Regulation of IGF-I production and proliferation of human leiomyomal smooth muscle cells by *Scutellaria barbata* D. Don in vitro: isolation of flavonoids of apigenin and luteolon as acting compounds

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Abstract

*Scutellaria barbata* D. Don (Lamiaceae) (SB) is a perennial herb, which is natively distributed throughout Korea and southern China. This herb is known in traditional Chinese Medicine as Ban-Zhi-Lian and traditional Korean medicine as Banjiryun, respectively. SB has been used as an anti-inflammatory and antitumor agent. We aimed to determine the expression of growth factor molecules for growth inhibition after treatment of SB in two different human myometrial smooth muscle cell (SMCs) and leiomyomal SMCs. Water-soluble ingredients of SB, myometrial SMCs, and the leiomyomal cell lines were used in vitro. SB significantly reduced cell numbers in culture and arrested cell proliferation, and also induced apoptosis, indicating that the presence of an intact apoptotic pathway was demonstrated in these cells by SB. Uterine leiomyoma is the most common benign smooth muscle cell tumor of the myometrium. The expression of insulin-like growth factor-I (IGF-I) was measured at the mRNA and protein level in myometrium and leiomyomal cells with and without treatment with a water extract of SB for 3 days. IGF-I mRNA expression was significantly higher in leiomyomal cells than in myometrium cells. The IGF-I protein was more abundant in leiomyomal cells than in myometrium. When SB was treated to the cells, the IGF-I protein concentrations in myometrial and leiomyomal cells from the SB-treated cells were similar. The results indicated that IGF-I expression is probably associated with a proliferation of leiomyomalous cells than myometrium. However, SB down-regulated the IGF-I expression where IGF-I contributes to the selective growth of the leiomyoma. Therefore, growth modulation of LMs by SB occurs via mechanisms dependent of apoptosis. The raw materials were extracted and subjected to functional isolation for the active molecules in the present assay systems. The five flavonoids were isolated and the chemical structures of resveratrol, baicalin, berberine, apigenin, and luteolin were determined. The effects of resveratrol, baicalin, and berberine on the above parameters have not been significantly evidenced, whereas apigenin and luteolin were effective. The anti-proliferative compounds apigenin and luteolin belong to the flavones, a class of flavonoids and are characterized as selectively inhibitors of the growth of LM cells. Our findings suggest that flavonoids of apigenin and luteolin are potentially useful for the development of therapeutic treatments of cancer. These data also suggest that SB reduces tumor volume with inducing a concomitant increase in the rate of apoptosis.

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Keywords: Scutellaria barbata D. Don (Lamiaceae); Cell proliferation; HCG; HCG-LH receptor; Leiomyoma; Uterus; Myometrium; Leiomyomal cells; IGF-I mRNA; Fibroids; Apoptosis; Flavonoids; Apigenin; Luteolon

Introduction

Uterine fibroids, or leiomyomata, are the most common tumors in the female genital tract. They are benign without detectable symptoms, these tumors may cause bleeding disturbances, pelvic discomfort or pain, and occasionally, recurrent abortion and infertility. The average decrease in volume of the fibroids following this treatment has been reported as >50%, whereas the effect on total uterine volume is less pronounced (Puzigaca et al., 1994). Antiprogestins have been shown to decrease the size of...
fibroids to a similar degree (Murphy et al., 1995). During pregnancy, fibroids often increase in size during the first two trimesters, whereas very few continue to grow during the last trimester and most fibroids even shrink (Lev-Toaff et al., 1987). The reason for this lack of growth response, despite high serum concentrations of sex steroids during late pregnancy, is unknown.

It was known that both hormone receptors and growth factors might be important for the differences in growth regulation. The insulin-like growth factors (IGFs) constitute a family of polypeptides with insulin-like and growth-promoting activities (Hummel, 1990). They are produced not only in the liver but also in several extrahepatic tissues, including the uterus. IGF-I and IGF-II are expressed and secreted in both fibroids and adjacent myometrium (Rein et al., 1990). In the rat uterus, the expression of IGF-I (but not of IGF-II) has been shown to be regulated by estrogens. Down-regulation of both IGF-I and IGF-II by GnRH agonists has been suggested (Rein et al., 1990). Membrane preparations from fibroids have more high affinty binding sites for only IGF-I than myometrium, not for IGF-II (Chandrasekhar et al., 1992). IGF-I binds mainly to the type 1 IGF receptor, whereas IGF-II binds to both the type 1 and the type 2 IGF receptors (Jones and Clemmons, 1995). Thus, we examined the expression of IGF-I in leiomyomas in comparison with that in the normal myometrium by means of mRNA and immunoblot analysis. Furthermore, to understand the role of SB in regulating the expression of IGF-I mRNA and protein expressions in leiomyomal cells, we examined whether SB could influence the levels of IGF-I mRNA and protein expression in leiomyoma cells cultured in vitro under a serum-free, phenol red-free condition.

