Effects of the Antlered Form of *Ganoderma lucidum* on Tumor Growth and Metastasis in Cyclophosphamide-Treated Mice

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Received September 25, 2007; Accepted January 31, 2008; Online Publication, June 7, 2008

We examined the alleviation of cyclophosphamide-induced immunodepression by the antlered form of *Ganoderma lucidum* (G. *lucidum* AF) and also evaluated the anti-tumor and anti-metastatic effects of G. *lucidum* AF in cyclophosphamide-treated mice. G. *lucidum* AF alleviated cyclophosphamide-induced decrease in body weight, natural killer (NK) activity, interferon (IFN)-γ production, and cytotoxic T lymphocyte (CTL) activity, and inhibited the abnormal increase and decrease in interleukine (IL)-4 level due to cyclophosphamide administration. Post-treatment with cyclophosphamide and G. *lucidum* AF significantly inhibited tumor growth in MM 46-bearing mice. When Lewis lung carcinoma cells were injected into mice after a cyclophosphamide administration, metastasis of these cells to the lung was increased, but G. *lucidum* AF suppressed it. The anti-tumor and anti-metastatic effects of the combination of G. *lucidum* AF and cyclophosphamide might influence the modulatory effects of G. *lucidum* AF on both cellular and humoral immunity. These findings suggest that G. *lucidum* AF would be beneficial in alleviating the reduction of immune response by chemotherapeutic anti-cancer drugs.

Key words: antlered form of *Ganoderma lucidum*; cyclophosphamide; anti-tumor activity; anti-metastatic activity; immunodepression

Although chemotherapeutic drugs are most frequently used for cancer therapy, they exhibit adverse effects, principally involving body weight loss, neutropenia, and immunosuppression, and may thus increase the risk of cancer metastasis and bacterial infection, despite their pharmacological benefits. Cyclophosphamide (CY) is one of the most commonly used chemotherapeutic agents.1) It has exhibited a synergistic anti-tumor effect in an MCA207 sarcoma-bearing model in combination with IL-12.2,3) Treatment with CY and IL-15 has also activated natural killer (NK) cells and tumor-specific αβ and γδ T cells,4) and IL-2 production has been found to increase the anti-tumor effect of CY.5)

β-glucan, a biological response modifier (BRM), has been reported to alleviate chemotherapeutic agent-induced immunodepression and to enhance anti-tumor activity. For example, the administration of lentinan from *Lentinus edodes* with CY completely inhibited the growth of Rous sarcoma and D2 fibrosarcoma.6) SCG from *Sparassis crispa* also increased cytokine production by splenocytes and inhibited CY-induced leukopenia in mice.7,8) Thus, β-glucan, which has a strong immunostimulatory effect, can be expected to exhibit synergistic anti-tumor activity and suppress the adverse effects in combination with CY.

*Ganoderma lucidum* (Fr.) Karst is a medical Chinese mushroom widely used in Asia, and has preventive and therapeutic effects against cancer,9,10) allergy,11,12) and hepatitis.13,14) We have previously reported the anti-tumor effects of the antlered form of G. *lucidum* (G. *lucidum* AF) in MM 46-bearing C3H/HeN mice. G. *lucidum* AF significantly countered the depression of activity of splenic CD8+ T cells and protected against a decrease in interferon γ (IFN-γ) production in regional lymph nodes in MM 46-bearing mice.15) Kouguchi et al. have found that G. *lucidum* AF activated phagocytosis of macrophages and induced Th1 cytokine production.16)
The β-(1→3)-d-glucan content of *G. lucidum* AF is high.\(^{(6)}\) *G. lucidum* AF can thus be expected to act as a good BRM in combination with chemotherapeutic agents for cancer therapy.

We examined in this study the alleviation by *G. lucidum* AF of CY-induced immunodepression by measuring the body weight, NK activity, mixed lymphocyte reaction (MLR), cytotoxic T lymphocyte (CTL) activity, and cytokine production. We then examined the synergistic anti-tumor and anti-metastatic effects of treatment with *G. lucidum* AF and CY by using syngeneic MM 46 tumor-bearing C3H/HeN mice and C57BL/6 mice injected into each group of mice. The control mice were monitored daily for 8 d. Splenocytes and lung lymphocytes were prepared 3, 5 and 7 d after CY administration without this CY treatment. Body weights were measured weekly without this CY treatment. Body weights were measured 3, 5 and 7 d after CY administration without this CY treatment. Body weights were measured weekly.

