an inverted microscope (Olympus Co., Japan) using a 10 × objective lens and by Image J tool program (by NIH; http://rsb.info.nih.gov/ij/), respectively. Anti-angiogenic activity was determined by measuring the area of the fraction formed by the tubes.

Matrigel plug assay. The *in vivo* Matrigel angiogenesis model described by Passaniti *et al*. (1992) was used with some modifications. Briefly, Matrigel (800 μl) con-

taining VEGF (150 ng/*ml*) and heparin (25 U) with or without GFPL were injected subcutaneously into either the left or right abdomen of 6-week-old C57BL/6 male mice. Five days after injection, the animals were sacrificed under diethyl ether and the Matrigel pellets were collected, mined and solubilized with 600 *μl* DDW. The extent of neovascularization was assessed by measuring the hemoglobin content using the Drabkin's Reagent Kit (Sigma). Five mice were used for each treatment group and the experiment was done at least three times.

Anti-cancer effect *in vivo*. The anti-cancer effect of GFPL was measured against murine tumors by the survival rate of tumor-induced mice. C57BL/6 (5 week male) mice were randomly divided into four groups consisting of ten mice per group and were intraperitoneally implanted with 0.1 *ml* of 1×10^5 B16F10 melanoma cells per mouse. Intraperitoneal injection of GFPL was supplied daily to experimental mice at a dose of 5 or 30 mg/kg until all control group mice, fed with 0.85% NaCl, died. The number of surviving mice was counted every day for 20 days. The results were expressed as Survival rate (%) = (MST of treated group/MST of control group) × 100 (MST: mean survival time).

A single dose toxicity test. ICR mice (4 week male/female) were used to investigate GFPL single dose toxicity. The mice were randomly divided into 3 treatment groups (n = 10 or 20) which were administered single oral doses of either 0 (0.85% NaCl), 2000, or 5000 mg/kg of GFPL. We daily examined number of deaths, clinical signs, body weight and gross findings for 14 days after GFPL administration.

Statistical analysis. The results were expressed as the mean (± S.E.M.) of the indicated number of experiments. The statistical significance was estimated using a student's t-test. Results with a $P < 0.05$ were considered statistically significant.

Results

Analysis of ginsenoside content in GFPL. The effect GFPL has upon the conversion of ginsenosides during fermentation was assessed by HPLC. As shown in Table 1, content of the minor ginsenosides (Rg3, Rh1 and Rh2) was extremely increased in GFPL compared to ginseng. Interestingly, ginsenoside Rg3, which is well known for distinct anti-tumor activity, was highly increased as much as 24 times over that of ginseng.

Table 1. The comparison of ginsenosides content

Ginsenosides	GFPL (*μg*/g)	Ginseng (*μg*/g)	The ratio of GFPL to ginseng
Rg3	2822	120	24-fold
Rh1	608	37	16-fold
Rh2	4519	238	19-fold

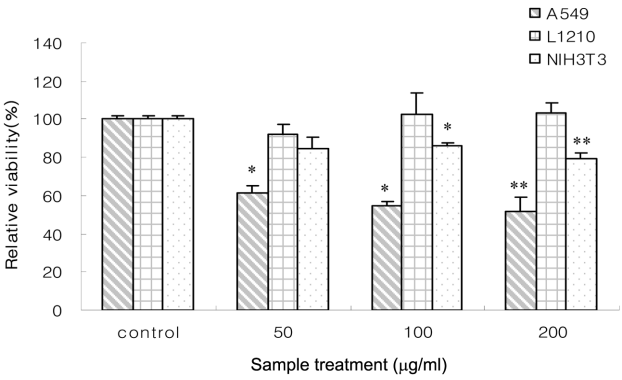

Fig. 1. Anti-proliferative effects of GFPL treatment on A549, NIH3T3 and L1210 cells. Cells were treated with various concentrations of GFPL. After 72 hrs incubation with GFPL, MTT assay was performed. Results were expressed as means ± S.E.M. of three separate experiments. *Significantly different from control group ($P < 0.05$), **Significantly different from control group ($P < 0.01$) based on student t-test.

The cytotoxicity of GFPL to A549 cells. GFPL inhibited A549 cell proliferation in a dose-dependence manner as shown in Fig. 1. NIH3T3 cells and L1210 cells are cell types generally used for evaluating toxicity of anti-cancer drugs. However, although GFPL decreases A549 cell proliferation, the viability of both NIH3T3 and L1210 cells is not significantly decreased. These results are similar to those of the tryphan blue dyed method (data not shown). The IC_{50} value of GFPL in A549 cells was affected by GFPL, as shown in Table 2. The IC_{50} represents a value of 50% inhibition in anticancer drug studies.