Scutellaria barbata D. Don (SB) is one of herbs from perennial plants, which is mainly distributed throughout Korea and southern China. This herb has been used as an anti-inflammatory and antitumor agent and as a diuretic (Jiangsu New Medical College, 1977). This herbal material is known in traditional Korean Medicine as ‘Ban-Ji-Ryun’ and has been used for hundreds of years in Asian countries. SB, which has been traditionally used for inflammation, hepatitis, tumors, and osteoarthritis in Korea and China, was found to be potent. This herbal material is known to contain a large number of alkaloids and flavones, frequently found as glucosides and other constituents, including phenethyl alcohols, sterols, and essential oils and amino acids (Jiangsu New Medical College, 1977). The active principles in SB have not been determined fully. By bioassay-guided separation, emodin, emodin 8-O-glucopyranoside, resveratrol (3,4’-5-trihydroxyxystilbene), baicalin, berberine, and resveratrol were isolated as active principles [unpublished results]. Apigenin and luteolin were also isolated from this plant as active constituents against the bacteria. These flavonoid congeners were selectively toxic to Staphylococcus aureus, including the MRSA and methicillin-sensitive S. aureus strains (Sato et al., 2000). SB has been used in Asian ethnic medicine for treating gynecological, lung, and rectal tumors. It was reported to be capable of enhancing macrophage function in vitro and inhibiting tumor growth in vivo (Wong et al., 1996). SB had anti-mutagenic effects and inhibited the damage to DNA in lymphocytes caused by the total particle material extracted from cigarette tar (Han et al., 1997). SB inhibited mutagenesis, DNA binding, and metabolism of aflatoxin B1 (AFB1) and benzo(a)pyrene (BaP) bioactivated by Aroclor 1254-induced rat S9 (Wong et al., 1992a, 1992b, 1993a). SB also inhibited the mutagenicity of AFB1 in Salmonella typhimurium TA100 using dexamethasone (DXM)-induced rat hepatic S9, on cytochrome P450-linked aminopyrine N-demethylase (APND) activity in DXM-induced hepatic microsomes and on the metabolism of AFB1 by DXM-induced S9. SB consistently inhibited the mutagenicity of AFB1 bioactivated by either non-induced or DXM-induced S9. The effects correlated with the inhibition of cytochrome P450-linked APND activity in DXM-induced microsomes and with an inhibition of DXM-induced S9 mediated metabolism of [³H]. Therefore, it was suggested that SB possess antimutagenic and antitumorigenic activity towards AFB1 through an inhibition of CYP3-mediated metabolism of AFB1 (Ducki et al., 1996; Wong et al., 1993b). Also, oral feeding with SB inhibited the growth of a murine renal cell carcinoma (Renca) in mice, showing that SB is capable of enhancing macrophage function in vitro and inhibiting tumor growth in vivo (Wong et al., 1996). Natural herbal materials such as SB have been used empirically in the treatment of cancer, but the mechanisms by which they bring about antitumor effects are unclear. Furthermore, although SB is presently being used as an anti-gynecological tumor agent, the effect of SB on human myometrial and leiomyomal smooth muscle cells (SMC) remains unknown.

Previously, our laboratory has examined the effect of SB on this tumor due to SB’s widespread use in clinical trials. We examined the ability of various herbal materials and preparations to inhibit the growth of leiomyomalous cells in vitro, attempting to gain a better understanding of the action mechanism of SB. The responses of both normal uterine myometrial SMC and leiomyomalous SMCs to SB treatment were analyzed. Cell cycle analysis using flow cytometry and Western blotting using antibodies against α-SMA, calponin h1 and the G1 cell-cycle-related gene products, cyclin E, cyclin-dependent kinase 2 (cdk2) and the cdk inhibitor, p27, clearly showed its effectiveness on anti-growth activity of leiomyomalous SMCs (Lee et al., 2004a, 2004b).

In the present study, we demonstrated that SB acts as an antagonist, inhibiting the growth of uterine LM cell lines in vitro. We report here that SB inhibits cell proliferation dependent of an apoptotic response. Additionally, this observation suggests that SB results in apoptosis and helps explain the observed rapid regrowth of these tumors after SB treatments. The present study also aimed to investigate whether SB affects expression of IGF-I mRNA in leiomyomalous cells compared with myometrium under SB treatment.
The present study also examined effects of SB on cell proliferation of cultured myometrial cells.

Materials and methods

Materials. Antibiotic solution (1 × 10^5 U/l penicillin and 50 mg/l streptomycin), FBS, progesterone, collagenase, monoclonal antibodies to human cytokeratin 19, human desmin and human vimentin were purchased from Sigma Co. (St. Louis, MO, USA). The 75-cm² flasks were purchased from Nunc Korea Co. (Seoul, Korea).

*Extraction of Scutellaria barbata D. Don.* Authentic plant material was purchased from local market and identified at the Oriental Medical Department, Dongguk University, Kyungju, Korea. Voucher specimen [OM-S37] is present at the Herbarium of Botany Department, Dongguk University, Kyungju, Korea. More specifically, stems of SB were cut into small pieces and 200 g of the material was boiled in 4 l of water until the liquid volume had been reduced by 50%. The fluid was then filtered through a 1-mm pore-size filter, the water-insoluble component was discarded, and the extract was lyophilized to a dry powder. Then, the extract was filtered. The filtrate was evaporated to dryness in an oven at 40 °C. The dried extract was weighed (yield, 1.5%).

A sample of the dry powder (2.5 g) was dissolved in 100 ml of milli-Q water at 37 °C and stirred for 60 min. The sample was then centrifuged at 4000 × g for 15 min, and the supernatant was passed though a 0.22-µm membrane bottle filter system (Corning, COstar, NY, USA). The water-insoluble component was discarded and the water-soluble ingredients were used in the experiments. The concentration of SB extract in the solution was calculated by lyophilizing the water-soluble component that passed through a 0.22-µm membrane bottle filter. The concentration of lipopolysaccharide in SB (600 µg/ml) was below 1.0 pg/ml.

*SB treatment.* SB were made by placing the required quantity of SB in boiling water; final concentrations were either 20 or 50 g/l. The SB was allowed to infuse for 10 min followed by straining to remove the pieces of SB. The solution was then allowed to cool prior to being added to the animals’ water bottles. Fresh SB was provided each day.

*Tissue collection.* Uterine leiomyomas and adjacent normal myometrial tissues were obtained from women with regular menstrual cycles who underwent abdominal hysterectomy for medically indicated reasons at Dongguk University Hospital. The use of uterine tissues for culture experiments was approved by the institutional review board. The patients ranged in age from 30 to 43 years with a mean age of 37 years, and none had received hormonal therapy for at least three cycles before surgery. Informed consent was obtained from each patient before surgery for the use of uterine tissues for the present studies. Each uterine specimen was studied by a pathologist for histological examination and dating of the endometrium. The study was approved by the ethical committee of Dongguk University Hospital, Korea, and a full and informed consent was obtained from all patients participating in the study. Fibroids and/or myometrial tissue were collected from five untreated premenopausal women.

