Apoptosis of Human Bladder Cancer Cells by an Ethanolic Extract of *Scutellaria Baicalensis* GEORGI Via Caspase and MAPK Signaling Pathways

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An ethanolic extracts of *Scutellaria Baicalensis* GEORGI are used to treat cancer, infectious diseases, and inflammation. In the present study, we investigated the effects of an ESBG on the growth and survival of 5637 cells, a human bladder carcinoma cell line. Cells were treated with different con-centrations of an ethanolic extract of *Scutellaria Baicalensis* GEORGI (ESBG), and cell death was assessed using a MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay. Analyses of the sub G1 peak, caspase-3 and -9 activities, and mitochondrial membrane de-polarizations were conducted to confirm cell death by apoptosis. ESBG had a cytotoxic effect on 5637 cells, and increased the sub G1 peak, caspase-3 and -9 activities, and mitochon-drial depolarization, indicating ESBG induced apoptosis. Furthermore, MAPK (mitogen-activated protein kinases) inhibitors suppressed this apoptosis. In an *in vitro* study, a combination of sub-optimal doses of ESBG and paclitaxel, 5-fluorouracil, or docetaxel noticeably suppressed tumor growth by 5637 cells. Our findings provide insight of the mechanisms underlying cellular apoptosis induced by ESBG, and suggest new therapeutic strategies for bladder cancer.

keywords : *Scutellaria Baicalensis* GEORGI, Human bladder carcinoma cell line, 5637 cells, Apoptosis

Introduction

Traditional herbal medicine (ThM) is an important complementary strategy for treating cancer[1]. ThM can reduce the toxicities of chemotherapy and radiotherapy, enhance the antitumor effects of these therapies, alleviate tumor-induced clinical symptoms and cancer associated pain, and prolong the survival of surgically treated and advanced stage cancer patients[2,3]. When combined with modern medicine, ThM could improve symptoms, enhance quality of life, prevent recurrence and metastasis, and prolong survival, and thus, we look forward reading the results of clinical studies on the use of ThM for cancer prevention and rehabilitation.

Scutellaria baicalensis GEORGI (*S. baicalensis*) is the dried root of labiatae Scutellaria baicalensis GEORGI[4]. Traditionally, *S. baicalensis* has been widely used to treat cancer, infectious diseases, and inflammation[5,6]. Its principal active constituents are the flavonoids baicalin, wogonoside, baicalein, and wogonin, which have been found to possess anti-hepatitis B virus, anti-inflammatory, antioxidant, anti-allergic, andanti-tumor properties[7-11].

Apoptosis is a form of programmed cell death in multicellular organisms and is associated with changes in cell morphology, such as, blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation[12,13]. However, the mechanisms by which exerts anticancer activity are poorly understood. In this study, we investigated the anticancer effect of an ethanolic extract of Scutellaria baicalensis GEORGI (ESBG) on bladder cancer using 5637 cells, a human bladder cancer cell line.

Materials and Methods

1. Preparation of the ESBG

Powdered Scutellaria Baicalensis root GEORGI (Catalog number: CA04-087) was obtained from the plant extract bank at the Korea Research Institute of Bioscience and Biotechnology (KRIBB) (Daejeon, Korea). The powder was then immersed in ethanol, sonicated for 15 minutes, and extracted for 72 hs. The extract so obtained was filtered through non fluorescent cotton and evaporated under reduced pressure at 45℃ in a rotary evaporator (N-1000

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SWD, Eyela, Japan) in 45℃. The condensed extract was then lyophilized by using a Modul Spin 40 dryer (Biotron Corporation, Calgary, Canada) for 24 hs. The yield of lyophilized powder obtained (defined as ESBG) was 12.3%. To produce a stock solution, ESBG was dissolved in dimethyl sulfoxide (DMSO; Jersey Lab Supply, Livingston, NJ, USA) at 100 mg/mL and stored at 4℃. This stock solution was diluted with medium to desired concentrations immediately prior to use.

