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Original Article

Inhibition of Proliferation of Prostate Cancer Cell Line DU-145 *in vitro* and *in vivo* Using *Salvia miltiorrhiza* Bunge.*

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ABSTRACT **Objective:** To investigate the antiproliferative activity of *Salvia miltiorrhiza* Bunge. (SM) on the castration-resistant prostate cancer (CRPC) cell line DU-145 *in vitro* and *in vivo*. **Methods:** Prostate cancer cell line (DU-145) and normal prostate cell line (RWPE-1) were treated with SM at different concentrations (3.125, 12.5, 25 and 50 μg/mL) to investigate the antiproliferative effects. DNA laddering analysis was performed to investigate the apoptosis of DU-145 cells. Molecular mechanism was investigated by Western blot analysis of p53, Bcl-2, prostate specific antigen (PSA), and androgen receptor (AR). Six-week-old male BALB/c nude mice were randomly divided into normal control group (*n*=101) and treated group (*n*=101) which administered 500 mg/kg SM for 2 weeks. Tumor volumes were measured. **Results:** Treatment with SM resulted in a dose-dependent decrease in cell number of DU-145 cells in comparison with RWPE-1. DNA laddering analysis indicated the apoptosis of DU-145 cells. Treatment with SM increased the expression of p53 and reduced the expression of Bcl-2 proteins. The levels of PSA were considerably reduced in SM-treated group compared to the controls, and a decrease in AR expression was observed when cells were treated with SM in the same pattern as a reduction in PSA. In the tumour xenograft study, SM given once a day for 2 weeks significantly inhibited tumour growth. **Conclusion:** SM might contribute to the anticancer actions such as induction of apoptosis and inhibition of proliferation of prostate cancer cells.

KEYWORDS apoptosis, prostate cancer, *Salvia miltiorrhiza* Bunge., Chinese medicine

Prostate cancer (PCa) is the most frequently diagnosed male malignancy in most industrialized countries.(1) Recently, Asian countries have reported high incidences of PCa and consequent mortality rates.(2) The incidence of this disease is also rapidly increasing in Korea. Westernization of dietary habits among Koreans may contribute significantly to the increased morbidity and mortality of PCa in Korea.(3) Epidemiological studies suggest that natural dietary ingredients used in Asian countries have anticarcinogenic potential against PCa.(4) While prostate-confined disease is curable by surgery, advanced PCa remains essentially incurable. Although the majority of patients with PCa initially respond to hormone therapy, almost all patients ultimately develop tumor progression despite castrate levels of testosterone, which is often referred to castration-resistant prostate cancer (CRPC),(5-7) for which there is no currently effective therapy. The heterogeneity of cancerous cells allows for the development and spread of androgen-independent cancerous cells which do not respond to androgen deprivation therapy.(8) Treatment options for CRPC remain limited, and the prognosis of patients with CRPC is poor, with a median survival of 12 to 18 months.(5) Moreover, for elderly patients the complications are often worse. Dietary considerations may have an effect on PCa. Certain foods have been associated with a decreased risk of developing cancer, especially fruits and vegetables.(9) It has been shown that these foods contain many bioactive compounds, which both *in vitro* and *in vivo* have profound effects on the behaviour of cancerous cells. Some of these effects include increasing rates of apoptosis and arresting the cell cycle.(10,11)

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Salvia miltiorrhiza Bunge. (SM) is a well-known plant used in Chinese medicine to treat various entities, such as cardiovascular diseases, hyperlipidemia, and acute ischemic stroke.[12,13] This extracts contain several ingredients including watersoluble phenolic acids and lipophilic tanshinones.[14] Recently, other studies found that these exhibit significant antitumor activity by different mechanisms in various types of tumor cells.

This study was designed to investigate the antiproliferative activity of SM on the hormone refractory prostate cancer cell line *in vitro* and the efficacy in inhibiting PCa xenograft growth in an athymic nude mouse model.

