Scutellaria baicalensis Georgi induces caspase-dependent apoptosis via mitogen activated protein kinase activation and the generation of reactive oxygen species signaling pathways in MCF-7 breast cancer cells

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Abstract. Scutellaria baicalensis Georgi extract (SBGE) is used in traditional herbal medicine and has also been used clinically to ameliorate the symptoms of various inflammatory diseases and cancer. In women, breast cancer is one of the most common diseases and numerous women succumb to it. The present study was undertaken to investigate the mechanism responsible for the SBGE-induced apoptosis of MCF-7 human breast cancer cells. SBGE was administered to cells at concentrations between 100 and 500 mg/ml, and cell viability was identified using an MTT assay. B-cell lymphoma 2 (Bcl-2) and Bcl-2 X-associated protein (Bax) family members were identified by western blotting, and the mRNA expression levels of the pro-apoptosis genes Fas, Fas ligand (FasL) and tumor necrosis factor (TNF)-α were assessed by reverse transcription-polymerase chain reaction. It was identified that SBGE treatment for 24 h inhibited MCF-7 proliferation and increased the sub-G1 phase ratio. SBGE suppressed mitochondrial membrane potentials and SBGE-induced apoptotic cell death was identified to be associated with downregulation of Bcl-2, but upregulation of Bax. SBGE-activated caspases 3 and 9, and increased reactive oxygen species generation. However, SBGE had no effect on the expression levels of Fas, FasL or TNF-α. Furthermore, mitogen-activated protein kinase and C-Jun N-terminal kinase inhibitors inhibited SBGE-induced cell death. These results suggested that SBGE be considered as an agent for the treatment of breast cancer.

Introduction

Breast cancer is the most common malignnant disease in women (1) and accounts for ~25% of cancer cases (2,3). Despite the number of breast cancer medicines, the prognosis of breast cancer remains poor (4). The main approaches used to treat breast cancer are surgery, radiotherapy, chemotherapy, hormone therapy and targeted therapy (5). However, recovery rates following application of these conventional methods are only 60-80% for primary cancer and ~50% for metastatic cancer (5,6).

Traditional Chinese medicine (TCM) has been used to treat various cancers (7). Scutellaria baicalensis Georgi (SBG) is a well-known TCM and is traditionally described as possessing ‘heat-clearing, dampness-drying, fire-purging, detoxicating and hemostasis-maintaining’ properties (8). In the modern era, it has been demonstrated to possess various properties, including antioxidant and anti-inflammatory effects (9,10), and to inhibit the proliferations of several tumors via apoptosis pathways (11-15). For example, SBG induces the apoptosis of human bladder 5637 cancer cells via the reactive oxygen species (ROS)-dependent activation of caspases (11), inhibits the spread of B16F10 mouse melanoma cells by inactivating the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway (12), suppresses the proliferation of ovarian cancer cells by suppressing the p38 mitogen-activated protein kinases (MAPK)-dependent pathway (13), inhibits the growth of human MCF10A cells via transforming epithelial-mesenchymal transition (14) and has been reported to inhibit pulmonary tumor metastasis by impairing the activations of MAPK and PI3K-Akt (15).
Apoptosis is one of the processes of programmed cell death with specific morphologic characteristics and biochemical features, including membrane blebbing, intracellular fragmentation associated with membrane enclosed cellular fragments (apoptotic bodies) and cellular shrinkage (16,17). Apoptosis-associated molecules serve key roles in the maintenance of physiological homeostasis, but can be abnormally expressed during tumor development (18,19). However, the effects of SBG on MCF-7 human breast adenocarcinoma cells have not been previously investigated. Therefore, the present study aimed to identify the mechanism responsible for the SBG-induced apoptosis of MCF-7 human breast cancer cells.

Materials and methods

Preparation of SBG extract (SBGE). Baicalin and wogonin were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Acetonitrile, methanol and water of high performance liquid chromatography (HPLC)-grade were obtained from Avantor Performance Materials (Center Valley, PA, USA). Trifluoroacetic acid was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Accurately weighed standard compounds were dissolved in methanol and diluted. SBGE was obtained from the plant extract bank at the Korea Research Institute of Bioscience and Biotechnology (cat. no. CA04-087; Daejeon, Republic of Korea). SBGE was dissolved in methanol and then filtered through a 0.2 mm syringe filter (Biofact Co., Ltd., Daejeon, Korea) prior to injection into the HPLC apparatus. The HPLC system used was an Agilent 1200 (Agilent Technologies, Inc.) equipped with a quaternary pump, autosampler, column oven and diode-array detector. HPLC data were acquired using Chemstation software (version B.03.02; Agilent Technologies, Inc.). Chromatographic separation was performed on a XDB C8 column (4.6x150 mm, 5 µm; Agilent Technologies, Inc.) at 35°C. The mobile phase consisted of water containing 0.1% trifluoroacetic acid and acetonitrile and the chromatographic gradient program was: 20% acetonitrile for 2 min, 20-60% acetonitrile from 2 to 10 min and held for 1 min. The column was then re-equilibrated with 20% acetonitrile. The flow rate was set at 0.8 ml/min and the injection volume used was 5 µl. Baicalin and wogonin were detected at 280 nm.