2. Cells

The human bladder (5637) carcinoma cell line, established at the Cancer Research Center, College of Medicine, Seoul National University, Korea, was used throughout the present study. Cells were propagated in RPMI-1640 medium (Gibco-BRL) supplemented with 10% heat-inactivated fetal bovine serum and 20 μg/ml of each of penicillin and streptomycin in a 5% CO_2 atmosphere at 37℃.

3. MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide) assay

Cell viability was assessed using a MTT assay. Briefly, 5637 cells were seeded into the wells of a 12-well culture plates and then cultured in RPMI-1640 for 72 hs. MTT solution (100 μL, 5 mg/mL in PBS (phosphate buffered saline)) was then added to each well, and plates were then incubated for 4 hrs at 37℃. After removing supernatants and shaking with 200 μL of DMSO (Jersey Lab Supply, Livingston, NJ, USA) for 30 mins, absorbances were measured at 570 nm. Experiments were repeated at least three times.

4. Caspase-3 and -9 assays

Caspase-3 and -9 assay kits were purchased from BioMol (Plymouth, PA, USA). After treatments, cells were centrifuged (1,000 g, 4℃, 10 minutes), washed with PBS, resuspended in ice-cold cell lysis buffer, incubated on ice for 10 mins, and centrifuged at 1,000 g (4℃, 10 mins). Supernatants were removed and aliquots (10 μL) were incubated with 50 μL of substrate (400 μM Ac-DEVD-pNA) in 40 μL of assay buffer at 37℃. Absorbance at 405 nm was read at different times, and pNA concentrations were determined using a plot of absorbance versus pNA concentration.

5. Flow cytometric analysis

Flow cytometry and propidium iodine (PI) staining was used to determine whether cell cycles were affected by ESBG[14,15]. Cells (1×10^6) were placed in an e-tube and ice-cold fixation buffer (ethyl alcohol; 700 μL) was slowly added with vortexing. Tubes were then sealed with parafilm, and incubated at 4℃ overnight. Samples were then spun at 106 g for 3 mins at 4℃, and supernatants were aspirated and discarded. Cell pellets were resuspended in 200 μL of PI staining solution (PI [5 mg/mL] containing 2 μL of RNase in PBS (196 μL)), spun at 20,817 g for 5 secs, and allowed to stand for 30 mins in the dark at room temperature. Samples were analyzed using a fluorescence-activated cell sorter (FACScan; Becton-Dickinson, Mountain View, CA, USA) at λ= 488 nm using Cell-Quest software (Becton-Dickinson). The DNA contents of normal cells are characterized by using two peaks representing the G1/G0 and G2/M phases. Cells in the sub-G1 phase have least DNA content, which is termed hypodiploid. Hypoploid DNA contents represent DNA fragmentation[16].

6. Assessment of mitochondrial membrane depolarization

Mitochondrial membrane depolarization was evaluated using a JC-1 fluorescence probe according to the manufacturer's instructions (Molecular Probes, Eugene, OR). 5637 cells were labeled with 2 μM JC-1 for 30 min at 37℃ and then analyzed by flow cytometry at an excitation wavelength of 488-nm using 530/30 or 585/42 nm bypass emission filters. The cells without red fluorescence were considered to exhibit mitochondrial membrane depolarization.

7. Statistical analysis

Results are presented as means ± SEMs (Standard Errors of Means). The significances of differences were determined using the Student's t-test. Statistical significance was accepted for p values < 0.05.

Results

1. ESBG inhibited the growth of human bladder cancer cells

Treatment with ESBG (50-400 μg/ml) for 24 h showed the cell viability of 5637 cells by 96.6 ± 2.2% at 50 μg/ml, 78.3 ± 3.1% at 100 μg/ml, 64.8 ± 5.4% at 200 μg/ml, 36.7 ± 4.6% at 300 μg/ml, and by 19.2 ± 4.1% at 400 μg/ml (Fig. 1A). Furthermore, treatment with ESBG for 72 hs showed the cell viability by 37.6 ± 9.1% at 50 μg/ml, 25.5 ± 3.7% at 100 μg/ml, 11.3 ± 3.5% at 200 μg/ml, 4.8 ± 2.5% at 300 μg/ml, and by 5.1 ± 2.0% at 400 μg/ml, respectively (Fig. 1B). These findings indicate that ESBG has a cytotoxic effect on 5637 cells.

