**Ganoderma lucidum** Extracts Inhibited Leukemia WEHI-3 Cells in BALB/c Mice and Promoted an Immune Response in vivo

Yung-Hsien Chang,1 Jai-Sing Yang,2 Jiun-Long Yang,3 Chang-Lin Wu,4 Shu-Jen Chang,5 Kung-Wen Lu,6 Jen-Jyh Lin,6 Te-Chun Hsia,6,7 Yi-Ting Lin,4 Chin-Chih Ho,4 W. Gibson Wood,8 and Jing-Gung Chung4,9,1

1Graduate Institute of Acupuncture Science, China Medical University, Taichung 404, Taiwan
2Department of Pharmacology, China Medical University, Taichung 404, Taiwan
3Graduate Institute of Chinese Pharmaceutical Science, China Medical University, Taichung 404, Taiwan
4Department of Biological Science and Technology, China Medical University, Taichung 404, Taiwan
5School of Pharmacy, China Medical University Hospital, Taichung 404, Taiwan
6Graduate Institute of Chinese Medical Science, China Medical University, Taichung 404, Taiwan
7Department of Internal Medicine, China Medical University Hospital, Taichung 404, Taiwan
8Department of Pharmacology, University of Minnesota, School of Medicine and Geriatric Research, Education and Clinical Center, VA Medical Center, Minneapolis, MN 55417, USA
9Department of Biotechnology, Asia University, Wufeng, Taichung 413, Taiwan

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**Ganoderma lucidum** (G. lucidum) is a medicinal mushroom having biological effects such as immunomodulation and anti-tumor actions. In China and many other Asian countries, *G. lucidum* is used as a folk remedy to promote health and longevity. Although many studies have shown that *G. lucidum* modulates the immune system, including, for example, antigen-presenting cells, natural killer (NK) cells, and the T and B lymphocytes, the effects of *G. lucidum* on the WEHI-3 leukemia cells in BALB/c mice are unclear. We attempted to determine whether *G. lucidum* would promote immune responses in BALB/c mice injected with WEHI-3 leukemia cells. The effects of *G. lucidum* on the survival rate of WEHI-3 leukemia cells injected into BALB/c mice were examined. It increased the percentages of CD3 and CD19, but decreased the percentages of Mac-3 and CD11b markers, suggesting that differentiation of the precursor of T and B cells was promoted but macrophages were inhibited. It decreased the weight of spleens as compared with control mice. It also promoted phagocytosis by macrophage from peripheral blood mononuclear cell (PBMC) and it also promoted natural killer cell activity. It decreased the percentage of leukemia cells in the spleens of mice before they were injected with WEHI-3 cells. Apparently, *G. lucidum* affects murine leukemia WEHI-3 cells in vivo.

**Key words:** Ganoderma lucidum; WEHI-3 cells; BALB/c mice; in vivo; phagocytosis

While there has been progress in treating leukemia, the discovery of more effective therapeutic agents is needed. Ganoderma lucidum (*G. lucidum*; GL) is a traditional Chinese medicine that has been used as a health tonic to promote longevity for more than two thousand years in China. GL has been reported to have potential immunomodulation and anti-tumor effects in both *in vitro* and *in vivo* models.1,2) Its major components are polysaccharides and triterpenes; GL polysaccharides exert anti-cancer effects indirectly by activation of the host’s immune responses, whereas GL triterpenes can kill cancer cells directly via direct cytotoxic effects3,4) and have been found to inhibit growth and cancer metastases in mice;5,6) polysaccharides increased levels of inflammatory cytokines7,8) and inhibited the growth of tumors in mice.5,6) Furthermore, GL also caused mitochondrial damage and apoptosis in human promyelocytic leukemia HL-60 cells.9) Overall, it has anti-proliferation effects via both promotion of cell cycle arrest and induction of apoptosis in several human cancer cell lines.10-15)

Although induction of apoptosis and possible signaling pathways involved in death receptor ligands (TNF-α and TRAIL) and the activation of caspase cascades in leukemia cells after treatment with GL have been reported,15) there is no available information to address the way GL affects leukemia cells through immune responses in vivo. The purpose of the present study was to determine whether GL would promote immune responses in BALB/c mice injected with WEHI-3 leukemia cells.

