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Induction of apoptosis in HT-29 colon cancer cells by crude saponin from Platycodi Radix

Jae-Yong Kim [a], Kyoung-Wuk Park [b], Kwang-Deog Moon [a], Mi-Kyung Lee [b], Jina Choi [c], Sung-Tae Yee [c], Ki-Hwan Shim [d], Kwon-Il Seo [b,*]

[a] Department of Food Science and Technology, Kyungpook National University, Daegu 702-701, Republic of Korea
[b] Department of Food and Nutrition, Sunchon National University, 413 Jungangno, Suncheon, Jeonnam 540-742, Republic of Korea
[c] Department of Biology, Sunchon National University, Suncheon 540-742, Republic of Korea
[d] Division of Applied Life Sciences, Graduate School, Institute of Agricultural and Life Sciences, Gyeongsang National University, Jinju 660-701, Republic of Korea

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ABSTRACT

This study examined the apoptotic effects of crude saponins acquired from the roots of *Platycodon grandiflorum* (SPR) in HT-29 human colon cancer cells.

SPR decreased HT-29 cell proliferation in dose- and time-dependent manners by inducing apoptosis via DNA fragmentation and poly (ADP-ribose) polymerase (PARP) cleavage. The apoptosis induced by SPR was associated with the activation of initiator caspases-8 and -9, as well as the effector caspase-3. SPR stimulated Bid cleavage, indicating that the apoptotic action of caspase-8-mediated Bid cleavage leads to the activation of caspase-9. SPR increased the expression of the pro-apoptotic protein, Bax, and decreased the expression of the anti-apoptotic protein, Bcl-2. SPR also increased the expression of the caspase-independent mitochondrial apoptosis factor, AIF, in HT-29 cells. These results indicate that SPR inhibits HT-29 cell proliferation by inducing apoptosis, which may be mediated via both caspase-dependent and -independent pathways.

1. Introduction

Colon cancer is the greatest contributor to cancer incidence and death in Western countries (Linsalata and Russo, 2008; Volate et al., 2005). Recently, colon cancer rates have been increasing in Asia due to the rapid increase in individuals adopting Westernized dietary patterns (Park et al., 2008). Diet is an important factor in cancer prevention; particularly in relation to the increasing incidence of colorectal cancer (Block et al., 1992). Epidemiologic studies have suggested that fruit and vegetable consumption reduces the risk of certain types of human cancers (Meyskens and Szabo, 2005). Recently, many natural foods were found to exhibit pharmacological effects, and may have potential uses in cancer chemotherapy (Hwang et al., 2007). Therefore, many plants have been examined to identify new and effective anticancer compounds, as well as to elucidate mechanisms of cancer prevention via apoptosis. Several studies have indicated that the saponins in plants are associated with a reduced risk of colorectal cancer (John et al., 2004; Rao and Sung, 1995; Xiao et al., 2007). Also, saponin-containing plants possess a broad range of bioactivities, and are commonly used in folk medicine for their health-promoting properties (Shi et al., 2004).

Platycodi Radix is the root of *Platycodon grandiflorum* A.DC, which is commonly known as Doraji in South Korea. In Korea as well as other Asian countries, Platycodi Radix has been used as a food material and a traditional oriental medicine for such conditions as bronchitis, asthma and pulmonary tuberculosis, hyperlipidemia, diabetes, and as a sedative (Lee et al., 2004, 2006; Han et al., 2001). Chemical analyses of Platycodi Radix have revealed that triterpenoid saponins are its main chemical components (Wen et al., 2006). And earlier studies examining the roots of *P. grandiflorum* have reported on the isolation and structural elucidation of these triterpenoid saponins (He et al., 2005; Kim et al., 2005; Wen et al., 2006), in which platycodins A, C, and D were determined as the primary saponins (Saeki et al., 1999).

And recently, the saponins, which are its major components, were shown to have biological activities, including anti-inflammatory effects (Kim et al., 2006), anti-hyperlipidemic effects (Zhao et al., 2005), inhibitory effects on pancreatic lipase (Xu et al., 2005), anti-obesity effects (Han et al., 2002; Park et al., 2007), apoptosis induction effects (Ahn et al., 2006; M.O. Kim et al., 2008), and immunomodulatory effects (Han et al., 2001). Although there have been several reports on the various chemical and

Abbreviations: DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; SPR, saponin from Platycodi Radix; SRB, sulforhodamin B.