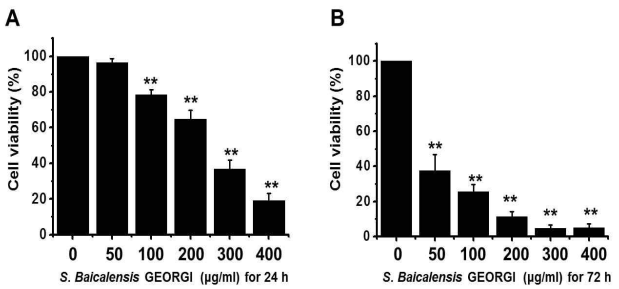

Fig. 1. Effect of an ethanolic extract of *Scutellaria Baicalensis* GEORGI (ESBG) on cytotoxicity in 5637 cells. 5637 cells were incubated with ESBG at the concentrations indicated (μg/ml). After (A) 24 hs or (B) 72 hs, cell viability was assessed using a MTT assay as described in Methods. Results are expressed as percentages (%) of non-treated controls and columns represent means ± SDs. **P<0.01.

2. ESBG increased the sub-G1 population

After treatment with ESBG for 24 hs, 5637 cells were stained with propidium iodide (PI), and cell cycle progression was assessed by flow cytometry. ESBG increased the number of cells in the sub-G1 peak in a dose-dependent manner, which is consistent with the induction of cell death (Fig. 2). After 24h of treatment with different concentrations of ESBG the following percentages were in the sub-G1 phase; 14.2% at 100 μg/ml, 33.0% at 200 μg/ml, 36.8% at 400 μg/ml, and 42.3% at 500 μg/ml; whereas only 4.2% of untreated cells were in this phase. At the same time, the following percentages were in the G0/G1 phase; 41.8% at 100 μg/ml, 35.7% at 200 μg/ml, 23.9% at 400 μg/ml, and 27.8% at 500 μg/ml; and 16.4% at 100 μg/ml, 10.3% at 200 μg/ml, 19.8% at 400 μg/ml, and 15.1% at 500 μg/ml were in the G2/M phase, which was lower than percentage of untreated cells in this phase (23.5%) (Fig. 2). These results suggested that ESBG has an anti-cancer effect and that it is closely associated with the induction of apoptosis.

3. ESBG induced apoptosis via a mitochondria- and caspase-dependent pathway in 5637 cells

Mitochondrial membrane depolarization (an early event of intrinsic apoptosis signaling) was elevated by ESBG. The mitochondrial membrane depolarization by ESBG was markedly increased by 13.1 ± 3.2% at 50 μg/ml, 19.4 ± 3.1% at 100 μg/ml, 27.6 ± 4.3% at 200 μg/ml, and 39.1 ± 3.6% at 300 μg/ml, and 51.5 ± 3.5% at 400 μg/ml by flow cytometry (n = 5; Fig. 3A). Also, because it is known that caspase activation is required for apoptotic cell death, we performed caspase activity assays to observe the activities of caspase-3 and -9. We found that caspase activities were dose dependently elevated in the presence of ESBG (at concentrations from 50 to 400 μg/ml) for 24hs, and that these activities were repressed by zVAD-fmk (a pan-caspase inhibitor; Fig. 3B). These observations suggest that ESBG-induced apoptosis is mediated by a mitochondria- and caspase-dependent pathway in 5637 cells.