*Cell culture.* Uterine leiomyoma tissues and adjacent normal myometrial tissues dissected from endometrial cell layers, washed in PBS, cut into small pieces, and digested in 0.2% collagenase (wt/vol) at 37 °C for 3–5 h (Rossi et al., 1992). The leiomyoma cells and myometrial cells were collected by centrifugation at 460 × g for 5 min and washed several times with DMEM containing 1% antibiotic solution. The isolated leiomyoma cells and myometrial cells were, respectively, plated in 75-cm² flasks at an approximate density of 5 × 10^5 cells/flask and subcultured for 120 h at 37 °C in a humidified atmosphere of 5% CO₂–95% air in DMEM supplemented with 10% FBS (vol/vol). The trypan blue exclusion test was used to determine the cell viability. Characterization of the cultured cells was examined using immunostaining with monoclonal antibodies to a muscle-specific protein desmin, to a class of intermediate filament protein present in fibroblast vimentin, and to a cytoskeletal protein for epithelial cells cytokeratin 19. Thereafter, the cultured cells were stepped down to serum-free conditions by incubating in serum-free DMEM in the presence or absence of progesterone or SB. Treatment with progesterone or SB was began when the cultured cells were at approximately 40–50% confluence, and monolayer cultures were maintained in serum-free DMEM for an additional 72 h.

*Preparation of cellular extracts and Western immunoblot analysis.* At the termination of cultures, cultured cells were incubated at 4 °C for 15 min in the presence of a lysis buffer consisting of 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris–HCl, and 2 mM phenylmethylsulfonylfluoride, pH 7.5. Cells were subsequently scraped off the plates, the extracts were centrifuged at 13,000 × g for 30 min, and the supernatants were collected. On the other hand, leiomyomas and adjacent normal myometrial tissues for protein extraction were collected immediately after hysterectomy. These tissue samples were homogenized at 4 °C in the above-noted lysis buffer (5 g tissue in 1–2 ml). Homogenates were subsequently centrifuged at 13,000 × g for 30 min, and the supernatants were collected. Protein estimation of the supernatants and total proteins in cell lysates was performed by the Bradford assay (Bradford, 1976) and Lowry method (Lowry et al., 1951).

Each of 15-µg aliquots of proteins extracted from cultured cells and uterine tissues was run on a 10% SDS-polyacrylamide gel under a reducing condition using 20–25 mA for the stacking gel and 30–35 mA for the separating gel.
Total nucleic acids (TNA) were prepared from samples. Blots were exposed overnight to the monoclonal antibody directed against IGF-I protein at a dilution of 1:80 in Tris buffer. The antigen–antibody complexes were detected with a secondary antibody using the ECL chemiluminescence detection system (Amersham Korea, Seoul, Korea). Control procedures for Western immunoblotting included the substitution of the primary antibody with nonimmune murine IgG and omission of the primary antibody. These controls prevented the appearance of immunoreactive IGF-I protein band of the membrane-bound proteins.

These experiments were repeated as followed with similar results, and the reported results are representative. Western blot analysis of leiomyoma and myometrium tissue extracts with a monoclonal antibody to IGF-I protein were performed six times using six different uterine specimens. On the other hand, experiments to investigate the effects of SB on IGF-I protein expression in cultured leiomyoma cells were performed four times.

**mRNA analysis: effect of SB extracts on IGF-I mRNA transcription.** Total nucleic acids (TNA) were prepared according to Durnam and Palmiter (1983) and TNA concentrations were determined by spectrophotometry. IGF-I mRNA values were determined by hybridization of TNA in solution as previously described (Durnam and Palmiter, 1983). A complementary RNA (cRNA) probe was synthesized using Riboprobe system (Promega, Seoul, Korea) with a 775-bp fragment of the gene coding for IGF-I as template (amplified by PCR). All samples were analyzed several times. The results presented represent a typical experiment where all samples in duplicate have been hybridized with the same probe within a week. The background in terms of cpm/vial without TNA, expressed as percentage of input values, was 2–5%. At least double background concentrations were reached, within a range where a linear relationship between increasing amount of TNA and cpm/µg TNA was achieved. We have chosen to relate IGF-I mRNA in all samples to their DNA concentrations, which is a more stable parameter than RNA or TNA content when proliferation differs.

**Assay of IGF-I: effect of SB extracts on IGF-I production.** Total IGF-I concentrations in cells were determined by radioimmunoassay with a commercial kit from Sigma Co. The cells was transferred to a prechilled tube and suspended in 10 volumes of buffer containing 10 mM Tris, 1.5 mM EDTA and 5.0 mM Na₂MoO₄, (sodium molybdate) adjusted to pH 7.4 with HCl. The suspension was centrifuged at 24,000 × g for 30 min at +4 °C and the supernatant was transferred to a prechilled tube and frozen at −70 °C. IGF-I was extracted from cell homogenate, as suggested by the manufacturer of the radioimmunoassay kit. The detection limit in serum using the standard procedure suggested by the manufacturer was 0.6 µg/l and the inter- and intra-assay coefficients of variation were 6% and 10%, respectively. Serial dilutions of extracts from leiomyomal and myometrial cells yielded dilution curves parallel to the standard curve.

**Flow cytometry.** Cells were plated at low density in T-150 flasks (Corning) and treated with the following: DF8 medium plus 5–100 µg/ml SB. After 48 h of treatment, 5-bromo-2′-deoxyuridine (BrdU, Sigma) was added to each flask at a final concentration of 10 µM, and cultures were incubated for 6 h at 37 °C before harvest by trypsinization. Growth and wash media were collected and processed to include any cells that might have detached during treatment. After isolation and rinsing, cells were resuspended in 3 ml PBS, fixed by the addition of 1.5 ml cold 100% ethanol, and stored at −20 °C overnight. Cells were permeabilized by incubation in 2.0 N HCl with 0.5% Triton X-100 (vol/vol; Sigma) at room temperature for 30 min. Cells were resuspended in 1 ml 0.1 M sodium borate, pH 8.5, to neutralize residual acid. A portion of the suspension was counted using a Coulter counter, and an aliquot of 1 × 10⁶ cells was incubated in a solution containing 50 µl 0.5% Tween-20 (vol/vol; Sigma) plus 1.0% BSA (wt/vol) in PBS and 20 µl fluorescein isothiocyanate (FITC)-labeled anti-BrdU (Becton Dickinson Immunocytometry Systems, San Jose, CA) for 30 min at room temperature. Cells were finally resuspended in 0.5 ml PBS containing 5 µg/ml propidium iodide (PI) and stored at 4 °C in the dark until analyzed on a fluorescence-activated cell sorter (FACS).

