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Induction of Cytotoxicity and Apoptosis in HT-29 Human Colon Carcinoma Cells by a Gleditsiae Semen Extract

Mi-Ran Cha, Ju-Young Kim, Ji-Hwan Hwang, and Hae-Ryong Park*

Department of Food Science and Biotechnology, Kyungnam University, Masan, Gyeongnam 631-701, Korea

Abstract Gleditsiae Semen (GS) has been used in both Korea and China as herbal medicine for the treatment of cephalalgia, catharsis, and other diseases. However, the apoptosis of GS against human cancer cells has not previously been investigated. The primary objective of this study was to determine the mechanisms inherent in GS-induced cytotoxicity and apoptosis, using methanolic extract of GS (GSE) in HT-29 human colon carcinoma cells. We found that GSE induced cytotoxicity in HT-29 cells in a dose-dependent manner, and this effect was verified via a lactate dehydrogenase release assay and a colony formation assay. In particular, HT-29 cells showed extensive cell death when treated with 50 μg/mL of GSE; the calculated IC_{50} value was 20 μg/mL. It induced characteristic apoptotic signs in HT-29 cells, including chromatin condensation and DNA fragmentation, occurring within 6-24 hr when the cells were treated at a concentration of 50 μg/mL. Interestingly, we detected the activation of caspase-3 and -9, but not caspase-8, and apoptotic bodies in GSE-treated HT-29 cells. Collectively, our results indicate that GSE induces apoptosis via a mitochondria-mediated apoptotic pathway, and these findings may be significant with regard to the development of a new drug for the treatment of human colon carcinoma cells.

Keywords: Gleditsiae Semen, herbal medicine, HT-29 cell, cytotoxicity, apoptosis

Introduction

In cases where an injury is caused by an extrinsic stimulus, most normal cells are repaired immediately, or undergo apoptosis to maintain equilibrium. However, when the maintenance of cell proliferation and cell death is disrupted for some reason, these cells invade other tissues and organs. From a physical point of view, changes in resonance and homeostasis may induce a disease state (1). Cancer in its multifarious forms is one of the principal causes of human death in the current era (2). Several primary types of cancer exist, and have been classified into three types: carcinomas, sarcomas, and lymphomas. These cell types tend to be associated with solid tumors. Solid tumors can be either benign or malignant. Moreover, they are condensed with many cancerous cells, and may form near blood vessels (3). Therefore, cores of cells are subjected to physiological stress conditions, and this arrangement renders anticancer agents somewhat ineffectual (4, 5). Thus, the complete treatment of a solid tumor is not simple.

Many people have been afflicted by cancer and a deep worldwide concern exists regarding its continuing threat (6). Cancer therapies and anticancer drugs have been continuously examined by researchers (7-11). Anticancer agents are associated with many problematic side effects, including fatigue (12), nausea and vomiting (13), hair loss (14), and reduced blood cell counts, in addition to drug resistance due to long-term overuse (15, 16). These agents have also been associated with serious cytotoxicity issues in normal tissues and cells. In an attempt to reduce these problems, a number of researchers have attempted to utilize natural products for the treatment of cancer. A host of natural products have been shown to have pharmacological applications, and may have some potential in chemotherapeutic uses (17, 18). Herbal medicines have been extensively tested because of their low toxicity and considerable medicinal value (19).

We investigated that Gleditsiae Semen (GS) exerts a cytotoxic and apoptotic effect against HT-29 human colon carcinoma cells. In our previous study, we described the preliminary evaluation of the cytotoxicity from GS (20). GS, the seed of *Gleditsia japonica* Miquel var. *koraiensis* Nakai, has traditionally been used in East Asian medicine. Studies regarding the splinters (21) and fruit (22, 23) of *G japonica* Miquel var. *koraiensis* Nakai have been investigated previously, and their biological activities have been elucidated. The major components of fruits and leaves of the activity tree are Gleditsia saponin (24) and flavanoids such as C-glycoside, respectively. However, the activities of the seeds of these useful plants had, until now, remained to be assessed.

The objective of this study was assessed the cytotoxic and apoptotic effects of a methanolic extract from GS (GSE) against HT-29 cells, and we propose mechanisms responsible for its cell death activity.