Cell culture and reagents. MCF-7 cells (human breast adenocarcinoma cells) were established at the Cancer Research Center, Seoul National University College of Medicine (Seoul, Republic of Korea). Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) and 20 µg/ml penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) in a 5% CO2 atmosphere at 37°C. SP600125 and PD98059 were purchased from Tocris Bioscience (Bristol, UK). All other reagents were supplied by Sigma-Aldrich (Merck KGaA).

Cell viability assay. Cell viability was checked using an assay. MCF-7 cells were treated with 100 µl MTT solution (5 mg/ml in PBS per well) and incubated for 4 h at 37°C. The absorbance was measured at a wavelength of 570 nm using a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Measurement of cell cycle. MCF-7 cells were placed in an Eppendorf tube and ethyl alcohol was slowly added with vortexing. Tubes were then sealed with parafilm and incubated at 4°C overnight. Samples were centrifuged for 5 min at 110 x g at 4°C and supernatants was aspirated and discarded. Cell pellets were resuspended in 200 µl propidium iodine (PI) staining solution [PI (5 mg/ml; 2 µl) containing RNase (2 µl in PBS 196 µl)] (20,21) and then centrifuged at 20,000 x g for 10 sec at 4°C. Following an incubation for 30 min in the dark at room temperature, samples were analyzed using a fluorescence-activated cell sorter (FACScan; BD Biosciences, Franklin Lakes, NJ, USA) at 488 nm using Cell-Quest Pro software (version 5.1; BD Biosciences).

Tetramethylrhodamine (TMRM) mitochondrial membrane potential (MMP) assay. To measure MMP, MCF-7 cells were incubated with 25 nM TMRM for 1 h, mounted on a coverslide in a chamber filled with complete culture medium and incubated at 37°C in a cell culture incubator. Fluorescent images of samples were observed and captured using a fluorescence microscope at excitation/emission wavelengths of 549/575 nm. The fluorescent intensities of images were measured using ImageJ v1.62 software (National Institutes of Health, Bethesda, MD, USA) and values were expressed as percentages of controls.

Western blot analysis. Western blotting was performed using the lysates of 5x10^5 MCF-7 cells. Briefly, total proteins were extracted using radioimmunoprecipitation assay buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) containing a protease inhibitor cocktail (cat. no. #5871; Cell Signaling Technology, Inc.). The samples were centrifuged at 18,000 x g and 4°C for 1 min, and protein concentrations were determined using the Bio-Rad Protein Assay kit II (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal amounts (20 µg) of protein from each sample were separated by 10% SDS-PAGE, transferred to a PVDF membrane and blocked with 5% non-fat dry milk at room temperature for 1 h. The membranes were washed twice with Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and incubated with specific primary antibodies against target proteins, including B-cell lymphoma 2 (Bcl-2; cat. no. NB100-56095; Novus Biologicals, LLC, Littleton, CO, USA), Bcl-2 X associated protein (Bax; cat. no. NB100-56098; Novus Biologicals, LLC, Littleton, CO, USA), Bcl-2 X-associated protein (Bax; cat. no. NB100-56098; Novus Biologicals, LLC, Littleton, CO, USA) and β-actin (cat. no. A2066; Sigma-Aldrich; Merck KGaA) antibodies, diluted to 1:100 with 5% skim milk at 4°C overnight. The membranes were rinsed twice with TBS-T and incubated with the appropriate secondary horseradish peroxidase-conjugated antibodies, including rat anti-rabbit IgG (cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) and rabbit anti-mouse IgG (cat. no. Ab6728; Abcam, Cambridge, MA, USA) diluted to 1:1000 with 5% skim milk, at room temperature for 1 h. The bands for proteins of interest were detected using the ECL Plus Western Blotting Detection Reagent (GE Healthcare Life Sciences, Pittsburgh, PA, USA) according to manufacturer's instructions.
Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNAs were isolated from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and equal amounts of RNA (2 µg) were converted to cDNA using AccuPower RT-PreMix (Bioneer Corporation, Daejeon, Korea) at 70°C for 5 min, 42°C for 1 h and 94°C for 5 min using oligo-dT primers. Specific DNA sequences were amplified using AccuPower PCR-PreMix (Bioneer Corporation) under the following thermocycling conditions: An initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 60°C and extension for 30 sec at 72°C, with a final extension for 10 min at 72°C. The PCR primers used in this study were as follows: oligo-dT, 5'-TTTTTTTTTTTTTTTTTTTTTTTTT-T reverse; Fas, 5'-ATGCTTGGGCATCTGGACCCCTCTCTCA-3' forward and 5'-TCTGCACTTTGATTCTGGTCCG-3' reverse; Fas ligand (FasL), 5'-ACTTCGGGGGTTCAATCTTGC-3' forward and 5'-TAGAACATCTCGGTGCTGTAA-3' reverse; tumor necrosis factor (TNF)-α, 5'-GTGGGACGAGGTGTCGACT-3' forward and 5'-AGGCCACCTGCTCTCCCTCCCTCC-TCC-3' reverse; and β-actin, 5'-CAAGAGATGGCCACCGCTGTCCT-3' forward and 5'-TCTCTTCTGATCTCCTGTGCAGCA-3' reverse. Amplified products were analyzed in 1% agarose gels under UV light and images were captured using the GelDoc-It TS Imaging system (UVP, Inc., Upland, CA, USA).

