Induction of Apoptosis by Ethanol Extracts of *Ganoderma lucidum* in Human Gastric Carcinoma Cells

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Abstract

*Ganoderma lucidum*, a well-known medicinal mushroom, is highly valued and commonly used in Oriental medicine. Although recent experimental data has revealed the proapoptotic potency of *G. lucidum* extracts, the underlying mechanisms of this apoptotic activity have not yet been studied in detail. In the present study, the effects of ethanol extracts of *G. lucidum* (EGL) on the growth of an AGS human gastric carcinoma cell line were investigated. We found that EGL treatment resulted in a dose- and time-dependent significant decrease in the viability of AGS cells. This decreased viability was caused by apoptotic cell death, with observed chromatin condensation and an accumulation of apoptotic fraction. EGL treatment induced the expression of death receptor-related proteins such as death receptor 5 and tumor necrosis factor-related apoptosis-inducing ligand, which further triggered the activation of caspase-8 and the cleavage of Bid. In addition, the increase in apoptosis that was induced by EGL was correlated with activation of caspase-9 and -3, downregulation of IAP family proteins such as XIAP and survivin, and concomitant degradation of poly (ADP-ribose) polymerase. Moreover the activity of Akt was downregulated in EGL-treated cells, and the phosphatidylinositol-3 kinase/Akt inhibitor LY294002 sensitized the cells to EGL-induced apoptosis. The results indicated that EGL induces the apoptosis of AGS cells through a signaling cascade of death receptor-mediated extrinsic, as well as mitochondria-mediated intrinsic, caspase pathways which are associated with inactivation of the Akt signal pathway.
1. Introduction

Apoptosis is the active process of programmed cell death that occurs during many important physiological conditions and has become an area of extensive study in cancer research for an ideal way of eliminating precancerous and/or cancerous cells [1,2]. Apoptosis is characterized by a series of distinct morphological and biochemical alterations to cells such as plasma membrane blebbing, cell shrinkage, cell surface expression of phosphatidylserine, depolarization of the mitochondria, chromatin condensation, and DNA fragmentation. In general, apoptosis can be initiated in two ways: by the death receptor (DR; extrinsic) pathway or by the mitochondria-dependent (intrinsic) pathway, leading to activation of caspases and consequent apoptosis in mammalian cells [3–5]. In the former case, plasma membrane DRs are involved and the apoptosis signal is provided by the interaction between the ligand and death receptor. Changes in mitochondrial integrity caused by a broad range of physical and chemical stimuli, however, can trigger the intrinsic pathway of apoptosis [2,6,7]. Recent evidence has also shown that phosphatidylinositol-3 kinase (PI3K)/Akt is modulated in response to a variety of stimuli, in particular, the activation of Akt signaling promotes survival [8,9].

As a traditional medicine, *Ganoderma lucidum* (靈芝, lingzhi in Chinese or reishi in Japanese), a polypore mushroom that grows on the lower trunks of deciduous trees, has been widely used as a tonic in promoting longevity and health in Korea and other Asian countries [10–13]. The pharmacological activities of *G. lucidum*, especially its intrinsic immunomodulating and anti-tumor properties, have been extensively documented [13–16]. Recently, several studies have demonstrated that *G. lucidum* extracts (EGL) interfere with cell cycle progression, induce apoptosis, and suppress angiogenesis in human cancer cells to act as anticancer agents [17–25]. However, the precise mechanisms of apoptosis induction of EGL are largely unknown in human cancer cells.

The present study attempts to elucidate the apoptotic potential of the ethanol extracts of EGL in an AGS human gastric carcinoma cell line. The underlying intracellular signal transduction pathways involved in regulating apoptosis were also investigated. The results of this study demonstrated that EGL induces the apoptosis of AGS cells through a signaling cascade of death receptor-mediated extrinsic and mitochondria-mediated intrinsic caspase pathways, which are associated with inactivation of the Akt signal pathway.

