Ganoderma lucidum inhibits tumour cell proliferation and induces tumour cell death

Yi-Zhen Xiea,1, Sen-Zhu Li a,1, Albert Yee c, David P. La Pierre c,d, Zhaoqun Deng c, Daniel Y. Lee c,d, Qing-Ping Wu a, Qi Chen a, Chong Li a, Zhi Zhang a, Jun Guo a, Zide Jiang b, Burton B. Yangc,d,∗

a Guangdong Institute of Microbiology, Guangdong Academy of Sciences, 100 Central Xian-Lie Road, Guangzhou, China
b Department of Plant Pathology, South China Agricultural University, 483 Wu-Shan Road, Guangzhou, China
c Sunnybrook Health Sciences Centre, 2075 Bayview Avenue, Toronto, Canada M4N 3M5
d Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ont., Canada

Received 18 March 2005; received in revised form 8 October 2005; accepted 11 October 2005

Abstract

Ganoderma lucidum, a traditional Chinese medicinal fungus, has been a favourite remedy in oriental medicine for centuries. The objective of this study is to analyze whether G. lucidum affects cancer cell proliferation and cell death. Malignant human breast carcinoma cells were used in our studies. Different preparations of G. lucidum spores were added to the cancer cells at a final concentration of 1 mg/ml followed by incubation of the cultures for two days. Treatment with G. lucidum resulted in tumour cells detachment from the tissue culture plates and death. The proliferation of the adherent cells was also inhibited. The experiments indicated that the inhibitory effects of G. lucidum on cancer cell growth were sporoderm-broken spores (broken by enzymatic method) > sporoderm-broken spores (broken by physical method) > intact spores > buffer control. The polysaccharides isolated from G. lucidum cultivated with wood-logs exhibited the greatest inhibitory effect on cell proliferation, which was concentration-dependent. These results were confirmed by trypan blue staining and MTT assay. The inhibitory effect of G. lucidum on cell proliferation appeared to occur through the Erk pathway: The expression of Erk was reduced in the presence of G. lucidum polysaccharide.

© 2006 Elsevier Inc. All rights reserved.

Keywords: Polysaccharide; Spore; Proliferation; Cancer; Erk activation

1. Introduction

Ganoderma lucidum is a favourite remedy in oriental medicine for centuries. Its fruiting body is called “Lingzhi” in China and “Reishi” in Japan. For hundreds of years, this mushroom has been regarded as a traditional Chinese medicine, or a folk medicine, used for the prevention and treatment of many human diseases. The major bioactive components in G. lucidum are G. lucidum polysaccharides, ganoderic acid (triterpene), and adenosine. While the polysaccharides are the major source of its biological activity and therapeutic use [1–5], ganoderic acid possesses interesting anti-tumour and anti-HIV-1 activities [6,7]. To date, more than 100 species of oxygenated triterpenes have been isolated from this fungus, many of which have been identified only in this species [8]. There have been a few reports on biological activities including inhibition of histamine release [9], immunomodulatory activity [10], cytokine production [11], and differentiation-inducing activity [12].

Traditionally, the fruiting body has been the only part of G. lucidum used for medical purposes. However, it has recently been recognized that the spores of G. lucidum are more potent [13]. As a result of their containing some unique components, the spores have been shown to be much more effective in disease treatment. Although the sporoderm-broken spores are more effective than the intact spores, there are far fewer publications on the roles of sporoderm-broken spores, and
the polysaccharides in the spores have been less studied due to the difficulty in breaking the sporoderm [14]. With the improvement of cultivation techniques, it has become possible to harvest spores. We have recently developed an enzymatic method to digest the sporoderm and obtain a large quantity of sporoderm-broken spores of *G. lucidum*, and we have isolated the polysaccharides from different types of fruiting bodies of *G. lucidum*.

This study was designed to investigate the roles of the sporoderm-broken spores on the growth of human tumour cells. We demonstrated that different preparations of *G. lucidum* spores were able to inhibit cancer cell proliferation and induced cell death. Nevertheless, the sporoderm-broken spores produced greater effects than the intact spores. Furthermore, sporoderm-broken spores generated by enzymatic methods produced the best results. Interestingly, the polysaccharides isolated from *G. lucidum* cultivated from wood-logs exhibited the greatest inhibitory effect on cell proliferation and cell death as compared with polysaccharides isolated from other sources. The inhibitory effect of *G. lucidum* on cell proliferation appeared to occur through the Erk pathway.

