Therapeutic Potential of *Ganoderma lucidum* in Cancer

Subjects: Biology

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Triterpenoids, such as ganoderic acid, and polysaccharides, including β-D-glucans, α-D-glucans, and α-D-mannans, are the main secondary metabolites of the medicinal fungus *Ganoderma lucidum*. There is evidence of the effects of ganoderic acid in hematological malignancies, whose mechanisms involve the stimulation of immune response, the macrophage-like differentiation, the activation of MAP-K pathway, an IL3-dependent cytotoxic action, the induction of cytoprotective autophagy, and the induction of apoptosis. In fact, this compound has been tested in twenty-six different human cancer cell types and has shown an anti-proliferative activity, especially in leukemia, lymphoma, and myeloma lines.

Keywords: Ganoderma lucidum ; reishi ; nutraceutical ; leukemia ; lymphoma ; hematological malignancies

1. Induction of Apoptosis

After the exposure to the F3 polysaccharide fraction of *G. lucidum*, four hematopoietic cell lines were examined for apoptosis, and they showed to be able to induce the expression of IL-1, IL-6, IFN-gamma, TNF-alpha in monocytes–macrophages, and T lymphocytes, which, in turn, exert a proapoptotic activity [1][2][3][4][5][6]; moreover, these polysaccharides may also have immune-enhancing properties, perhaps as a consequence of their ability to stimulate cytokine production [7][8][9][10].

Among hematological malignancies, research focused on the anti-cancer effects of *G. lucidum* in cells from acute lymphoblastic leukemia, myeloid leukemia (HL-60, U937, K562, THP-1, NB4), non-Hodgkin's lymphoma (NCEB-1, SUDHL6), and multiple myeloma (RPMI8226, ARH77, U266, NCI-H929); in detail, HL-60 myeloid leukemia cells exposed to 100 g/mL of F3 for 72 h became multinucleated and showed an increased DNA content with the result of 29% of G2/M arrest compared with the 12% of control cells. It is well known that apoptosis starts with a series of cascading events such as the depolarization of the mitochondrial membrane potential [11]; in fact, *G. lucidum* has the capability to alter the mitochondrial transmembrane electrical potential of HL-60 and U937 myeloid cells. When cells lose their electrical potential, the fluorescence of the kit used for experiments changes from red to green in a dose-dependent manner, indicating that apoptosis is in course [12].

A dynamic analysis of gene expression in THP-1 monocytic leukemia cells with *G. lucidum* treatment was conducted at various time points in order to determine whether F3 has similar interactions with death receptors that drive apoptotic pathways in leukemia cells. THP-1 cells experienced cell shrinkage, one of the signs of cell death, following a 48 h F3 treatment. Moreover, it was possible to observe nuclear chromatin condensation, which results in the deterioration of genomic DNA, as a marker of early-stage cell death; in fact, the proportion of chromatin-condensed cells significantly increased in the F3-treated culture [13].

Apoptosis pathways have been studied by scientists, who focused on six genes of the NF-κB signaling pathway, observing that they are up-regulated following F3 therapy, especially for those that concern MYD88, TRADD, interleukin 1 beta (IL1B), interleukin 1 alpha (IL1A), NFKBIA, and NFKB1. In detail, IL1B at low concentrations strongly elicited apoptotic responses as demonstrated by caspase-8 activation and DNA fragmentation [14]; soon after TNF engages TNFR1, NF-B is strongly activated, producing a pro-survival signal that must be suppressed in many cell types for TNF to induce apoptosis.

2. Stimulation of the Immune Response

The most effective antigen-presenting cells are called dendritic cells (DCs), and they have a special capacity to bridge innate and adaptive immunity; the present approach to examine the biology and differentiation of circulating DCs is mostly based on the differentiation of monocytes into circulating DCs using the cytokines GM-CSF and IL-4. Researchers have

shown that *G. lucidum* polysaccharides can promote the maturation of human dendritic cells, boost monocytic leukemic cell proliferation, and trigger dendritic cell differentiation from monocytic leukemic blasts [15].

In leukemic cells from mice, polysaccharides from *G. lucidum* improve T-cell and B-cell surface markers and potentiate phagocytosis of macrophages and NK cell activity; in fact, NK cells help to get rid of altered tumor cells and are involved in non-specific antiviral and anticancer defense in human cancer cells [16][17]. Recent findings showed that NK cell activity was enhanced following treatment with *G. lucidum* extracts, while the percentages of Mac-3 and CD11b genes were reduced, and CD3 and CD19 were, instead, elevated, suggesting that F3 may increase the amount of T and B cells, respectively [18].