2. Materials and Methods

2.1. Cell culture, preparation of ethanol extracts of EGL and cell viability assay

Human gastric carcinoma AGS cell line was purchased from the American Type Culture Collection (Rockville, MD, USA). AGS cells were cultured in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin in a 37°C incubator with 5% CO₂. The preparation of ethanol extracts of EGL occurred at Dongeui University Oriental Hospital, Busan, Korea. The freeze-dried and milled fruiting body of *G. lucidum* (200 g) was extracted with 25% ethanol (4L) at room temperature for 10 hours using a blender. The extracts were filtered through a Whatman #2 filter (Whatman, Maidstone, UK) concentrated to 500 mL under vacuum, and then kept at −20°C. The EGL solution obtained was directly diluted in the medium before assay. Measurement of cell viability was determined using the 3-(4,5-diethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes. For morphological studies, cells were treated with EGL for 72 hours and photographed directly using an inverted microscope (Carl Zeiss, Oberkochen, Germany).

2.2. Nuclear staining with DAPI

Untreated control and EGL-treated AGS cells were harvested and washed with phosphate-buffered saline (PBS). The cells were fixed with 3.7% paraformaldehyde (Sigma-Aldrich) in PBS for 10 minutes at room temperature. Fixed cells were collected by centrifugation and washed twice with PBS. The cells were fixed with 3.7% paraformaldehyde (Sigma-Aldrich) in PBS for 10 minutes at room temperature. The cells were washed two more times with PBS and analyzed via a fluorescence microscope (Carl Zeiss).

2.3. Measurement of apoptosis by annexin-V fluorescein isothiocyanate and propidium iodide double staining

The magnitude of the apoptosis elicited by EGL treatment was determined using an Annexin-V fluorescein isothiocyanate Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA). In brief, the cells were washed with PBS and resuspended in annexin-V binding buffer containing 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl₂ according to the manufacturer’s protocol. Aliquots of the cells were incubated with annexin-V fluorescein isothiocyanate, mixed, and incubated for
15 minutes at room temperature in the dark. Propidium iodide (PI) at a concentration of 5 μg/mL was added to identify the necrotic cells. The apoptotic cells were measured by a fluorescence-activated cell sorter analysis in a flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.4. Protein extraction, gel electrophoresis, and western blot analysis

Total cell lysates were lysed in an extraction buffer [25 mM Tris-Cl (pH 7.5), 250 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 1% Nonidet P-40, 0.1 mM sodium orthovanadate, 2 μg/mL leupeptin, and 100 μg/mL phenylmethylsulfonyl fluoride]. Protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). For western blot analysis, proteins (30–50 μg) were separated by 8–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then electrotransferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). The membranes were blocked with 5% skim milk for 1 hour and then subjected to immunoblot analysis with the appropriate antibodies. Proteins were subsequently visualized by the enhanced chemiluminescence method according to the recommended procedure (Amersham Co., Arlington Heights, IL, USA). Primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and Calbiochem (Cambridge, MA, USA). Peroxidase-labeled donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin were purchased from Amersham Co.

2.5. In vitro caspase activity assay

The activities of caspase-3, -8, and -9 were determined by colorimetric assay kits from R&D Systems (Minneapolis, MN, USA) following the manufacturer’s protocol. Briefly, the cells were lysed in a lysis buffer for 30 minutes in an ice bath. The supernatants were collected and incubated at 37°C with the supplied reaction buffer, which contained dithiothreitol and the substrates Asp-Glu-Val-Asp-p-nitroaniline for caspase-3, Ile-Glu-Thr-Asp-p-nitroaniline for caspase-8, and Leu-Glu-His-Asp-p-nitroaniline for caspase-9. The optical density of the reaction mixture was quantified using a spectrophotometer at a wavelength of 405 nm.

2.6. Statistical analysis

Analysis of variance, with a Bonferroni post hoc test, and graphical presentations were performed by GraphPad Prism version 5 (Hearne Scientific Software, Melbourne, Australia). Statistical significance was confirmed when p values were less than 0.05 or 0.01.

3. Results

3.1. Growth inhibition and apoptosis induction by EGL treatment in AGS cells

EGL-induced cell cytotoxicity was determined from the effects of various EGL concentrations on AGS cell viability using an MTT assay. The data showed that treatment with EGL decreased the viability of AGS cells in a concentration- and time-dependent manner (Figure 1). Further experiments were performed to determine if this inhibitory effect of EGL on cell viability was the result of apoptotic cell death. Direct observation using an inverted microscope demonstrated that the cells treated with EGL showed many morphological changes compared with control cells (Figure 2A). In particular, cell shrinkage, cytoplasm condensation, and formation of cytoplasmic filaments appeared after 1.5% EGL treatment for 72 hours. Morphological analysis with DAPI staining revealed nuclei with concentration-dependent chromatin condensation and the formation of apoptotic bodies in cells cultured with EGL. In contrast, very few apoptotic cells were observed in the control culture (Figure 2B). Therefore, flow cytometry analysis with annexin V and PI staining was used to determine the magnitude of apoptosis elicited by EGL. As shown in Figures 2C and 2D, the annexin V-positive cells
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increased with concentration and length of exposure to EGL, compared with the untreated control cells. These results demonstrated that the cytotoxic effects observed in response to EGL are associated with the induction of apoptosis in AGS cells. A good correlation between the extent of apoptosis and the inhibition of growth was observed.

