Anti-Tumor Activities of the Antlered Form of *Ganoderma lucidum* in Allogeneic and Syngeneic Tumor-Bearing Mice

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We investigated the anti-tumor effects of a dry powder preparation of the antlered form of *Ganoderma lucidum* (*G. lucidum* AF, *rokkaku-reishi* in Japanese), a variant type of *G. lucidum*, not only in allogeneic Sarcoma 180-bearing ddY mice, but also in syngeneic MM 46-bearing C3H/He mice. *G. lucidum* AF inhibited tumor growth and elongated the life span when orally administered to mice by free-feeding of a 2.5% *G. lucidum* AF-containing diet. It also showed anti-tumor activity in spite of post-feeding after tumor inoculation. *G. lucidum* AF significantly countered the depression of splenic CD8+ cells and protected the decrease in interferon-gamma (IFN-γ) production in regional lymph nodes of MM 46-bearing mice, indicating that the anti-tumor activity of *G. lucidum* AF might be caused by its immunostimulating action. These results suggest that the ingestion of *G. lucidum* AF can be useful for the prevention and curing of cancer.

**Key words:** antlered form of *Ganoderma lucidum*; anti-tumor activity; syngeneic tumor; cytokine production; immunodepression

*Ganoderma lucidum* (Fr.) Karst (*reishi* in Japanese), an Oriental fungus, is a famous traditional Chinese medicine used in China, Japan, and other Asian countries.1 It has been prescribed for the prevention and treatment of various diseases, such as hypertension,2 hyperlipidemia,3, 4 diabetes,5, 6 hepatitis,6, 7 allergy,8, 9 and cancer.10–12 Recently, active components, such as β-D-glucans and triterpenoids, which exhibited anti-tumor effects, have been isolated from *G. lucidum*, and many experimental data in vitro and in vivo have been reported.10–19 Anti-tumor activities of edible and medical mush-

rooms in vivo have been reported,20 but Sarcoma 180 tumor-bearing mice have been used in many studies. Sarcoma 180 tumor is an allogeneic tumor, and so it cannot be denied that the anti-tumor mechanism in this model depends on the allograft rejection of the host animal. Therefore, it is indispensable to use a syngeneic tumor to elucidate the anti-tumor activity and its immunological mechanism of active compounds.

The antlered form of *Ganoderma lucidum* (*G. lucidum* AF, *rokkaku-reishi* in Japanese) is a variant type of *G. lucidum* rarely found in nature. Recently, a cultivation technology was developed, and *G. lucidum* AF is artificially cultivated in China, Korea, and Japan. It has been reported that *G. lucidum* AF contained large amounts of β-D-glucans,21 and that the triterpenoids in *G. lucidum* AF were much larger than those in the normal type of *G. lucidum*.17 It is expected that the anti-tumor activity of *G. lucidum* AF might be much stronger than that of the normal type of *G. lucidum*.

Kohguuchi et al.21 found that *G. lucidum* AF can activate macrophages and induce Th1-associated immune activities in vivo, but no information has been reported as to anti-tumor activity and the immunological mechanism of *G. lucidum* AF in a syngeneic tumor-mice model.

In the present study, we investigated the anti-tumor effects of *G. lucidum* AF by oral administration (p.o. and free-feeding) not only in allogeneic Sarcoma 180 tumor-bearing ddY mice, but also in syngeneic MM 46 mammary carcinoma-bearing C3H/He mice, and then elucidated the immuno-stimulating effect of *G. lucidum* AF in syngeneic MM 46 tumor-bearing mice using splenocytes and lymphocytes from regional lymph nodes.

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* Abbreviations: G. lucidum AF, antlered form of *Ganoderma lucidum*; p.o., post orally; i.p., intraperitoneally; s.c., subcutaneously; IFN-γ, interferon-gamma; IL-4, interleukin-4; FBS, fetal bovine serum; PBS, phosphate-buffered saline; Con A, concanavalin A; ELLISA, enzyme-linked immunosorbent assay; CD, cluster of differentiation; BRM, biological response modifiers
Materials and Methods

**Materials.** We used *G. lucidum* AF in this study, grown commercially in China, Korea, and Japan. All *G. lucidum* AF samples (Chinese 4, Japanese 3, and Korean 1) were purchased from Shinwa Bussan (Osaka, Japan). They were crushed to a 200-mesh path equivalent. A *G. lucidum* AF-containing diet was prepared from AIN-93M chow (Oriental Yeast, Tokyo). Sarcoma 180 cells were kindly donated by Dr. M. Hayashi (Kitasato Institute for Life Sciences and School of Pharmaceutical Sciences, Kitasato University, Tokyo). MM 46 cells were from the collection of the Institute of Medical Mycology, Teikyo University.

