Induction of Apoptosis by *Gagamhwanglyeonhaedog-tang* through Activation of Caspase-3 in Human Leukemia Cell Line HL-60 Cells

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Gagamhwanglyeonhaedog-tang(GHH), a Korean genuine medicine, is a newly designed herbal drug formula based on the traditional oriental pharmacological knowledge for the purpose of treating tumorous diseases. Apoptosis is an evolutionarily conserved suicide program residing in cells. It leads to cell death through a tightly regulated process resulting in the removal of damaged or unwanted tissue. In the present study, the apoptosis inducing activities of the decocted water extract of GHH were studied. Results of the 3- [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay showed that GHH had a strong cytotoxic effect on HL-60 cells. The number of live cells was less than 20 % after exposure to 1 mg/ml GHH for 48 hr. GHH increased cytotoxicity of HL-60 cells in a dose- and time-dependent manner. Cell apoptosis by GHH was confirmed by flow cytometric analysis of the DNA-stained cells. The percentage of apoptotic cells increased to 28 %, 31 % and 37 % 24 hr and 37 %, 44 % and 81 % 48 hr after treatment with 0.01, 0.1 and 1 mg/ml GHH, respectively. Flow cytometric analysis of GHH treated HL-60 cells showed increase of hypodiploid apoptotic cells in a dose- and time- dependent manner. DNA fragmentation also occurred in apoptosis and was characterized by a ladder pattern on agarose gel. In addition, GHH (0.01 and 0.1 mg/ml) increased the secretion of tumor necrosis factor-alpha in 24 and 48 hr. The author showed that GHH-induced apoptosis was accompanied by activation of caspase-3. These results suggest that GHH induces activation of caspase-3 and eventually leads to apoptosis.

Key words : Gagamhwanglyeonhaedog-tang, Caspase-3, Apoptosis

Introduction

Although Korean genuine medicines have long been used effectively in treating many diseases in Asian communities, the pharmacological mechanisms of most medicines used have not been defined. Hwanglyeonhaedog-tang, a traditional oriental medicine, has been used for treatment of various allergic diseases in Korea. Hwanglyeonhaedog-tang is a prescription composed of four oriental medicinal herbs including Rhizoma Coptidis, Radix Scutellariae, Fructus Gardeniae and Cortex Phellodendri. Hwanglyeonhaedog-tang water extract suppresses immunoglobulin E-mediated anaphylactic reaction[1]. However, Gagamhwanglyeonhaedog-tang (GHH) is a newly designed herbal medicine formula based on the traditional oriental pharmacological knowledge for the purpose of treating tumorous diseases, composed of nine oriental medicinal herbs including Rhizoma Coptidis, Radix Scutellariae, Fructus Gardeniae, Radix Bupleuri, Radix Salviae, Radix Glycyrrhizae, Herba Hedyotis, Flos Lonicerae and Fructus Forsythiae.

Apoptosis possesses unique morphologic and biochemical features which distinguish this mechanism of programmed cell death from necrosis. Apoptosis is an evolutionarily conserved suicide program residing in cells. It leads to cell death through a tightly regulated process resulting in the removal of damaged or unwanted tissue[2-3]. Recently, interest has focused on the manipulation of the apoptotic process for the treatment and prevention of cancer. Thus, much effort has been directed toward the search for substances that influence apoptosis and understanding their mechanism of action. Like carcinogenesis, apoptosis may conceptually be divided into several stages or phases that include initiation, propagation and execution[4-6]. Since the execution machinery of apoptosis appears to be evolutionally conserved, it seems possible that it plays an especially important role in governing both the accuracy and efficiency of the apoptotic pathway[7-9]. The execution phase of apoptosis involves the activation of caspases and the subsequent cleavage of several cellular substrates such as

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· Received : 2004/04/12 · Revised : 2004/06/11 · Accepted : 2004/07/13

PARP (poly-ADP-ribose polymerase), gelsolin, actin, lamins, and fodrin. Human caspases 11 have been described, and a previous study demonstrated that activation of the caspase cascade is involved in chemical- and agent-induced apoptosis[10-12]. Caspase 9 is an apoptosis initiator which is activated by binding with Apaf-1, a homolog of CED (caspase essential for developmental apoptosis) -4, to induce its oligomerization[13-15]. Activated caspase 9 then cleaves and activates executioner caspase-3, which exists as an inactive caspase-3 in the cytoplasm and is proteolytically activated by multiple cleavages of caspase-3 to generate the cleaved fragments in cells undergoing apoptosis[16-18]. After caspase-3 activation, specific substrate for caspase-3 such as PARP is cleaved which are important for the occurrence of apoptosis[19-21]. PARP that is required for DNA repair, is a 116-kDa protein, which converts NAD to nicotinamide and protein-linked ADP-ribose polymers[22-24]. In response to growth factor withdrawal or upon exposure to a variety of chemotherapeutic compound.