Cell suspensions were analyzed using a Coulter EPICS Elite flow cytometer (Coulter) equipped with a 488-nm argon laser, and subsequent data analysis was performed on 1–2.0 × 10⁶ cells for each treatment using Coulter Elite software (Coulter). The lower limit for FITC fluorescence, and thus BrdU incorporation, was set based on control values for background fluorescence from previous experiments. Gating of events into G0/G1, S, and G2/M phases of the cell cycle was based on the PI fluorescence frequency histogram for cells grown in DF8 medium. Using these parameters and restrictions based on the forward scatter vs. PI fluorescence profile of SB-treated cells, the criteria for apoptosis were established. These limits remove debris and nonviable cells from analysis of the less than diploid (sub-2N) population.

**DNA ladders: effect of SB extracts and compounds on DNA fragmentation.** Human uterine LM cells were plated at low density in T-150 flasks and treated with the following: fresh DF8 medium plus 5–100 µg/ml SB. After 48 h of continuous treatment, cells were rinsed and trypsinized, collecting both growth and rinse media to ensure that detached cells were retained for analysis. Cells were lysed in 2–3 ml lysis buffer [100 mM NaCl, 50 mM Tris, 10 mM EDTA, 0.5% SDS (wt/vol), and 0.5 µg/ml proteinase K, pH 8.1] for 1 h at 37 °C. Nucleic acids were then isolated by phenol-chloroform extraction and ethanol precipitation. The
resulting pellet was resuspended in 1.5 ml TE buffer (10 mM Tris and 1 mM EDTA, pH 7.5). RNA was digested by adding 4 U/ml ribonuclease A and incubating for 1 h at 37 °C. The resulting genomic DNA was precipitated as before, and the pellet was resuspended in TE buffer. Samples were electrophoresed for approximately 18 h on a 1% agarose (Life Technologies, Grand Island, NY) gel at 20 V. Gels were subsequently stained in a 5 μg/ml solution of ethidium bromide before photography.

SB effects on cell growth in serum-free medium. Cells were plated in 24-well plates in triplicate at 500 cells/well for each growth condition. After 72 h, the cells were treated with either serum-free medium or 10% FBS or a series of EA concentrations (5–100 μg/ml 10% FBS medium). Cells were counted every 24 h from 48 to 120 h as described above. The rate of cellular proliferation was determined by adding [3H]thymidine (Amersham Life Science, Arlington Heights, IL) to identical cultures 24 h before harvest. Nucleic acids were then isolated by lysing cells in a buffer of 1.0% SDS (wt/vol) and 0.3 N NaOH before trichloro-acetic acid precipitation. Incorporation was determined by scintillation and expressed as counts per min/cell. Cell number after 120 h and [3H]thymidine incorporation were subjected to log transformation and Student’s unpaired t test.

Determination of baicalin from SB stems. SB stems (1.0 kg) were cut into small pieces, immersed, and extracted with acetone (10 l × 2) at room temperature for 2 weeks. After filtration, the residues were then reflux-extracted with 50% aqueous ethanol (4.0 l × 2). Acetone and 50% aqueous ethanol extracts were concentrated under reduced pressure to 0.2 and 1.0 l, respectively. Ethanol was added into the concentrated 50% aqueous ethanol extracts and produced a large amount of yellow precipitate. A portion of the precipitate (1.0 g) was recrystallized with aqueous ethanol to obtain baicalin (0.7 g). The acetone extracts were subjected to column chromatography on silica gel (10 cm i.d. × 30 cm) eluted with CHCl3 and CHCl3–MeOH (10:1 to 1:1 gradient) to yield a total ten fraction. CHCl3 elute was coated with Celite 545 (Merck) and rechromatographed on silica gel (2 cm i.d. × 30 cm) eluted with hexane-acetone (10:1 to 2:1 gradient) to yield some baicalin (0.3 g). A portion of the CHCl3–MeOH (10:1) elute (0.8 g) was subjected to gel permeation chromatography on Sephadex LH-20 (2 cm i.d. × 45 cm) eluted with MeOH to yield baicalein (0.5 g). Each compound was identified by direct comparison of its spectroscopic data with authentic samples. Purity tests of baicalin, resveratrol, and baicalein were performed by HPLC equipped with a 280-nm detector and LiChrospher 100 RP-18e column (4 mm i.d. × 125 mm) (Ducki et al., 1996; Lee et al., 2004a). The mobile phase was composed of CH3CN-0.1 M H3PO4 (28:72) and the flow rate was 1.0 ml/min. The purity of the compound was more than 99.5%.