Materials and Methods

Materials Gleditsiae Semen (GS) was obtained from Kumkang Pharm Co., Masan, Korea. A lactate dehydrogenase (LDH) release assay kit was obtained from Wako Pure Chemical Industries (Osaka, Japan). RPMI 1640 medium, fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). All organic solvents and other chemicals were of analytical grade or complied with the requirements for cell culture experiments.

Preparation of the methanolic extract from Gleditsiae

*Corresponding author: Tel: 82-55-249-2689; Fax: 82-55-249-2995
E-mail: parkhy@kyungnam.ac.kr
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Semen Each 5 g of GS was extracted with 100 mL of methanol for 3 days at room temperature and filtered through Whatman No. 1 filter paper (Advantec, Tokyo, Japan). The methanol was then removed by evaporation *in vacuo*, and a dried methanol extract was obtained. The methanolic extract from GS was called GSE. The GSE was then dissolved in dimethyl sulfoxide (DMSO) at a concentration 5 mg/mL for experiments.

Cell culture and treatments HT-29 human colon carcinoma cells were obtained from Korean Cell Line Bank (KCLB). The cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL), streptomycin (100 mg/mL), and 2 mg/mL $NaHCO_3$ in a humidified 37°C incubator gassed with 5% CO_2. GSE were dissolved in DMSO and added to the culture medium so that the final concentration of DMSO was less than 1%.

Lactate dehydrogenase release assay Cytotoxicity was determined by measuring the release of lactate dehydrogenase (LDH). HT-29 cells were pretreated with various concentrations of GSE for 24 hr, and the supernatant was used to assay LDH activity. The reaction was initiated mixing 50 μL of cell-free supernatant with potassium phosphate buffer containing nicotinamide adenine dinucleotide (NADH) and sodium pyruvate in a final volume of 100 μL in a 96-well plate. A colorimetric assay was performed, according to which the amount of formazan salt is proportional to LDH activity in the sample. The intensity of red color formed in the assay and measured at 540 nm was proportional to LDH activity and to the number of damaged cells. Data were normalized to the activity of LDH released from vehicle-treated cells (100%) and expressed as percentage of the control (obtained in separate plating).

Colony formation assay HT-29 cells were seeded at 5.0×10^4 cells/well in 24-well plates, incubated overnight, and treated with different concentrations of GSE for 24 hr. The cells were then diluted in new medium, replated at 1.0×10^3 cells/well in 6-well plates, and cultured under normal growth conditions for 7 or 8 days at 37°C in a humidified atmosphere containing 5% CO_2 to form colonies. The colonies were fixed with 10% formaldehyde, stained with 0.01% crystal violet, and counted. Cell survival (mean values with 95% confidence intervals from triplicate determinations) was calculated by normalizing the survival of control cells as 100%. IC_{50} values (concentration required for 50% inhibition of colony formation) were determined from the dose-response curves of colony formation inhibition.

Cell staining HT-29 cells were washed twice with phosphate-buffered saline (PBS) and then fixed in PBS containing 10% formaldehyde for 2 hr at room temperature. Fixed cells were washed with PBS, and stained with Hoechst 33342 (Sigma, St. Louis, MO, USA) for 1 hr at room temperature. The cells were washed twice more with PBS and the Hoechst-stained nuclei were visualized by using a fluorescence microscope.

DNA fragmentation assay The HT-29 cells treated with 50 μg/mL of GSE for different periods, as described in the Fig. 3. Genomic DNA was extracted from cocultures after 6, 12, and 24 hr using a commercial kit (DNeasy, Qiagen, Valencia, CA, USA) according to the manufacturer's protocol (25). Cells were washed with PBS and resuspended in a lysis buffer. The DNA was collected by centrifugation (8,000×g) and dissolved in DNA rehydration solution. Qualitative evaluation of DNA fragmentation was carried out by electrophoresis of the DNA in 1.2 % agarose gel (100 V, 30 min) and then staining with ethidium bromide.