Caspase assay. Caspase 3 and 9 assay kits (BioMol Cellular Activity Assay Kit Plus, Enzo Life Sciences, Inc., Farmingdale, NY, USA) were used. Cells were centrifuged at 1,000 x g at 4°C for 10 min, washed with PBS and resuspended in ice-cold cell lysis buffer. Samples were then centrifuged at 10,000 x g for 10 min at 4°C and supernatants were removed. Supernatant samples (10 µl) were then incubated with 50 µl substrate (400-1M Ac-DEVD-pNA) in 40 µl assay buffer at 37°C. Absorbances at 405 nm were read at several time-points for each sample. pNA concentrations in samples were determined using a standard plot of absorbance vs. pNA concentration. zVAD-fmk (Calbiochem; EMD Millipore, Billerica, MA, USA) was used as the pan-caspase inhibitor.

Measurement of ROS production. ROS generation in MCF-7 cells was quantified using 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA; Molecular Probes; Thermo Fisher Scientific, Inc.). Briefly, following the various pharmacological treatments, cells were treated with 10 µl DCF-DA at 37°C for 30 min and washed with PBS. Fluorescence was measured using a FACSscan system (BD Biosciences) at excitation/emission wavelengths of 488/525 nm.

Statistical analysis. Data are expressed as the mean ± standard error. One-way analysis of variation followed by Tukey’s post hoc test was used for multiple comparisons. The statistical analysis was performed using Prism version 6.0 (GraphPad Software Inc., La Jolla, CA, USA) and Origin version 8.0 (OriginLab, Northampton, MA, USA) software. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of standard compounds in SBGE. Baicalin and wogonin were detected on the HPLC chromatogram of SBGE at retention times of 7.5 and 12.5 min, respectively (Fig. 1).

Apoptosis by SBGE in MCF-7 cells. To determine whether SBGE suppresses MCF-7 cell growth, MTT assays were performed following the culture for 24 h of cells containing different concentrations of SBGE. Cell viabilities were identified to be markedly reduced by SBGE treatment. Culture for 24 h in the presence of 100, 200, 300 or 500 µg/ml of SBGE in culture medium inhibited cell survival by 33.7±7.3, 78.4±5.4, 81.4±4.5 and 85.5±7.0%, respectively, as determined by MTT assay (n=5; Fig. 2A). In addition, to determine whether SBGE induces apoptosis, cell cycle and mitochondrial membrane potential analysis were conducted by flow cytometry and fluorescence microscopy, respectively. Cells were treated with SBGE for 24 h (at concentrations between 100 and 500 µg/ml; Fig. 2B and C). The sub-G1 phase ratio was significantly and dose-dependently increased by SBGE. More specifically, the sub-G1 phase was markedly increased by 6.2±2.1% at 100 µg/ml, 13.3±3.5% at 200 µg/ml, 17.8±4.1% at 300 µg/ml and 57.1±6.2% at 500 µg/ml compared with untreated cells (n=5; Fig. 2B). In addition, mitochondrial membrane potentials were suppressed by SBGE. SBGE-induced mitochondrial membrane potential was decreased by 18.5±11.3% at 100 µg/ml, 21.7±7.7% at 200 µg/ml, 39.5±11.6% at 300 µg/ml and 48.2±8.0% at 500 µg/ml compared with untreated cells (n=5; Fig. 2C). These results suggested that SBGE has an anti-cancer effect and that this effect is closely associated with apoptotic induction.