**Materials and Methods**

*G. lucidum* AF. *G. lucidum* AF cultivated in China was purchased from Shinwa Bussan (Osaka, Japan) and crushed to a 200 mesh-path equivalent. A *G. lucidum* AF (2.5%)-containing diet was prepared from AIN-93M feed which contained 2.5% of reduced cornstarch (Oriental Yeast, Tokyo). There was no significant difference in the change in body weight between the control (AIN-93M) diet-fed mice and 2.5% *G. lucidum* AF diet-fed mice.

**Animals.** C3H/HeN, C57BL/6, and Balb/c mice (6 weeks old, male) were purchased from Charles River Laboratories Japan (Yokohama, Japan). The animals were housed and fed on a commercial diet (CE-2) and tap water *ad libitum* for 1 week before the experiments at 25 ± 1°C and 60 ± 5% humidity under a 12 h light-dark cycle. The animals were subsequently fed specific diets in accordance with the protocol described next. All experiments were performed in accordance with the “Guidelines for the Care and Use of Experimental Animals of the Japanese Association for Laboratory Animals.”

CY-induced immunodepression. C57BL/6 mice (n = 12 per group) were fed on the AIN-93M diet or 2.5% *G. lucidum* AF-containing AIN-93M diet for 1 week, and then 150 mg/kg of CY was intraperitoneally injected into each group of mice. The control mice (n = 12 per group) were fed on the AIN-93M diet or 2.5% *G. lucidum* AF-containing AIN-93M diet for 1 week without this CY treatment. Body weights were monitored daily for 8 d. Splenocytes and lung lymphocytes were prepared 3, 5 and 7 d after CY administration. The spleen was passed through a 70-μm nylon mesh, and then washed with a hemolysis buffer (155 mM NaCl, 10 mM KHCO\(_3\), and 0.1 mM EDTA) and phosphate-buffered saline (PBS). The lung was passed through a 70-μm nylon mesh, and non-parenchymal cells were fractionated by using Lympholyte M (Cedarlane Laboratories, Ontario, Canada). The prepared splenocytes and lung lymphocytes were suspended in RPMI1640 (Nacalai Tesque, Kyoto, Japan) containing a 10% FBS and 1% antibiotic-antimycotic cocktail, and then assayed as described next.

**NK activity (PINK method).** Yac-1 cells, which are target cells of NK cells, were suspended in PBS (1 × 10\(^6\) cells/ml) containing 40 μg/ml of 3,3’-diocetylxylo-carboxycamine perchlorate (DiO; Sigma, St. Louis, MO, USA) and incubated at 37°C for 10 min. The stained cells were washed with RPMI1640 and resuspended in 10% FBS-containing RPMI1640 (5 × 10\(^5\) cells/ml). The DiO-stained target cells (100 μl), splenocytes at various concentrations (100 μl), and 25 μg/ml of propidium iodide (PI; Molecular Probes, Eugene, OR, USA) were added to round-bottomed 96-well multiplates (BD Bioscience, Franklin Lakes, NJ, USA) which were centrifuged at 200 × g and cultured at 37°C in 5% CO\(_2\) for 4 h. The rate of spontaneous cell death was determined by culturing the target cells alone without adding effector cells. After the reaction, the DiO\(^+\)PI\(^+\) cells (dead target cells) were counted by using Epics XL (Beckman Coulter). The NK activity was calculated by using the following equation:

\[
\text{NK activity} (\%) = \frac{(\text{DiO}^+\text{PI}^+)/\text{(DiO}^+\text{PI}^- + \text{DiO}^+\text{PI}^+)} \times 100 - \text{rate of spontaneous cell death (\%).}
\]