METHODS

Preparation of Materials and Cell Culture

The dried SM was extracted with 95% ethanol for 24 h by 50 ℃. The extracts were filtered and concentrated in vacuo and then lyophilized. A venture company developing oriental herbal medicines, the Korea Biomedical Science Institute (Seoul, South Korea), developed this product. Normal prostate cell line, RWPE-1 (ATCC, Manassas, VA, USA) and androgen-independent prostate cancer cell line, DU145 (Korean Cell Line Bank, Seoul, South Korea) were cultured 24 h in RPMI 1640 supplemented with 10% FBS, 300 mg/L L-glutamine, 25 mmol/L HEPES, and 25 mmol/L $NaHCO_3$. RWPE-1 and DU-145 cells were seeded (5×10^3 cells per well) in 96-well plates (100 mL per well).

Cell Viability Assay

Cell growth and viability were evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) assay. After 24 h of growth in RPMI-1640 medium, the cells were treated with various concentrations of SM extract (3.125–100 μg/mL) for 48 h. The medium was removed at the end of the treatment, and 0.5 mg/mL of MTT was added to the medium. After 4 h, DMSO (200 mL) was added to each well and the optical density was read at 570 nm. Cell sensitivity to drug treatment was expressed as the drug concentration that yielded 50% cell inhibition (IC_{50}). All of the experiments were performed in triplicate.

DNA Laddering Analysis

RWPE-1 cells and DU-145 cells (5×10^5 cells/mL) treated with the SM (50, 100 μg/mL) for 48 h were washed with phosphate-buffered saline. Then 200 μL binding/lysis buffer was added and the total volume adjusted to 400 μL, and mixed. After incubating at 15–25 ℃ for 10 min, 100 μL isopropanol was added, and shaken. To a filter tube, a collection tube was attached and subsequently the samples were added to the filter tube and centrifuged at 800 r/min for 1 min. And 500 μL washing buffer was added and centrifuged at 800 r/min for 1 min twice, and centrifuged a third time at 13,000 r/min for 10 s. Then 100 μL elution buffer was added, and centrifuged at 800 r/min for 1 min. Finally, electrophoresis was performed on 1% agarose gel at 75 V voltage for 1.5 h, and assessed under ultraviolet light.

Cell Cycle Analysis

DU-145 cells (5×10^5 cells/mL) treated with SM (100 μg/mL) were counted and fixed in 80% chilled ethanol for cell cycle analysis (treated group) and compared with non-treatment control group (control group). Flow cytometric measurement of cellular DNA content were performed with ethanol fixed cells using the intercalating DNA fluorochrome, propidium iodide (PI). Measurements were made with an argon laser-based Flowcytometer (FACS-Calibur Becton Dickinson San Jose, CA, USA) using the Argon laser (488 nm) for excitation.

Western Blot Analysis

DU-145 cells (5×10^5 cells/mL) treated with the SM (100 μg/mL) were extracted by centrifuging at 4 ℃, 2,000 r/min, for 5 min, and lyzing by the reaction with 100 μL lysis buffer for 30 min at room temperature. The supernatant was extracted and quantified by use of the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) and then electrophoresed on an SDS-PAGE gel. After transfer to a nitrocellulose membrane, the membrane was blocked with 5% skim milk and then incubated with antibody against p53 (1:1,000, Abcam), Bcl-2 (1:1,000, Abcam, Cambridge, UK), prostate specific antigen (PSA, 1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and androgen receptor (AR, 1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Tumour Model in Nude Mice

In order to study the inhibitory effects on tumor growth *in vivo*, DU-145 tumor xenografts were established. Six-week-old male BALB/c nude mice,

weighing 18–22 g, provided by Samtaco Bio Co. (Osan, South Korea), were used in this experiment. After 1-week adjustment period, the animals were maintained as 5 per plastic cage. This study was approved by the Institutional Animal Care and Use Committee of the Catholic University of Korea, Seoul St. Mary's Hospital (IRB approval No. CUMC-2011-0026-02). Experimental animals were divided into the normal control group (*n*=10) and 500 mg/kg SM orally administered group (*n*=10). After 2 weeks, a total of 20 mice were inoculated subcutaneously on the lower back above the tail after alcohol preparation of the skin, using a sterile 22-G needle with 0.1 mL of a cell suspension of 2×10^6 DU-145 cells. Tumour volumes were measured every 3 days using a calliper and calculated according to the formula $(L\times W^2)/2$, that L and W stands for length and width, respectively.[15]

Statistical Analysis

All experiments were performed on 3 separate cultures. All data was presented as the mean ± standard deviation ($\bar{x}\pm s$). Overall comparisons between groups were performed using SPSS program (version 12.0). ANOVA analysis of *Duncan* test was performed to detect differences over the time course and between the groups. P<0.05 was considered to be statistically significant.