Caspases colorimetric assay Activities of caspase-3, -8, and -9 were determined by a colorimetric assay using activation kits from R&D Systems (Wiesbaden-Nordenstadt, Germany), following the manufacturer's protocol (26). The kits used synthetic tetrapeptides labeled with *p*-nitroanilide (pNA). Briefly, 50 μg/mL of 24 hr GSE-treated or untreated cells were lysed in the supplied lysis buffer. The supernatants were collected and incubated with the supplied reaction buffer containing dithiothreitol and DEVD-pNA (specific for caspase-3) or IETD-pNA (specific for caspase-8) and LEHD-pNA (specific for caspase-9) as a substrate at 37C. The reaction was measured by changes in absorbance at 405 nm using the ELISA reader (Tecan Spectra, Wetzlar, Germany). Data were expressed as a fold increase in caspase activity of apoptotic cells over that of non induced cells.

Statistical analysis All data are the means of three determinations and the data were analyzed using the SPSS package for Windows (Version 11.5; Chicago, IL, USA). The data were evaluated by one-way analysis of variance (ANOVA) followed by Scheffe's test. Caspase activity was determined, and statistical significance was evaluated using Student's *t*-test. The differences were considered significant at $p<0.05$.

Results and Discussion

Cytotoxic activities of GSE on HT-29 cells To characterize the cytotoxicity occurring in GSE-treated HT-29 cells, the cells were incubated with GSE, and morphological alterations were verified via a phase-contrast microscope. As shown in Fig. 1, after 24 hr of incubation with various concentrations of GSE, many of the cells showed cytoplasmic shrinkage, and either detached from each other, or floated in the medium.

Next, we attempted to determine the cytotoxic effects of the GSE via a cytoplasmic LDH release assay, evaluating the degree to which plasma-membrane damage occurred in HT-29 human colon carcinoma cells because of exposure to GSE. As shown in Fig. 2A, the cytotoxic effects of GSE on the HT-29 cells were evaluated via measurements of the extent of LDH leakage into the medium. The loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage (27, 28). The HT-29 cells were exposed to various concentrations of GSE for 24 hr. As expected, GSE induced cell death occurred in a dose-dependent manner, as evidenced by a 25-90% increase in

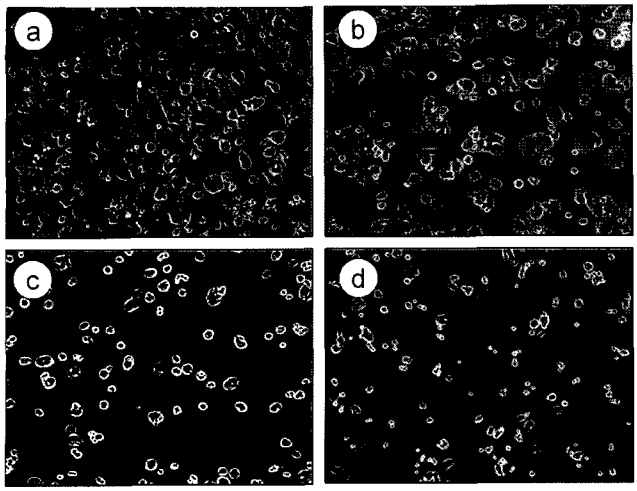

Fig. 1. GSE-induced cell death in HT-29 cells. The cells were exposed to various concentrations of GSE, and morphological changes were monitored for 24 hr (a, control; b, 10 μg/mL; c, 25 μg/mL; d, 50 μg/mL). Photographs were taken with a phase-contrast microscope at 200× magnification. The results are representative of two independent experiments.