**Materials and Methods**

**Materials and reagents.** Crude extracts of GL were kindly offered by Dr. Yung-Hsien Chang (China Medical University, Taichung, Taiwan), 1

1 To whom correspondence should be addressed. Present address: Department of Biological Science and Technology, China Medical University, No. 91, Hsin-Shih Road, Taichung 404, Taiwan; Tel: +886-4-2205-3366 ex. 2161; Fax: +886-4-2205-3764; E-mail: jgchung@mail.cmu.edu.tw

Abbreviations: Con A, concanavalin A; CD marker, cluster of differentiation marker; FBS, fetal bovine serum; *G. lucidum*, Ganoderma lucidum; NK cell, natural killer cell; i.p., intraperitoneally; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cell
Taiwan). RPMI 1640, fetal bovine serum, penicillin-streptomycin, and L-glutamine were obtained from Gibco BRL (Invitrogen, Grand Island, NY, USA).

BALB/c mice. Fifty male BALB/c mice approximately 22–28 g in weight at 8 weeks of age were obtained from the Laboratory Animal Center, College of Medicine, National Taiwan University (Taipei, Taiwan).

Murine leukemia WEHI-3. Murine myelomonocytic leukemia cell line WEHI-3 was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were immediately plated onto 75-cm² cell culture flasks at 37 °C under a humidified 5% CO₂ atmosphere, and were grown in RPMI-1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml Penicillin, and 100 μg/ml Streptomycin.

GL treatment. Fifty BALB/c mice were randomly divided into 5 groups (10 mice per group), and were kept on a 12-h light/dark cycle at 25 °C. GL (3 and 6 mg/kg/d) was administered by gavage. Groups I and II were control and were given only distilled deionized water (DDW). Group III was injected with WEHI-3 cells (1 × 10⁵ cells/100 μl) i.p. Group IV was injected with WEHI-3 cells (1 × 10⁵ cells/100 μl) i.p., and then treated with GL (3 mg/kg) in DDW. Group V was injected with WEHI-3 cells (1 × 10⁵ cells/100μl) i.p. and then treated with GL (6 mg/kg) in DDW. The mice were treated daily for 2 weeks and then sacrificed.16,17

Liver and spleen tissues. All the mice (control and experimental groups) were weighed before whole blood was drawn. The tissues of liver and spleen were isolated and weighed for each animal.17,18

Histopathology. Spleen samples from each group (control and experimental animals) were fixed in 4% formaldehyde and embedded in paraffin. Sections (5 mm) were stained with hematoxylin and eosin following standard procedures.17–19

Immunofluorescence staining of blood cells. Approximately 1 ml of blood was collected from each animal, and the red blood cells were lysed with ammonium chloride, and then centrifuged at 1,500 rpm (1,000 × g) at 4 °C for 15 min to isolate white blood cells. The isolated cells were further examined for cell surface markers such as T cells (CD3), and B cells (CD19), monocytes, and macrophage (CD11b and Mac-3) (WEHI-3 is a myelomonocytic leukemia cell line) were stained with anti-CD3-FITC, CD19-PE, CD11b-FITC, and Mac-3-PE antibodies (BD Biosciences, San Jose, CA). Stained cells of each sample were washed with PBS and then analyzed to determine cell surface marker levels by flow cytometry (FACS Calibur™, Becton Dickinson, Franklin Lakes, NJ) as described previously.17,20,21

T and B cell proliferation examination. Splenocytes (1 × 10⁶/well) were isolated from the spleens of each mouse and 100 μl of RPMI-1640 medium was added. This was placed in 96-well plates and spleenocytes (1 × 10⁵) were plated on 96-well plates and splenocytes (1 × 10⁵) were added to each well, and this was incubated at 25 °C for 12 h. NK cell activation was determined following standard procedures.17–19

Phagocytic determination. Macrophages were isolated from the PBMC of each mouse (control and experimental groups). About 100 μl of PBMC and 50 μl of E. coli-FITC were added and this was shaken in a shaker bath for 30 min at 37 °C. The supernatant was discarded and the pellets were mixed with DNA staining, as described previously.17,20,21 according to the PHAGOTEST kit manufacturer’s instructions (Orpegen Pharma Gesellschaft für Biotechnologische, Heidelberg, Germany). Each sample was analyzed by flow cytometry with CellQuest software.