Determination of berberine from SB stems. SB stems (100 mg) were extracted with 2% hydrochloric acid (10 ml) by ultrasonicating for 30 min at 40 °C, and cobalt thiocyanate reagent (2 ml) and dichloroethane (10 ml) were added. The mixture was shaken at 280 rev./min for 10 min and centrifuged at 2500 rev./min for 10 min. The aliquot (0.1 ml) of the organic layer was evaporated to dryness under the stream of nitrogen gas and the residue was dissolved in methanol (2 ml) to destroy the complex. Protoberberine-type alkaloids was extracted by Soxhlet extraction, ultrasonication or shaking with methanol or methanol–hydrochloric acid mixture (100:1). In this study, methanol was inappropriate as an extraction solvent because it hindered the complex formation of protoberberine-type alkaloids with cobalt thiocyanate. Ultrasonication of the samples with 2% hydrochloric acid for 30 min was enough for the extraction of berberine and palmatine from crude drugs. Protoberberine-type alkaloids formed green protoberberine–cobalt complexes, which were freely soluble in dichloromethane and dichloroethane compared to chloroform, ethyl acetate, and benzene and had a specific absorbance at 625 nm. Therefore, dichloroethane was used as extraction solvent of protoberberine–cobalt complexes because of low volatility compared to dichloromethane. Addition of cobalt thiocyanate reagent and dichloroethane to 2% hydrochloric acid extracts of SB resulted in the selective extraction of protoberberine-type alkaloids as green protoberberine–cobalt complexes into the dichloroethane layer. The aliquot (5 μl) was injected onto HPLC. The HPLC system consisted of a SpectraSystem P4000 pump (Thermo Separation Products, CA), a Rheodyne 7125 injector (Cotati, CA), and a SpectraSystem UV3000 detector with a 6-l microbore cell. Data handling was performed by a PC1000 software program. The analytical column was a Capcell Pak UG 120 column (250 × 2 mm i.d., Shiseido, Tokyo, Japan) equipped with a Capcell Pak UG 120 guard column (10 × 2 mm i.d., Shiseido). The mobile phase was acetonitrile-phosphate buffer (50 mM, pH 4.5) containing sodium octanesulfonate (10 mM) (34:66) and the flow rate was 0.2 ml/min. Detection wavelength was 254 nm and column temperature was 30 °C. For the recovery test, berberin were added to SB, in which their contents had already been determined by HPLC. Limit of quantitation (LOQ) was evaluated at a signal-to-noise ratio of 5:1.

Ion-pair HPLC methods (Misaki et al., 1982) and reversed-phase HPLC (Lee et al., 1999) were reported for the simultaneous determination of protoberberine-type alkaloids. Sodium dodecyl sulfate was used as an ion-pair reagent at a concentration range of 17–50 mM at pH 2.2. In this study, a narrowbore HPLC method was chosen due to higher column efficiency, increased detectability and lower solvent consumption. Capcell Pak UG 120 column, pH-stable octadecyl silica column, was used as the stationary phase. Effects of pH and the concentration of sodium octanesulfonate in mobile phase on capacity factor of
berberine was evaluated to achieve satisfactory resolution; 34% acetonitrile in 0.05 μM phosphate buffer (pH 4.5) containing 10 mM sodium octanesulfonate was found to be the best.

Determination of resveratrol from SB stems. HPLC and column chromatography were used to separate and purify resveratrol from the SB stems, as described below. The HPLC system was from Shimadzu, equipped with a HPLC pump, a variable wave-length monitor, a LC controller, column oven and a solvent conditioner. The system was equipped with UV monitor operating at 254 nm, recorder and a manual injection valve with a 10-ml loop. Two hundred grams of dry mass of SB stems were extracted by 2.0-l methanol, the mixture was centrifuged and the supernatant was washed with light petroleum (b.p. 60–90 °C). The remaining methanol phase was evaporated to form a syrup. The syrup was then dissolved and fractionated in 200 ml EtOAc. Resveratrol existed in the EtOAc phase and EtOAc solution was vacuum evaporated at 40 °C, and 18.93-g residue of EtOAc was obtained. In separation process, the column was filled with the upper organic stationary phase, then the sample solution was injected through the injection valve. The effluent from the outlet of the column was continuously monitored with a UV detector at 254 nm. Peak fractions were separately collected for analysis. A solvent system consisting of chloroform/methanol/water (4:3:2) was used and five fractions were obtained. Each fraction was analyzed by thin-layer chromatography (TLC) with precoated silica G-25 UV254 plates. TLC plates were developed with hexane/EtOAc/formic acid (30:10:0.5), and detected at 254 nm. Fraction 5, a completely separated peak, was supposed to be resveratrol, 546.4 mg dried resveratrol was obtained from the peak.

Identification of the two active chemical ingredients of apigenin and luteolin from the ethanolic extract of SB stems. Target substances were extracted from aerial parts of S. barbata, as basically described in previous reports by Zhang et al. (2003) and Sato et al. (2000). The extracts were analyzed by thin-layer chromatography (TLC) on precoated silica gel 60 F254 plates (Merck, Darmstadt, Germany) with chloroform/methanol, 9:1 (v/v) as the mobile phase. The material was coarsely ground before extraction. A total of 2.0 kg of the material was extracted three times with 50% methanol, 9:1, to afford SB-2 (43 mg). Purity was monitored by TLC and by HPLC on LiChrosorb Si 60 (4.0 mm × 25 cm; Merck) with n-hexane/ethyl acetate, 1:1, as the mobile phase. Detection was performed at 254 nm.

Statistical analysis. The Mann–Whitney U test was used to analyze differences between the individual groups. P < 0.05 was considered to be statistically significant.

Results

Growth arrest by SB treatment

The human uterine LM cell is of confirmed smooth muscle origin, expresses functional estrogen receptors, and has been shown to proliferate in response to exogenous 17β-estradiol in culture. To determine the effects of SB on the growth of human uterine LM cell, cells were grown under culture conditions that maximally or minimally stimulated cell growth (complete DF8 medium) and in medium containing 5–100 μg/ml SB (Table 1). After 72 h of exposure, differences in cell number between all treatment groups and cells grown in complete medium were statistically significant (P < 0.01). Treatment with 100 μg/ml SB in 10% FBS medium resulted in an additional decrease in cell number, inhibiting human uterine LM cell growth to 85% of the control value. Although this process did not occur in cultures treated with SB, cells did appear highly vacuolated within 24 h of SB exposure. Neither of these changes was witnessed in cultures grown in complete medium.

Induction of apoptosis under conditions that arrest cell proliferation

The growth inhibition of human uterine LM cells induced by SB treatment suggested that SB could be modulating the rate of programmed cell death in these cells. To investigate the mechanism of SB effect this system, the proliferative and apoptotic responses of cell to SB were analyzed by flow cytometry and DNA laddering.