3.2. Modulation of apoptosis-related proteins by EGL in AGS cells

To elucidate the apoptotic pathways activated by EGL, we used Western blot analyses to measure the expression of the death receptors and corresponding pro-apoptotic ligands, as well as the expression of the Bcl-2 and IAP family members. As indicated in Figure 3, no significant changes of Fas, Fas ligand (FasL) and DR4 protein levels were noted in AGS cells treated with EGL, however, EGL increased the expression levels of DR5 and necrosis factor-related apoptosis-inducing ligand (TRAIL) proteins in a concentration- and time-dependent manner. When AGS cells were treated with EGL, a clear decrease in anti-apoptotic Bcl-2 protein expression was observed without alteration of anti-apoptotic Bcl-XL, and pro-apoptotic protein Bax and Bad expression. However, treatment with EGL significantly induced the cleavage of Bid, a BH3-only proapoptotic member of the Bcl-2 family, in a concentration- and time-dependent manner. In addition, the levels of IAP family proteins, such as XIAP and survivin, but not cIAP-1 and cIAP-2, were significantly downregulated after EGL treatment.

3.3. Activation of caspases and degradation of poly(ADP ribose) polymerase protein by EGL treatment

Next, experiments were performed to characterize the role of caspase activation in EGL-mediated apoptosis in AGS cells. The immunoblotting results showed that the levels of active caspase-3, -8, and -9 proteins were increased in a concentration-dependent manner in EGL-treated AGS cells (Figure 4A). Furthermore, to monitor the enzymatic activity of these enzymes during EGL-induced apoptosis, the
in vitro caspase activity was measured following treatment with EGL using specific fluorogenic peptide substrates for each caspase. As shown in Figure 4B, the activities of these caspases were significantly increased in a concentration-dependent fashion as compared with untreated control cells. Subsequent Western blot analyses showed the progressive proteolytic cleavage of poly(ADP ribose) polymerase protein (PARP) in AGS cells after EGL treatment (Figure 4A), suggesting that the activation of caspase is involved in the EGL-induced apoptotic pathway.

3.4. PI3K/Akt inhibitor sensitizes EGL-induced apoptosis

Recent studies have reported that EGL can cause cell cycle arrest and apoptosis. These observations suggest that the growth inhibitory effect of the extracts occurs through the blockage of the G1/S or G2/M phase, and that these cells do not enter cell cycle progression and die through apoptosis [17–21,23–25]. While some mechanisms of cell death related to these extracts have been suggested [17,20–24], the signaling pathway by which they occur have not been elucidated in human gastric cancer cells. Therefore, the aim of this study was to determine the capacity of EGL to induce apoptosis, and to identify the biochemical mechanisms involved in a human gastric carcinoma AGS cell line. Our present study demonstrated that EGL inhibits AGS cell growth by the induction of apoptotic cell death through modulation of several apoptosis related proteins and activation of caspase. Furthermore, the inhibition of the PI3K/Akt pathway enhanced EGL-induced cytotoxic and apoptotic effects.

Apoptosis is endogenous programmed cell death that can be triggered by various stimuli, including DR-mediated signaling (via the DR/extrinsic pathway) and intracellular stress (via the mitochondrial/intrinsic pathway). Mitochondria are major regulators of extrinsic, as well as intrinsic, apoptotic pathways, and they undergo a series of consequential changes during apoptosis. Mitochondrial function is controlled by several factors, such as the Bcl-2 and IAP protein family [1,3–5]. The Bcl-2 family significantly regulates apoptosis, either as an activator (e.g., Bax, Bad, etc.), or as an inhibitor (e.g., Bcl-2, Bcl-xL, etc.). It has been suggested, therefore, that the Bax/Bcl-2 ratio is a key factor in the regulation of the apoptotic process [26,27]. The IAP family functions by binding to and inhibiting several PI3K for survival signaling. As shown in Figure 5A, the levels of total Akt protein remained unchanged by EGL treatment; however, phosphorylation levels were markedly decreased in a concentration- and time-dependent manner. Thus, the involvement of Akt signal pathways in EGL-induced apoptosis was examined using the PI3K/Akt inhibitor LY294002 to determine if the inhibition of Akt phosphorylation was responsible for the induction of apoptosis. As shown in Figures 5B and 5C, the combined treatment with EGL and LY294002 significantly decreased the phosphorylation levels of Akt and increased the cleavage of PARP proteins and apoptosis. This indicates that EGL-induced apoptosis is associated with the downregulation of the PI3K/Akt signaling pathway.