The NO production in macrophage cells by GFPL. We investigated whether NO production was increased in Raw 264.7 cells when stimulated with either GFPL or LPS (200 ng/*ml*). It was found NO production was induced when assayed 24 hrs after the administration of GFPL or LPS. Importantly, the production of NO by GFPL was increased by three times over that of ginseng and significantly compared to a negative control (Fig. 2). GFPL itself induced NO production as much as a LPS positive control, thereby demonstrating GFPL is able to stimulate macrophages in a related immune response.

Table 2. The IC_{50} value of GFPL in A549 cells

	IC_{50} value (*μg*/*ml*)
GFPL	362.5

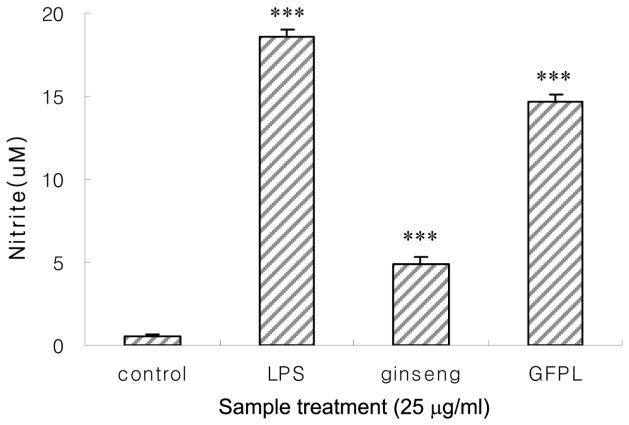

Fig. 2. Induction of nitrite in Raw 264.7 cell by GFPL. This panel shows the changes in NO production in macrophages stimulated with GFPL. After 24 hrs incubation at 37°C, nitrite levels in the culture medium were assayed using Griess reagent and absorbance measured at 540 nm. Results were expressed as means ± S.E.M. of three separate experiments. ***Significantly different from medium alone ($P < 0.001$) based on student t-test.

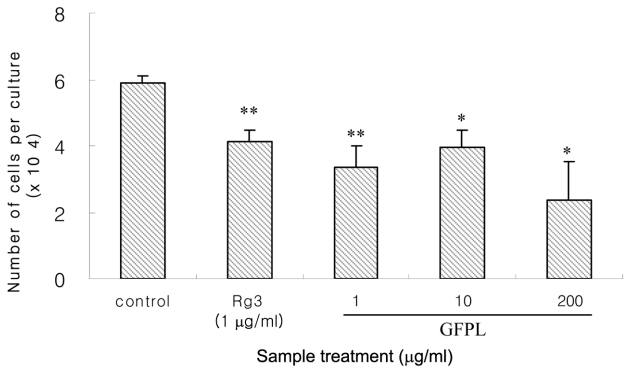

Fig. 3. Anti-proliferative effect of GFPL. HUVEC were incubated with various concentrations of GFPL or Rg3 for 48 hrs. Viable cells were counted using the trypan blue exclusion method. Values was expressed as means ± S.E.M. of triplicate experiments. *Significantly different from control group ($P < 0.05$), **Significantly different from control group ($P < 0.01$) based on student t-test.

Inhibitory effect of GFPL on HUVEC proliferation. The number of HUVEC (passage 3) in 24-well plate culture increased in a time-dependent fashion throughout the observation period, from day 1 to day 4. The effect of GFPL on cell proliferation was analyzed by direct cell counting (Fig. 3), which showed GFPL inhibited the proliferation of HUVEC in a dose-dependent manner. At a concentration of 200 $\mu g/ml$, GFPL inhibited proliferation