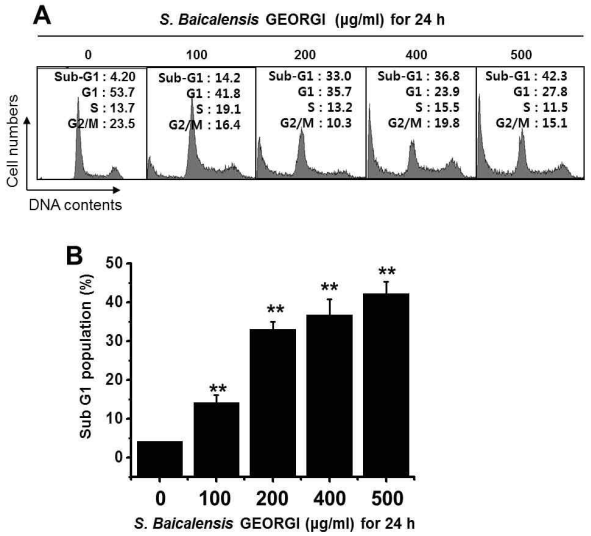

Fig. 2. ESBG increased 5637 cell apoptosis. 5637 cells were incubated with ESBG at the indicated concentrations (μg/ml). (A) After 24 hr, sub-G1 peaks were measured using a FACScan as described in Methods. (B) Results are expressed as percentages (%) of non-treated controls and columns represent means ± SDs. **P<0.01.

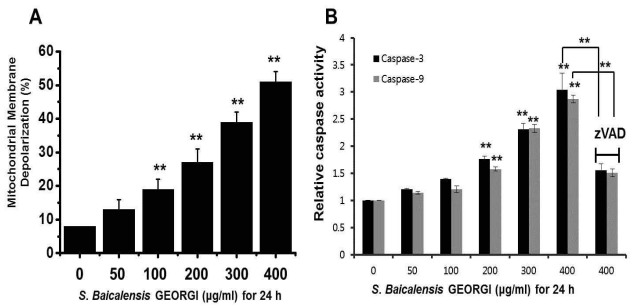

Fig. 3. ESBG treatment increased mitochondrial membrane depolarization and caspase-3 and -9 activities. 5637 cells were incubated with ESBG at the indicated concentrations (μg/ml). (A) After 24 hs, mitochondrial membrane depolarization was measured using a FACScan as described in Methods. (B) Caspase 3 and 9 activities were measured using enzyme assays. Specific activities were determined using four samples per group. Results are expressed as percentage (%) of non-treated controls and column represent means ± SDs. **P<0.01.

4. ESBG regulated apoptosis through MAPK (mitogen-activated protein kinase) cascades

To investigate the relationship between the regulation of MAPK pathways and the cytotoxicity of cancer cell by ESBG, 5637 cells were pretreated with the MAPK inhibitors PD98059 (inhibitor of ERK1/2), SB203580 (inhibitor of p38), or SP600125 (inhibitor of JNK) and then treated with ESBG (300 μg/ml) for 24 hs (Fig. 4). Each of the MAPK inhibitors significantly diminished the cytotoxicity effects of ESBG in

5637 cells. Treatment with these inhibitors reduced cell death by the following percentages PD98059 by 38.3 ± 3.2%, SB203580 by 32.6 ± 1.5%, and SP600125 by 38.8 ± 3.3% as compared with ESBG. In contrast, the cytotoxicity effects of ESBG were not enhanced by LY294002 (a specific inhibitor of Akt). To confirm these results, we used the aqueous extract of Magnolia officinalis in 5637 cells. The aqueous extract of Magnolia officinalis has the anti-cancer activity through p38 MAP kinase pathway[17]. Magnolia officinalis has a cytotoxic effect on 5637 cells and the p38 MAP kinase inhibitor (SB203580) significantly diminished the cytotoxicity effects of Magnolia officinalis in 5637 cells (Fig. 4). Also, to confirm the involvement of apoptosis in this pathway, we experimented the sub-G1 phase by flow cytometry. The sub-G1 was markedly decreased with MAPK inhibitors not with LY294002 (Fig. 5). Taken together, these data suggest that ESBG exerts the cytotoxicity effect on 5637 cells by modulating MAPK signaling pathways resulting in apoptosis.