RESULTS

Inhibitory Effect of SM on Growth of DU-145 Cells

SM inhibited the growth of DU-145 cells in a dose-dependent manner. At 48 h, the survival rates were 71.7%±4.1%, 25.3%±2.0%, 4.6%±0.3% and 8.9%±0.3% in the 3.125, 12.5, 25 and 50 μg/mL SM-treated groups, respectively. IC_{50} of SM against DU-145 was estimated to be 6.25–12.5 μg/mL (Figure 1).

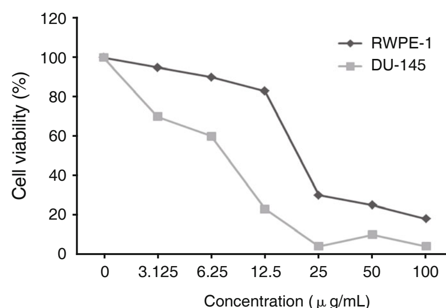

Figure 1. Result of Cell Viability Assay

DNA Laddering Analysis

To characterize the mechanism of cell death, DNA laddering analysis was performed. As evident in Figure 2, the migration of fragmented DNA as a laddering pattern was observed by electrophoresis in DU-145 cells although it was not observed in RWPE-1 cells.

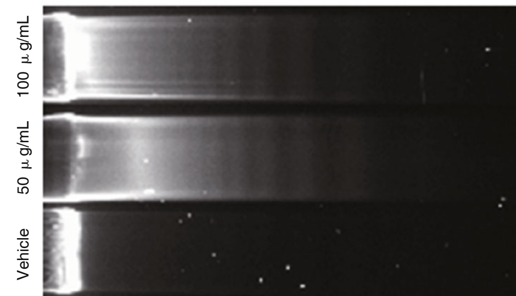

Figure 2. Result of DNA Ladering Analysis in DU-145 Cells

Cell Cycle Analysis

After treatment with SM, the percentage of DU-145 cells was decreased in G_0/G_1 phase and increased in G_2/M phase significantly (P<0.05); and the increases in the percentage of apoptosis were induced in the treated group, which suggested that SM played its inhibitory roles mainly via the induction of cell cycle arrest and apoptosis (Figure 3).

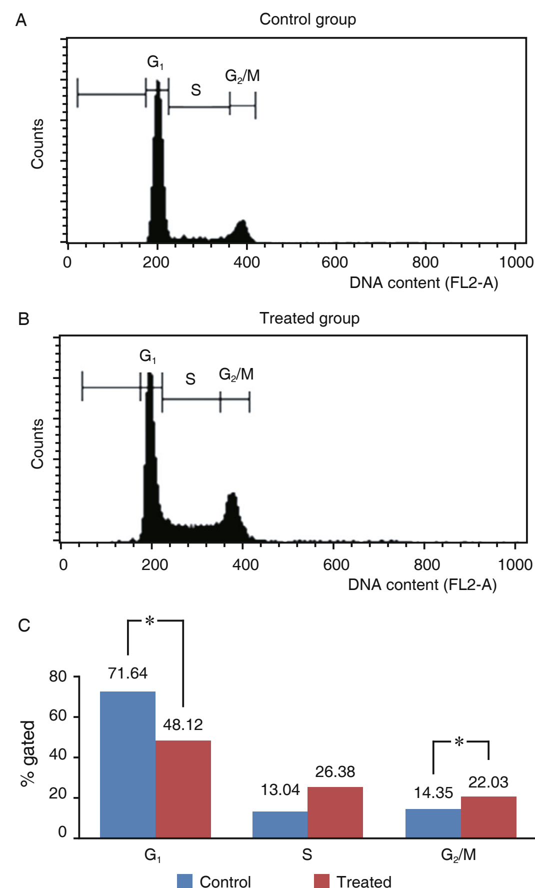

Figure 3. Effect of SM on Cell Cycle in DU-145 Cells

Note: *P<0.05, compared with control group

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Expression of p53, Bcl-2, PSA and AR

Compared with the control group the expression of p53 was significantly increased while significant decrease was seen in Bcl-2 expression in the treated group (P<0.05). AR and PSA were constitutively expressed in untreated DU-145 cells. The levels of PSA were significantly reduced in SM-treated group compared to the controls (P<0.05). A decrease in AR expression was observed when cells were treated with SM in same patterns as a reduction in PSA (P<0.05, Figure 4).