**Animals.** Male ddY and C3H/He mice (5–6 weeks old) were purchased from Japan SLC (Shizuoka, Japan) and Charles River Japan (Yokohama, Japan) respectively. These animals were housed and put on a commercial diet (AIN-93M) and tap water *ad libitum* for 1 week before experimentation at 25 ± 1 °C and 60 ± 5% humidity under a 12 h light-dark cycle. Experiments were performed according to the “Guidelines for the Care and Use of Experimental Animals” of the Japanese Association for Laboratory Animals.

**Anti-tumor tests.** Allogeneic tumor model: Sarcoma 180 cells (1 × 10⁶ cells) were inoculated intraperitoneally (i.p.) into ddY mice for passage culture. Tumor cells were collected from the peritoneal cavity 7 d after inoculation and then suspended in MEM medium. The cells were subcutaneously (s.c.) inoculated into the left chest of ddY mice (1 × 10⁶ cells/0.1 ml/mouse). *G. lucidum* AF (0.005, 0.05, or 0.5 g/kg/d) was suspended in 0.6 ml of distilled water and post orally (p.o.) administered to mice for 19 consecutive d starting from the day before inoculation. Water was administered to the control mice. These animals were housed and put on a commercial AIN-93M diet with or without 2.5% *G. lucidum* AF in this study, and Charles River Japan (Yokohama, Japan) respectively. The cells were washed with a hemolytic buffer (155 mM NaCl, 10 mM KHCO₃, 0.1 mM EDTA) and phosphate-buffered saline (PBS) in that order. The cells were suspended in RPMI 1640 medium containing 10% FBS and a 1% antibiotic-antimycotic mix cocktail (Nacalai Tesque).

**Preparation of splenocytes and lymphocytes.** Splenocytes and lymphocytes were prepared from spleens and regional (inguinal) lymph nodes from MM 46 tumor-bearing mice killed 28 d after tumor inoculation, as described above. After removing the spleen or the regional (inguinal) lymph node from a mouse, each single-cell suspension of splenocytes or lymphocytes was prepared by passing it through a 70 μm nylon mesh. The cells were washed with a hemolytic buffer (155 mM NaCl, 10 mM KHCO₃, 0.1 mM EDTA) and phosphate-buffered saline (PBS) in that order. The cells were suspended in RPMI 1640 medium containing 10% FBS and a 1% antibiotic-antimycotic mix cocktail (Nacalai Tesque).

**Flow cytometric analysis.** CD4⁺ and CD8⁺ cells were stained for 45 min using CY-CHROMÉ™ conjugated anti-mouse CD4 monoclonal antibody (BD Pharmingen, San Diego, CA) and FITC-conjugated anti-mouse CD8 monoclonal antibody (Immunootech, Marseille, France) respectively. The cells were washed with PBS, and CD4 and CD8 expression in lymphocytes was analyzed using Epics XL (Beckman Coulter, Fullerton, CA). The analysis was based on 15,000 cells gated as lymphocytes, and calculated using EXPO32 software.

**Assay for cytokine production.** Splenocytes and lymphocytes (5 × 1⁶ cells/ml each) were stimulated with 2.5 μg/ml of concanavalin A (Con A) (Wako Pure Chemical Industries, Osaka, Japan). After incubation for 24 h at 37 °C, the culture supernatant was collected and the levels of IFN-γ and IL-4 production were determined using an OptEIA™ kit (BD PharMingen).

**Statistical analysis.** The statistically significant differences for each value were tested by one-way ANOVA, followed by Fisher’s PLSD as a *post-hoc* test. Cumulative survival curves were constructed using Kaplan–Meier methods, and significant differences were tested by the log-rank test.

**Results**

**Anti-tumor activity of *G. lucidum* AF in Sarcoma 180 tumor-bearing mice**

The anti-tumor activities of *G. lucidum* AF from eight different growing-districts (Chinese 4, Japanese 3, and Korean 1) were tested using Sarcoma 180 tumor-bearing mice. Each sample was orally administered to mice daily by gastric gavage at a dose of 0.5 g/kg/d from one
day before tumor inoculation. There was no correlation between the anti-tumor activity and the total β-glucan content of *G. lucidum* AF in this experiment, although the total β-D-glucan contents were more than 40% in all of the eight different district-derived *G. lucidum* AF samples (data not shown). A kind of Chinese *G. lucidum* AF (from Sang Tong) exhibited the highest activity, inhibiting tumor growth by 48% 18 d after inoculation. Hence we used this Chinese *G. lucidum* AF in all the experiments described below.

