Full Paper

Induction of Apoptosis by Korean Medicine Gagam-whanglyun-haedoktang Through Activation of Caspase-3 in Human Leukemia Cell Line, HL-60 Cells

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Received May 24, 2004; Accepted November 19, 2004

Abstract. Gagam-whanglyun-haedoktang (GWH) 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. In the present study, apoptosis inducing activities of the decocted water extract of GWH were studied. Results of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showed that GWH had a strong cytotoxic effect on HL-60 cells. The number of live cells was less than 20% after exposure to 1 mg/ml GWH for 48 h. GWH increased cytotoxicity of HL-60 cells in a dose- and time-dependent manner. The percentage of apoptotic cells by flow cytometric analysis of the DNA-stained cells increased to 28%, 31%, and 37% at 24 h and to 37%, 44%, and 81% at 48 h after treatment with 0.01, 0.1, and 1 mg/ml GWH, respectively. DNA fragmentation also occurred in apoptosis and was characterized by a ladder pattern on agarose gel. In addition, GWH increased the secretion of tumor necrosis factor-α. GWH-induced apoptosis was accompanied by activation of caspase-3. These results suggest that GWH induces activation of caspase-3 and eventually leads to apoptosis.

Keywords: gagam-whanglyun-haedoktang, apoptosis, cancer, human leukemia cell, caspase-3

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. Whanglyun-haedoktang, a traditional oriental medicine, has been used for treatment of various allergic diseases in Korea. Whanglyun-haedoktang is a prescription composed of four oriental medicinal herbs including Coptis japonica MAKINO, Scutellaria baikalensis GORY, Gardenia jasminoides ELLIS, and Cortex Phellodendri amurense RUPR. Whanglyun-haedoktang water extract suppresses immunoglobulin E-mediated anaphylactic reaction (1).

However, Gagam-whanglyun-haedoktang (GWH) 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 Coptis japonica MAKINO, Scutellaria baikalensis GORY, Gardenia jasminoides ELLIS, Bupleurum chinense DC, Salvia miltiorrhiza BGE, Glycyrrhiza uralensis FISCH, Hedychites diffusa WILLD, Lonicerae japonica THUNB, and Forsythia suspensa Vahl.

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. 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 poly-ADP-ribose polymerase (PARP), gelsolin, actin, lamins, and fodrin. Human caspases 1 – 10 have been described, and previous studies demonstrated that activation of the caspase cascade is involved in chemical- and agent-induced apoptosis (10 – 12). Caspase 9 is an apoptosis initiator that is activated by binding with Apaf-1, a homolog of caspase essential for developmental apoptosis-4 (CED-4), to induce its oligomerization (13 – 15). Activated caspase 9 then cleaves and activates executor 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, a 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 that converts nicotinamide adenine dinucleotide (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 compounds, PARP is cleaved to generate an 85-kDa fragment (25, 26). Abnormalities of the tumor necrosis factor (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 ability of the Korean medicine prescription GWH to induce apoptosis was investigated in HL-60 cells. The following traditional criteria for this purpose have been proposed: 1) tetrazolium-based colorimetric assay was performed for cytotoxicity test; 2) a DNA ladder must be demonstrated by agarose gel electrophoresis; 3) TNF-α secretion was assayed by enzyme-linked immunosorbent assay (ELISA). There are several new assays potentially useful for determining the basis of biochemical events associated with apoptosis such as a flow cytometry and caspase-3 activation.

Materials and Methods

Reagents

RPMI-1640 and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium 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. (Santa Cruz, CA, USA). Human recombinant TNF (rTNF)-α and TNF-α antibody were from R&D Systems (Minneapolis, MN, USA).

Preparation of GWH

An extract of GWH was prepared by decocting the dried prescription of herbs with boiling distilled water (130 g/l). The duration of decoction was about 3 h. The decoction was filtered, lyophilized, and kept at 4°C. The yield of extraction was about 10% (w/w). The ingredients of 130 g GWH include 10 g of Coptis japonica MAKINO (Ranunculaceae), 10 g of Scutellaria baikalensis GORY (Labiatae), 10 g of Gardenia jasminoides ELLIS (Rubiacae), 15 g of Bupleurum chinense DC (Umbelliferae), 15 g of Salvia miltiorrhiza BGE (Labiatae), 10 g of Glycyrrhiza uralensis FISCH (Leguminosae), 30 g of Hedyotis diffusa WILLD (Rubiaceae), 15 g of Loniceraceae japonica THUNB (Loniceraceae), and 15 g of Forsythia suspensa VAHL (Oleaceae). These plant materials were obtained from Gwangju Oriental Medical Center, Wonkwang University, Korea, and their voucher specimens have been deposited in the Herbarium in the College of Pharmacy, Wonkwang University.