MLR and CTL activity. Splenocytes (1 × 10\(^7\) cells/ml) from BALB/c mice were incubated in an RPMI1640 medium containing 50 μg/ml of mitomycin C (Kyowa Hakko Industry, Tokyo, Japan) at 37°C for 30 min. The medium was removed by centrifugation, and the cells were washed 4 times with the RPMI1640 medium. One ml of the mitomycin C-treated splenocytes (2.5 × 10\(^6\) cells/ml) and 1 ml of splenocytes (5 × 10\(^6\) cells/ml) from CY-treated C57BL/6 mice were mixed in a 12-well multiplate (BD Bioscience) and cultured at 37°C in 5% CO\(_2\) for 4 d. The non-adhering viable cells were fractionated by using Lympholyte M and then counted. CTL activity was measured by a modified PINK method, using P-815 cells as the targets.

**Flow cytometric analysis.** NK1.1\(^+\), CD4\(^+\) and CD8\(^+\) cells were respectively stained for 45 min with the PI-conjugated anti-mouse NK1.1 monoclonal antibody (Immunotech, Marseille, France), PI-conjugated anti-mouse CD3 monoclonal antibody (Immunotech), and FITC-conjugated anti-mouse CD8 monoclonal antibody (Immunotech). The cells were washed with PBS, and the NK1.1, CD3 and CD8 expression in lymphocytes was determined by using Epics XL (Beckman Coulter, Fullerton, CA, USA). The analysis was based on 15,000 cells gated as lymphocytes, the calculations being performed with EXPO32 software.

**Th1 and Th2 cytokine measurements.** Splenocytes (5 × 10\(^6\) cells/ml) from C57BL/6 mice were cultured
with a 10% FBS-containing RPMI1640 medium in 24-well multiplates (BD Bioscience). Concanavalin A (Con A; 2.5 μg/ml, Wako Pure Chemicals, Osaka, Japan) was added, and the cells were cultured for 24 h at 37 °C. The culture supernatant was collected, and IFN-γ and IL-4 were measured by using an OptEIA ELISA kit (BD Pharmingen, San Diego, CA, USA).

Anti-tumor model. MM 46 mammary carcinoma (1 × 10⁶ cells/ml) was inoculated intraperitoneally into C3H/HeN mice for a passage culture. The tumor cells were collected from the peritoneal cavity 7 d after inoculation. The cells were suspended in a 5% FBS-containing MEM medium (Nacalai Tesque) and then subcutaneously inoculated (2 × 10⁶ cells/0.2 ml/mouse) into the inguinal region of C3H/HeN mice. The mice were divided into four equal groups (10 animals/group) according to the tumor size 7 d after inoculation. Three groups were then administered with AIN-93M or a 2.5% G. lucidum AF-containing AIN-93M diet, and then 50 mg/kg or 150 mg/kg of CY (Endoxan®, Shionogi Pharmaceutical Co., Osaka, Japan) diluted with saline was intraperitoneally injected 14 d after inoculation. The body weight was measured 7, 14, 21, and 28 d after inoculation, and the tumor weight was determined 28 d after inoculation. To the other group was administered AIN-93M alone without CY as the control. The average tumor diameter was calculated by using a slide caliper as follows:

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\text{Tumor diameter (mm)} = \sqrt{\text{the largest diameter (mm)} \times \text{the smallest diameter (mm)}}
\]

Anti-metastatic model. The LLC metastasis experiment on CY-treated C57BL/6 mice was performed as shown in Fig. 1. LLC cells were subcutaneously injected into C57BL/6 mice. The solid tumor was excised 10 d later, and then treated with a 0.25% trypsin-Hanks solution at 37 °C. The viability of the cells was confirmed to be greater than 80%. The LLC cells were adjusted to 1 × 10⁶ cells/ml with a Hanks solution, and then 0.2 ml of the tumor cell solution was injected into the tail vein of C57BL/6 mice at 0.2 ml/head 3, 5 and 7 d after the CY administration (150 mg/kg). The control mice were fed with the AIN-93M diet for 1 week without CY administration. The lungs were excised 14 d after LLC inoculation and fixed with Bouin’s solution (a saturated picric acid aqueous solution:formalin: glacial acetic acid = 15:5:1). Metastases in the lung were determined by measuring the number of nodules in the fixed tissue.