* Corresponding author. Tel.: +82 61 750 3655; fax: +82 61 752 3657.
E-mail address: seoki@sunchon.ac.kr (K.-I. Seo).

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biological properties of the saponins, their apoptotic mechanism remains to be elucidated.

The conventional methods for isolating crude saponins from Platycodi Radix have used butanol extraction. However, due to its high toxicity to humans, crude saponins obtained by butanol extraction can not be used for medicinal purposes or as health food materials. Therefore, with an aim to use the saponins of Platycodi Radix as functional food material with anti-cancer effects, we attempted to prepare non-toxic, edible crude saponins using a previously reported method that employed Diaion HP-20 resin (Kim et al., 2005; Park et al., 2005).

In the present study, we investigated the anti-proliferation effects of these Diaion HP-20 resin-obtained saponins in HT-29 human colon cancer cells. Specifically, SPR's induction of apoptosis and mechanism of induced apoptosis were examined.

2. Materials and methods

2.1. Isolation of crude saponins

The crude saponins were isolated from three year-old roots of *P. grandiflorum* collected from Gyeongsangbuk-do South Korea. Dried roots (500 g) were ground into powder and then extracted three times in 80% ethanol by boiling for 3 h at 65 °C. The concentration of the solvent gave 102 g of a brown syrup. The EtOH extract was suspended in 2 L of H_2O and poured into a Diaion HP-20 column. The column was eluted with an additional 5 L of H_2O. The column eluates were combined and concentrated under reduced pressure to give 30 g of syrupy residue. The Diaion HP-20 column was further washed with an additional 5 L of 20% EtOH. The eluate was then concentrated under reduced pressure to give 0.5 g of brown syrupy residue. Finally, the column was washed out with EtOH, and the washings were concentrated under reduced pressure to give 3.1 g of brown powder (Fr. C), which is crude saponin (SPR) used to the study (Kim et al., 2005).

2.2. Cell culture

The HT-29 (human colon cancer cell) cell lines were obtained from the Korea Cell Line Bank, Seoul National University. HT-29 cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) containing 10% fetal bovine serum (FBS) with 100 U/mL of penicillin and 100 μg/mL of streptomycin in 5% CO_2 at 37 °C.

2.3. Cell proliferation

Cell proliferation was determined by sulforhodamin B (Sigma, St. Louis, USA) assay. The cancer cells were seeded at a concentration of 1×10^5 cells/well in 24-well tissue culture plates and incubated with various concentrations of SPR for different time periods. After treatment, the medium was aspirated and 10% trichloro-acetic acid was added. After 1 h of incubation at 4 °C, the plates were washed five times with D.W. and air dried. The cells were stained with 0.4% (w/v) SRB at room temperature for 1 h and then washed five times using 1% acetic acid. Bound SRB was solubilized with 10 mM Tris and absorbance was measured at 540 nm (Moon et al., 1998).

2.4. Lactate dehydrogenase activity

Cytotoxicity was studied by determining the lactate dehydrogenase activity in cell free supernatants using LDH-cytotoxicity assay kit (USA) following the instruction of manufacturer.

2.5. Detection of DNA fragmentations

After treatment with SPR, HT-29 cells were lysed in buffer containing 10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, pH 8.0, 0.5% Triton X-100, 20% SDS added to 10 mg/mL proteinase K and the mixture was incubated for 4 h at 55 °C. The mixture was then extracted with phenol: chloroform: isoamyl alcohol (25:24:1), and the DNA was precipitated with 2 vol of cold absolute ethanol. The pellets were incubated with TE buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA, pH 8.0) and RNase (2 mg/mL) for 1 h at 37 °C. Then, separation by electrophoresis was performed on 2% agarose containing ethidium bromide. The DNA bands were examined using a UV Transilluminator Imaging System (Wan et al., 2005).