2. Materials and methods

2.1. Materials

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), Hank’s balanced salt solution (HBSS), and trypsin/EDTA, were purchased from invitrogen (Burlington, ON, Canada). The ECL Western blot detection kit was from Amersham. Antibodies against Erk and β-actin were from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody was from Sigma (St. Louis, MO). Tissue culture plates were from Nunc Inc. All chemicals were from Sigma. Jurkat cells were obtained from the American Type Culture Collection (Rockville, MD).

High quality and purity of *G. lucidum* spores and the fruiting body of *G. lucidum* were identified and selected for use by the experts from the Center for Research and Development of Edible Fungi, Guangdong Institute of Microbiology, Guangdong Academy of Sciences. The sporoderm of *G. lucidum* spores were broken using physical methods by grinding the spores with an ultra smashing machine or enzymatic methods. The enzymatic method was conducted by inoculating the heat-treated *G. lucidum* spores with *G. lucidum* mycelia, which were obtained from germinating spores. The mycelia released different types of enzymes which digested the sporoderm of the spores. In this way, the bioactive components were slowly released.

The polysaccharide of *G. lucidum* was extracted from the fruiting body using hot water, concentrated and powdered. These procedures were performed at the Center for Research and Development of Edible Fungi, Guangdong Institute of Microbiology.

Commercial products of *G. lucidum* were either purchased from the Greater Toronto Area or provided by Guangdong Yue-Wei Edible Fungi Technology Co. Ltd.

2.2. Cell counting

Cells were maintained in DMEM containing penicillin, streptomycin, and 10% fetal bovine serum (FBS). The cells were seeded on 12-well tissue culture plates at a density of 1 × 10^5 cells/well in DMEM containing 10% serum. The isolated components, total extract of sporoderm-broken spores of *G. lucidum* were added to the cultures at different concentrations (0–2 mg/ml). The buffer (PBS) used to dissolve the products of *G. lucidum* served as a control. The cultures were maintained in a tissue culture incubator at 37 °C containing 5% CO₂ for two days. Cells were harvested and the cell number was determined with a Coulter counter as described [15,16].

2.3. Trypan blue staining

Cells were seeded on 12-well tissue culture plates at a density of 1 × 10^5 cells/well in DMEM containing 10% serum. *G. lucidum* products were added to the cultures, which were maintained in a tissue culture incubator at 37 °C containing 5% CO₂ for two days. Cells were harvested and mixed with trypan blue (invitrogen) in a 1:1 ratio for exclusion staining [17,18]. Dead cells were stained as blue, while living cells were not stained by the dye due to the presence of the intact cell membrane. The number of living cells was determined with a Coulter counter. Each experiment was repeated three times.

2.4. MTT assay

Cell growth was determined either by direct cell counting or by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. Cells were seeded on 96 well plates at a density of 1 × 10^5 cells/well in DMEM containing 10% serum. *G. lucidum* products were added at different concentrations to the cultures, which were maintained in a tissue culture incubator at 37 °C containing 5% CO₂. The number of metabolically active cells was measured using an MTT cell proliferation kit I (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. Briefly, MTT labeling solution was added to the culture medium at the end of each experiment and the cells were further incubated for 3h. Mitochondrial dehydrogenase activity reduced the yellow MTT dye to a purple formazan, which was then solubilized by overnight incubation with
MTT solubilizing reagent (10% SDS in 0.01 HCl) and absorbance was read at 595 nm on an enzyme linked immunosorbent assay (ELISA) plate reader (Bio-Tek Instruments Inc.).

2.5. Protein extraction and Western blot analysis

After being treated with polysaccharide, the cells were washed twice with ice-cold phosphate buffer saline (PBS) and lysed with RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate and 0.1% SDS) containing 1 mM DTT, 1 mM Na3VO4, 5 mM NaF, 100 mM EDTA, 10 mg/ml aprotinin, and 100 mM PMSF. The buffer system was 1x-Tris/glycine buffer (Amresco) containing 1% SDS. Separated proteins were transblotted onto a nitrocellulose membrane (Bio-Rad) in 1x TG buffer (Amresco) containing 20% methanol. The membrane was blocked in TBST (10 mM Tris–Cl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 10% non-fat dry milk powder (TBSTM) for 1 h at room temperature, and then incubated for 4 °C overnight with primary antibody, prepared in TBSTM. The membranes were washed with TBST (3 × 30 min) and then incubated for 1 h in TBSTM with goat anti-mouse secondary antibody conjugated to horseradish peroxidase. After washing as above, the bound antibody was visualized with an ECL kit according to the manufacturer’s instructions. The Western blot analysis technique is routinely used in our laboratory [19–22].