The proliferative status and cell cycle distribution of human uterine LM cells grown in complete medium containing 10 to 100 μg/ml SB for 48 h were examined using FACS to measure cell proliferation based on BrdU incorporation and to determine the total DNA content of each cell using PI staining. Quantitation of BrdU incorporation into newly synthesized DNA indicated that media
Cells were grown under the indicated conditions for 48 h prior to harvest, staining, and DNA content determination by flow cytometry. Values represent the percent of total events after gating to remove debris and nonviable cells.

containing SB inhibited the proliferation of human uterine LM cells compared to that of cells grown in complete medium (Table 2). During the 6-h BrdU exposure, only 16% of cells grown in the presence of 20 μg/ml SB had undergone DNA replication. SB, 50 μg/ml, also inhibited proliferation, reducing the number of labeled cells to 15% of the control value. In comparison, 82% of human uterine LM cells were grown in complete medium and only 14% of those exposed to 100 μg/ml SB had incorporated BrdU into newly synthesized DNA, indicating that SB treatment was effective at arresting cell growth. The primary effect of SB treatment was to arrest cells in G0/G1 and prevent entry into S phase (Table 2). Approximately 72% of cells in each of these conditions remained in G0/G1, compared to 50% in complete medium, with a concomitant reduction in S phase from 29% to approximately 17%. SB, 10 μg/ml, also inhibited the growth of cells by limiting the exit of cells from G1, but in addition, produced a subpopulation of cells containing a sub-2N DNA content, indicative of the apoptotic process that made up 25% of the total cell population. Induction of a substantial sub-2N population did not occur when cells were grown in SB. Fewer than 6% of cells treated with the highest dose of SB were determined to be apoptotic by this method.

The presence of a DNA ladder upon electrophoresis of genomic DNA results from the induction of endonuclease activity during apoptosis and has become a hallmark of this process. DNA extracted from human uterine LM cells grown in complete medium or grown in SB-containing medium for 48 h was electrophoresed to detect the presence of oligonucleosome-sized fragments. The DNA of cells grown in complete medium was of high mol wt and displayed little mobility upon electrophoresis (Fig. 1). Cells that were growth arrested by 100 μg/ml SB prominently displayed a DNA ladder. Treatment with 20 and 50 μg/ml SB induced a characteristic DNA ladder (Fig. 1A). Interestingly, LM cells grown with each 10 μM of apigenin and luteolin have clearly shown their DNA fragmentation ladder (Fig. 1A). These observations indicate that human uterine LM cells maintained their ability to undergo apoptosis, and SB treatment up to concentrations of 100 μg/ml inhibited cell growth largely by mechanisms that SB uses an apoptotic response. In a similar fashion, apigenin and luteolin treatments induce their apoptotic responses.

mRNA expression and protein concentration of IGF-I in human myometrium and leiomyomal cells

It was known that Western blot examinations of leiomyomas and normal myometrial tissues from the same individual uterus demonstrated that IGF-I mRNA and protein were abundantly present in the leiomyoma cells, but was scarcely present in normal myometrial smooth muscle cells.

mRNA levels which specifically hybridize with IGF-I mRNA from total RNA of leiomyomal and myometrial cells revealed that IGF-I mRNA was highly expressed in both leiomyomal and normal myometrial tissues from the same individual. IGF-I mRNA expression and protein concentration of IGF-I in human myometrium and leiomyomal cells...
from leiomyoma cells cultured for 72 h under serum-free conditions in the presence of SB showed that the cultured leiomyoma cells contained hybridizable IGF-I mRNAs. In contrast, the addition of SB (40 μg/ml) to the serum-free medium resulted in a somewhat decreased expression of IGF-I mRNA in the cultured leiomyoma cells (P < 0.05). However, treatment with SB did not affect the expression of IGF-I mRNA in cultured normal myometrial cells. IGF-I mRNA values are shown in Fig. 2. The IGF-I mRNA values were significantly higher in leiomyomal cells than in myometrium.

Protein concentration of IGF-I in human myometrium and leiomyomal cells

Similarly, IGF-I proteins were higher in leiomyomal cells than myometrium, as shown in Table 4. Western blot analyses of protein extracts from leiomyomal and myometrial cells revealed that IGF-I protein was abundantly present in leiomyomal cell extracts, whereas in myometrial cell extracts, IGF-I protein amounts were very low (Table 4). Western immunoblotting of proteins extracted from leiomyoma cells cultured for 72 h under serum-free conditions in the absence or presence of SB showed that the cultured leiomyoma cells contained immunoreactive IGF-I protein. In contrast, the addition of SB (40 μg/ml) to the serum-free medium resulted in a somewhat decreased expression of IGF-I protein in the cultured leiomyoma cells relative to that in the leiomyoma cells in control cultures (P < 0.05). Unlike the cultured leiomyoma cells, treatment with SB affected the expression of IGF-I protein in cultured normal myometrial cells (Fig. 3). Although the concentrations of IGF-I were higher in leiomyomal cells than in myometrium, no such difference was seen in the SB-treated. A significant positive correlation between IGF-I mRNA and tissue IGF-I protein was observed in the total cell extracts of leiomyomal cells. No significant correlation was seen for the myometria.

Identification of the three inactive chemical ingredients from SB stems

For SB preparation, the raw materials were extracted. The chemical structures of resveratrol, baicalin, and berberine are shown in Fig. 4. These compounds are flavonoids. The extraction and isolation of each compound from the SB stems was described in Materials and methods, and the purity of each compound was more than 99.5%, by HPLC analysis (data not shown). The effects of resveratrol, baicalin, and berberine on the physiological activity of the above parameters have not been significantly evidenced, indicating that some other different molecules are mixed in the crude extracts of the SB.
Identification of the two active chemical ingredients from SB stems

The diethyl ether extract showed notable anti-proliferative activity against the LM cells. A combination of silica gel (Wako gel C-300, Kiesel gel 60, and LiChrosorb Si60) and reversed-phase (ODS-Q3) column chromatographies of the diethyl ether extract afforded anti-proliferative active compounds (yield from the plant, 0.02% and 0.0010%, respectively). Molecule-1 compound showed a molecular ion peak at m/z 270 in the electron ionization (EI)-mass spectrum. Molecule-2 compound showed a molecular ion peak at m/z 286 in the EI-mass spectrum. By comparing the data from Sato et al. (2000), these compounds were identified as flavone derivatives of apigenin and luteolin (Fig. 5). The anti-proliferative compounds belong to the flavones, a class of flavonoids. In the present study, apigenin and luteolin are characterized as selectively inhibiting the growth of LM cells (Table 5).