3. Macrophage-Like Differentiation through Caspase and p53 Activation

The change in cell morphology from a monocytic phenotype to a macrophage-like phenotype and the increased cell adherence after receiving *G. lucidum* treatment for 24 and 48 h both supported the influence of F3 on cell differentiation; in fact, the decrease of nitroblue tetrazolium (NBT) and the evidence of cell cycle arrest in the G0/G1 phase indicate the impact of differentiation into cells that resemble macrophages. Moreover, the upregulation of CD11b, CD14, CD68, and MMP-9 and the downregulation of MPO strongly showed the influence of macrophage differentiation in THP-1 cells treated with F3.

Multiple experimental models have shown that wild-type p53 and p21 promote cellular differentiation of monocytes; in fact, when F3 is applied to THP-1 cells, p53 and p21 expressions are gradually increased during macrophage differentiation, and macrophage differentiation markers are decreased when p53 activity is inhibited. Additionally, the accumulation of cell cycle progression in the G0/G1 phase may be partially influenced by the actions of p53 and p21. According to these results, the p53-dependent mechanism controls cell differentiation and cell cycle arrest [19], and Mac-3 decreased, while macrophage phagocytosis increased because F3 stimulated the differentiation of monocytes into activated peritoneal macrophages [12][20][21][22][23][24].

One of the main pathways leading to apoptosis is the mitochondrial/caspase-mediated signaling cascade, and mitochondrial outer-membrane permeabilization (MOMP) plays a significant part in this route: cytochrome C is released into the cytoplasm due to MOMP collapse, which sets off the caspase cascade and apoptosis that follows. Researchers found that GT treatment increased the levels of procaspase-9, procaspase-3, cytochrome C, and PARP, which in turn caused apoptosis in K562 cells; furthermore, the production of FasL receptor, Fas protein, and caspase-8 regulates the death-receptor-mediated extrinsic pathway, which in turn promotes apoptosis. Following FasL activation, caspase-8 activation causes the proteolytic cleavage of mitochondrial-associated Bid to cause apoptotic cell death. Bid, a proapoptotic protein that is a substrate for caspase-8 targets the permeabilization of the mitochondrial membrane, linking the intrinsic and extrinsic apoptotic pathways.

4. Activation of MAP-K Pathway

Since MAPK activation causes its downstream substrates to be successively phosphorylated, including transcription factors, protein kinases, and constitutive proteins, it is believed to be one of the key regulators of cell proliferation and differentiation; in fact, targeting MAPK signaling pathways has emerged as one of the key therapeutic approaches to halt tumor growth, in particular for concerning ERK, JNK (c-jun N-terminal kinase), and p38 MAPK. Therefore, using these kinases and the genes they regulate, the targeting effect of *G. lucidum* on MAPK pathways in leukemia cells has been studied [25]: ERK/MAPK signaling transduction is a traditional pathway that is also involved in the pathogenesis of leukemia, and a constitutive activation was found in some acute leukemic cells; this statement is supported by previous studies finding that untreated HL-60 cells both in vitro and in vivo had overexpression of p-ERK1/2.

It was hypothesized that *G. lucidum* may affect HL-60 cell proliferation by suppressing the ERK/MAPK pathway because it was found that when it was administered in high concentrations, the level of ERK1/2 phosphorylation was down-regulated [26]. Moreover, the expression of cyclin D1, an ERK-regulated downstream protein, is significantly downregulated, and, as a result, the G1/S phase transition that cyclin D1 controls is repressed. In addition to controlling cyclin D1 in acute leukemic cells, the ERK/MAPK pathway also works with members of the Bcl-2 proteins to prevent apoptosis; among them, the protein Bax can form a heterodimer that, in turn, controls cell apoptosis, but the ratio of Bcl-2 to Bax determines how sensitive it is to death signals. According to research, blocking ERK1/2 activity increased the activity of caspases such as caspase-3 while having no effect on the levels of the pro-apoptotic gene Bax or the production of the Bcl-2 protein: all these events lead to apoptosis.

