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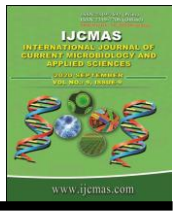

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In-vitro Analysis of *Ganoderma lucidum* Extract Induces Cell Cycle Arrest and Apoptosis in MCF-12 Human Breast Cancer Cell

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A B S T R A C T

Ppharmalogical and clinical application of water extracts of *Ganoderma lucidum* have been extensively documented, in short is known regarding its alcohol extract. In this research work, the anti-tumor effect of an alcohol extract of *Ganoderma lucidum* was investigated using MCF-12 cells. We found that the alcohol extract of *Ganoderma lucidum* inhibited cell proliferation in a time and dose dependent manner, which might be mediated through up-regulation of p21/Waf1 and down-regulation of cyclin D1. Further, this compound can directly induce apoptosis in MCF-12 cells, which might be mediated through up-regulation of a proapoptotic Bax protein and not by the immune system. Our research suggests that there are multiple mechanisms underlying the anti-tumor effects of *Ganoderma lucidum*.

Introduction

An increasing number of natural chemical compounds have been identified to be potent in the treatment of various major human diseases including cancer (1– 4). Many of these substances are found in oriental herbaceous plants. Recently, some chemo-preventive extracts of herbaceous plants have been shown to be anti-tumorigenic (5– 8). Carcinogenesis is a multistage or multi-mechanism process, involving the irreversible alteration of a stem cell followed by the clonal proliferation of the initiated stem cell from which the acqui sition of the invasive and metastatic phenotypes are generated (the "progression" phase) (9). Cancer prevention or therapy may be accomplished at different steps of this process by different mechanisms. Mixed extract of herbaceous plants might contain different chemopreventive chemotherapeutic compounds with more than one mechanism of action, thus possessing a combination of different chemopreventive or chemotherapeutic effects. It is therefore very important to identify combination effects of mixed extract of herbaceous plants.

Ganoderma lucidum, an oriental fungus, has been widely used as a medical drug product in China and other Asian countries. The fruiting bodies and cultured mycelia of *Ganoderma lucidum* have been demonstrated to possess anti-tumor activities, (10 –18), neuroactive effects immunomodulatory activities (19 – 20). Although numerous studies on the effects of water ex- tracts of GL have been reported, (10 –15) in short is known regarding its alcohol extracts. It has been reported that the alcohol extract of *Ganoderma lucidum* can induce cell cycle arrest at transition from G1 to S phase in HeLa-cells17 this work was design to further investigate the anti-tumor effect of alcohol extract of *Ganoderma lucidum* and elucidate the potential mechanisms using an in vitro system. We found that the alcohol extract of *Ganoderma lucidum* was able to induce cell cycle arrest at G1 phase and induce apoptosis in MCF-12 cells in a time-dependent manner. The mechanisms might be mediated through up-regulation of the expression of p21/Waf1-a cell cycle inhibitor, down-regulation cyclin D1, and up regulation of a pro-apoptotic BAX protein pathway.

Materials and Methods

Cell culture

MCF -12 cells (Manassas, ATCC, VA) were cultured in D-media4 supplemented with 10.5% FBS (Carlsbad, CA, Invitrogen).

Preparation of ethanol-soluble and acidic components from *Ganoderma lucidum*

Fruiting bodies of *Ganoderma lucidum* (1 kg) were extracted with ethanol (90%, 10l) for 24 hr at room temperature to give 30 g of solid extract. The ethanol extract was suspended in chloroform and extracted with a saturated $NaHCO_3$ solution 3×. The $NaHCO_3$ phase was then collected and adjusted to pH 3 with cold HCl solution (7N). The resulting precipitates were extracted by chloroform, and 10.5 g ethanol-soluble and acidic components (ESAC) were obtained in the form of yellow solid.

Cell proliferation assay

The effects on cell proliferation were measured by MTT assay, which is based on the ability of live cells to convert tetrazolium salt into purple formazan. In short, the cells were seeded in 96-well micro- plates and incubated overnight. Then the cells were treated with different concentrations of the test compound (100, 250, 350, 450 and 550 µg/ml) or its vehicle, ethanol (0.8%) for 12, 24, 36 or 48 hr. At the end of these times, 20 µl of MTT stock solution (5 mg/ml, Sigma, St. Louis, MO) were added to each well and the plates were further incubated for 6 hr at 36°C. The supernatant was removed and 100 µl of DMSO were added to each well to solubilize the water-insoluble purple formazan crystals. The absorbency at a wavelength of 570 nm was measured with Multiscan MCC 340 microplate reader (Titertek, Huntsville, AL). All the measurements were performed in triplicate.