SBGE induces apoptosis via a mitochondrial- and caspase-dependent signaling pathway in MCF-7 cells. To determine whether SBGE-induced MCF-7 cell apoptosis is regulated by Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic), western blotting was used following the exposure of the cells to various concentrations (between 100 and 500 µg/ml) of SBGE for 24 h. The results revealed that Bcl-2 expression was markedly inhibited by SBGE, whereas that of Bax was upregulated (Fig. 3A).

As the Fas/FasL system serves a key function in death receptor-mediated apoptosis, the involvement of the Fas/FasL/TNF-α system was examined in SBGE-treated cells by RT-PCR. The results demonstrated that Fas/FasL and TNF-α expression levels were unchanged by SBGE (Fig. 3B). In addition, as caspase activation is required for apoptosis, caspase activity assays were performed to assess the activities of caspases 3 and 9 in MCF-7 cells. It was identified that caspase activities were increased following treatment with SBGE (between 100 and 500 µg/ml) for 24 h, and that these activities were suppressed by zVAD-fmk (Fig. 3C). These results suggested that SBGE-induced apoptosis is mediated by a mitochondrial- and caspase-dependent signaling pathway in MCF-7 cells.

SBGE induces apoptosis via the e-Jun N-terminal kinase (JNK) and MAPK signaling pathway in MCF-7 cells. To investigate the association between the regulation of MAPK
pathways and the inhibition of MCF-7 cell proliferation by SBGE, cell viabilities were measured following the treatment of cells with SBGE at various concentrations (between 100 and 500 µg/ml) for 24 h with or without SP600125 (a JNK inhibitor) or PD98059 (a MAPK inhibitor) using the MTT assay. Co-treatment with 10 µM SP600125 or PD98059 markedly inhibited SBGE-induced cell death, particularly when cells were co-treated with 200 µg/ml SBGE. More specifically, co-treatment with 100, 200, 300 or 500 µg/ml SBGE and SP600125 inhibited cell survival by 7.9±3.5, 19.5±4.9, 34.2±2.8 and 48.7±3.5, respectively, as determined by the MTT assay (n=6; Fig. 4A). Co-treatment with 100, 200, 300 or 500 µg/ml of SBGE and PD98059 in culture medium induced cell survival by 6.4±2.8, 21.1±3.5, 34.8±2.7 and 46.4±4.5%, respectively (n=6; Fig. 4B). These results suggested that JNK and MAPK are involved in SBGE-induced apoptosis of MCF-7 cells.

SBGE-induced apoptosis is mediated by the generation of intracellular ROS in MCF-7 cells. AS intracellular ROS serve a key role in apoptosis, whether SBGE is able to generate ROS in MCF-7 cells was studied. To investigate whether ROS generation was directly associated with SBGE-induced apoptosis, intracellular ROS were investigated using the fluorescent dye DCF-DA. As indicated in Fig. 5A, when the cells were exposed to SBGE, levels of intracellular ROS increased. Furthermore, when cells were treated with various concentrations (between 100 and 500 µg/ml) of SBGE for 24 h, ROS generation significantly and dose-dependently increased, as demonstrated by flow cytometry (Fig. 5B).

Discussion

SBG is used as a medicinal plant in traditional herbal medicine, particularly in China (22). The major materials isolated from *S. baicalensis* are flavonoids, glycosides and the glucuronides of flavonoids, including baicalin, baicalein and wogonin. (22). Certain flavonoids possess various pharmacological activities, including anti-oxidant, anti-apoptotic, anti-allergic and anti-inflammatory effects (22-24). In addition, *S. baicalensis* and its flavonoids have been demonstrated to exhibit anti-cancer effects in various types of cancer cells (11-15,25-30). Although it has been reported that *S. baicalensis* inhibits the proliferations of several cancer cell lines *in vitro* (11-15,25-30), its anti-cancer activity and associated mechanisms in human breast adenocarcinoma MCF-7 cells remain unclear. Therefore, the present study investigated the underlying mechanisms of this phenomenon and identified SBGE-induced apoptotic signaling via mitochondrial- and caspase-dependent pathways via ROS generation in human breast cancer cells.