2.6. Statistical analysis

Differences among treatment means were determined by Student’s t-test. P < 0.05 was considered significant.

3. Results and discussion

3.1. G. lucidum products inhibit cancer cell growth

Products of the micro-organism G. lucidum are known to help in improving conditions of cancer patients [23]. G. lucidum is widely taken by patients with different types of cancers in advanced stages, when the cancers become too advanced for surgery, chemotherapy, or radiotherapy, especially in oriental countries. For those who have gone through one of these therapeutic treatments, G. lucidum is used as a dietary supplement for an alternative therapy. The outcomes have been encouraging. In clinical studies at the Sloan-Kettering Cancer Center, it was reported that applications of G. lucidum should be studied and considered for (1) chemoprophylaxis of cancer in individuals at high risk for developing cancer; (2) adjuvant use in the prevention of metastasis or recurrence of cancer; (3) palliation of cancer-related cachexia and pain; and (4) adjunctive use with concurrent chemotherapies to reduce side-effects, maintain leucocyte counts, and allow a more optimal dosing of chemotherapeutics. We hypothesize that G. lucidum improves the health of cancer patients by modulating the activities of cancer cells. The objective of this study is to analyze whether various preparations of G. lucidum affect cancer cell proliferation and cancer cell death.

We have thus developed a model of cancer cell culture to study the effect of G. lucidum on mediating cell activities. Using human breast malignant carcinoma cells MT-1 [24], maintained as monolayer cultures on tissue culture plates, we examined a large number of G. lucidum products obtained commercially. These products were imported from China (Mainland and Hong Kong), Japan, the United States, and New Zealand or produced in Canada. The G. lucidum products were suspended in PBS (20 mg/ml), followed by incubation at 100 °C for 60 min. The preparations were added to the cell cultures at the amount of 50 μl/well (1 mg/ml), followed by incubation at 37 °C for two days. The addition of PBS alone served as a control. Treated

![Fig. 2](image.png) The effect of sporoderm-broken spores on cancer cell growth. (A) Human malignant breast carcinoma cells (MT-1), maintained as monolayer cultures on tissue culture plates in 1 ml medium containing 10% FBS, were used in our studies. Intact G. lucidum spores and sporoderm-broken spores were suspended in PBS (20 mg/ml), followed by incubation at 100 °C for 60 min. The supernatant and mix (the mixture of supernatant and pellet) of the preparations were added to the cell cultures at the amount of 100 μl/well, followed by incubation at 37 °C for two days. Addition of PBS alone served as a control. Treated with G. lucidum products, some tumour cells detached from the tissue culture plates and died. The experiments indicated that the sporoderm-broken spores of G. lucidum produced greater inhibitory effects on cancer cell growth than intact G. lucidum spores. The supernatant exhibited a lower inhibitory effect on tumour cell growth than the mixture. (B) Statistical analysis of the growth inhibition indicated that the sporoderm-broken spores of G. lucidum produced very significant inhibitory effects on cell growth (n = 3, **p < 0.01).
with *G. lucidum* products, some tumour cells detached from the tissue culture plates and died (Fig. 1). The experiments indicated that the *G. lucidum* products exerted a direct effect on the cancer cells. This cellular model thus allows us to test the effect of *G. lucidum* products on cancer cell activities. Our experiments also indicated that all *G. lucidum* products had some inhibitory effect on cancer cell growth. This provided direct evidence of how *G. lucidum* products might improve the conditions of cancer patients. However, different *G. lucidum* products exerted very different effects on cancer cell growth. This indicated that it is crucial to improve the quality of *G. lucidum* products to produce a better outcome for patients taking the products.

### 3.2. Effect of *G. lucidum* spores on cancer cell growth

Recent studies have indicated that the spores of *G. lucidum* have better outcome in disease treatments than the fruiting body [13]. This may be due to the presence of the glucan complex in the spores [25]. The expended chains of sulfated glucan possess higher anti-tumour activity [26]. As such, the sporoderm-broken spores of Ganoderma have much higher bioactivities than the whole spores in anti-tumour growth in an animal model [27], since the bioactive substances in the spores are not well utilized *in vivo* when the sporoderm is not broken. The whole spores have been found in the dejecta of animals and humans who take the whole spores during treatment [27]. When the sporoderm is not broken, the spores have little effect on HeLa cells *in vitro* [28].