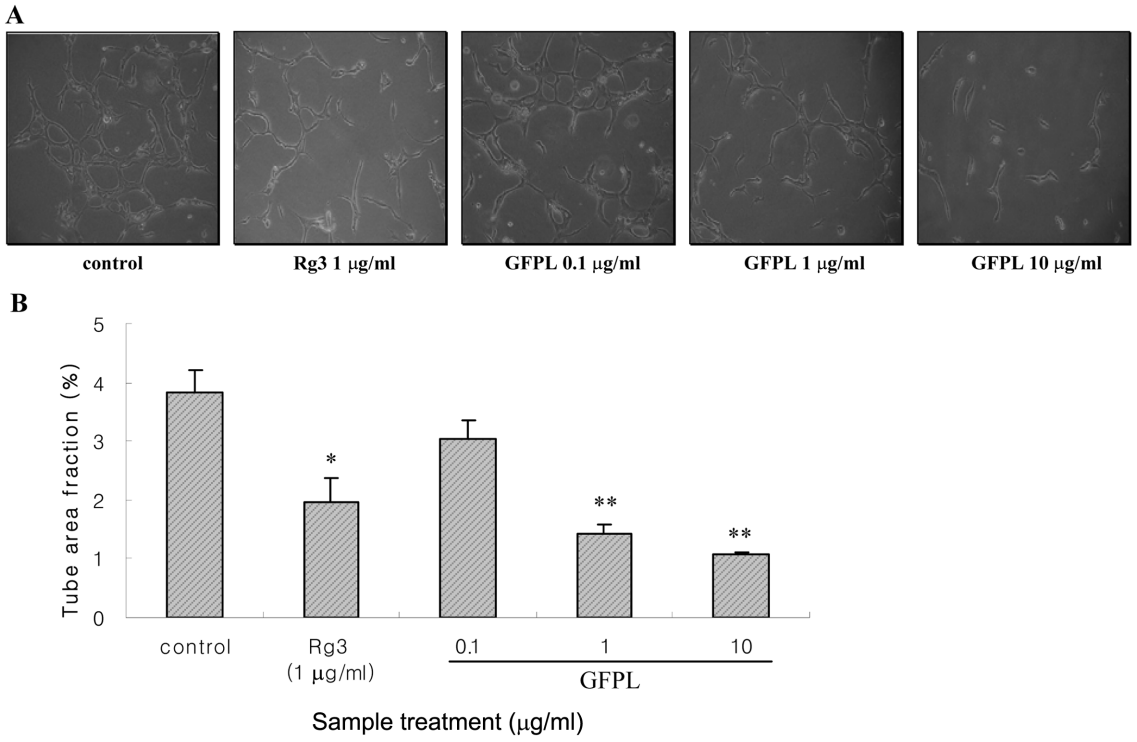

Fig. 4. Inhibition of tube formation by GFPL. HUVEC were treated with various concentrations of PLFG (0.1~10 $\mu g/ml$) for 24 hrs. A, photomicrographs depict the alignment of HUVEC under defined treatment conditions; B, five microscopic fields were counted for each treatment and mean tube area fraction was analyzed. The data represented means ± S.E.M. from triplicate experiments. *Significantly different from control group ($P < 0.05$), **Significantly different from control group ($P < 0.01$) based on student t-test.

of HUVEC more than twice as much as ginsenoside Rg3 alone.

GFPL decreases tube formation of HUVEC *in vitro*. HUVEC that were plated on Matrigel underwent significant morphological changes, forming a tubular network within 24 hrs (Fig. 4A). Treatment with GFPL (0.1~10 $\mu g/ml$) strongly prevented tube formation in a progressive manner dependent upon dose. A GFPL dose of 10 $\mu g/ml$ decreased the average total tube area by 72% compared to the control. In fact, the total area of the tubules was significantly decreased by this treatment (Fig. 4B). As a positive control, the effect of ginsenoside Rg3 on tube formation in matrigel-cultured HUVEC was examined. Ginsenoside Rg3 (1 $\mu g/ml$) reduced tube formation by 48%, a magnitude lower than the reduction caused by an identical treatment concentration of GFPL (48% vs. 62%).

Anti-angiogenic effects of GFPL *in vivo*. The effect of GFPL on angiogenesis was measured *in vivo* using the Matrigel plug assay. Five days after subcutaneous injection of Matrigel (800 μl) containing VEGF and heparin into C57BL/6 mice, the hemoglobin content in the Matrigel pellets was measured to assess the degree of vascularization. As shown in Fig. 5, when added at a concentration of 50 $\mu g/ml$, GFPL significantly reduced hemoglobin content from control levels. These results demonstrate that GFPL has the potential capacity of inhibiting vascularization of Matrigel pellets implanted in mice.