Statistical analysis. One-way ANOVA was performed, followed by Fisher’s PLSD as a post-hoc test for 3-group comparisons. Student’s t-test was used for 2-group comparisons.

![Fig. 1. Scheme for the LLC Metastasis Model.](image)

**Fig. 2. Effects of G. lucidum AF on CY-Induced Body Weight Loss.**

C57BL/6 mice were fed on an AIN-93M diet or a 2.5% G. lucidum AF-containing AIN-93M diet for 1 w, and then 150 mg/kg of CY was intraperitoneally (i.p.) injected into each group of mice. Body weights were monitored daily for 8 d. Each value is the mean ± S.E. (n = 12). Asterisks indicate significant differences from the CY-treated group (*p < 0.05, **p < 0.01; Student’s t-test).
5 d after CY administration was highest in the *G. lucidum* AF-fed group, intermediate in the CY-treated group, and lowest in the control group, with significant differences being observed among these groups (Fig. 3B), although the proportion of NK1.1+ cells among splenocytes in these groups was nearly the same (6.4%, 7.3%, and 5.2%, respectively).

Lung lymphocytes were prepared 3, 5 and 7 d after CY administration, and the NK activity was measured. As shown in Fig. 4A, no significant differences were apparent in lung NK activity among the groups 3 d after CY administration. *G. lucidum* AF feeding alleviated the decrease in lung NK activity 5 d after CY administration (Fig. 4B), the activity in the *G. lucidum* AF-fed group 7 d after CY administration being higher than the control NK activity (Fig. 4C).

MLR was performed as described in the Materials and Methods section. The numbers of non-adhering cells 5 and 7 d after CY administration were significantly lower than in the control mice. However, the cell numbers recovered to the control level by 7 d after CY administration in the *G. lucidum* AF-fed groups (Fig. 5A).

When then CTL activities were measured by using the non-adherent cells after MLR, no significant differences were apparent among the groups 5 d after CY administration (Fig. 5B), although the CTL activity in the *G. lucidum* AF-fed group was significantly higher than that in any of the other groups 7 d after CY admin-
istration (Fig. 5C). The ratios of CD3+ and CD8+ cells among splenocytes in the control, CY-treated, and G. lucidum AF-fed groups at 7 d after CY administration were 16.9% and 4.3%, 3.8% and 1.4%, and 9.4% and 4.4%, respectively.

G. lucidum AF alleviated the decrease in ConA-induced IFN-γ production 7 d after CY administration (Fig. 6A). It also inhibited the abnormal increase (d 5) and decrease (d 7) in IL-4 level due to CY administration (Fig. 6B). In addition, the IFN-γ production by splenocytes not stimulated with Con A was beneath the quantifiable limit (data not shown).

Anti-tumor effects of G. lucidum AF and CY in MM 46 tumor-bearing mice

It was confirmed in a preliminary experiment that the anti-tumor effect of CY was not significant at a dose of 50 mg/kg or less (data not shown). We therefore evaluated the anti-tumor effect of CY (50 mg/kg) with or without G. lucidum AF with CY (50 mg/kg and 150 mg/kg). As shown in Fig. 7A, after the administration of CY at a dose of 150 mg/kg, the body weight of the mice decreased as a side effect, although no such body weight reduction was apparent in the other groups. Treatment with CY (50 mg/kg) plus 2.5% G. lucidum AF and CY (150 mg/kg) significantly inhibited the tumor growth from 17 d and 21 d after MM 46 tumor cell inoculation (Fig. 7B), and the respective final tumor weight was also decreased to about 47% and 50% of the control level (Fig. 7C).