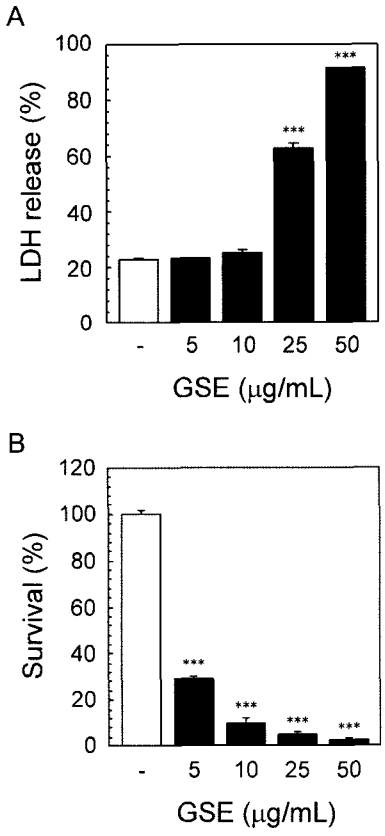

Fig. 2. Effects of GSE on cell viability and cytotoxicity in HT-29 human carcinoma cells. The cells were exposed to the indicated concentrations GSE for 24 hr. Cell viability and cell death were measured using a LDH release assay (A), and colony formation assay (B). The LDH release assay, data were normalized to the activity of LDH released from vehicle-treated cells (100%) and expressed as percentage of the control (obtained from a separate plating). Data (means±SD of triplicate determinations) are representative of at least three independent experiments. ***Significant vs. control untreated cells ($p<0.001$).

LDH release from GSE-treated HT-29 cells.

To clarify these results further, we conducted a colony formation assay (29). In this assay, the low concentration of 5 μg/mL GSE showed good activity. The highest GSE concentration (50 μg/mL) caused an extensive reduction in cell viability, as shown by a 95% inhibition of colony formation (Fig. 2B). Taken together, these results indicate that GSE may induce typical apoptosis in HT-29 human colon carcinoma cells, including cell shrinkage and chromatin condensation.

Apoptosis induction by GSE To determine whether the cytotoxic activity of GSE was due to apoptosis, HT-29 cells were treated for 24 hr with various concentrations of GSE. Cells stained with Hoechst 33342 (10 μM) revealed marked chromatin condensation and apoptotic body formation when examined using a fluorescence microscope (Fig. 3A), confirming that GSE induced apoptosis in HT-29 cells.

To assure whether GSE induces apoptosis in human colon cancer cells, we determined DNA fragmentation (Fig. 3B). When the cells were treated with 50 μg/mL of GSE for various periods from 6 to 24 hr, we observed an increase in DNA fragmentation shown by the time dependent appearance of DNA ladders, suggesting the occurrence of apoptosis. These findings indicate that GSE induced endonucleolytic DNA cleavage and might result in the apoptosis of human colon cancer cells.

Examination of the contributions of the apoptotic pathways For effective apoptosis, the activation of a family of caspases is an absolute requirement. The observed activation of caspase-8 and caspase-9 in this study proved the involvement of the caspase-dependent apoptotic pathway (26). In our study, we used a colorimetric assay to measure caspase-8 and -9 activities after the HT-29 cells had been treated for 24 hr with 50 μg/mL of GSE. The enzyme activity of a component of the extrinsic pathway, caspase-8, was evaluated in GSE-treated cells. The activation of this protease cell death effector is induced via the stimulation of Fas or tumor necrosis factor receptor 1 (TNFR1) cell surface receptors, as is the subsequent apoptosis (30). However, GSE was not found to exert any effects on the activation of caspase-8 (Fig. 4A).

Subsequently, in the mitochondria, when apoptosis occurs, cytochrome *c* is released into the cytoplasm, after which cytochrome *c*, Apaf-1, and procaspase-9 form a complex. The activated caspase-9 cleaves the caspase-3 proenzyme. As shown in Fig. 4B, GSE induced caspase-9 activity by approximately three-fold compared with the controls. Collectively, our results showed that GSE induced apoptosis in HT-29 cells, and that this apoptotic activity involved a mitochondrial pathway.

We also determined that the caspase-3 activity performs a central function in apoptosis in the mitochondrial pathway (31). We attempted to determine whether the caspase-3 activity is inherent to the apoptotic process triggered by GSE. To verify whether the caspase pathways are involved in GSE-induced apoptosis, caspase-3 assayed. We found a significant elevation in the activity of this enzyme in HT-29 cells treated with GSE (Fig. 4C).