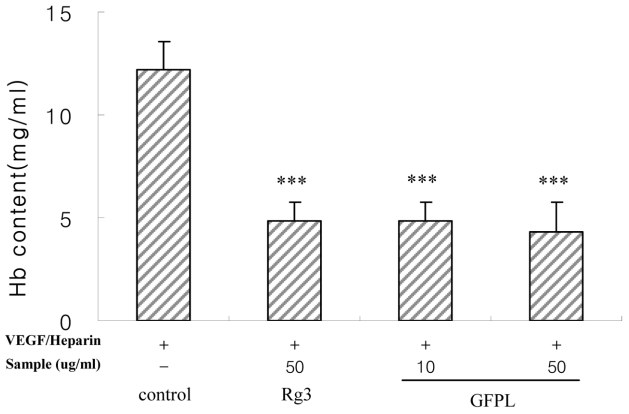

Fig. 5. Inhibitory effect of GFPL on angiogenesis in subcutaneous implanted Matrigel plugs in mice. GFPL, Rg3 or vehicle (saline sol.) was added to a mixture containing VEGF, heparin and Matrigel. Five days after injection, the hemoglobin content in the Matrigel pellets was measured to assess the degree of neovascularization. The data represented means ± S.E.M. The mice numbers for vehicle controls, GFPL and Rg3 were 5, 5 and 4, respectively. ***Significantly different from medium alone ($P < 0.001$) based on student t-test.

Anti-cancer effects of GFPL in mice transplanted with cancer cells. The anti-cancer effects of GFPL were investigated by the intraperitonieal injection of B16/F10 melanoma cancer cells in C57BL/6 mice. After induction of cancer, GFPL was administrated daily through intraperitoneal injection at a dose of 5 or 30 mg/kg until all control group mice died. Control group mince showed significant increases in body weight, while variations in GFPL group mice were only slightly increased against control mice (Fig. 6A). The survival rate was increased more than 13% in mice treated with both concentrations of GFPL, compared to control group in a dose dependent manner (Fig. 6B).

Oral toxicity of single GFPL dose in mice. To test if GFPL had any adverse effect, and therefore its feasibility as an anti-cancer treatment, the oral toxicity analysis of a single GFPL dose was performed with ICR mice of both sexes. Mice were administrated orally with 2,000 or 5,000 mg/kg doses of GFPL. No GFPL-related deaths or clinical morbidities were produced (see Table 3). Overall, there was no GFPL-related effect on body weight gain, food consumption or water consumption. Gross necropsy and histopathology furthere revealed no evidence of specific toxicity related to GFPL. Therefore, the median

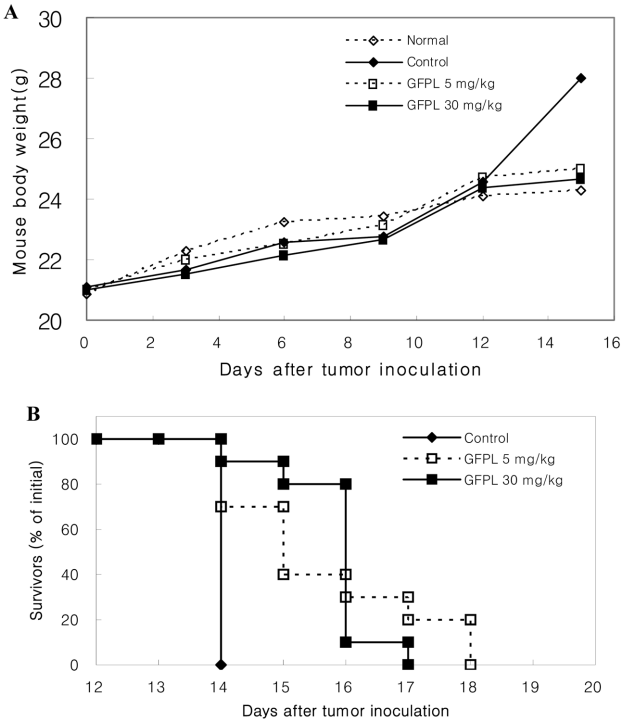

Fig. 6. Anticancer effects of GFPL in mice with transplanted with cancer cells. A, changes in body weight of B16F10 tumor-bearing mice. The body weight per day was expressed in grams (g); B, effect of intraperitoneal administration of GFPL on the growth of induced melanoma.

Table 3. Single oral toxicity in male and female ICR mice administered with GFPL for 14 days[a]

Sex	Dose (mg/kg)	Mortality[a]	Body weight[b]	Clinical signs[c]	Gross findings[d]
Male	0	0/5	NSC	NAD	NGL
	2000	0/10	NSC	NAD	NGL
	5000	0/10	NSC	NAD	NGL
Female	0	0/5	NSC	NAD	NGL
	2000	0/10	NSC	NAD	NGL
	5000	0/10	NCS	NAD	NGL

[a]Values are expressed as the numbers of dead animals/total of animals.
[b]NSC: no statistically significant weight change.
[c]NAD: no abnormality detected.
[d]NGL: no gross lession.

lethal dose (LD_{50}) values of male and female ICR mice treated with GFPL are estimated to be more than 5000 mg/kg/day, which is much more than the experimental dose in our study.