Effect of G. Lucidum AF on lung metastasis in CY-treated mice

The lung metastasis experiment was performed as shown in Fig. 1. The number of metastases increased when LLC was injected into the tail vein of C57BL/6 mice 3 d or 5 d after CY administration. The number of metastases was similar to the control level when LLC was injected 7 d after CY administration. Pre-feeding the G. lucidum AF-containing diet suppressed the CY-induced metastasis of LLC. In particular, the number of nodules in the G. lucidum AF-fed group injected with LLC 7 d after CY administration was significantly lower than that in the AIN-93M diet group (Fig. 8A). Figure
Fig. 6. Changes of Cytokine Production from Splenocytes in CY-Treated C57BL/6 Mice.
C57BL/6 mice were fed on an AIN-93M diet or a 2.5% G. lucidum AF-containing AIN-93M diet for 1 w, and then 150 mg/kg of CY was i.p.-injected into each group of mice. Splenocytes were prepared 5 and 7 d after CY administration. The control mice were fed on the AIN-93M diet or 2.5% G. lucidum AF-containing AIN-93M diet for 1 w without the CY treatment, and then splenocytes were similarly prepared. The IFN-γ (A) and IL-4 (B) production was measured as described in the Materials and Methods section. Each value is the mean ± S.D. (n = 4). a, b, and c: significant difference between groups with different letters (p < 0.05, Student’s t-test (control) or one-way ANOVA followed by Fisher’s PLSD as a post-hoc test (5 and 7 d after CY administration)).

Fig. 7. Anti-Tumor Effects of G. lucidum AF and CY in Syngeneic MM 46 Tumor-Bearing Mice.
C3H/HeN mice were subcutaneously inoculated with MM 46 mammary carcinoma (2 × 10⁶ cells/0.2 ml/mouse) into the inguinal region. The mice were divided into 4 equal groups according to tumor size 7 d after inoculation. The G. lucidum AF (●) groups were then administered with the 2.5% G. lucidum AF-containing AIN-93M diet. CY was intraperitoneally injected 14 d after the inoculation. Changes in the mean body weight (A), tumor diameter (B), and tumor weight 28 d after tumor inoculation (C) were measured. Each value is the mean ± S.E. (n = 10). Asterisks indicate significant difference from the control group (*p < 0.05, **p < 0.01; one-way ANOVA, followed by Fisher’s PLSD as a post-hoc test).
8B shows typical findings for lung metastasis in the CY-induced mice with and without G. lucidum AF feeding.

**Discussion**

*G. lucidum* AF is a medical Chinese mushroom widely used in China, Korea, and Japan. We have reported in a previous study\(^\text{15}\) that G. *lucidum* AF exhibited anti-tumor activity against syngeneic MM 46 tumor-bearing C3H/HeN mice when a diet containing more than 2.5% of it was administered. We examined in this present study, the anti-tumor and anti-metastatic activities of G. *lucidum* AF (a 2.5%-containing diet) in CY-treated MM 46-bearing C3H/HeN mice and C57BL/6 mice which exhibit Th1-derived anti-tumor immunity.

We confirmed in a preliminary study that 150 mg/kg of CY suppressed the growth of MM 46 under the same experimental conditions as in a previous study,\(^\text{15}\) although anti-tumor effect was not enhanced even if the 2.5% G. *lucidum* AF-containing diet was combined with 150 mg/kg of CY (data not shown), since 150 mg/kg of CY itself had high anti-tumor activity. In the present study, we therefore examined the effectiveness of a concomitant administration of G. *lucidum* AF with CY at a non-effective dose (50 mg/kg), and confirmed a significant anti-tumor effect at an almost similar level to that with 150 mg/kg of CY (Fig. 7). On the other hand, 150 mg/kg, but not 50 mg/kg of CY caused body weight reduction. These findings suggest that the administration of G. *lucidum* AF may enable the application of smaller doses of CY and may thus alleviate the side effects of chemotherapy.

