Immunoactivation and Antiproliferation of Tumor Cells by Active Compounds of *Ganoderma lucidum*

HUA FAN[1] JINGSONG ZHANG[1,2] QINGJIU TANG[1,2] CHANGYAN ZHOU[1,2] WIE JIA[1,2] MARTIN ZIMMERMAN-KORDMANN[1] & WERNER REUTTER[1]

[1]Institut für Molekularbiologie und Biochemie, Campus Benjamin Franklin, Charité - Universitätsmedizin Berlin, Arnimallee 22, 14195 Berlin-Dahlem, Gemany; and [2]Shanghai Academy of Agricultural Sciences, 2901 Beidi Road, Shanghai 201106, China. E-mail: hua.fan@charite.de

Abstract: *Ganoderma lucidum*, a traditional Chinese medicine, harbors a well-known capacity to modulate immunoactivity and inhibit tumour cell growth. In this study, the bioactive fractions responsible for stimulation of immunoactivity and for induction of the apoptosis of tumor cells were isolated from *G. lucidum* by different chromatographic methods and their functional mechanisms were investigated by experiments *in vitro* and *in vivo*. A bioactive fraction, GLIS, was isolated from the fruiting body of *G. lucidum*. GLIS is a proteoglycan and has a carbohydrate:protein ratio of 11.5:1. GLIS activated both mouse spleen lymphocytes (MSLs) and human peripheral blood lymphocytes (HPBLs) and stimulated the proliferation of these lymphocytes. After stimulation with GLIS, both MSLs and HPBLs showed a significantly increased production of IFN-γ and IL-2. The percentage of B cells was increased. The B cells were enlarged, expressed CD71 and CD25 on the cell surface, and showed a significant increase in the secretion of immunoglobulins. In addition, GLIS also markedly triggered the activity of human peripheral NK cells for cytotoxicity of leukemia K562 cells. Moreover, GLIS stimulated mouse bone marrow-derived macrophages (MBMMs) to increase the secretion of IL-1ß, TNF-α and reactive nitrogen intermediate (RNI) production. The percentage of phagocytosis of the macrophages was significantly enhanced in the presence of GLIS. The crude extract from *G. lucidum* inhibited the proliferation of different tumor cell lines, and fractions LZ-2-2 and LZ-DW-2-a-3 were found to be the inhibitory fractions. They arrested SW620 tumor cells in the G0 phase, inhibited the cell cycle from G2/M to G1 phase and induced apoptosis of SW620 cells in a dose-dependent manner. In conclusion, GLIS is a new immune stimulating factor from *G. lucidum*. It simulates both humoral and cellular immune activities. LZ-2-2 and LZ-DW-2-a-3 are new apoptotic inducers of tumor cells. *G. lucidum* is a useful complementary medicine for tumor therapy.

Key words: *Ganoderma lucidum* (GL), immunoactivation, proteoglycan, anticancer, apoptosis

1 Introduction

Fungi are an important source of material in Traditional Chinese Medicine (TCM). Extracts from about 200 species of fungi have been shown to stimulate immunoactivity and to inhibit the growth of different kinds of tumors.[1-5] *Ganoderma lucidum* (GL), called 'Lingzhi' in China and 'Reishi' in Japan, is a lamella-less basidiomycetous fungus belonging to the family Polyporaceae. The use of this bracket fungus dates back more than 4000 years and is recorded in the TCM book "Benzhao Gangmu" written by Li Shizhen (1518-1593) in the Ming dynasty. It has been used in both Chinese and Japanese traditional medicine for the treatment of different diseases .[6]

Many studies on the various therapeutic effects of GL have been reported. Extracts of GL generally support health and show a hepatoprotective effect.[7] Hsu et al.[8] reported that the administration of GL extracts enhanced the recovery of body weight and the normalization of hemograms of mice suffering from radiation

damage. Recently, many studies have demonstrated that extracts of *G. lucidum* has therapeutic effects on the activation of immunosystem, inhibition of tumor cell growth and protection of radiation damage.[9-12] It has been also reported that the extracts of *G. lucidum* have anti-HIV-1,[13] anti-platelet aggregation,[14] hepatoprotective,[15] anti-microbial,[16] anti-inflammatory[17] and anti-allergic activities.[18,19]

Although many studies have been reported on the therapeutic effects of GL extracts, the active compounds have not been structurally characterized, and the mechanism of their functions is not known. The search for active ingredients and the investigation of the functional mechanisms of natural products used in TCM are becoming increasingly important. In order to understand the therapeutic mechanisms of *G. lucidum*, we have been involved in research on medicinal properties of *G. lucidum* for the past eight years. Different chromatographic methods were used for separating biologically active compounds. Two experimental systems have been established in our laboratory for high-throughput screening and investigation of the molecular mechanisms of the active fractions *in vitro*. One fraction, GLIS, with immunoactivation activity and two fractions with activity for inducing the apoptosis of tumor cells were isolated and characterized, and their functional mechanisms investigated by molecular biological and cell biological methods.