Experimental procedures

1. Materials

These plants materials were obtained from Jeonju Oriental Medical Center, Wonkwang University, South Korea.

Table 1. Prescription Contents of Gagamhwanglyeonhaedog-tang(GHH)

Herbal name	Herb medicine name	Scientific name	Amount(g)
黃連	Rhizoma coptidis	*Coptis japonica MAKINO.*	10
黃芩	Radix scutellariae	*Scutellaria baikalensis GEORGI.*	10
梔子	Fructus gardeniae	*Gardenia jasminoides ELLIS.*	10
柴胡	Radix bupleuri	*Bupleurum chinense DC.*	15
丹蔘	Radix salviae miltiorrhizae	*Salvia miltiorrhiza BGE.*	15
甘草	Radix glycyrrhizae	*Glycyrrhiza uralensis FISCH.*	10
白花蛇舌草	Herba oldenlandiae diffusae	*Hedyotis diffusa ROXB*	30
金銀花	Flos lonicerae	*Lonicera japonica THUNB.*	15
連翹	Fructus forsythiae	*Forsythia koreana NAKAI.*	15
TOTAL AMOUNT			130

2. Methods

1) Chemicals

RPMI-1640 and fetal bovine serum (FBS) were purchased from Gibco/BRL(Burlington, Ontario, Canada). Dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium Bromide (MTT), isopropanol, RNase, sodium EDTA, propidium iodide (PI) and other reagents were purchased from Sigma Chemical (St. Louis, MO, USA). Anti-human caspase-3 antibody was obtained from Santa Cruz Biotechnology, Inc. (Califonia, USA). Human recombinant TNF-α (rTNF-α) and TNF-α antibody were from R&D System Inc. (USA).

2) Preparation of extract of GHH

An extract of GHH was prepared by decocting the dried prescription of herbs with boiling distilled water. The duration of decoction was about 3 hr. The decoction was filtered, lyophilized and kept at 4 ℃. The ingredients of 130 g GHH include 10 g of Rhizoma Coptidis, 10 g of Radix Scutellariae, 10 g of Fructus Gardeniae, 15 g of Radix Bupleuri, 15 g of Radix Salviae, 10 g of Radix Glycyrrhizae, 30 g of Herba Hedyotis, 15 g of Flos Lonicerae and 15 g of Fructus Forsythiae. These ingredients correspond to parts of the following plants: Coptis japonica Makino (Ranunculaceae), Scutellaria baikalensis Gorgy (Labiatae), Gardenia jasminoides Ellis (Rubiaceae), Bupleurum chinense DC. (Umbelliferae), Salvia miltiorrhiza Bge. (Labiatae), Glycyrrhiza uralensis Fisch. (Leguminosae), Hedyotis diffusa Willd. (Rubiaceae), Lonicerae japonica Thunb. (Loniceraceae) and Forsythia suspensa Vahl. (Oleaceae), respectively.

3) Cell culture

HL-60 cells were obtained from Korean Cell Line Bank (KCLB, South Korea). HL-60 cells were maintained in RPMI 1640 medium containing 10 % heat-inactivated FBS supplemented with 100 unit/ml penicillin and 100 unit/ml streptomycin at 37 ℃ under 5 % CO_2 in the air.

4) MTT assay

Cell viability was assessed by MTT staining as described by Mosmann[27], with some modifications. In brief, cells were seeded in 4-well plates (500 ㎕/well at a density of 4×10^5 cells/ml) and exposed to various concentrations of GHH for 24 and 48 hr. At the end of treatment, MTT solution (5 mg/ml in PBS) was added (50 ㎕/well), and cells were incubated for a further 4 hr at 37 ℃, and the formazan crystals formed were centrifuged and the pellets dissolved by the addition of DMSO. Absorption was measured by a spectrometer at 540 nm.

5) DNA extraction and electrophoresis

The characteristic ladder pattern of DNA break was analysed by agarose gel electrophoresis. Briefly, DNA from the HL-60 cells (3×10^6 cells/each group) was isolated by a Genomic DNA extraction kit (iNtRON BIOTECH NOLOGY Co., South Korea). Isolated genomic DNA was subjected to 1.5 % agarose electrophoresis at 100 V for 1 hr. DNA was visualized by staining with ethidium bromide under UV light.