MM 46 carcinoma is moderately antigenic in C3H/HeN mice,\(^\text{17}\) and is attacked by NK cells.\(^\text{18}\) Thus, cellular immunity, including NK and CTL activity, is very important in manifesting the anti-tumor effect on MM 46 tumor growth. The population of CD3\(^+\) and CD8\(^+\) cells was down-regulated, and the mitogenic activity after MLR was also down-regulated 7 d after the CY treatment (Fig. 5A). However, the MLR-induced CTL activity was equal to the control level (Fig. 5C). These findings suggest that, although the T cell function was reduced, the CTL activity was maintained at the control level. On the other hand, despite the CD3\(^+\) population being down-regulated, the functions of T cells such as the CD8\(^+\) population, mitogenic activity (Fig. 5A), and IFN-γ induced activity (Fig. 6A) were maintained at the control levels by G. *lucidum* AF. These findings suggest that the CD8\(^+\) T cells were
highly activated, and maintained the general T cell functions and enhanced the CTL activity (Fig. 5C) in the *G. lucidum* AF-fed mice. The finding of up-regulation of the CD8$^+$ population by *G. lucidum* AF agrees with that of a previous study that used *G. lucidum* AF-fed MM 46-bearing C3H/HeN mice.15) These findings suggest of a previous study that used *G. lucidum* adminstered Furthermore, Vetvicka *G. lucidum* functions and enhanced the CTL activity (Fig. 5C) in the highly activated, and maintained the general T cell activity against MM 46 carcinoma of the $\beta$-(1→3)-$\beta$ glucan-containing polysaccharide, lenturon, was correlated with the augmentation of cellular and humoral immune reactions to the MM 46 antigen.17)

Various immune mechanisms inhibit LLC metastasis. For example, IL-2-activated NK cells and MHC-restricted CTL inhibited metastasis by LLC.5,22) Macrophage-activating $\beta$-glucans such as schizophyllan inhibited metastasis by the infiltration of CTL and macrophages into the tumor and regions with metastasis.28) In this study, we confirmed that *G. lucidum* AF tended to inhibit the increase in LLC metastasis induced by CY administration (Fig. 8). One mechanism for the inhibition of LLC metastasis by *G. lucidum* AF might involve lung NK activity, since, on d 7, the lung NK activity in the *G. lucidum* AF-fed group was significantly higher than that in the CY-administered group (Fig. 4C). However, no correlation was apparent between the anti-metastatic activity and lung NK activity by d 3 or d 5 after CY administration. Kimura *et al.* have reported that the triterpenoid fraction of *G. lucidum* inhibited angiogenesis and metastasis in LLC-implanted C57BL/6 mice,29) and Wang *et al.* have reported that a lanostane triterpenoid ganoderic acid Me purified from *G. lucidum* mycelia could inhibit lung metastasis of LLC through the up-regulation of IL-2 and IFN-$\gamma$, and activate NK activity.30) Furthermore, since the amount of the triterpenoid fraction in *G. lucidum* AF is higher than that in the normal type of *G. lucidum*,31) the triterpenoid fraction in *G. lucidum* AF might also be expected to exhibit anti-metastatic activity. The combination of several bioactive components in *G. lucidum* AF, such as $\beta$-glucans and triterpenoids, produced the anti-metastatic effects in CY-treated mice.

Lin has recently reviewed the broad immunomodulatory functions of *G. lucidum*, including promotion of the function of antigen-presenting cells, the mononuclear phagocyte system, humoral immunity, and cellular immunity.32) *G. lucidum* also inhibits the formation of oxidative stress-induced invasive cancers by inhibiting the transcription of NF-$\kappa$B and AP-1 mediated by the phosphorylation of extracellular signal-regulated protein kinase.33) Combinations of *G. lucidum* AF and various chemotherapeutic drugs can thus be expected to reduce adverse effects and to enhance anti-tumor effects, suggesting that immunotherapy with *G. lucidum* AF would be superior to anti-cancer therapy with a single recombinant cytokine.

In conclusion, *G. lucidum* AF ingestion before CY administration enhanced the anti-tumor and anti-metastatic effects by inhibiting the decrease in NK activity and stimulating the recovery of T cell functions. Chemotherapeutic drugs exhibit severe adverse effects that are difficult for patients, impairing their QOL and increasing the possibility of infection. Although it is difficult to completely cure cancer by the ingestion of such foods as *G. lucidum* AF, the combination of *G. lucidum* AF and chemotherapeutic drugs may yield
a more effective cancer treatment. Clinical studies will be needed for application of the accumulating evidence for the anti-tumor efficacy of *G. lucidum* AF.

**References**