2 Results

2.1 Isolation and characterization of GLIS, LZ-2-2 and LZ-DW-2-a-3

GLIS, LZ-2-2 and LZ-DW-2-a-3 were isolated from crude extracts of fruiting bodies of *G. lucidum* by a series of different chromatographic steps. HPLC-analysis showed that GLIS has the molecular weight of 2000 kDa, while LZ-2-2 and LZ-DW-2-a-3 are smaller with molecular weights < 5 kDa. Analysis of the content of carbohydrate and protein in GLIS showed a carbohydrate to protein ratio of 11.5 to 1, indicating that GLIS is a proteoglycan.

2.2 Activation and proliferation of lymphocytes stimulated by GLIS

Lymphocytes were prepared from the spleens of normal (MSLs) and tumor bearing mice (TMSLs), and human peripheral blood (HPBLs). After stimulation with GLIS, the rate of proliferation of MSLs and TMSLs was increased by between two- and five-fold in a dose-dependent manner. TMSL responded more strongly than MSLs to this stimulus. The rate of proliferation of HPBLs was increased by only 50 to 100%. It was observed that some of the lymphocytes were enlarged after stimulation with GLIS. Flow cytometry analysis showed that most of the enlarged cells in both MSLs and HPBLs are B cells. The percentage of B cells in MSL populations was increased from 25% to 72%. They could be labelled with anti-CD25 and anti-CD71 mAbs, indicating that these B cells were activated and in proliferation.[22]

2.3 Secretion of cytokines and production of immunoglobulins by lymphocytes after stimulation with GLIS

The secretion of different cytokines following stimulation of lymphocytes with GLIS was analyzed by ELISA. Only very low levels of IL-2 were found in MSL culture media 24 h after exposure to GLIS, whereas the production of IL-2 was increased significantly after 48 h stimulation by GLIS. IL-2 production by TMSLs showed only a slight increase after different periods of stimulation by GLIS. In comparison with MSLs, HPBLs secreted significant amounts of IL-2 and markedly increased IFN-γ after stimulation with GLIS. In contrast, GLIS had no significant influence on IL-4 production by MSLs.

To study the response of B cells to GLIS, the production of total immunoglobulins by MSLs and TMSLs was analyzed by ELISA. Both MSLs and TMSLs produced significant amounts of immunoglobulins after stimulation by GLIS. Immunoglobulin production increased daily until the eighth day. TMSLs produced much more immunoglobulins (up to 2,700 ng/ml) than MSLs (up to 700 ng/ml).[22]

2.4 Increase in cytolytic activity of NK cells stimulated by GLIS

The effect of GLIS on the cytolytic activity of human peripheral blood NK cells was studied by LDH release assay. Human leukemia cell line K562 was used as target. The result showed that the cytolytic activity of NK cells was increased dramatically (up to 60%) after stimulation with GLIS in a dose-dependent manner.

2.5 Enhancing the activity of macrophages stimulated by GLIS

Mouse bone marrow-derived macrophages were prepared. After stimulation with GLIS, the macrophages were elongated, spread out and produced a large amount of IL-1ß , TNF-γ and reactive nitrogen intermediate (RNI). The percentage of phagocytosis of macrophages was increased from 25% to 80% in the presence of GLIS. In addition, GLIS also triggered the activation of macrophages for tumor cytotoxicity.[20]

2.6 Inhibition of proliferation of tumor cells by LZ-2-2 and LZ-DW-2-a-3

The antiproliferative capacity of crude extract of *G. lucidum* was tested using 11 tumor cell lines. The crude extract strongly inhibited the proliferation of human T-cell leukemia Jurkat, human myelogenous leukemia K562, human colon carcinoma SW620 cells, LS180 and human pancreas tumor QGP-1 cells. The extract did not inhibit the growth of neuroendocrine carcinoid BON, human epithelioid carcinoma Panc-1 and human hepatocellular carcinoma HUH7 cells. A detectable but less-marked inhibitory effect on cell proliferation was observed in the case of human breast adenocarcinoma MCF7 and human colon adenocarcinoma Caco-2 cells.[21, 23, 24]