6) Cell cycle distribution

Cells were harvested and washed once with cold PBS. Then cell pellets were suspended in 500 × of PI solution containing 50 ㎍/ml PI, 0.1 % (w/v) sodium citrate and 0.1 % RNase. Cell samples were incubated at 4 ℃ in a dark for at least 30 min, and analyzed using flow cytometer (FACS

Calibur, Becton Dickinson) and Cell Quest software.

7) Assay of TNF-α secretion

TNF-α concentration in the cell-derived culture supernatants was measured by a modified ELISA, as described[28]. The ELISA was devised by coating 96-well plates of human monoclonal antibody with specificity for human TNF-α. Before use and between subsequent steps in the assay, coated plates were washed with PBS containing 0.05 % tween-20. All reagents used in this assay were incubated for 2 hr at 37 °C. Recombinant human TNF-α were diluted and used as a standard. Serial dilutions starting from 1 pg/ml were used to establish the standard curve. Assay plates were exposed sequentially to rabbit anti-TNF-α antibody, and phosphatase conjugated goat anti-rabbit IgG antibody and avidine peroxidase, and p-nitrophenyl phosphate and ABTS substrate solution containing 30 % H_2O_2. The plates were read at 405 nm.

8) Western blot analysis

Western blotting was performed according to the methods previously described[29]. Whole cell lysates were made by boiling cells in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol). Proteins in the cell lysates were separated by 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose paper. The membrane was blocked with 5 % skim milk in 0.1 % PBS-tween 20 for 1 hr at room temperature and incubated with anti-caspase-3 antibody. After washing in 0.1 % PBS-tween 20 three times, the blot was incubated with secondary antibodies for 30 min and the antibody specific proteins were visualized by the enhanced chemiluminescence detection system (ECL; Amersharm Pharmacia Biotech. Braunschweig, Germany) according to the recommended procedure.

9) Statistical analysis

Each experiment was performed at least in triplicate. The results were expressed as mean ± S. D. for the number of experiments. Statistical significance was compared between each treated group and control by the Student's t-test. Results with $P<0.01$ were considered significantly from control group.

Results

1. Effect of GHH on cell viability

Initially, the author used the MTT assay as an indirect measure of viability of HL-60 cells exposed to GHH. As shown in Fig. 1, GHH had a strong cytotoxic effect on HL-60 cells. The number of live cells was less than 20 % after exposure to 1 mg/ml GHH for 48 hr. GHH increased the cytotoxicity of HL-60 cells in a dose- and time- dependent manner.

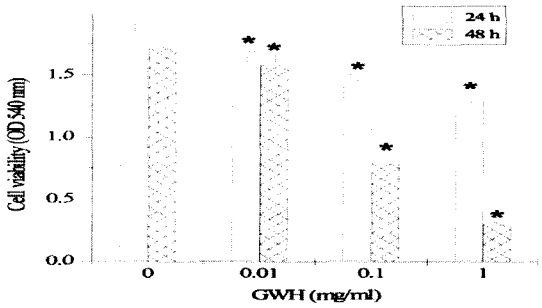

Fig. 1. Effect of GWH on cell viability in HL-60 cells. The cells (4×10^5 cells/ml) were treated with various concentrations of GWH for 24 and 48 h. The value represented the mean ± S. D. from four independent experiments. *$P<0.01$; significantly different from the control value.

2. Effect of GHH on cell cycle distribution and DNA fragmentation

The author examined DNA fraction of HL-60 cells treated with GHH. Cell apoptosis from GHH was confirmed by flow cytometric analysis of the DNA-stained cells. The percentage of apoptotic cells increased to 28, 31 and 37 % in 24 hr and 37, 44 and 81 % in 48 hr after treatment with 0.01, 0.1 and 1 mg/ml GHH, respectively (Fig. 2).