Apoptosis can be initiated through two signaling pathways: Extrinsic (death receptor-mediated) and intrinsic (mitochondria-mediated) pathways (31,32). The extrinsic pathway is initiated by extracellular signals, including FasL (33). The intrinsic pathway is regulated by Bcl-2 family proteins (anti-apoptotic proteins) (34). Notably, Bax protein shares regions of homology with Bcl-2 (35,36). Additionally, the downregulation of Bcl-2 proteins and the upregulation of Bax proteins may induce the destruction of the mitochondrial outer membrane (37-39). The caspases are major executors of apoptotic processes and belong to a group of enzymes called cysteine proteases (40). In the current study, Bcl-2 expression was inhibited by SBGE, whereas Bax expression was upregulated. However, Fas/FasL and TNF-α expression levels were unchanged, which suggested that SBGE induces cell death via the intrinsic pathway in MCF-7 human breast cancer cells. There are a number of breast cancer cell line types including, MCF-7, HBL100, MDAMB231, BT-20 and SKBR3 (41). In the present study, however, the effects of SBGE were investigated only in MCF-7 cells. The authors are now investigating the effects of SBGE on other breast cancer cell line types.

Transient receptor potential (TRP) channels were first cloned from *Drosophila* species and constitute a superfamily of proteins that encode a diverse group of Ca<sup>2+</sup>-permeable nonselective cation channels (NSCCs) (42,43). Among the TRP channels, TRP melastatin type 7 (TRPM7) channel expression is essential for cell survival in breast adenocarcinoma cells and thus is also a potential therapeutic target in breast cancer (44). Therefore, the effects of SBGE on TRPM7 channels were investigated. However, no effects were observed on overexpressed TRPM7 channels (data not shown). Thus, it is hypothesized that the TRPM7 channel is not involved in SBGE-induced anti-cancer effects in MCF-7 cells.

A number of cancer-associated components of the MAPK signaling pathways have been identified in Ras and B-Raf, which participate in the extracellular signal regulated kinase (ERK) signaling pathway (45,46). This kinase cascade presents novel opportunities for the development of novel cancer therapies designed to be less toxic than conventional chemotherapeutic drugs (47). The ERK signaling pathway serves a function in several steps of tumor development and also
induces the expression of matrix metalloproteinases, thereby promoting the degradation of extracellular matrix proteins and consequent tumor invasion (48). Therefore, components of signaling pathways activated by MAPK have received great attention as potential targets for the development of novel therapeutic drugs for cancer (49).
There is considerable interest among oncologists to find anticancer drugs in TCM. In the past, clinical data demonstrated that certain herbs possessed anticancer properties (50, 51); however, western scientists have doubted the validity of TCM due to the lack of scientific evidence (52). Recently, experiments have demonstrated that elements of TCM may possess an anticancer role, and clinical trials have demonstrated that TCM could improve survival, increase tumor response, improve the quality of life and reduce chemotherapy toxicity (52-56). Therefore, the authors of the present study suggest that when combined with chemotherapy, TCM could raise the efficacy level and lower toxic reactions.

In conclusion, the present study demonstrated that SBGE induces Bcl-2 protein downregulation and Bax protein upregulation, and activates caspases 3 and 9, leading to apoptosis. It was also identified that SBGE-induced cell death depends on the ROS-mediated JNK/MAPK signaling pathway. Therefore, SBEG may cause cell death via the intrinsic pathway in human breast cancer MCF-7 cells. These findings implicate SBEG as a useful potential anticancer agent.

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References


Figure 4. Effect of SBGE on the MAPK signaling pathway in MCF-7 cells. An MTT assay was used to determine cell viabilities in the presence of (A) SP600125 (a JNK inhibitor) or (B) PD98059 (a MAPK inhibitor). Cells were co-treated with the indicated concentrations of SBGE plus SP600125 or PD98059 (10 µM) for 24 h. Data are presented as the mean ± standard error. **P<0.01, ***P<0.001 vs. untreated cells. #P<0.05. SBGE, Scutellaria baicalensis Georgi extract; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase.

Figure 5. SBGE enhances ROS accumulation in MCF-7 cells. (A) To assess ROS production, cells were stained with DCF-DA and intracellular ROS levels were measured by flow cytometry. (B) Intracellular ROS was detected in MCF-7 cells treated with the indicated concentrations of SBGE for 24 h. ROS levels are expressed as percentages of those of untreated cells. Data are presented as the mean ± standard error. **P<0.01, ***P<0.001 vs. untreated cells. Ns, not significant; SBGE, Scutellaria baicalensis Georgi extract; ROS, reactive oxygen species; DCF-DA, 2',7'-dichlorodihydrofluorescein diacetate.