As shown in Fig. 1, *G. lucidum* AF significantly inhibited the growth of Sarcoma 180 tumor at a dose of 0.05 or 0.5 g/kg/d when administered daily by gastric gavage. An ordinary type of commercially available *G. lucidum* did not show anti-tumor activity at either 0.05 g/kg/d (data not shown) or 0.5 g/kg/d (Fig. 1A).

In the preliminary experiments, the anti-tumor activity at 1 g/kg/d *G. lucidum* AF was weaker than that at 0.5 g/kg/d. No adverse effects of *G. lucidum* AF was observed up to 1 g/kg/d (data not shown).

**Anti-tumor and life span-elongation effects of *G. lucidum* AF in Sarcoma 180 tumor-bearing mice**

We examined the anti-tumor effect of *G. lucidum* AF by free feeding in Sarcoma 180 tumor-bearing mice, since oral administration by gastric gavage can induce experimental stress in mice. A control diet (AIN-93M) or the *G. lucidum* AF diet (0.156, 0.625, or 2.5%) was given. There was no difference between the groups in food intake. A mouse took about 75 mg/d (2.5 g/kg/d) in the 2.5% *G. lucidum* AF-fed group. In the 2.5% *G. lucidum* AF-fed group, tumor growth was significantly inhibited from 15 d after tumor inoculation. The lower amounts of *G. lucidum* AF groups also showed a repression tendency against tumor growth as compared with the control group (Fig. 2A). In the *G. lucidum* AF-fed groups, body weights were slightly lighter than in the control group, but were not significantly different. No adverse effect of *G. lucidum* AF was observed up to 10% (data not shown).

Survivals of Sarcoma 180 tumor-bearing mice, with or without a 2.5% *G. lucidum* AF-containing diet, were monitored up to 100 d after tumor inoculation. There was no difference between the groups in food intake, but food intake reduction was observed starting about 30 d after tumor inoculation. The life span of mice fed the *G. lucidum* AF diet was significantly prolonged as compared with the control group. The survival periods giving 50% lethality were 54 and 64 d for the control and *G. lucidum* AF-fed mice respectively (Fig. 2B).

**Anti-tumor and life span-elongation effects of *G. lucidum* AF in MM 46 tumor-bearing mice**

*G. lucidum* AF also exhibited anti-tumor activity against a syngeneic MM 46 tumor in C3H/He mice in spite of post-feeding from 7 d after tumor inoculation. The control diet (AIN-93M) or *G. lucidum* AF diet (0.156, 0.625, 2.5%) was given as described in “Materials and Methods,” and there was no difference between the groups in food intake. As shown in Fig. 3A, *G. lucidum* AF significantly reduced tumor weight 28 d after inoculation when orally administered at a dose of 2.5% *G. lucidum* AF-containing diet.

Survivals of MM 46 tumor-bearing mice, with or without the 2.5% *G. lucidum* AF diet, were monitored up to 100 d after tumor inoculation. The life span of the mice fed *G. lucidum* AF diet was significantly prolonged as compared with the control group. The survival periods giving 50% lethality were 49 and 62 d for the control and *G. lucidum* AF-fed mice respectively (Fig. 3B). They were not significantly different in body weight.
weight, although food intake reduction was observed starting about 42 d after tumor inoculation.

**Immunological function of G. lucidum AF in MM 46 tumor-bearing mice**

We checked the activity of immunocompetent cells isolated from MM 46 tumor-bearing mice fed the G. lucidum AF diet. Splenocytes and lymphocytes were prepared from spleens and regional (inguinal) lymph nodes from MM 46 tumor-bearing mice killed 28 d after inoculation, as described in Fig. 3A. Splenocytes and lymphocytes from the normal mice were similarly prepared, as a control.

The populations of CD4<sup>+</sup> and CD8<sup>+</sup> cells in both splenocytes were decreased by MM 46 inoculation. G. lucidum AF significantly recovered the reduction of CD8<sup>+</sup> cells in splenocytes. No changes in cell populations were detected in any of the groups of regional lymph nodes (Fig. 4).

IFN-γ and IL-4 production by Con A-stimulated splenocytes or lymphocytes were also measured. G. lucidum AF significantly protected the decrease in IFN-γ production in splenocytes and lymphocytes in regional lymph nodes, respectively. The IL-4 level in regional lymph nodes was also recovered by feeding the G. lucidum AF-containing diet (Fig. 5).