Cell culture

HL-60 cells were obtained from Korean Cell Line Bank (Seoul, 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°C under 5% CO₂ in air.

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 µl/well at a density of 4 × 10⁵ cells/ml) and exposed to various concentrations of GWH for 24 and 48 h. At the end of treatment, MTT solution (5 mg/ml in PBS) was added (50 µl/well), and cells were incubated for a further 4 h at 37°C, 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.

**DNA extraction and electrophoresis**

The characteristic ladder pattern of DNA breakage was analyzed 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 Biotechnology, Sungnam, Korea). Isolated genomic DNA was subjected to 1.5% agarose electrophoresis at 100 V for 1 h. DNA was visualized by staining with ethidium bromide under UV light.

**Cell cycle distribution**

Cells were harvested and washed once with cold PBS. Then cell pellets were suspended in 500 µl of PI solution containing 50 µg/ml PI, 0.1% (w/v) sodium citrate, and 0.1% RNase. Cell samples were incubated at 4°C in a dark for at least 30 min and analyzed using flow cytometer (FACS Calibur) and Cell Quest software (Becton Dickinson, Sunnyvale, CA, USA).

**ELISA of TNF-α**

TNF-α concentration in the cell-derived culture supernatants was measured by a modified ELISA, as previously 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 h 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, phosphatase conjugated goat anti-rabbit IgG antibody and avidine peroxidase, and p-nitrophenyl phosphate and full name (ABTS) substrate solution containing 30% H₂O₂. The plates were read at 405 nm.

**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 h 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; Amersham Pharmacia Biotech., Braunschweig, Germany) according to the recommended procedure.

**Statistical analyses**

Each experiment was performed at least in triplicate. The results were expressed as the 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**

**Effect of GWH on the cell viability**

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

**Effect of GWH on the cell cycle distribution and DNA fragmentation**

The author examined DNA fraction of HL-60 cells treated with GWH. Cell apoptosis from GWH was confirmed by flow cytometric analysis of the DNA-stained cells. The percentage of apoptotic cells increased

![Fig. 1. Effect of GWH on the cell viability in HL-60 cells. The cells (4 × 10^6 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 negative control value (0 mg/ml).](image-url)
GWH Induces Apoptosis

To 28%, 31%, and 37% at 24 h and 37%, 44%, and 81% at 48 h after treatment with 0.01, 0.1, and 1 mg/ml GWH, respectively (Fig. 2). Flow cytometry analysis of GWH 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).

**Effect of GWH on the TNF-α secretion**

In this study, we investigated the ability of GWH to secrete TNF-α in HL-60 cells. The level of TNF-α was quantified using the ELISA method. Both 0.01 and 0.1 mg/ml GWH increased the secretion of TNF-α at 24 and 48 h (Fig. 4).

**Effect of GWH on the 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 caspase-3 is involved in GWH-induced apoptosis, caspase-3 activation was assayed by Western blot analysis. As shown in Fig. 5, there was no important change at 24 h but caspase-3 was significantly activated compared with the control at 48 h.

Discussion

Apoptosis includes cell shrinkage and loss of contact with neighboring cells, formation of cytoplasmic vacuoles, plasma and nuclear membrane blebbing, chromatin condensation, and formation of apoptotic bodies (30). These apoptotic bodies are rapidly cleaned from the local tissue by macrophages (31). The preponderance of apoptotic nuclei was most likely blocked at the G1 and G2 phase, which is in agreement with the DNA fluorescence flow cytometry profiles. We have also noted DNA fragment ladder formation, a characteristic gel electrophoretic band pattern associated with apoptosis. A DNA ladder, the biochemical hallmark of apoptosis, is degradation of DNA by endogenous DNase, which cuts the internuclosomal regions into double-stranded DNA fragments of 180–200 base pairs (32).

The cytotoxic effect, the integrity of genomic DNA,
the morphological changes, the hypodiploid cells, and the TNF-α secretion in HL-60 cells were examined. GWH potently inhibited the cell viability and caused the digestion of genomic DNA into ladders in a time-dependent manner, associated with a decrease in intact DNA. Apoptotic bodies increased in a time-dependent manner. An increase of hypodiploid cells was detected dose- and time-dependently. The present results strongly suggest that the cytotoxic mechanism of GWH may be attributable to the induction of apoptosis in HL-60 cells.

TNF-α participated in host defense against pathogens. TNF-α also modulates the immune response by triggering the production of a number of other regulatory cytokines such as IL-1, IL-6, IFNs, transforming growth factors, and granulocyte-monocyte colony stimulating factor (33) and is harmful to microorganisms or cancer cells (34). Exposure of HL-60 cells to 0.01 and 0.1 mg/ml GWH resulted in an increase in TNF-α secretion. This findings suggest that GWH induces secretion of TNF-α in HL-60 cells. TNF-α is a potential candidate in accounting for the GWH-induced cytotoxicity measured in HL-60 cells.