To screening the biological active compounds, the crude extract was divided into two parts by dialysis, and then further fractionated using different chromatographic procedures. The different fractions obtained were tested for their anti-proliferation effect on human colon carcinoma SW620 cells. We found that the inhibitory effects were present in the LZ-2-2 and LZ-DW-2-a-3 fractions.

2.7 Induction of apoptosis of SW620 colon carcinoma cells by LZ-2-2 and LZ-DW-2-a-3

Light microscopy revealed that the SW620 cells formed apoptotic bodies *in situ* after incubation of with LZ-2-2 or LZ-DW-2-a-3. To confirm if the SW620 cells underwent apoptosis, the cells were stained with Annexin V-FITC conjugate. Fluorescence microscopy revealed that some cells had been labelled by Annexin V-FITC suggesting that these cells had undergone apoptosis induced by LZ-2-2 or LZ-DW-2-a-3.

SW620 cells were treated with different concentrations of crude extract, LZ-2-2 or LZ-DW-2-a-3 and DNA fragmentation was measured by flow cytometry. Our results showed that LZ-2-2 and LZ-DW-2-a-3 induced apoptosis of SW620 cells in a dose-dependent manner. At a concentration of 600 µg/ml, crude extract resulted in 28%, while LZ-2-2 and LZ-DW-2-a-3 resulted in 39.5% and 46.1% cell apoptosis, respectively.

2.8 Influence of LZ-2-2 and LZ-DW-2-a-3 on the cell cycle of SW620 cells

In order to investigate the influence of LZ-2-2 and LZ-DW-2-a-3 on the cell cycle of SW620 cells during their apoptotic processes, the distribution of cellular DNA content was analyzed by flow cytometry. After SW620 cells were treated with LZ-2-2 or LZ-DW-2-a-3, the percentage of cells in the G1 phase decreased in a dose-dependent manner; the percentage of cells in the G2/M phase was only slightly reduced, whereas those in the S phase decreased. The results suggest that SW620 cells were arrested in the G0 phase, and could not transit from

G2/M phase to G1 phase.

3 Conclusions

G. lucidum is rich in polysaccharides. A biological active proteoglycan fraction, GLIS, was isolated from crude extracts of fruiting bodies of *G. lucidum*. GLIS could stimulate both humoral and cellular immune responses and thereby increase immune activity. GLIS stimulates the lymphocytes to secrete cytokine, such as IL-2 and IFN-γ and to produce significant amounts of immunoglobulins. The phagocytotic activity of macrophages and activity of NK cells were increased markedly after stimulation by GLIS. Two biological active fractions, LZ-2-2 and LZ-DW-2-a-3, were shown to inhibit the proliferation of tumor cells. They could induce the apoptosis of colon carcinoma SW620 cells. *G. lucidum* is a useful complementary medicine for tumor therapy.

Acknowledgement

This work was supported by the Deutsche Forschungsgemeinschaft Bonn (Graduiertenkolleg 276/2), the Sonnenfeld-Stiftung Berlin, Fonds der Chemischen Industrie, Frankfurt/Main and Fonds der Gesellschaft für Biologische Krebsabwehr e. V. (GfBK).