2.6. Assay of caspase activities

This assay was based on the ability of the active enzyme to cleave the chromophore from the enzyme substrate, Ac-DEVD-pNA (for caspase-3), Ac-IETD-pNA (for caspase-8), and Ac-LEHD-pNA (for caspase-9). The cells were seeded at a density of 2×10^6 cells in a 100 mm dish, and cultured for 24 h in DMEM. After culturing, the cells were treated with the indicated concentrations of SPR for 24 h, and then collected by centrifugation. The cells were incubated with the peptide substrate in lysis buffer for 30 min on ice, and centrifuged at 10,000*g* for 5 min at 4 °C. The protein content of the supernatant was measured using BCA protein assay reagent before analysis of the caspases-3, -8, and -9 activities. The supernatant that contained 50 μg of protein was mixed with DTT in 2× reaction buffer and a 10 μM concentration of the different substrates. After incubation, the release of p-nitroaniline was monitored at 405 nm (Kuo et al., 2005).

2.7. Assay for caspase inhibitor activity

The cells were seeded at a density of 5×10^5 cells/well, and cultured for 24 h in DMEM (Dulbecco's Modified Eagle Medium). The cells were preincubated with z-VAD-fmk for 2 h, treated with the indicated concentrations of SPR for 24 h. For the growth inhibition analysis and measurement of sub-G1 DNA content, the cells were collected and fixed in ice-cold 70% ethanol in media, and stored at 4 °C overnight. After resuspension, the cells were washed and incubated with 1 μL of RNase (1 mg/mL) (Sigma, St. Louis, USA), 20 μL of propidium iodide (1 mg/mL) (Sigma, St. Louis, USA), and 500 mL of PBS at 37 °C for 30 min. After staining, flow cytometry was used to analyze the cell cycle phase and sub-G1 DNA content (S.Y. Kim et al., 2008).

2.8. Western blot analysis

The cells were seeded at a density of 2×10^6 cells in a 100 mm dish, and cultured for 24 h in DMEM (Dulbecco's Modified Eagle Medium). After culturing, the cells were treated with the indicated concentrations of SPR for 24 h, and collected by centrifugation. The pellets were lysed by lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 30 mM $Na_4P_2O_7$, 1 mM PMSF, 2 μg/mL aprotinin) for 30 min on ice. The protein content of the supernatant was measured using BSA protein assay reagent before analysis. The protein samples were loaded at 10 μg of protein/lane and separated by SDS–PAGE in 12% gel at 100 V of constant voltage/slab for 1.5 h. Following electrophoresis, the proteins were transferred onto nitrocellulose membranes. After blocking with 2.5% and 5% bovine serum albumin (BSA) for 1 h at 37 °C, the membranes were incubated with first antibody (anti-PARP, anti-Bid, anti-Bax, anti-Bcl-2, and anti-AIF) at 4 °C overnight. Finally, the membranes were treated with horseradish peroxidase-coupled secondary antibodies for 1 h at 4 °C. The membranes were washed with T-TBS after each antibody binding reaction. Detection of each protein was performed using an ECL kit (Wan et al., 2005).

2.9. AIF translocation

The HT-29 cells were seeded in 6-well plates at seeding densities of 5×10^5 cells, and then treated with SPR for 24 h. After harvesting, the cells were washed twice with PBS and then blocked with blocking buffer (2% BSA in T-TBS) for 1 h. The cells were incubated with AIF primary antibody overnight at 4 °C, followed by anti-rabbit secondary antibody for 1 h. AIF translocation was analyzed under a fluorescence microscope (Olympus Optical Co. Ltd. Japan) (Kang et al., 2004).

2.10. Statistical analysis

The data were analyzed by Student's *t*-test to evaluate significant differences. A level of $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. SPR-induced cell death

The effects of SPR on the inhibition of HT-29 cell growth and proliferation were determined via SRB and LDH assays.

To determine the optimal dose of SPR for colon cancer cell inhibition, HT-29 cell growth was examined following the 24 h treatment of various SPR doses. Cell viability decreased with increasing SPR concentration, and the IC_{50} value of SPR was 37.07 μg/mL (data not shown). In order to determine the time-dependent inhibition induced by SPR, HT-29 cells were treated with the various indicated concentrations of SPR for 24–48 h and SRB assays were performed (Fig. 1). As Fig. 1 shows, the anti-proliferation effects of SPR occurred in a treatment time-dependent manner.

Furthermore, the cytotoxic effects of SPR were determined by measuring the extent of LDH leakage by the cells into the medium. The loss of intracellular LDH and its release into the culture