The Cytotoxic Effect of the Gleditsiae Semen Extracts on Human Colon Carcinoma Cells

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Abstract
The present study describes the preliminary evaluation of the cytotoxicity from Gleditsiae Semen extracts. G. Semen was extracted with methanol, ethanol, and acetone, and then cytotoxic effect of these extracts was measured by the MTT reduction assay and phase-contrast microscopy on the HT-29 human colon carcinoma cells. Among these extracts, methanol extract showed the highest cytotoxic activity on the HT-29 cells. The methanol extract was further fractionated with n-hexane, diethyl ether, ethyl acetate, and water layer according to the degree of polarity. The water layer showed the highest inhibitory activity on the growth of HT-29 cells, but the other fractions indicated the low cytotoxic activity. In addition, water layer also showed the cytotoxic activity against SW620 human colon carcinoma cells. Based on these results, we suggest that extracts of G. Semen may contain bioactive materials and are potential candidates as chemotherapeutic agents against human colon carcinoma cells.

Keywords: Cytotoxicity, Gleditsiae Semen, MTT reduction assay, HT-29 cells, SW620 cells

Introduction
In cases where an injury is caused by an extrinsic stimulus, most normal cells are repaired immediately, or undergo apoptosis to maintain equilibrium. 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). Many people have been afflicted by cancer and a deep worldwide concern exists regarding its continuing threat (3). Cancer therapies and anticancer drugs have been continuously examined by researchers (4, 5). Anticancer agents are associated with many problematic side effects, including fatigue, nausea and vomiting, hair loss, and reduced blood cell counts, in addition to drug resistance due to long-term overuse (6). 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 (7). Herbal medicines have been extensively tested because of their low toxicity and considerable medicinal value.

We investigated that Gleditsiae Semen (GS) exerts a cytotoxic effect against HT-29 human colon carcinoma cells. In our previous study, we described the preliminary evaluation of the cytotoxicity from GS. GS, the seed of Gleditsia japonica Miquel var. koraiensis Nakai, has traditionally been used in East Asian medicine. Studies regarding the splinters (9) and fruit (10) of G. japonica Miquel var. koraiensis Nakai have been investigated previously, and their biological activities have been elucidated. 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, South 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 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 Gleditsiae Semen 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 NaHCO₃ in a humidified 37°C incubator gassed with 5% CO₂. 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 a separate plating).

**Colony formation assay**  HT-29 cells were seeded at $5.0 \times 10^4$ 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 $1.0 \times 10^3$ 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.

**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. 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 LDH release from GSE-treated HT-29 cells.
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.

To clarify these results further, we conducted a colony formation assay. 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.

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 ± S.D of triplicate determinations) are representative of at least three independent experiments. *** significant vs. control untreated cells (p<0.001).

In conclusion, our results clearly demonstrate that GSE significantly inhibits cell proliferation in HT-29 human colon carcinoma cells. These results confirm the potential of GS, a natural product, as an agent of chemotherapeutic activity. We determined that GSE reactivates may be a potential candidate for a novel anticancer drug based on a natural source.

References