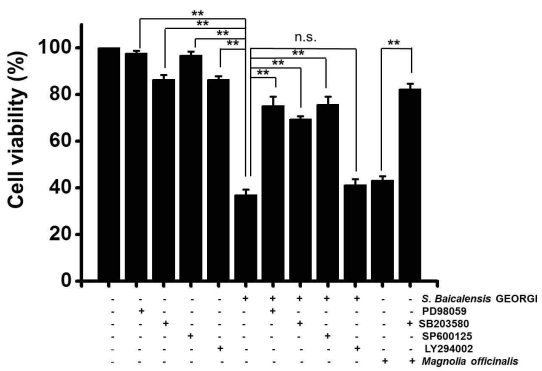

Fig. 4. Effects of MAPK and Akt inhibition on ESBG-induced 5637 cell death. Cells were pretreated with the indicated MAPK inhibitors (SB203580 (20 μM), SP600125 (20 μM), or PD98059 (50 μM)) or Akt inhibitor (LY294002 (20 μM) for 1 h and then treated with ESBG for 24 hs. Cell viabilities were determined using a MTT assay. Results are expressed as percentages (%) of non-treated controls and columns represent means ± SDs. **P<0.01. n.s. = Not significant versus ESBG-treated cells

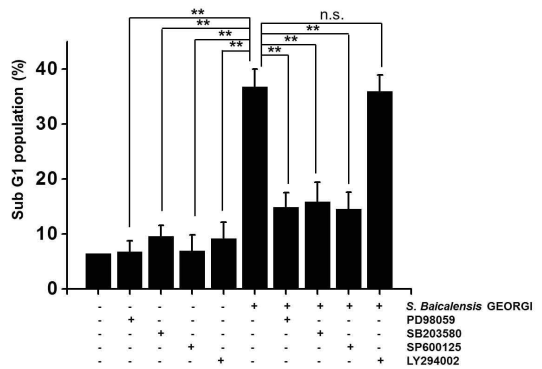

Fig. 5. ESBG increased 5637 cell apoptosis through MAPK pathway. After 24 hr, sub-G1 peaks were measured using a FACScan as described in Methods. Results are expressed as percentages (%) of non-treated controls and columns represent means ± SDs. **P<0.01.

5. ESBG increased the chemosensitivity of 5637 cells in vitro.

We also investigated whether ESBG enhances the sensitivity of 5637 cells to chemotherapeutic agents, that is, paclitaxel, 5-fluorouracil, cisplatin, ectoposide, doxorubicin, and docetaxel in vitro. Combinations of ESBG plus a chemotherapeutic agents were found to suppress cell growth more than each agent alone (Fig. 6). In particular, paclitaxel, 5-fluorouracil, and docetaxel plus ESBG markedly suppressed cell growth. These results suggest that ESBG increases the chemosensitivity of 5637 cells.

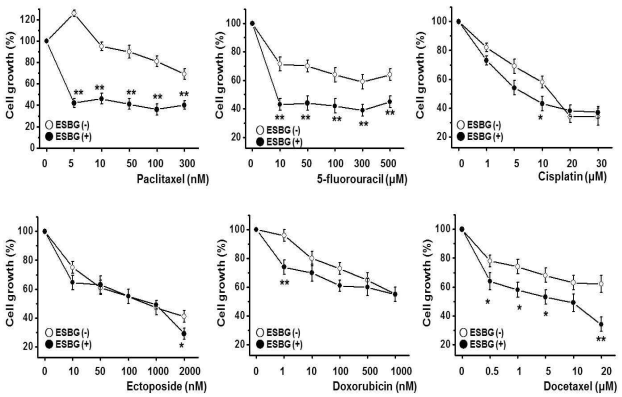

Fig. 6. ESBG (50 μg/ml) increased cellular chemosensitivity. 5637 cells were co-treated with ESBG and chemotherapeutic agents, that is, paclitaxel, 5-fluorouracil, cisplatin, ectoposide, doxorubicin, or docetaxel at the indicated concentrations prior to MTT assay. Results are expressed as percentages (%) of non-treated controls and columns represent means ± SDs. *P<0.05. **P<0.01.