Cell cycle analysis

MCF-12 cells in exponential phase of growth were treated with the alcohol extract of *Ganoderma lucidum* (550 µg/ml) for the indicated times, then harvested by trypsinization, and washed 2× with ice-cold PBS and fixed by 75 % ethanol at —25°C for at least 30 min. The fixed cells were then washed 2 times with ice-cold PBS and stained with 50 µg/ml of propidium iodide in the presence of 100 µg/ml of RNase for 30 min. Cell cycle distribution was analyzed using FACS Calibur. Data from 10,000 cells per sample were collected and analyzed using the Cell Fit Cell analysis program.

Apoptosis assay

The apoptotic effect of the alcohol extract of *Ganoderma lucidum* on MCF-12 cells were analyzed by nuclear DNA staining and DNA fragmentation assay. For nuclear DNA staining, control and compound-treated cells were fixed in 4% para-formaldehyde in PBS for 20 min, washed with PBS, stained with Hoechst 33258 at 1 µg/ml in PBS for 20 min. Stained cells were washed 2× with PBS. The changes in nuclei were observed with a fluorescent microscope (Olympus, Melville, NY) through UV-filter. For DNA fragmentation assay, either detached and attached control or com- pound-treated cells were harvested by scraping and centrifugation. The cells were then lysed with lysis buffer (5 mM Tris [pH 8.0] 20 mM EDTA, 0.5% Triton X-100) on ice for 45 min. Fragmented DNA in the supernatant fraction after centrifugation at 14,000 rpm (45 min at 4°C) was extracted 2× with phenol-chloroform-isoamyl alcohol (25:24:1, v/v) and once with chloroform and then precip-itated with ethanol and 3 M sodium acetate overnight at —20°C. The DNA pellet was washed once with 70% ethanol and resus-pended in Tris-EDTA buffer (pH 8.0) with 100 µg/ml RNase and incubated at 37°C for 2 hr. The DNA fragments were separated by 1.8% agarose gel electrophoresis and visualized under UV light.

Western blot analysis

MCF-12 cells were grown in a 10 cm dish. When density reached 85-90 % then the confluence cells were treated with alcohal extact of *Ganoderma lucidum* (500 µg/ml) for the indicated times. The cells was then washed once with ice-cold PBS and lysed with lysis buffer (25% SDS containing 2 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide, 0.1 mM sodium orthovanadate, 1.5 mM leupeptin, 1 mM antipain and 5 mM sodium fluoride) for 10 –20 min. The lysates were sonicated 3× at 10 sec intervals, aliquoted and stored at —25°C. The protein concentration was determined by the Bio-Rad DC protein assay (Bio-Rad Laborato- ries, Hercules, CA). Equal amounts of protein (20 µg/lane) were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were subsequently incubated with the corresponding initial (Primary) antibodies, as indicated, a rabbit anti- p21Cip1/WAF1 polyclonal antibody, a mouse anti-Bcl-2 mono- clonal antibody (Zymed Laboratories Inc., South San Francisco, CA); a mouse anti-p53 monoclonal antibody, a rabbit anti-Bax antibody (Sigma); a mouse anti-cyclin D1 antibody, a rabbit anti- cdk4 polyclonal antibody, a mouse anti-E2F-1 monoclonal anti- body, a mouse anti-PARP monoclonal antibody (Santa Cruz Bio- technology, Santa Cruz, CA); a rabbit anti-caspase-7 polyclonal antibody (Chemicon International Inc., Temecula, CA). Antibody detector was detected with the respective secondary antibody, ether or anti-rabbit IgG or anti-mouse IgG antibodies linked to horseradish peroxidase (Zymed). Antibody-bound proteins were detected by the ECL western blotting analysis system (Amersham Pharmacia Biotech, UK).

Results and Discussion

Alcohol extract of *Ganoderma lucidum* inhibited proliferation of MCF-12 cells

To evaluate the effects of alcohol extract of *Ganoderma lucidum* on the growth of MCF-12 cells, the cells were treated with a series of concentrations of alcohol extract of *Ganoderma lucidum* from 100 –550 µg/ml for 0, 12, 24, 36 and 48 hr. Cell growth was determined by MTT assay. As shown in Figure 1, alcohol extract of *Ganoderma lucidum* inhibited the proliferation of MCF-12

cells in a time and dose dependent manner. After 48 hr of treatment, at 500 µg/ml, this alcohol extract caused nearly a 70% inhibition of cell growth compared to the control. This concentration was used in all further experiments.

Alcohol extract of *Ganoderma lucidum* induced cell cycle arrest at G1 phase in MCF-12 cells

To investigate the basis of the antiproliferative properties of alcohol extract of *Ganoderma lucidum*, cell cycle analyses were performed with flow cytometry. As shown in Figure 2, cells accumulated in the G1 phase, and the number of the cells in G1 phase increased gradually from 24– 48 hr after treatment with the test compound. The number of cells in S and G2/M phase de-creased in same manner.