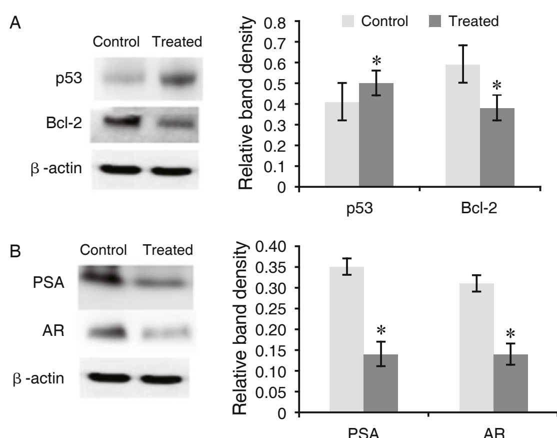

Figure 4. Western Blot Results

Note: *P<0.05, compared with control group

Tumour Growth of Xenografts

The athymic nude mice did not exhibit any considerable changes in body weight during the experiment. In the first analysis at 4 weeks postinoculation, initial detection time and average tumor volume were not significantly different between the two groups. However, after 8 weeks of postinoculation, the average tumor volume in the normal control group increased and reached the volume of 317.3 mm^3, while the average tumor volume was only 214.9 mm^3 in mice treated with SM. At 12 weeks postinoculation, the tumor volume in SM administered group had grown up to 313.3 mm^3 (Figure 5A) although that in control group had grown up to 822.3 mm^3 in the same period. The observed differences for tumor development in SM-treated mice compared with vehicle-treated mice were statistically significant at 8–12 weeks postinoculation (P<0.01, Figure 5B).

DISCUSSION

Overall, the major findings of the present study are that SM significantly inhibits advanced human PCa growth, which is accompanied by a increased apoptosis

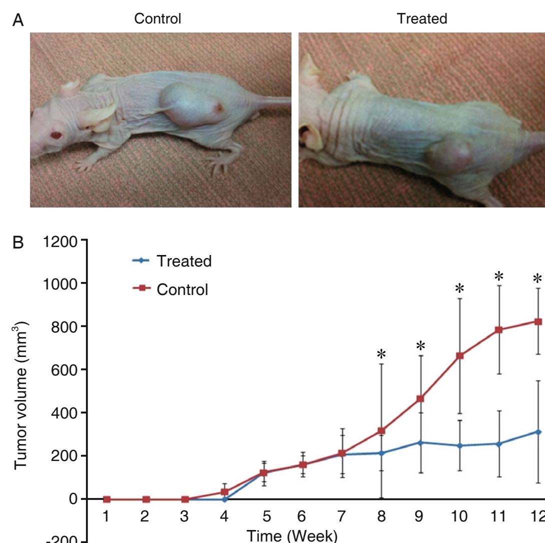

Figure 5. Effects of SM on Tumorigenecity of DU-145 Tumor Xenograft in Athymic Nude Mice

Note: *P<0.01, compared with treated group

without any apparent signs of toxicity in nude mice; and itexert strong inhibition on DU-145 cell growth. The cancer chemopreventive effect was also evident by the results in which mice were fed for 2 weeks before the implantation of the tumor xenograft.

SM contains several constituents including watersoluble phenolic acids and lipophilic tanshinones. Tanshinone Ⅰ was also shown to induce cancer cell apoptosis in human myeloid leukemia cells[16] and non-small cell lung cancer.[17] Although various mechanisms were proposed to explain the antitumor effects, such as inactivation of the PI3K/Akt/survivin signaling pathways,[16] reductions of interleukin (IL)-8, Ras-mitogen-activated protein kinase and Rac1,[17] this issue has not been clarified. Cryptotanshinone is the major tanshinone isolated from SM, and have anti-inflammatory, anti-oxidant and anti-tumor activities. Cryptotanshinone also exhibits potent anti-angiogenic activity.[18] However, the precise mechanism of antitumor activity of cryptotanshinone is unknown.