Numerous cell genes, including c-myc and p53, participate not only in the advancement of the cell cycle but also in the control of cell apoptosis: in vitro studies showed increased levels of phosphorylated c-myc and p53 proteins after cells were treated with *G. lucidum*, suggesting that it was the cause of the c-myc- and p53-induced apoptosis [27]. The MAPK signaling pathways, which mediate significant physiological responses like proliferation, differentiation, and death are among the oldest signal transduction pathways and the most evolutionarily conserved signaling regulators. Though they are members of a vast MAPK family, JNK, p38 MAPK, and ERK are functionally distinct subgroups: the ERK cascade is regularly linked to pro-survival activity in a variety of cell types, while the JNK and p38 families seem to have proapoptotic effects; in detail, JNK promotes the transcription of c-Jun target genes by phosphorylating and activating c-Jun.

5. Induction of Cytoprotective Autophagy

In myeloid leukemia and solid tumors, a dynamic and self-catabolic process known as autophagy is involved in the breakdown and recycling of macromolecules and organelles. During these events, the activating enzymes Atg7 and Atg3 combine the cytosolic form of LC3-I with phosphatidylethanolamine to create LC3-II [28][29][30][31]. Another essential protein known as p62 or sequestosome 1 (SQSTM1) directly attaches to LC3-II through a particular sequence motif to produce autophagosomes, which then go through self-degradation during autophagy. Because it may interact with signaling proteins, the autophagy adaptor protein p62/SQSTM1 serves as a hub for signaling and represents an adaptive survival strategy in adult acute myeloid leukemia cells.

The results of previous investigations showed that *G. lucidum* treatment of K562 cells induced autophagy by increasing the accumulation of LC3-II and the expression of P62/SQSTM1; in fact, real-time protein-chain reaction (RT-PCR) results revealed an elevation of LC3 mRNA expression levels. In tumor suppression, autophagy and apoptosis-associated cell-death pathways are crucial and share some essential signals that influence cell survival or death; these two biological processes have the ability to inhibit one another.

It has been possible to identify the anticancer activities of substances obtained from natural sources and traditional Chinese medicine extracts using the pharmacological autophagy inhibitors 3-MA and CQ, which could block autophagy and increase caspase-3 cleavage, demonstrating that *G. lucidum*-mediated cytoprotective autophagy prevented apoptosis in K562 cells.

Beclin-1 is regarded as a key protein in the autophagy process, and its expression may be cytoprotective or encourage cell apoptosis; it is known to be inhibited under normal circumstances by Bcl-2 protein and separates from Bcl-2 in stress response, which causes autophagy to be triggered. In a time-dependent experiment, researchers found that incubation with *G. lucidum* significantly elevated Beclin-1/Bcl-2 expression levels, indicating the activation of autophagy in K562 cells [32][33]. Autophagy is strictly regulated by upstream modulators, namely, the PI3K/AKT/mTOR signaling pathway; natural bioactive substances stimulate autophagy via this mechanism in cancer cells; in fact, it was observed that blocking EFGR and the PI3K/AKT/mTOR signaling cascade may help cancer cells engage in autophagy: there is evidence of a significant reduction of the levels of EFGR, PI3K, phosphorylated PI3K, and AKT protein expression after F3 incubation [34].

6. Ganoderma lucidum and Hematological Malignancies

The effects of *G. lucidum* on various hematological malignancies have been investigated largely, and the antitumor effects of *G. lucidum* using a panel of 26 human cancer cell lines have been evaluated, including acute lymphoblastic leukemia (Blin-1, Nalm-6, Jurkat), myeloid leukemia (HL-60, U937, K562, THP-1, NB4), Burkitt's lymphoma (Daudi, Ramos), non-Hodgkin's lymphoma (NCEB-1, SUDHL6), multiple myeloma (RPMI8226, ARH77, U266, NCI-H929), prostate cancer (LNCaP, PC-3, DU145), colorectal cancer (HT-29), breast cancer (MCF-7, MDA-MB-231), non-small cell lung cancer (NCI-H520), and pancreatic cancer (PANCI, ASPC1, BxPC-3). The 26 cancer cell lines used were cultured for 96 h in the presence of 50 and 100 µg/mL *G. lucidum* extract, obtained by aqueous extraction from the fruiting bodies and enriched in ganoderic acid C2 (GA-C2), and their growth was evaluated. Subsequently, the six hematological cell lines (HL-60, U937, K562, Blin-1, Nalm-6, and RPMI8226) were more sensitive to GL and achieved a growth inhibition of 50%, so they were further studied. The effective dose to inhibit growth by 50% (ED50) was calculated for each cell line and ranged from 26 to 63 µg/mL. Cell cycle analysis was performed on HL-60, U937, RPMI8226, Nalm-6, and Blin-1 cells incubated for 72 h with or without *G. lucidum* extract at a concentration of 100 µg/mL. The results showed a G2/M arrest, especially in HL-60 cells (29% in treated vs. 12% in control cells). The increase in cells in the G2/M phase was minimal in RPMI8226 cells (20% in treated vs. 16% in control cells) and Nalm-6 cells (15% in treated vs. 12% in control cells), but there was no increase in the number of cells in the G2/M phase in Blin-1 or U937 cells. Apoptosis was then assessed by the Annessin V assay, and four cell lines (HL-60, U937, Blin-1, and RPMI8226) were treated with *G. lucidum* at concentrations of 50, 100, 150, and 200 µg/mL for 72 h. *G. lucidum* induced apoptosis in each of the cell lines in a dose-dependent manner, with the best