Discussion

The roots of *S. baicalensis* GEORGI is one of the most widely used traditional oriental medicines[18], and it is used for heat-clearing and dehumidifying traditional oriental medicines. *S. baicalensis* has various pharmacologic properties[18]. For example, may inhibit the proliferation of lymphocytic leukemia, lymphoma, and myeloma cells[19] and has a neuroprotective effect on spinal cord injury in rats[20]. In addition, it inhibits the activities of cyclooxygenase and 5-lipoxygenase, and thus, ameliorates inflammation[21]. Recently, Shin et al. (2014)[22] *S. baicalensis* suggested could attenuate OVA-induced food allergy symptoms by regulating cellular systemic immune responses, and that it might prevent for food allergies. However, no study has been per-formed to investigate the effect of *S. baicalensis* GEORGI on the growth of human bladder cancer cells. Therefore, in the present study, we investigated the anti-cancer effects of an ethanolic extract of *S. baicalensis* GEORGI (ESBG) in 5637 cells and the mechanisms involved.

We found that ESBG treat-ment reduced 5637 cell viability in a concentration dependent manner (Fig. 1) by inducing apoptosis, which was confirmed by increases in the sub G1 population, mitochondrial membrane depolarization, and enhanced caspase activities (Fig. 2 and 3). Also, this apoptosis was inhibited by MAPK inhibitors (Fig. 4 and 5). Furthermore, combinations of sub-optimal doses of ESBG and paclitaxel, 5-fluorouracil, and docetaxel noticeably suppressed 5637 cell growth more that these drugs alone (Fig. 6).

The anti-carcinogenic effects of ESBG were confirmed by caspase activity assays. The activations of initiator caspases, such as, caspase-9 and -10, by pro-apoptotic signals leads to the downstream activations of effector caspases, such as, caspase-3 and -6[23,24]. In the present study, ESBG induced the activations of caspase-9 and -3 (Fig. 3), indicating ESBG induces apoptosis via a caspase-dependent pathway in 5637 cells. On the other hand, MAPKs regulate cellular processes, such as, proliferation and apoptosis[25]. In particular, the pharmacological modulation of MAPK signals influences apoptotic responses to anti-tumor agents[26]. In the present study, we found that the inhibition of MAPK signaling by specific protein inhibitors (ERK inhibitor: PD98059, p38 inhibitor: SB203580, or JNK inhibitor SP600125) protected cells from the cytotoxic effects of ESBG, which suggests that the activation of MAPK cascades inhibits the proliferation of 5637 cells (Fig. 4). However, pretreatment with LY294002 (a specific inhibitor of Akt) had no effects on ESBG-induced cell death (Fig. 4).

Chen et al. suggested that transient receptor potential vanilloid type 1 (TRPV1) channels were expressed in 5637 bladder carcinoma cells and capsaicin induced cell death through increased reactive oxygen species (ROS) generation and decreased mitochondrial membrane potential, whereas these effects could be partially blocked by TRPV1 antagonist, capsazepine[27]. Therefore, we will investigate the involvement of ion channel, especially TRP channel in ESBG-induced apoptosis in future.

There are two limitations of this study. First, the 5637 cells, human bladder carcinoma cell lines were used in our study. However, there are so many bladder carcinoma cell lines (for example, HT-1376, SCaBER, SW 780 and so on). Therefore, future studies may be warranted to substantiate these findings in other bladder carcinoma cell lines and bladder carcinoma tissue. Second, we decreased the highest dose of ESBG from 50 μg/ml to 400 μg/ml in experiments. The reason is that high concentration of ESBG (400 μg/ml) could decrease of cell viability significantly, so that it was difficult to collect enough viable cells to perform these analysis.

Our study shows that ESBG induced 5637 cell cytotoxicity and apoptosis, as evidenced by an accumulation in the sub G1 phase. Furthermore, ESBG induced apoptosis was found to be associated with activations of caspases, and mitochondrial dysfunction. In addition, specific inhibitors of MAPK significantly reduced ESBG-induced apoptosis in 5637 cells. These findings suggest that ESBG should be considered a potential therapeutic agent for the treatment of bladder cancer.

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