Caspase-3 is the final executioner enzyme associated with cell death during stimuli-induced apoptosis (35). Once activated, caspase-3 is free to initiate the various processes involved in apoptosis (36). Activated caspase-3 is found only in cells undergoing apoptosis and consists of p18 and p12 subunits that are derived from a 32 kDa proenzyme by cleavage at multiple aspartic acid sites (37). Protease caspase-3 can cleave and inactivate PARP, an enzyme that is used for DNA repair (38). Houttuynia cordata Thunb extract possibly causes mitochondrial damage, leading to cytochrome c release into the cytosol and activation of caspases resulting in PARP cleavage and execution of apoptotic cell death in HL-60 cells (39). A Chinese herbal medicine Takrisodeskyeum (TRSDY), known to exert anti-tumoral activity in Korea, induces hydrogen peroxide generation, which, in turn, causes activation of caspase-3, degradation of PARP and D4-GDI, and eventually leads to apoptotic cell death (40). Apoptosis of HL-60 cells induced by squamocin requires caspase-3 activation and is related to stress-activated protein kinase (SAPK) activation (41). Wogonin and fisetin had effects on the induction of apoptosis in hepatocellular carcinoma cells SK-HEP-1; activation of caspase 3 cascade, induction of p53 protein, and alternative expression of p21 (Waf/Cip-1) protein were involved (42). In this study, caspase-3 activity in HL-60 cells increased following treatment with GWH. From this, we demonstrated the requirement for caspase-3 activation in GWH-induced cell death. We speculate that activation of caspase-3 by GWH may contribute to the treatment of tumor diseases.

As is already described in the Materials and Methods section, GWH consists of 9 different herbs. Other studies reported that each medicinal herb has a different effect. Salvia miltiorrhiza Bge. has been widely used in clinics to improve blood circulation, relieve blood stasis and eliminate swelling, and so on. In addition, it has been reported to have vasodilator, hypotensive, anticoagulant, and antibacterial activities and have a beneficial effect in rats with chronic renal failure (43, 44). In a rat model of adenine-induced chronic renal failure (CRF), extract was demonstrated to improve renal function by decreasing the serum levels of urea nitrogen and creatinine and also inhibited the increase in the mean glomerular volume and the basement membrane thickness (45). Extract can inhibit mesangial cells proliferation, which may be related to the decrease of endothelin release (46). Glycyrrhiza uralensis Fisch is used in approximately 74% of traditional herbal medicines (47). Glycyrrhizin is a major constituent of it and other components include glabric acid and flavonoids such as liquiritin, licoricone, licoflavone, licoricidin, and formononetin, as well as putrescine, glycyrol, isoglycyrol, glycyrin, glycyocumarin, and deoxyglycyrrhetol (47). Glycyrrhiza uralensis Fisch has been shown to possess several pharmacological effects including anti-inflammatory activity, (48), estrogenic effects (49), and others. In general, anti-estrogenic effects work protectively against estrogen-dependent cancers. This has been confirmed for uterine endometrial cancer in animals (50). There are some reports of chemopreventive effects of Glycyrrhiza uralensis Fisch on skin carcinogenesis, (51, 52) although the role of the anti-estrogenic action was not established. Glycyrrhiza uralensis Fisch has inhibitory effects on E2-related endometrial carcinogenesis in mice through suppression of estrogen-induced c-fos/jun-expressions (53). Coptis japonica Makino has been shown to possess anti-inflammatory and anti-inflammatory activities, (54 – 56) including antimicrobial, antimalarial, and antifungal activities. Furthermore, these alkaloids exhibit a wide variety of pharmacological effects via their inhibition of enzymes and/or their interaction with proteins (57, 58). A methanol extract of Coptis japonica MAKINO effectively enhanced the outgrowth of neurite in PC12 cells induced by nerve growth factor (NGF) and protoberberine alkaloids potentiated the NGF-induced differentiation of neural cells (59). Even though those herbs are frequently used to treat the various diseases, their pharmacological effects are not the same as those of GWH. GWH was composed on the
basis of the theory of Korean medicine to maximize its efficacy. There are several reports in the literature about apoptosis of cells exposed to pesticides (60, 61). Most of the herbs used in this study, however, were gathered in the wild where is believed to be pesticides free. Some of the herbs were proven to be pesticides free from pesticide residue analysis. In conclusion, we found that GWH induces apoptosis in HL-60 cells. However, further studies are needed to clarify the precise pharmacological mechanism.

Acknowledgment

This work was supported by Wonkwang University in 2003.

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