results for HL-60 cells (approximately 92% apoptotic cells at the concentration of 200 µg/mL, after 72 h) and Blin-1 cells (approximately 42% apoptotic cells at the concentration of 200 µg/mL, after 72 h); U937 and RPMI8226 cells showed less apoptosis (32% and 21%, respectively, at the concentration of 200 µg/mL, after 72 h). The effect of *G. lucidum* on the expression of cell cycle and apoptosis-related proteins was investigated by Western blot analysis on U937 cells treated with increasing doses of *G. lucidum* extract (50, 100, 150, and 200 µg/mL) for 48 and/or 72 h, and an upregulation of p21 WAF1 and p27 KIP1 by *G. lucidum* extract was detected [12].

Another in vitro study, conducted by Calviño and colleagues, evaluated the effect of two aqueous extracts (E1, unboiled, obtained after centrifugation of fruiting bodies in sterile water at room temperature for 5 min; E2, boiled, obtained by resuspending the pellet in sterile water and boiling for 5 min) and a methanolic extract (E3, obtained after fruiting bodies were disrupted and resuspended in 10% ethanol; the solvent evaporated, and the compound was resuspended in dimethyl-sulfoxide) of *G. lucidum* on a murine model of interleukin-3 (IL-3)-modulated lymphoma, lymphoma DA-1 cells. A total of two cell lines were employed: DA-1 cells were kept at 37 °C and 5% CO_2 in Iscove MDM medium with 10% fetal calf serum, 2 mm l-glutamine, and 2.5×10^{-5} m β-mercaptoethanol, and the WEHI-3B cell line was used as a source of conditioned medium that contained interleukin-3, which is essential for the survival of DA-1 cells. Cells were incubated with IL-3 and exposed to different concentrations of E1, E2, and E3 extracts or to 100 µm etoposide, which was used as a positive control for toxicity and induction of apoptosis, for 13, 19, and 24 h. Etoposide induced a decrease in cell viability with viability values of 70%, 58%, and 51% at 13, 19, and 24 h, respectively. Extracts E1 and E3 showed an effective reduction in cell viability of up to 70% and 36%, respectively, after 24 h of treatment. In contrast, extract E2 had no significant effect on viability. Treatment-induced apoptosis was then assessed and measured by quantifying subdiploid DNA. Extract E1 showed a similar level of time-dependent induction of cell death as a 100 µm etoposide. Extract E3 also showed significant induction of subdiploid DNA in DA-1 cells, although it was less than E1. In contrast, extract E2 showed very limited induction of cell death in DA-1 cells. Finally, Western blot analysis was used to assess the levels of certain proteins, as p53, Bax, Bcl-2, and Mdm-2 are involved in apoptosis processes after exposure to the different types of *G. lucidum* extract. In particular, an increase in p53 and a reduction in Mdm2 were observed in DA-1 cells treated with E1 and E3, but not in cells treated with E2, as was a reduction in the levels of Bcl-2 and NF-kB for all three extracts and activation of caspase 3 in cells exposed to E1 and E3 [35].