4. Discussion

Recent studies have reported that EGL can cause cell cycle arrest and apoptosis. These observations suggest that the growth inhibitory effect of the extracts occurs through the blockage of the G1/S or G2/M phase, and that these cells do not enter cell cycle progression and die through apoptosis [17–21,23–25]. While some mechanisms of cell death related to these extracts have been suggested [17,20–24], the signaling pathway by which they occur have not been elucidated in human gastric cancer cells. Therefore, the aim of this study was to determine the capacity of EGL to induce apoptosis, and to identify the biochemical mechanisms involved in a human gastric carcinoma AGS cell line. Our present study demonstrated that EGL inhibits AGS cell growth by the induction of apoptotic cell death through modulation of several apoptosis related proteins and activation of caspase. Furthermore, the inhibition of the PI3K/Akt pathway enhanced EGL-induced cytotoxic and apoptotic effects.

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Activation of the mitochondrial pathway leads to the release of Smac/Diablo, which removes the IAP blockage of caspase activation [2,6,7]. The present data shows that EGL-induced apoptosis was related to the downregulation of pro-apoptotic Bcl-2 (Figure 3), which suggests that EGL increased the Bax/Bcl-2 ratio and induced mitochondrial dysfunction, leading to apoptosis in AGS cells. Furthermore, exposure of AGS cells to EGL caused a downregulation of IAP family proteins such as XIAP and survivin (Figure 3).  

Cell surface Fas/FasL and TRAIL/DR systems are key signaling transduction pathways of the extrinsic pathway of apoptosis in cells. Binding FasL to Fas receptors or TRAIL to DRs, leads to receptor oligomerization and the formation of the death-inducing signaling complex, followed by the activation of caspase-8, and then cleavage of Bid (tBid). The tBid can translocate to mitochondria and bind to Bax, leading to a conformational change of Bax and to the activation of caspase-9, and concomitant activation of caspase-3 [6,7]. Thus, caspase-3 is the most important executioner of apoptosis. Significant evidence indicates that caspase-3 is either partially or totally responsible for the proteolytic cleavage of many key proteins including PARP. PARP is important for cell viability, and the cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis [30]. To further gain the mechanical insights of EGL-induced apoptosis of AGS cells, the catalytic activity of caspases and the levels of Bid were investigated. The present data demonstrated that EGL increased the levels of DR5 and TRAIL and the enzymatic activity of extrinsic and intrinsic caspase cascades such as caspase-8 and -9, and decreased the levels of total Bid expression (Figures 3 and 4). In addition, caspase-3 was activated and PARP proteins were cleaved in EGL-treated AGS cells (Figure 3).  

Although the PI3K/Akt signal pathways play a critical role in regulating cell survival and death in many physiological and pathological settings, numerous studies have indicated that these pathways are more often associated with cell survival through activation of anti-apoptotic downstream effectors [8,9]. Therefore, whether EGL-induced apoptosis is associated with these pathways was also investigated. EGL treatment caused the downregulation of Akt activation, and the combined exposure with LY294002, a PI3K/Akt inhibitor, made the cells more sensitive to EGL-induced apoptosis (Figure 5). These results indicate that the PI3K/Akt signaling may have a survival role in response to EGL-induced apoptosis.  

In summary, the results of this study demonstrate that EGL triggers apoptosis of AGS cells through activation of the intrinsic caspase pathway along with the DR-mediated extrinsic pathway. Moreover, the inactivation of Akt may play an important role in EGL-induced apoptosis. Although, it is still unclear if *G. lucidum* can induce apoptosis through other pathways, the results provide new information on the possible mechanisms for the anticancer activity of *G. lucidum*.
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References

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