Alcohol extract of *Ganoderma lucidum* induced apoptosis in MCF-12 cells

To determine if exposure to alcohol extract of *Ganoderma lucidum* causes cell death by apoptosis in human breast cancer cells, Hoechst 33258 staining and DNA laddering were performed. The test compound was capable of inducing apoptosis in MCF-12 cells in a time-dependent manner, which was characterized by nuclear shrinkage, chromatin condensation and significant DNA fragmentation. The most significant effect was observed 36 hr after the treatment.

Effects of alcohol extract of *Ganoderma lucidum* on the levels of P53, P21, cyclin D1, CDK4 and E2F in MCF-12 cells

To elucidate the mechanisms of alcohol extract of *Ganoderma lucidum* induced cell cycle arrest in MCF-12 cells, Western blot was performed to evaluate p21/Waf1-a cell cycle inhibitor, cyclin D1, cyclin-dependent protein kinase 4 (cdk4) and transcription factor E2F. The p21 level was increased 12 hr after treatment and remained elevated up to 48 hr (Fig. 2). In contrast, the levels of cyclin D1, cdk4 and E2F decreased in a time-dependent manner. No change was observed in protein expression of p53.

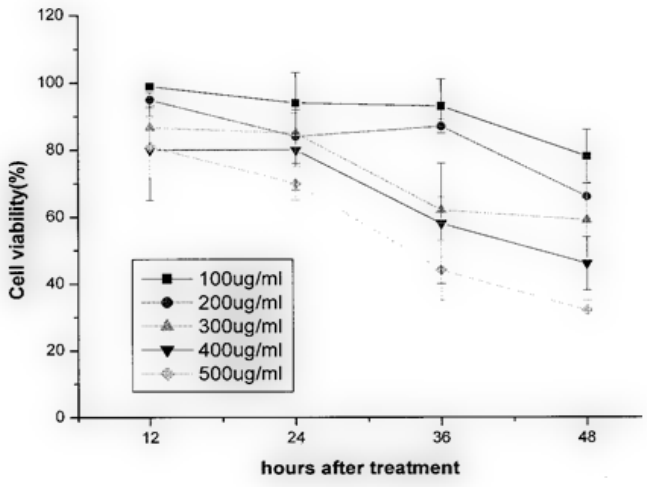

Figure.1 Inhibition of proliferation by G*anoderma lucidum*. Cells were treated with various concentrations of test compound for indicated times, and cell viability was determined by the MTT assay

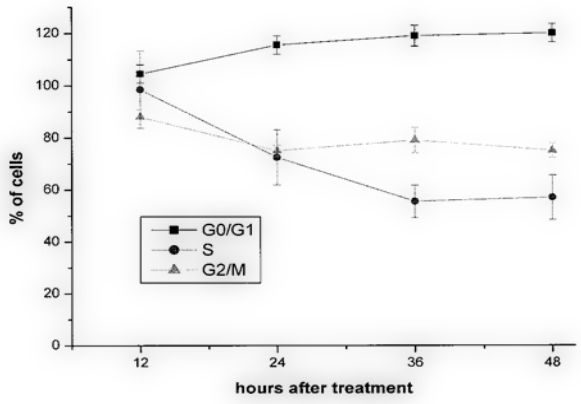

Figure.2 Cell cycle distribution in MCF-12 cells treated with *Ganoderma lucidum*

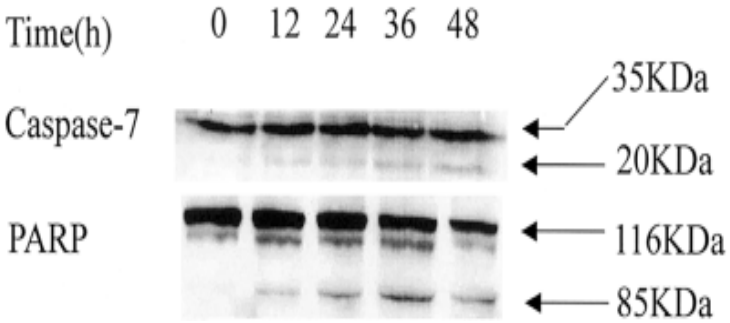

Figure.3 Effect of alcohol extract of *Ganoderma lucidum* on expression of caspase-7 and PARP in MCF-12 cells

Effects of alcohol extract of *Ganoderma lucidum* on the levels of P53, bax and Bcl-2 in MCF-12 cells

To explore the potential signaling pathways underlying *Ganoderma lucidum* alcohol extract-induced apoptosis, we evaluated transcription factor p53 and Bcl-2 family proteins by Western blot. The Bax protein level was increased 12 hr after treatment and remained elevated up to 48 hr. No change was observed in protein expression of Bcl-2 (Fig. 2). The results suggested that *Ganoderma lucidum* induced apoptosis in MCF-12 cells might be medi- ated through up-regulation of Bax pathway.