Discussion

From natural plants, several polyphenolic compounds showed their biological activities such as anti-inflammatory (Yang et al., 1998) and anticarcinogenic activities (Lin et al., 1998). Medicinal plants have been used as traditional remedies for hundreds of years. Among these herbs, Scutellaria barbata D. Don (SB), which has been traditionally used for hepatitis, inflammation, osteomyelitis, and gynecological diseases in Korea and China, was found to show potent growth inhibitory activity against leiomyomal cells. The water extract from the stems of SB was found to inhibit cell proliferation of leiomyomal SMCs. SB has been used in Chinese and Korean medicine as a remedy for treating inflammation, suppurative dermatitis, allergic diseases and gynecological diseases (Lin et al., 1998). By bioassay-guided separation, emodin, emodin 8-O-glucopyranoside, resveratrol, baicalin, and berberine were isolated as active principles. The plant has been reported to contain a large number of flavonoids, frequently found as glucosides and other constituents, including phenethyl alcohols, sterols, and essential oils and amino acids, although the active principles in SB have not been determined fully. Therefore, the raw materials were extracted and subjected to functional isolation for isolation of the active molecules in the present assay systems. The five flavonoids were isolated and the chemical structures of resveratrol, baicalin, berberine, apigenin, and luteolin are determined. The effects of resveratrol, baicalin, and berberine on the above parameters have not been significantly evidenced, whereas apigenin and luteolin were effective, indicating that these different molecules with different activities are mixed with the crude extracts of the SB. The anti-proliferative compounds belong
to the flavones, a class of flavonoids. These naturally occurring compounds are ubiquitous in vascular plants and possess a wide variety of cellular and biochemical effects (Brandi, 1992; Havsteen, 1983; Pathak et al., 1991). Originally, the molecules have been reported to have the antibacterial effects (Sato et al., 2000). The flavonoids have not been associated with serious toxicity (Fullas et al., 1994) and have already been used in medicine (Havsteen, 1983; Pathak et al., 1991). In the present study, apigenin and luteolin are characterized as selectively inhibiting the growth of LM cells. Our findings suggest that apigenin and the related flavonoids are potentially useful for the development of therapeutic treatments of cancer.

SB treatment of human uterine LM cell inhibited cell growth. Growth inhibition in cultures treated with SB-containing media occurred by blocking cells in G0/G1 of the cell cycle and reducing cell proliferation by greater than 80%. SB treatment induced a substantial apoptotic response in cells from 12 to 72 h after treatment. Human uterine LM cells were shown to undergo a maximum apoptosis after 48 h of SB treatment. Therefore, the ability of SB to modulate the growth dynamics of uterine LM cells occurs by mechanisms involving modulation of cell proliferation and apoptotic cell death. Flow cytometry indicated that SB treatment decreased the percentage of proliferating cells in each of these cultures. This was demonstrated by a decrease in both BrdU incorporation and the number of cells in S phase of the cell cycle. Additionally, the proportion of cells in G0/G1 increased with a concomitant decrease in G2/M. This occurrence indicated that treatment of cells with SB induced a G1 block in the cell cycle. Under all treatment conditions that arrested cell growth, there remained a significant subpopulation of cells with a G2/M DNA content. Whether this is due to the presence of an additional G2 arrest or to the presence of tetraploid cells in the population is not clear. Human uterine LM cells have a bimodal chromosome distribution, thus cells in G1 with a 4N DNA content could contribute to the percentage of cells falling within parameters for G2/M. The growth inhibition in SB-treated cultures (86%) was observed, however, the primary cause of growth arrest due to SB exposure was inhibition of cell proliferation by blocking the exit of cells from the G1 phase of the cell cycle.

The results also revealed a marked increase in the fraction of cells in the G1 phase of the cell cycle after SB treatment. This suggests that the anti-proliferative effect of SB on SMC from human myometrium and leiomyoma is related either to a block of the G1-S transition or to a G1 arrest. The transition from G1 to S is controlled by the concerted actions of protein kinases, the activities of which are modulated by families of regulatory proteins in both a positive (cyclins) and a negative [cyclin-dependent kinase inhibitors (CDI)] manner. A family of CDI plays a major role in the cell cycle machinery (Sherr and Roberts, 1995).

In this study, our analysis of Western blotting showed: (i) a suppression of PCNA after SB treatment that was consistent with the results obtained using the MTT method; and (ii) that SB increased the expressions of α-SMA, calponin h1, and p27. In contrast, no marked change in the expressions of cyclin E and cdk2 was observed in cells treated with SB after 2 days incubation. It is possible that the other G1 cell-cycle-related molecules or an increased activity of cyclin-associated kinase may have participated in the block of the G1-S transition or G1 arrest induced by SB treatment.