Extracts of *G. lucidum* E1, E2, and E3 were also used in another study conducted on a human leukemia cell line NB4. In this case, cells were treated with *G. lucidum* extracts E1, E2, E3, or GA-C2 for 19 h, using etoposide as a positive control. Treatment of NB4 cells with the E1 extract reduced cell viability to 77% compared to untreated control cells; the E2 extract also resulted in a reduction in cell viability, albeit less than E1; the E3 fraction showed a dose-dependent reduction in viability, up to a maximum value of 68%; in contrast, GA-C2 had no effect on cell viability. The induction of DNA fragmentation, an expression of apoptosis, was then investigated: extracts E1 and E3 produced significant DNA fragmentation, exceeding 40%; a lower effect was produced by treatment with GA-C2; while extract E2 did not result in significant DNA fragmentation. Furthermore, changes in some proteins involved in apoptosis were studied by Western blot in NB4 cells treated with E3 extract. The results showed a reduction in p53, Akt, Erk, NFκB, and Bcl-2 and an increase in Bax in treated cells compared to controls [36].

Wang and colleagues investigated the effect of active lipids from *G. lucidum* spores dissolved in ethanol on THP-1 cells, human acute monocytic leukemia cell lines, and HL-60 cells, human promyelocytic leukemia cells. The active lipids were obtained as follows: samples of *G. lucidum* spores were manually ground for four hours in a glass mortar, and then they were ultrasonically extracted using CH2Cl2 for two hours. After filtering the solution, the residue was extracted twice more using CH2Cl2. After combining the filtrates, the solvent was eliminated using a rotary evaporator. Finally, the lipids of *G. lucidum* spores were dissolved in ethanol. Cells were incubated with active lipids from *G. lucidum* spores at different concentrations (0, 0.25, 0.5, 1, and 2 mg/mL) for 48 h or at a concentration of 1 mg/mL for 0, 12, 24, 48, and 96 h. It was found that active lipids from *G. lucidum* spores induced the decrease in viability of THP-1 and HL-60 cells, as determined by trypan blue staining, in a dose- and time-dependent manner. Apoptosis was assessed by flow cytometry using FITC-conjugated Annexin V and PI in both cell lines, which showed high rates of apoptosis in a dose- and time-dependent manner after exposure to active spore lipids. Protein expression was then assessed by Western blot, and it was found that treatment with active lipids significantly decreased P-Akt production in THP-1 cells in a time-dependent manner, supporting the conclusion that apoptosis induced by active lipids in *G. lucidum* spores is mediated by downregulation of P-Akt; expression of P-ERK1/2 decreased and P-JNK1/2 increased in a time-dependent manner, whereas P-p38 MAPK expressions were not affected. Caspase-3, -8, and -9 activity were then measured in THP-1 cells incubated in the presence of active spore lipids, which showed a dose- and time-dependent up-regulation of these caspases, demonstrating that these caspases are involved in the apoptosis of THP-1 cells induced by active lipids of *G. lucidum* spores [37].

Other studies have been carried out on THP-1 cell lines, one of which evaluated the potential role in the induction of leukemic cell differentiation after exposure to a polysaccharide moiety isolated from the water-soluble residue of the *G. lucidum* polysaccharide, termed F3, which had been shown in a previous study to stimulate the inflammatory response through the expression of IL-1, IL-6, IL-12, and TNF-α [13]. F3 was obtained from the water-soluble residue of reishi and purified by gel filtration chromatography using a Sephacryl S-500 column with 0.1 N Tris buffer as an eluent. High-throughput microarrays were used to screen and study the dynamic patterns of gene expression, and the possible biological functions and physiological role of F3-treated leukemia cells were analyzed using a bioinformatics approach. THP-1 cells were treated with 30 μg/mL F3 and cell adhesion, a hallmark of macrophage differentiation, was examined by phase-contrast microscopy. F3 treatment resulted in the adhesion of approximately 45% of the cells; it significantly increased the reduction of NBT, a functional assay to assess the ability to produce superoxide during macrophage differentiation, and it also increased the population of THP-1 cells in the G0/G1 phase from 46 to 80% after treatment. Specific cellular markers and enzyme activities related to macrophage differentiation were analyzed, and overregulation of CD11b, CD14, CD68, and metalloproteinase-9 (MMP-9) and under-regulation of myeloperoxidase (MPO), a marker of the myeloid lineage, were noted. In addition, activation of p53 and caspase-3, -7, -8, and -9 was documented in THP-1 cells exposed to the F3 fraction. These data suggest that F3 polysaccharides from *G. lucidum* might have the potential to induce differentiation of leukemic cells into macrophage-like cells [19].

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