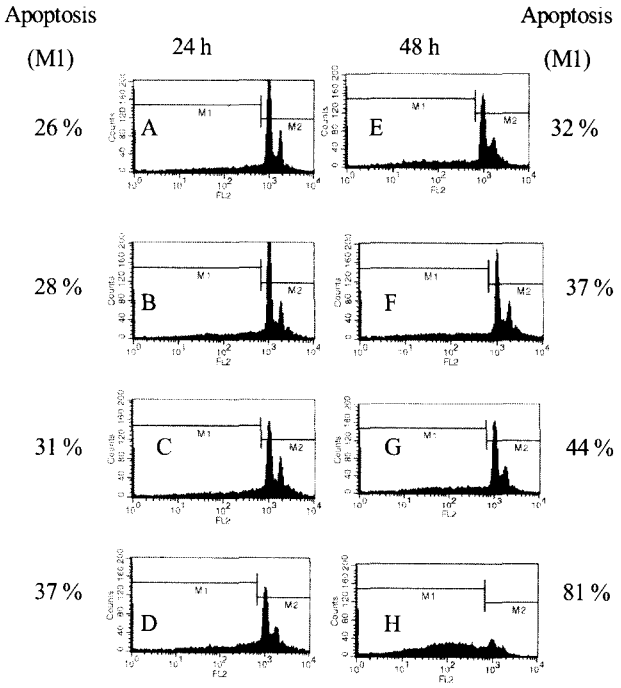

Fig. 2. Effect of GWH on cell cycle distribution in HL-60 cells. The cells (1.5×10^6 cells/ml) were treated with various concentrations of GWH for 24 and 48 h. The cells were stained with PI solution and analysed for DNA content by flow cytometry (see Materials and Methods). A and E, Control; B and F, 0.01 mg/ml; C and G, 0.1 mg/ml; D and H, 1 mg/ml.

Flow cytometry analysis of GHH treated HL-60 cells showed an increase of hypodiploid apoptotic cells in a dose- and time- dependent manner. DNA fragmentation also occurred in apoptosis and was characterized by a ladder pattern on agarose gel (Fig. 3).

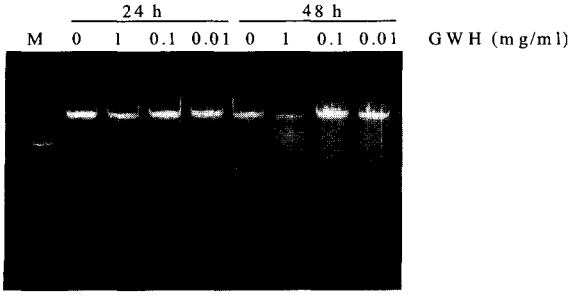

Fig. 3. Effect of GWH on DNA fragmentation in HL-60 cells. HL-60 cells (3×10^5 cells/ml) were treated with various concentrations of GWH for 24 and 48 h. Twenty μg of DNA was electrophoresed in a 1.5 % agarose gel, stained with ethidium bromide, and photographed under UV illumination. M, 100 base pair.

3. Effect of GHH on TNF-α secretion

Abnormalities of the TNF-α have been linked to several human diseases, including cancer. Novel treatment strategies for cancer are emerging based on an understanding of the function of TNF-α. The advantage of these strategies is their potential to selectively target cancer cells, while sparing normal cells. Combining these new strategies with currently available treatments such as chemotherapy and radiation therapy is under investigation, with promising results. In this study, the author investigated the ability of GHH to secrete TNF-α in HL-60 cells. TNF-α secretion was quantified by the ELISA method. Both 0.01 and 0.1 mg/ml GHH increased the secretion of TNF-α at 24 and 48 hr. However, GHH 1 mg/ml decreased the secretion of TNF-α at 24 hr (Fig. 4).

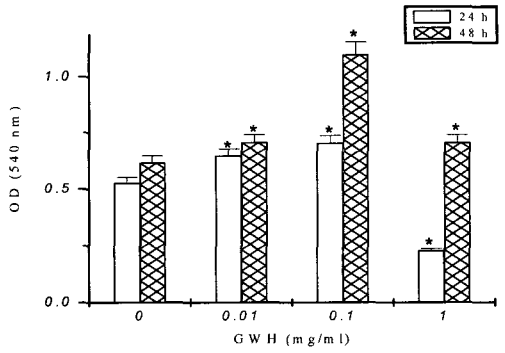

Fig. 4. Effect of GWH on TNF-(secretion in HL-60 cells. The cells (4×10^5 cells/ml) were incubated for 24 and 48 h in medium alone or in medium with various concentrations (0.01, 0.1 and 1 mg/ml) of GWH. The supernatants were collected and frozen at -80°C until assayed for TNF-(concentration. Each data value indicates the mean ± S. D. of three separate experiments. *P<0.01: significantly different from the control value.

4. Effect of GHH on caspase-3 activation

Extrinsic apoptotic cell death is receptor-linked and initiates apoptosis by activating caspase 8. Intrinsic apoptotic cell death is mediated by the release of cytochrome c from mitochondrial and initiates apoptosis by activating caspase 3. To determine whether GHH-induced apoptosis is involved in caspase-3, caspase-3 activation was assayed by western blot analysis. As shown in Fig. 5, there was no important change in 24 hr but caspase-3 was significantly activated compared with control in 48 hr (Fig. 5).