Natural killer cell activity. Splenocytes (2.5 × 10⁶) in 1 ml of RPMI-1640 medium were cultured on 24-well plates. YAC-1 cells were prepared following the guidelines (Sigma-Aldrich Corp., St. Louis, MO). In brief, YAC-1 cells in 15-ml tubes were washed twice with serum-free RPMI-1640 medium, then PKH-67/Dil.C buffer (Sigma-Aldrich) was added with thorough mixing for 2 min at 25 °C then 2 ml PBS was added. After 1 min, four milliliter of RPMI-1640 medium was added. Then the cells were incubated for 10 min at 1,200 rpm at 25 °C. The YAC-1 cells (2.5 × 10⁶) were plated on 96-well plates and splenocytes (1 × 10⁵) were added to each well, and this was incubated at 25 °C for 12 h. NK cell activation was determined by flow cytometry, as described previously.18,22

Statistic analysis. Data were expressed as mean ± SD, and the differences between control and GL treated groups were analyzed by Student’s t-test. *p < 0.05, **p < 0.001 were used as the significance levels.

Results

Effects of GL extracts on the survival of leukemic BALB/c mice

WEHI-3 leukemia mice were treated with 0, 3, or 6 mg/kg/d of GL for 2 weeks. The percent survival rate for each group is shown in Fig. 1. The control group (group I, not injected with WEHI-3 cells) had 100% survival. Group II (injected with WEHI-3 cells) had the lowest survival rate (30%). Group III (injected with WEHI-3 cells, then treated with 3 mg/kg of GL) had a higher survival rate than Group II. Group IV (injected with WEHI-3 cells, then treated with 6 mg/kg of GL) had the highest survival rate as compared with groups II and III (Fig. 1). For example, the survival rates of groups I, II, III, and IV treated for 28 d were 100, 50, 40 and 30% respectively.

Representative pictures of livers and spleens from BALB/c mice injected with WEHI-3 cells and/or treated with GL for 2 weeks

The BALB/c mice were injected with WEHI-3 cells for 2 weeks and/or co-treated with GL, blood was
collected, and the animals were sacrificed for examination of liver and spleen tissues. They were individually photographed and weighed (Fig. 2A). The weights of spleens are presented in Fig. 2B, and the pathological examination of the spleens is presented in Fig. 2C. The results indicate that GL decreased the weight of spleen tissues and mice in the control and GL treated groups after exposure to 3 and 6 mg/kg of GL, and the percentages of spleen weights decreased 20% and 19% respectively. As Fig. 2C indicates in the spleen tissues, there was marked expansion in red pulp (R) but the white pulp (W) showed little change. The neoplastic cells contained large irregular nuclei accompany with clumped chromatin and prominent nucleoli, abundant clear and light eosinophilic cytoplasm. Also, the number of megakaryocytes increased. In addition, the red pulp of the spleen indicated that some leukemic cells had already metastasized. If these leukemic cells had metastasized to the liver, immature ones entering the leukocyte were be found. Hematoxylin and eosin (HE) staining of the spleen section revealed infiltration of immature myeloblastic cells into splenic red pulp, which was reduced in the GL extracts-treated WEHI-3/BALB/c mice.

GL extracts affected cell surface markers of white blood cells from BALB/c mice

Blood was collected from the mice and the surface markers CD3, CD19, Mac-3, and CD11b were analyzed by flow cytometry. Percentage changes in cell markers of white blood cells are shown in Fig. 3A–D. GL increased the levels of CD3 (Fig. 3A) and CD19 (Fig. 3B) but reduced Mac-3 levels (Fig. 3C), and had little if any effect on CD11b (Fig. 3D).

GL extracts affected the cell proliferation in Con A-stimulated splenocytes from WEHI-3 injected BALB/c mice in vitro

Splenocytes were isolated from each group of animals following treatment with various doses of GL. Con A or LPS was used to promote proliferation of T and B cell. The results are shown in Fig. 4A and B, which indicate that Con A stimulated T cell proliferation at 72 h of treatment but LPS stimulated B cell proliferation at 120 h of treatment because B cells numbers in PBMC were less than T cells ones. Therefore, LPS-stimulated B cell proliferation needs longer periods of time. In this study, treatment of the non-stimulating group showed no significant differences (Fig. 4B), and the reason may be that GL extracts lacked pharmacological action in splenocytes from WEHI-3 leukemic mice in the 120 h treatment. The results also indicated that the GL extracts promoted proliferation only of Con A-stimulated splenocytes and had effects in the LPS-stimulated ones. Consequently, it only promoted the T cell proliferation after treatment with GL extracts.