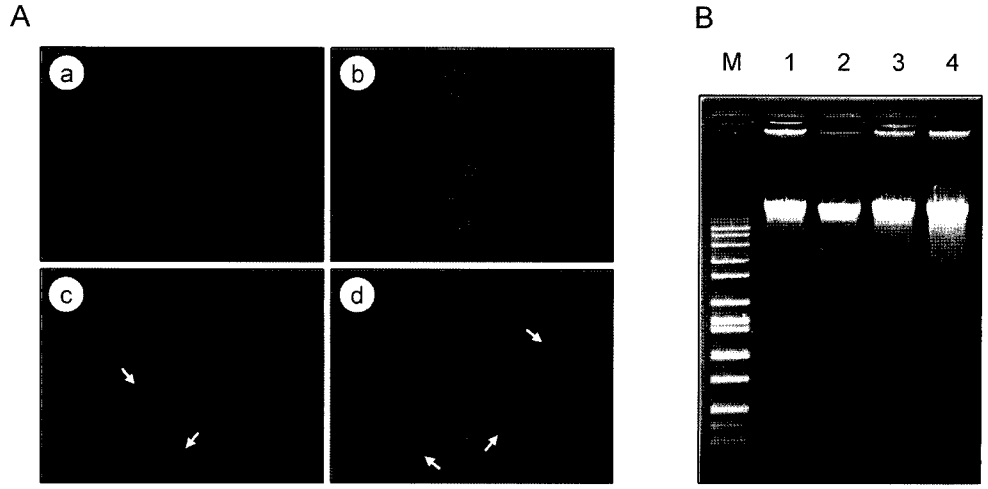

Fig. 3. Apoptosis induced by GSE in HT-29 cells. Cells were collected for the following different experiment for apoptosis induction (A) Morphological features of HT-29 cells treated with various concentration of GSE (a, control; b, 10 μg/mL; c, 25 μg/mL; d, 50 μg/mL). The arrow indicates apoptotic cell. (B) DNA fragmentation analysis. Cells treated with 50 μg/mL of GSE for different times. The data are representative of three independent experiments. M, DNA marker; lane 1, control (untreated); lane 2, GSE-treated for 6 hr; lane 3, GSE-treated for 12 hr; lane 4, GSE-treated for 24 hr.

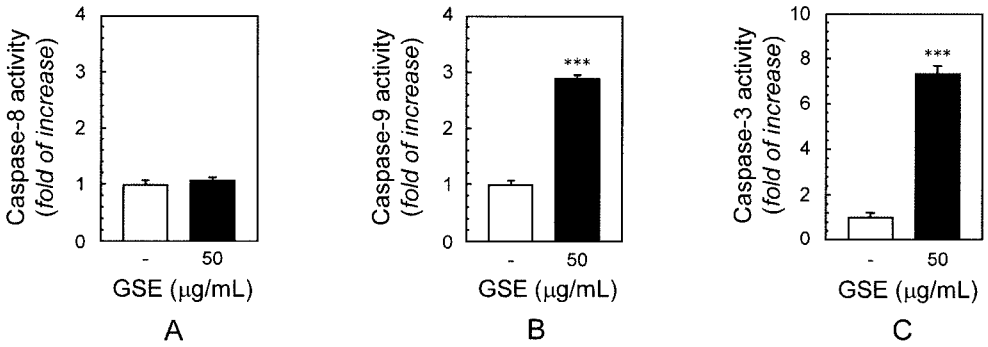

Fig. 4. Effects of GSE on caspase activity in HT-29 cells. The cells were treated with or without 50 μg/mL of GSE for 24 hr and aliquots were incubated with IETD-pNA, LEHD-pNA, and DEVD-pNA for *in vitro* caspase-8 (A), caspase-9 (B), and caspase-3 (C) respectively, at 37°C. Data represent the relative values±SD of three independent experiments. ***Significant vs. control untreated cells (p<0.001).

In conclusion, our results clearly demonstrate that GSE significantly inhibits cell proliferation and induces apoptosis in HT-29 human colon carcinoma cells. GSE induced apoptosis via a mitochondrial pathway, and involved the activation of caspase-9 and -3. These results confirm the potential of GS, a natural product, as an agent of chemotherapeutic activity. We determined that GSE reactivates the apoptotic cascade in colon carcinoma cells, and may be a potential candidate for a novel anticancer drug based on a natural source.

Acknowledgments

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