Using the cellular model described above, we examined the effect of intact spores and the sporoderm-broken spores of *G. lucidum* on cancer cell growth. Human malignant breast carcinoma cells were maintained as monolayer cultures on tissue culture plates as above. Intact *G. lucidum* spores and sporoderm-broken spores were suspended in PBS (20 mg/ml), then incubated at 100 °C for 60 min. The supernatant and mixture, containing supernatant and pellet, of the preparations were added to the cell cultures at the amount of 100 μl/well, then incubated at 37 °C for two days. The addition of PBS alone served as a control. The experiments indicated that the sporoderm-broken spores of *G. lucidum* inhibited the growth of cancer cells more than intact *G. lucidum* spores (Fig. 2A). The supernatant exhibited lower inhibitory effect on tumour cell growth than the mixture. Perhaps, the spore surface-binding molecules exerted some inhibitory effect on tumour cell growth. Statistical analysis of the growth inhibition indicated that the sporoderm-broken spores of *G. lucidum* produced very significant inhibitory effects on cell growth (Fig. 2B). It is not clear whether enzymatic treatment produced some active components inhibiting cancer cell growth. We have also observed that *G. lucidum* oil fraction prepared from the enzymatic preparation of the sporoderm-broken spores exhibited greater effects on cell death as compared with oil prepared from the physical preparation of the sporoderm-broken spores (unpublished data).

We further tested the effect of the sporoderm-broken spores generated by physical and enzymatic methods. The latter allows slow digestion of the spores, resulting in the release of bioactive components. Our experiments indicated the inhibitory effects on cancer cell growth were sporoderm-broken spores (enzymatic method) > sporoderm-broken spores (physical method) > intact spores > buffer control (Fig. 3A), which was statistically significant (Fig. 3B). Our study suggests that breaking the sporoderm of *G. lucidum* spores is essential to improve the quality of the *G. lucidum* products. Furthermore, the methods used to break the sporoderm are also important. Enzymatic treatment allows the sporoderm to be digested and the bioactive components to be released. However, it should be pointed out that extensive digestion should be avoided, as it would cleave the bioactive components.
3.3. Effect of *G. lucidum* polysaccharides on cancer cell growth

It is known that the major bioactive components in *G. lucidum* are polysaccharides, ganoderic acid (triterpene), and adenosine. The polysaccharides of *G. lucidum* significantly improved the immune parameters of patients with advanced cancers [23]. The polysaccharide fraction of Ganoderma can suppress the activity of colon cancer cells and may act as a potent chemopreventive agent for colon carcinogenesis [29,30]. The anti-tumour activities of the polysaccharides appear to be due to the promotion of the expression of TNFα and IFNγ [31].

We tested if the polysaccharides could function directly in the cancer cells. Polysaccharides were prepared from different sources, including the fruiting body grown in wood-logs, mycelia, and the wild type fruiting body obtained from the mountains in South China. Human malignant breast carcinoma cells were cultured as monolayer, to which polysaccharides of *G. lucidum* were added at a final concentration of 0.5 mg/ml, followed by incubation at 37 °C for two days. The growth inhibition was examined under a light microscope and cell numbers were counted. The experiments indicated that the inhibitory effects of polysaccharides of *G. lucidum* from different sources were as follows: wood log > mycelium = wild type > buffer control (Fig. 4A) and the difference was statistically significant (Fig. 4B). The inhibitory effect of polysaccharides was concentration-dependent (Fig. 4C).

It has been reported that the polysaccharides are composed of heteroglycans and glycans, with or without a protein core [32,33]. A similar structure of polysaccharide was also isolated from mycelium of *G. lucidum* [34]. After repeated isolation and purification through DEAE-Sephadex and DEAE-Cellulose, two active polysaccharides, named GLMB0 and GLMB1, were isolated from the mycelium of *G. lucidum*. The structures of polysaccharides isolated from spores appear to be similar [25,35]. Recently, a polysaccharide, named Lzps-1,
Fig. 5. The inhibitory effect of polysaccharides on the growth of different cancer cells. Human breast carcinoma cells (MT-1) and human leukemia cells (Jurkat) were maintained as monolayer cultures on tissue culture plates, to which the *G. lucidum* products were added at different concentrations (mg/ml), followed by incubation at 37 °C for two days. These products were produced by the Center for Research and Development of Edible Fungi, Guangdong Institute of Microbiology, Guangdong Academy of Sciences. Treated with sufficient concentrations of *G. lucidum* product, some tumour cells detached from the tissue culture plates and died.