**Discussion**

We demonstrated, for the first time, the anti-tumor activities of G. lucidum AF, a variant type of G. lucidum, by oral administration not only in allogeneic Sarcoma 180 tumor-bearing mice, but also in syngeneic MM 46 mammary carcinoma tumor-bearing mice (Figs. 1, 2 and 3). G. lucidum AF inhibited the increase
in tumor weight and elongated the life span of the tumor-bearing mice when orally administered by free feeding (Figs. 2, 3). Furthermore, we found that G. lucidum AF protected the tumor-bearing mice from immunodepression due to tumor growth (Fig. 4, 5).

It has been expected that G. lucidum AF has strong anti-tumor activity because of the high content of β-D-glucan in its fruiting body. β-D-glucan, a major component of G. lucidum AF, exhibited immuno-stimulating activities in vivo and in vitro. Kohguchi et al. found that the β-D-glucan content in G. lucidum AF was 40.1%, and that total percentage of β-D-(1,3)-glucan in the β-D-glucan was 85.3%. They also indicated that the oral administration of G. lucidum AF resulted in Th1-associated immuno-potentiating activities in BALB/c mice, but there was no correlation between the anti-tumor activity and β-D-glucan content of G. lucidum AF in this study, although all the total β-D-glucan contents were more than 40%. We should take notice that the anti-tumor activity of G. lucidum AF could not be predicted by the total β-D-glucan content. We used Chinese G. lucidum AF in all experiments, which exhibited the highest activity of eight samples.

Recently, various structures and bioactivities of β-D-glucans derived from G. lucidum have been reported, e.g., protein-bound β-glucan, α-D-(1→3)-glucan, β-D-(1→6)-glucan with β-(1→3) or β-(1→4) branches, and β-D-(1→3)-glucan with β-(1→6) branches. The cytotoxic actions of triterpenes, such as ganoderic acid, lucidenic acid, and ganoderiol, in G. lucidum AF have been also studied vigorously. Further study should be done to determine the active component responsible for the anti-tumor activity with oral administration of G. lucidum AF.

We examined the anti-tumor activity of G. lucidum by oral administration with diet, in order to avoid physical and mental stress by gastric gavage. G. lucidum AF showed anti-tumor activity in Sarcoma 180 tumor-bearing mice by free feeding from 1 d before tumor inoculation. G. lucidum AF also exhibited anti-tumor activity against a syngeneic MM 46 tumor in C3H/He mice and elongated the life span of tumor-bearing mice, in spite of post-feeding from 7 d after tumor inoculation. These results suggest that ingestion of G. lucidum AF is useful not only for prevention, but also for curing cancer.

The mechanism of the anti-tumor effect of G. lucidum AF remains to be clarified. At this point, we consider as follows: The immunological mechanism may be related to inhibition of MM 46-tumor growth, because MM 46 carcinoma in C3H/He mice is moderately antigenic, and its growth can be inhibited without cytotoxicity by immuno-stimulating agents, including mushroom β-D-glucans such as lentinan and PSK. In this study, G. lucidum AF significantly countered the depression of splenic CD8+ cells and protected against the decrease in IFN-γ and IL-4 production in regional lymph nodes in MM 46 tumor-bearing mice. In addition, in a recent study, in vivo depletion of IFN-γ abrogated the anti-tumor vaccination effect, indicating that IFN-γ might play an important role in immunomodulating...
anti-tumor action. These findings suggest that one of the anti-tumor mechanisms of *G. lucidum* AF might be due to its IFN-γ-inducible activity and CTL activation under the cancer condition.

In summary, we confirmed the anti-tumor activity of *G. lucidum* AF not only against allogeneic tumors, but also against syngeneic tumors. We also suggest that the anti-tumor effects of *G. lucidum* AF might be explained by the relieving activity from tumor-induced immunodepression. BRMs derived from mushrooms are generally treated with chemotherapeutic agents to counteract their side effects, such as myelosuppression or leukocytopenia.31,32 *G. lucidum* AF can clinically alleviate chemotherapeutic agent-induced immunodepression. Currently, the anti-tumor activities of concomitant administration of *G. lucidum* AF and chemotherapeutic agents are under investigation in our laboratory. Finally, the present study suggests that *G. lucidum* AF might be useful for the prevention of relapse and as a co-adjuvant in the medical treatment of cancer.

References