Effects of alcohol extract of *Ganoderma lucidum* on the caspase-7 and PARP expression and cleavage in MCF-12 cells

To assess the role of caspase-7 in this apoptotic process, the expression of caspase-7 was examined by Western blot analysis. As shown in Figure 3, the 35 kD proenzyme caspase-7 was cleaved to its active 20 kD form 12 hr after treatment in a time-dependent manner. The 116 kD PARP protein was cleaved to 85 kD fragment in a time-dependent manner to a maximum level at 36 hr.

This research work has shown that alcohol extract of *Ganoderma lucidum* inhibited cell

proliferation and induced apoptosis in human breast cancer cells. Furthermore, we demonstrated that alcohol extract of *Ganoderma lucidum* induced cell growth arrest through up-regulation of p21, down-regulation of E2F and cdk4 induced apoptosis through increasing the function of Bax protein expression and activation of caspase-7. Importantly, the alcohol extract of Gano-derma lucidum has no such effects on non-tumorigenic WB cells, indicating that it might be selectively cytotoxic for the tumor cells (data not shown). This can be examined in normal human breast epithelial cells and other breast cancer cell lines in further studies.

In case of mammalian cells, replication is regulated in an orderly fash- ion from G1 to S to mitosis by phase-specific oscillations in the level of cyclins, cyclin-dependent protein kinases (cdks) and cdk inhibitors. Cell cycle progression is accelerated by cyclins and cdks, and decelerated by cdk inhibitors (such as p16, p21 and p27), p53, retinoblastoma tumor suppressor protein (RB) and by ARF. The D-type cyclins (cyclin D1, D2 and D3) are involved in regulation of transition from G1 to S during cell cycle. Their critical function is to activate cdk4 and cdk6.22 Recent studies have shown that alcohol extract of *Ganoderma lucidum* can induce significant reduction in LNCaP cell growth. 18 It has been reported that the alcohol extract of *Ganoderma lucidum* can inhibit cell growth by preventing the transition from G1 to S phase in HeLa cells.17 Consistent with these previous reports, in this study we also observed that the alcohol extract of *Ganoderma lucidum* was capable of inducing cell cycle arrest at G1 phase in MCF-12 cells. Further mechanistic study suggested that this process might be mediated through up-regulation of p21 and down-regulation cyclin D1, which inhibited or inactivated cdk4 and led to the dephos- phorylation of RB. This in turn resulted in the inactivation of the transcription factors E2F, involved in the expression of genes required for DNA synthesis and cell cycle progression. Many investigators have suggested that the anti-tumor effect of *Ganoderma lucidum* might be mediated by host responses.10 –12 Wang et al.10 reported that the anti-tumor activities of water extract-po- lysaccharides of *Ganoderma lucidum* (PS-G) was mediated by cytokines released from activated macrophages and T lymphocytes, especially TNF-a and IFN-µ. These cytokines in turn in-duced apoptosis and differentiation in the treated leukemic cells. PS-G alone, however, can not directly induce apoptosis in the treated leukemic cells. Our results for the first time demonstrate that the alcohol extract of *Ganoderma lucidum* is directly capable of inducing apoptosis in human breast cancer cells, not through immune system. Our results further show that this apoptotic pro- cess might be mediated through up-regulation of BAX, a member of Bcl-2 family. The proapoptotic Bax protein led to mitochondrial dysfunction and the release of cytochrome c (Cyt c) from the mitochondria. The released Cyt c then interacts with specific adapter, such as Apaf-1, which in turn proteolytically converts procaspases to active caspases. One key step of this cascade in MCF-12 cells was activation of caspase-7, which cleaves several substrates including the PARP, a nuclear enzyme involved in DNA repair and maintenance of genome integrity and post-translational ribosylation of proteins, whereby apoptosis occurs. We found that the inactive caspase-7 precursor was cleaved to the 20 kD subunit forming the active protease during apoptosis. This occurred in conjunction with cleavage of 116 kD PARP to 85 kD proteolytic fragments. Our results suggest that caspase-7 may be the main effector caspase in caspase-3-deficient MCF-12 cells during apo-ptosis. The results are consistent with other reports using MCF-12 cell system.23–25

In short, alcohol extract of *Ganoderma lucidum* inhibits cell proliferation through down-regulation cyclin D1 and up-regulation of p21, and induces apoptosis through over expression of Bax protein in human breast cancer cells. Our research work thus suggests multiple mechanisms underlying the anti-tumor effects of *Ganoderma lucidum.*

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