Table 5

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sub-2N(%)</th>
<th>G1(%)</th>
<th>S(%)</th>
<th>G2/M(%)</th>
<th>BrdU Inc. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (10% FBS)</td>
<td>1.1 ± 0.1</td>
<td>51.2 ± 3.5</td>
<td>28.5 ± 1.8</td>
<td>21.3 ± 1.1</td>
<td>83.2 ± 5.4</td>
</tr>
<tr>
<td>Resveratrol 5 μM</td>
<td>1.3 ± 0.2</td>
<td>51.9 ± 5.2</td>
<td>27.7 ± 2.5</td>
<td>22.4 ± 2.4</td>
<td>82.4 ± 7.9</td>
</tr>
<tr>
<td>Resveratrol 10 μM</td>
<td>1.3 ± 0.3</td>
<td>52.2 ± 5.3</td>
<td>26.9 ± 3.3</td>
<td>21.9 ± 3.2</td>
<td>84.2 ± 6.4</td>
</tr>
<tr>
<td>Baicalin 5 μM</td>
<td>1.3 ± 0.3</td>
<td>53.2 ± 6.3</td>
<td>26.3 ± 1.3</td>
<td>21.5 ± 2.4</td>
<td>85.2 ± 4.5</td>
</tr>
<tr>
<td>Baicalin 10 μM</td>
<td>1.5 ± 0.2</td>
<td>53.3 ± 4.4</td>
<td>26.7 ± 3.4</td>
<td>22.3 ± 2.5</td>
<td>73.4 ± 4.3</td>
</tr>
<tr>
<td>Berberine 5 μM</td>
<td>1.4 ± 0.2</td>
<td>53.3 ± 5.3</td>
<td>27.4 ± 2.5</td>
<td>23.3 ± 3.2</td>
<td>78.3 ± 6.7</td>
</tr>
<tr>
<td>Berberine 10 μM</td>
<td>1.2 ± 0.2</td>
<td>54.2 ± 5.3</td>
<td>28.2 ± 3.5</td>
<td>22.5 ± 4.3</td>
<td>67.5 ± 5.7</td>
</tr>
<tr>
<td>Apigenin 5 μM</td>
<td>4.5 ± 0.3</td>
<td>69.3 ± 4.6</td>
<td>17.4 ± 2.3</td>
<td>12.1 ± 1.2</td>
<td>16.3 ± 1.3</td>
</tr>
<tr>
<td>Apigenin 10 μM</td>
<td>5.2 ± 0.4</td>
<td>72.2 ± 5.7</td>
<td>16.2 ± 1.5</td>
<td>10.6 ± 3.1</td>
<td>14.3 ± 2.4</td>
</tr>
<tr>
<td>Luteolin 5 μM</td>
<td>4.3 ± 0.4</td>
<td>68.4 ± 6.3</td>
<td>17.2 ± 2.6</td>
<td>11.7 ± 2.3</td>
<td>15.9 ± 2.3</td>
</tr>
<tr>
<td>Luteolin 10 μM</td>
<td>4.9 ± 0.3</td>
<td>72.1 ± 5.2</td>
<td>18.5 ± 2.3</td>
<td>9.5 ± 0.9</td>
<td>15.0 ± 1.6</td>
</tr>
</tbody>
</table>

Cells were grown under the indicated conditions for 48 h prior to harvest, staining, and DNA content determination by flow cytometry. Values represent the percent of total events after gating to remove debris and nonviable cells.
Interestingly, in human uterine LM cells, SB demonstrated an additional growth inhibitory effect (86% of control). Although the mechanisms of growth suppression by SB remain unknown, apoptosis was enhanced by SB, indicating that inhibition of proliferation is the primary response of human uterine LM cells to this antiestrogen as well as to apoptosis. The response of the human uterus to SB treatment appears to be complex and regulated at the level of the individual cell types within the organ. Presently, SB is treated for clinical patients in traditional Korean medicine, however, no systematic study of the effect of SB on the myometrium has been undertaken. In mature intact rats, SB treatment reduces uterine wet weight. The growth of the myometrium during periods of increased estrogen secretion, such as pregnancy, is primarily due to cellular hypertrophy, resulting in an increase in intracellular volume (Hendrickson and Kempson, 1980). Uterine LM growth is similarly stimulated by estrogen and affected by hormonal changes during the menstrual cycle (Friedman et al., 1990); however, in fibroids, this hormone appears to stimulate cell proliferation as well. The treatment of LMs with GnRH analogs reduces tumor volume by reducing cell size. This idea is supported by observations that cellularity, or the number of cells per given area of microscopic specimen, increases after treatment with GnRH agonists (Cohen et al., 1994). Additionally, cell loss has not been observed in microscopic sections of GnRH-treated LMs (Adamson, 1992). However, results from our current studies indicate that transformed myometrial cells are sensitive to the induction of apoptosis upon SB treatment and explain the rapid increase in tumor volume following the termination of therapy. The fact that transformed myometrial cells appear to remain competent for the induction of apoptosis could be instrumental in the development of novel therapeutic techniques.

The present study demonstrates that uterine leiomyomal cells have higher IGF-I mRNA expression and higher cellular concentration of IGF-I protein than the corresponding myometrial cells. Previously, IGF-I mRNA expression has been more highly expressed in fibroids than in myometrium (Van der Ven et al., 1994). Also, there was no difference between the tissues (Giudice et al., 1993). These discrepancies might partly be due to the fact that fibroids are individual tumors where the expression of IGF-I varies considerably between leiomyomal cells from the same patient. In the present study, about 2-fold difference in mRNA expression was observed between leiomyomal cells from the myometrium cells. It has been suggested that the IGF-I mRNA expression in fibroids is influenced by estrogen (Rein et al., 1990). This was supported by significant correlations between serum estradiol and IGF-I mRNA levels in both myometrium and fibroids (Stjernholm et al., 1996). The differences between leiomyomal cells and myometrium were pronounced for cellular concentrations of IGF-I than for IGF-I mRNA. This may partly be explained by the fact that the capacity of membrane preparations to bind IGF-I is higher in leiomyoma cells than in myometrium, which may favor accumulation of IGF-I (Chandra-sekhar et al., 1992). The capacity to bind IGF-I is determined by the concentration of type I IGF receptors (Boehm et al., 1990). The high IGF-I and IGF-I mRNA values observed in both leiomyomatous and myometrial cells is in accordance with previous observations (Englund, 2000; Vollenhoven et al., 1993). The alterations may also be part of more specific responses of the uterus.

In conclusion, we have demonstrated greater abundance of IGF-I mRNA and protein in leiomyomas relative to the normal myometrium of the same individual uterus. IGF-I expression in leiomyoma cells was down-regulated by SB, indicating that SB may participate in inhibition of leiomyoma growth. Data indicate that reduction of proliferation in SB-treated is associated with decreased amounts of IGF-I mRNA and protein. From the raw materials, the five compounds were isolated and the chemical structures of resveratrol, baicalin, berberine, apigenin, and luteolin were determined. Of them, two flavonoids of apigenin and luteolin were inhibitory for the growth of LM cells, suggesting that flavonoids of apigenin and luteolin are potentially useful for the development of therapeutic treatments of cancer. These observations suggest a role for SB in growth inhibition of leiomyomal cells. Taken together, the present study suggests that SB inhibited leiomyoma cell growth mediated by IGF-I in human uterus.

Acknowledgment

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References