Our results revealed that SM induced DNA fragmentation, supporting apoptosis induction. The role of p53 pathway in apoptosis signaling is currently being investigated. Bcl-2 inhibits apoptosis and prolongs the lifespan of a variety of cell types. Overexpression of Bcl-2 can inhibit a variety of stimuli-induced apoptosis events. There is a hydrophobic groove on the surface of the antiapoptotic Bcl-2 family proteins that can bind Bcl-2 homology 3 domains exposed on the surface of

proapoptotic proteins, forming heterodimers. Bcl-2 and BH3 interact to regulate apoptosis. Small molecules that mimic the BH3 domain can effectively inhibit the antiapoptotic action of Bcl-2 family proteins and induce the release of proapoptotic proteins such as Bax and Bak.[19] In this study, we showed that SM can effectively inhibit the expression of Bcl-2, which promotes apoptosis in PCa cells.

Although androgen blockade treatment for PCa is effective, the antitumor effects can be temporary. Virtually castration-sensitive phenotype will convert to castration-resistant phenotype. The major reason is that AR signaling remains intact and is often hyperactive in castration-resistant prostate cancer. Taken together with suggested mechanism for developing this castration-resistant cancer growth including mutation,[20] amplification[21] and activation[22] of AR, androgen signaling through AR plays an important role in promoting the development of androgen-independent PCa.[23] Targeting AR signaling and reducing AR expression for androgen independent PCa may represent an attractive approach to target androgen signaling in PCa. Actually in the recent reports, AR signalings such as synthetic siRNA, AR antisense, geldanamycin analogs and selective AR modulators have been the focus of research, which have demonstrated that down-regulation of AR expression is sufficient to slow prostate tumor growth and induce apoptosis.[24-26]

In this study, we demonstrated that SM decreased the expression of AR and PSA in concomitant with induction of apoptosis in DU-145 cell, which means that apoptosis resulted from administration of anthocyanin was mediated by suppressing AR and activating p53 signaling. In view of the importance of the AR in prostate cancer pathogenesis, our study, which indicated the suppression of AR expression, may suggest important clinical possibilities that diminishing the expression of androgen receptor by anthocyanin could be helpful not only for reducing prostate cancer burden but also for preventing progress after androgen blockade treatment.

Because PCa has long latency and its risk increases with age, chemopreventive strategies could be applied to effectively prevent or delay its progression. However, the outcomes from the recent selenium and vitamin E cancer prevention trial (SELECT trial) have been disappointing.[27] In the prostate cancer prevention trial (PCPT trial), finasteride, a 5a-reductase inhibitor, reduced the risk of prostate cancer by 24.8%, but was initially associated with increased risk of high-grade disease by 25.5%.[28] Although reanalysis indicated that high-grade cancer was not associated with finasteride, the results may need further confirmation from another clinical trial other than the Reduction by Dutasteride of prostate cancer events (REDUCE) trial.[29] Therefore, there is an urgency to identify more promising safe and efficacious agents for prostate cancer chemoprevention. The use of plants for medicinal purposes is as old as human history. The medicinal use of herbal products is growing in the United States and many other countries. In Korea, traditional medical systems rely primarily on botanicals as a mainstay of therapy or prevention. Plants and other botanicals have also been the basis for most modern pharmaceutical drugs.

Our results from the systematic *in vitro* and *in vivo* studies strongly suggest that SM may have favorable effects and may serve as a promising chemopreventive agent against PCa progression.

The results from this study provided promising experimental evidence to support that SM may be a novel efficacious agent for the chemoprevention and/or therapy of PCa progression by induction of apoptosis and inhibition of proliferation of PCa cells.

Conflict of Interest

The authors have nothing to disclose.

Author Contributions

WJB drafted the manuscript, and participated in every part of the experiments. USH participated in the design of the study and helped in the experiments and drafting of the manuscript. KSK and JBC participated in the design, biochemical assays and statistical analysis of the study. SHH participated in the experiments, coordinated among the authors and helped to draft the manuscript. JYL, TKH, WZP and SYH participated in the design of the study and drafting of the manuscript. SWK participated in the design of the study and experiments of the study.

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