Discussion

Ginsenoside Rg3 has been shown to exhibit anti-cancer activities in many *in vivo* models, including inhibition of the invasion and metastasis of tumor cells (Mochizuki *et al*., 1995) as well as the overall inhibition of angiogenesis (Pan *et al*., 2002). Patrick *et al*. recently demonstrated the angio-suppressive properties of ginsenoside Rg3 in several different angiogenesis assay systems, further providing evidence of its anti-cancer ability (Patrick *et al*., 2006). However, the content of ginsenoside Rg3 in natural ginseng is less than that produced through microbial conversion. Therefore it was practical to attempt the microbial conversion of ginsenosides in ginseng using Phellinus linteus, which itself is known to enrich anti-cancer ginsenosides and have anti-tumoral activities (Lee *et al*., 2006).

Different concentrations of ginseng extract fermented with *Phellinus linteus* (GFPL) inhibited the growth of A549 cells, but not the growth of NIH3T3 and L1210 cells. NIH3T3 cells were used for a negative control while L1210 cells are sensitive to general anti-cancer drugs. These data suggest that GFPL may potentially be effective as a lung cancer therapy drug by carefully preparing its concentrations.

To study NO production in macrophage cells treated with GFPL, we carried out an MTT assay and a Griess reagent assay after GFPL exposure. The Raw 264.7 cells treated with GFPL did not show any difference with respect to viability compared to untreated cells (data not shown). This result suggests that the GFPL does not exert any direct cytotoxic effect on the macrophages. However, NO production in cells treated with GFPL was increased to levels comparable to ginseng. NO has recently been recognized as an important messenger in diverse pathophysiological functions, including neuronal transmission, vascular relaxation, immune modulation, and cytotoxicity against tumor cells (Lowenstein *et al*., 1994). The induction of NO has been identified as the major event involved in the destruction of tumor cells by activated macrophages (Moncada *et al*., 1991; Lorsbach *et al*., 1993; Duerksen-Hughes *et al*., 1992), and can furthermore lead to cytotoxic effects on malignant cells (Duerksen-Hughes *et al*., 1992; Stuehr *et al*., 1989). Because of the pivotal role of NO in the anti-microbial and anti-tumor activities of macrophages, significant focus has been placed on developing therapeutic agents that regulate NO production (Poderoso *et al*., 1999). Following these previous reports, GFPL is assumed to activate host innate and adaptive immune systems by releasing mediators with cytotoxic activity. Finally, *Phellinus linteus* is used not only a source for microbial conversion but also acts as an effective anti-tumor adjuvant.

Results from the present study clearly demonstrate GFPL exerts inhibitory effects on proliferation and capillary tube formation in HUVEC and the in vivo Matrigel plug assay in a dose-dependent manner. Related, abnormal angiogenesis has been implicated in various diseases including brain tumors and other cancers, thereby leading to several angiogenic inhibitors being currently investigated in clinical trials for cancer therapy (Liekens *et al*., 2001; Bikfalvi and Bicknell, 2002; Puduvalli, 2004). Considering this, it is reasonable GFPL could be a novel and potent anti-angiogenic agent by inhibiting the proliferation, migration and differentiation of endothelial cells.

The survival rate of the mice with B16F10 murine melanoma was prolonged when treated with GFPL in a dose-dependent manner compared to control mice. B16F10 murine melanoma cells are well-known as a highly metastatic-malignant melanocyte neoplasm, which is supported by a report in which Bae *et al*. inhibited tumor growth and induced cell apoptosis in B16F10 melanoma-allografted mice using crude water-extract of *Phellinus gilvus* (Bae *et al*., 2005). They observed *in vivo* inhibition of tumor growth at a dose of 100 mg/kg (i.p.), which is similar to the effect of adriamycin at a dose of 0.1 mg/kg. On the other hand, Han et al. showed endo-polysaccharide from mycelial cultures of *P. linteus* inhibited tumor growth at a dose of 100 mg/kg (i.p.), probably through immuno-stimulation in a B16F10 melanoma model (Han *et al*., 1999). These results cumulatively indicate that GFPL significantly inhibits growth of melanoma tumor cells in mice.

In testing the single toxic dose of GFPL, there was no abnormal effect observed between mice treated with GFPL and the control group. We estimated the single toxic dose of GFPL is above 5000 mg/kg/day in mice.

In conclusion, GFPL can inhibit tumor growth *in vitro*

and *in vivo* not only by an induced humoral immune response, but also by direct cytotoxic action against tumor cells. Therefore, GFPL may have potential clinical utility in cancer prevention without concerns for safety.

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