Fig. 6. The polysaccharide of *G. lucidum* inhibits cancer cell proliferation. Human breast carcinoma cells (A) and human leukemia cells (B) were maintained as monolayer cultures on tissue culture plates, to which the *G. lucidum* products were added at different concentrations (mg/ml), followed by incubation at 37 °C containing 5% CO₂ for two days. The number of metabolically active cells was measured using MTT assay. Data represent means ± S.E.M. (n = 8 wells). The experiment has been repeated three times and similar results were obtained (n = 3, **p < 0.01).
was obtained from *G. lucidum* spores through water extract. Its molecular weight was estimated by HPGPC to be 8000. Its structure was confirmed to be glucan. The total polysaccharides, Lzps, exhibit anti-tumour activity against sarcoma 180 and Lewis lung cancer in mice and enhanced the activity of natural killer cells. Lzps-1 is the first reported polysaccharide obtained from *G. lucidum* spore Lzps that exerts anti-tumour activity [36].

We also tested the effect of the polysaccharides on human malignant breast carcinoma cells and human leukemia cells. Treated with sufficient concentrations of *G. lucidum* polysaccharides, the tumour cells detached from the tissue culture plates.
and died (Fig. 5). These results indicate that \textit{G. lucidum} can function in different types of tumours.

3.4. \textit{G. lucidum} reduces cancer cell proliferation and survival

We further tested how \textit{G. lucidum} products inhibited cancer cell growth and induced cancer cell death, using an MTT assay. Human malignant breast carcinoma cells and human leukemia cells were treated with different concentrations of the polysaccharides derived from \textit{G. lucidum} fruiting body cultivated with wood-logs, followed by incubation at 37 °C containing 5% CO₂. The number of metabolically active cells was measured using the MTT assay. The experiment indicated that the number of metabolically active cells decreased when treated with the polysaccharides as compared with the untreated cells (Fig. 6). These results indicate that the polysaccharides reduced the growth rate of cancer cells by inhibiting cell growth. We further validated these results with trypan blue staining. Our experiments indicated that the cancer cells exhibited a lower rate of survivability when treated with the polysaccharides than the untreated cells (Fig. 7). These results were consistent with the reduced rate of growth and proliferation and increased cell death when treated with polysaccharides.

3.5. \textit{G. lucidum} modulates Erk signal pathway

Epidermal growth factor receptor (EGFR) has been documented as a prototypic receptor tyrosine kinase (RTK) which modulates cell proliferation. The classic signalling cascade downstream of EGFR is the extracellular regulated kinase (Erk) pathway [37]. To examine whether \textit{G. lucidum} was able to regulate the EGFR downstream signalling pathway, human malignant breast carcinoma cells were incubated with the polysaccharide of \textit{G. lucidum} at different concentrations for 24 h. Cell lysate was prepared and subjected to Western blot analysis probed with anti-Erk and anti-actin (for loading control) antibodies. We detected strong signals of Erk expression in the control cells. Cells treated with the polysaccharide expressed a lower level of Erk protein, and a maximum reduction was observed when the cells were treated with the polysaccharides for 24 h (Fig. 8). Little difference was detected in actin expression between the cells treated with polysaccharides and those not treated. These results correlated with those of proliferation assay, suggesting that the polysaccharides were able to regulate Erk expression and modulate this signal pathway.

4. Conclusion

In this study, we examined the role of \textit{G. lucidum} in anti-cancer cell growth and induced cancer cell death. We demonstrated that \textit{G. lucidum} products directly modulate on tumour cell growth and cell death. The sporoderm-broken spores of \textit{G. lucidum} produced greater inhibitory effects on cancer cell growth than intact \textit{G. lucidum} spores, and spores broken by enzymatic methods produced the best results in the inhibition of cancer cell growth. The polysaccharides appear to exert a major effect on tumour cell growth, and those obtained from wood-log-cultured \textit{G. lucidum} appeared to be the best. The inhibitory effect of \textit{G. lucidum} on cell proliferation is mediated, at least in part, through the Erk signal pathway.

Acknowledgements

This work was funded by an operating grant from The Department of Sciences and Technologies of Guangdong Province, China, and by Grant NHP-78380 from the Natural Health Product Directorate (Health Canada/Canadian Institutes of Health Research Partnership), and Grant MOP-74469 from the Canadian Institutes of Health Research to B.B.Y.

References