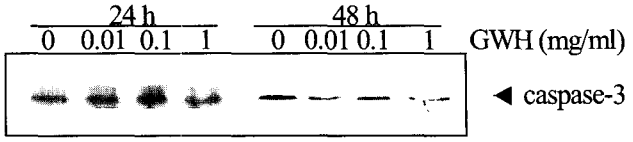

Fig. 5. Effect of GWH on caspase-3 activation in HL-60 cells. Fifty μg of total protein was resolved by 10% SDS-PAGE, transferred to nitrocellulose membrane, and analysed by Western blotting using anti-caspase-3 antibody.

Discussion

Apoptosis is the most common and distinct form of cell death involving a series of steps and acts as physiological suicide mechanism to preserve tissue homeostasis through proper cell turnover[30-32]. There is ample evidence that naturally occurring compounds and many chemotherapeutic agents with antitumor effects can trigger the apoptosis of cancer cells 33, 34). Therefore, one of the attractive methods for cancer chemoprevention or chemotherapy is dietary or pharmaceutical production to induce death of tumor cells through apoptosis. The author investigated the cytotoxic effect of GHH in HL-60 cells. In the present study, the author found that GHH potently inhibited cell viability (Fig. 1). Thus, the author expected that GHH-induced cytotoxicity is the result of apoptosis. To characterize cell death induced by GHH, the integrity of genomic DNA, morphological changes, and the hypodiploid cells in HL-60 cells were examined. On analysis of DNA integrity by agarose electrophoresis, the results showed that GHH caused the digestion of genomic DNA into ladders, the biochemical hallmark of apoptosis, in time- dependent manner, associated with a decrease in intact DNA. Endogenous DNase, which degradates DNA, cuts the internuclosomal regions into double-stranded DNA fragments of 180-200 base pairs[35]. Morphological alternations in GHH-treated HL-60 cells were detected under microscopic observation. Under GHH treatment for 24 and 48 hr, HL-60 cells showed swelling and rounded morphology and the appearance of significant apoptotic bodies around the rounded cells were detected. These apoptotic bodies are rapidly cleaned from the local tissue by macrophages[36]. Results of flow cytometry analysis showed that an increase of hypodiploid cells was detected in GHH-treated HL-60 cells. Our present results strongly suggest that the cytotoxic mechanism of GHH may be attributable to induce apoptosis in HL-60 cells. As reported in Fig. 4, in vitro, exposure of HL-60 cells to 0.01 and 0.1 mg/ml GHH resulted in a increase in TNF-α. This fact suggests that GHH induces release of TNF-α in HL-60 cells and TNF-α is a potential

candidate in accounting for the GHH-induced cytotoxicity measured in our in vitro model. Also, to clarify mechanism of GHH-induced apoptosis, the author investigated caspase-3 activation. Caspase-3 is the final executioner enzyme associated with cell death during stimuli-induced apoptosis[37]. Once activated, caspase-3 is free to initiate the various process involved in apoptosis[38]. Activated caspase-3 is found only in cells undergoing apoptosis and consists of p18 and p12 sub.

However, in order to clarify the hypothesis of this study, further experiments are necessary. For example, it is possible to examine if GHH has cytotoxicity on normal cells, not cancer cells. Also, further studies are needed to clarify the in vivo effect.

Conclusion

In this study, the ability of Korean medicine prescription, GHH, on apoptosis inducing effects was investigated in HL-60 cells.

GHH had a strong cytotoxic effect on HL-60 cells. GHH increased the cytotoxicity of HL-60 cells in a dose- and time-dependent manner. In this study, cell apoptosis from GHH was confirmed by flow cytometric analysis of the DNA-stained cells. Flow cytometry analysis of GHH treated HL-60 cells showed an increase of hypodiploid apoptotic cells in a dose- and time- dependent manner. DNA fragmentation also occurred in apoptosis and was characterized by a ladder pattern on agarose gel. In this study, the author investigated the ability of GHH to secrete TNF-α in HL-60 cells. TNF-α secretion was quantified by the ELISA method. Extrinsic apoptotic cell death is receptor-linked and initiates apoptosis by activating caspase-8. Intrinsic apoptotic cell death is mediated by the release of cytochrome c from mitochondrial and initiates apoptosis by activating caspase-3. There was no important change in 24 hr but caspase-3 was significantly activated compared with control in 48 hr.

Thus, the author expected that GHH-induced cytotoxicity is the result of apoptosis.

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