GL extracts affected phagocytosis by macrophages in the PBMC from BALB/c mice

PBMCs were collected from each animal and were analyzed for phagocytosis by macrophages. The data indicate (Fig. 4) that the GL extracts stimulated phagocytic activity by macrophages, and that these effects were dose-dependent.
Several studies have indicated that *Ganoderma lucidum* (GL) extracts have numerous biological effects, including anti-cancer activity,\textsuperscript{4,11,15,24} and GL mycelia have been found suppress the proliferation of human and mouse carcinoma cell lines.\textsuperscript{25,26} Moreover, many studies have indicated that GL extracts have anti-cancer activity\textsuperscript{27} and that lucidinic acids B isolated from GL induces apoptotic cell death in human leukemia HL-60 cells by activation of caspase-9/-3 through the mitochondria-dependent pathway.\textsuperscript{28} However, the immune functions of leukemic mice after exposure to GL extracts still did not exact clear. The purpose of the

**Discussion**

Several studies have indicated that *Ganoderma lucidum* (GL) extracts have numerous biological effects, including anti-cancer activity,\textsuperscript{3,11,15,24} and GL mycelia have been found suppress the proliferation of human and mouse carcinoma cell lines.\textsuperscript{25,26} Moreover, many

Fig. 3. GL Affected Cells Markers of White Blood Cells from BALB/c Mice.

The mice were injected with WEHI-3 cells i.p. for 2 weeks and treated without and with various doses of GL for 2 weeks. Blood was collected from the animals, and was analyzed for cell markers such as CD3 (A), CD19 (B), Mac-3 (C), and CD11b (D) by flow cytometry, as described in "Materials and Methods." Each point is mean ± S.D. *p < 0.05 and **p < 0.001 (n = 10).

Fig. 4. GL Extracts Affected the Cell Proliferation of Con A-Stimulated Splenocytes from WEHI-3 Injected BALB/c Mice in Vitro.

Splenocytes were isolated from each group of WEHI-3 injected BALB/c mice after treatment with various doses of GL extracts, and then Con A (A) stimulation for 72 h or LPS (B) stimulation for 120 h for T and B cell proliferation examination with a CellTiter 96 assay kit, as described in "Materials and Methods." Each point is mean ± S.D. ***p < 0.001 (n = 10).
present study was to determine whether GL extracts would promote immune response and inhibit leukemia in mice after injection with WEHI-3 cells. The results indicated that GL extracts can: (i) increase the survival rate of WEHI-3 leukemic mice; (ii) enhance the cell surface marker of T cells, and B cells in WEHI-3 leukemic mice; (iii) promote the proliferation of splenocytes from WEHI-3 leukemic BALB/c mice; and (iv) increase phagocytosis of macrophage and NK cell activity in WEHI-3 leukemic mice. It has been reported that NK cells are involved in nonspecific anti-virus and anti-tumor defense in human cancer cells, and that NK cells contribute to the elimination of transformed tumor cells. 200 Our results (Fig. 6) indicated that after treatment with GL extracts, NK cell activity increased. The rationale for using WEHI-3 leukemic mice is that this model has been used as an animal model of leukemia in vivo and application for other anti-cancer agents.

Our data indicate that GL extracts significantly decreased the average size and weight of the spleens of BALB/c mice injected with WEHI-3 leukemia cells. The GL extracts also decreased the percentages of Mac-3 and CD11b, but it promoted CD3 and CD19 in the blood. The stimulating effects of GL extracts on the CD3 and CD19 markers indicate it may promote the numbers of T and B cells respectively. GL extracts increased phagocytosis by macrophages in PBMC and promoted natural killer cell activity of the blood. These effects were confirmed with agents promoting immune responses that tend to increase phagocytosis by macrophages and the activities of natural killer cells. GL promoted the differentiation of monocytes to activated peritoneal macrophages, this shows that Mac-3 decreased while macrophage phagocytosis increased. In this study, GL extracts decreased leukemia-related spleen growth. One of the notable characteristics of the WEHI-3 leukemia model is elevation of peripheral monocytes and granulocytes with immature morphology and enlarged and infiltrated spleens as compared with normal mice. 200 Overall, our findings indicate that GL extracts promoted immune responses in a BALB/c mouse orthotopic model of leukemia in vivo.

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References