References

[1] Pezzuto JM. Plant-derived anticancer agents. Biochem. Pharmacol. 1997, 53:121-33.
[2] Chang R. Functional properties of edible mushrooms. Nutr. Rev. 1996, 54:S91-93.
[3] Chaux P, Hammann A, Martin F, *et al. In vitro* cytotoxicity of *Ganoderma lucidum* on oral cancer cells. Eur. J. Immunol. 1993, 23:2517-25.
[4] Wang SY, Hsu ML, Hsu HC, *et al.* The anti-tumor effect of *Ganoderma lucidum* is mediated by cytokines released from activated macrophages and T lymphocytes, Int. J. Cancer, 1997, 70:699-705.
[5] Yun TK, Kim SH, Lee YS. Trial of a new medium-term model using benzo(a)pyrene induced lung tumor in newborn mice. Anticancer Res. 1995, 15:839-845.
[6] Maruyama H, Yamazaki K, Murofushi S, *et al.* Antitumor activity of *Sarcodon aspratus* (Berk.) S. Ito and *Ganoderma lucidum* (Fr.) Karst. J. Pharmacobiodyn. 1989, 12:118-123.
[7] Kim DH, Shim SB, Kim NJ, *et al.* Beta-glucuronidase-inhibitory activity and hepatoprotective effect of *Ganoderma lucidum.* Biol. Pharm. Bull. 1999, 22:162-164.
[8] Hsu HY, Lian SL, Lin CC. Radioprotective effect of *Ganoderma lucidum* (Leyss. ex. Fr.) Karst after X-ray irradiation in mice. Am. J. Chin. Med. 1990, 18:61-69.
[9] Li D, Yang SL, Lian B. Exploration of clinical study of antileukemia cell drug-resistance by traditional Chinese medicine. Chung Kuo Chung Hsi I Chieh Ho Tsa Chih, 1995, 15:636-7.
[10] Pisha E, Chai H, Lee IS, *et al.* Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. Nat. Med. 1995, 1:1046-51
[11] Hoessel R, Leclerc S, Endicott JA, *et al.* Indirubin, the active constituent of a Chinese antileukaemia medicine, inhibits cyclin-dependent kinases. Nat. Cell Biol. 1999, 1:60-7.
[12] Wasser SP, Weis AL. Therapeutic effects of substances occurring in higher Basidiomycetes mushrooms: a modern perspective. Crit. Rev. Immunol. 1999, 19:65-96.
[13] el-Mekkawy S, Meselhy MR, Nakamura N, *et al.* Anti-HIV-1 and anti-HIV-1-protease substances from *Ganoderma lucidum.* Phytochemistry, 1998, 49:1651-1657.
[14] Su CY, Shiao MS, Wang CT. Differential effects of ganodermic acid S on the thromboxane A2- signaling pathways in human platelets. Biochem Pharmacol. 1999, 58:587-595.
[15] Kim DH, Shim SB, Kim NJ, *et al.* Beta-glucuronidase-inhibitory activity and hepatoprotective effect of *Ganoderma lucidum.* Biol. Pharm. Bull. 1999, 22:162-164.
[16] Yoon SY, Eo SK, Kim YS, *et al.* Antimicrobial activity of *Ganoderma lucidum* extract alone and in combination with some antibiotics. Arch. Pharm. Res. 1994, 17:438-442.
[17] Lin JM, Lin CC, Chiu HF, et al. Evaluation of the anti-inflammatory and liver-protective effects of *Anoectochilus formosanus*, *Ganoderma lucidum* and *Gynostemma pentaphyllum* in rats. Am. J. Chin. Med. 1993, 21:59-69.
[18] Tasaka K, Mio M, Izushi K, *et al.* Anti-allergic constituents in the culture medium of *Ganoderma lucidum.* (II). The inhibitory effect of cyclooctasulfur on histamine release. Agents Actions, 1988, 23:157-160.
[19] Tasaka K, Akagi M, Miyoshi K, *et al.* Anti-allergic constituents in the culture medium of *Ganoderma lucidum.* (I). Inhibitory effect of oleic acid on histamine release. Agents Actions, 1988, 23:153-156.
[20] Tang QJ, Zhang JS, Pan YJ, *et al.* Activation of mouse macrophages by the alkali-extracted polysaccharide from spore of *Ganoderma*

lucidum. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi, 2004, 20:142-4.
[21] Tang QJ, Zhang JS, Pan YJ, *et al.* Immuno-stimulation and proliferation-inhibition for tumor cells by several crude extracts from medicinal fungi. Acta Edulis Fungi, 2003, 10:1-6.
[22] Zhang JS, Tang QJ, Zimmerman-Kordmann M, *et al.* Activation of B lymphocytes by GLIS, a bioactive proteoglycan from *Ganoderma lucidum.* Life Sci. 2002, 71:623-638.
[23] Zhang JS, Fan H, Li YX, *et al.* Effects of four extracts from medicinal fungi on different tumor cells. Nat. Prod. Res. Devel. 2000, 12:27-31.
[24] Li YX, Fan H, Zhang JS, *et al.* In vitro evaluation of anticancer activity of extracts from Chinese medicinal herbs. J. China Pharmaceutical University, 1999, 30:37-42.