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Preliminary Report

Inhibitory effects of *Scutellaria barbata* D. Don on human uterine leiomyomal smooth muscle cell proliferation through cell cycle analysis

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Abstract

Scutellaria barbata D. Don (SB) is one of the herbs belonging to perennial plants, which is known in traditional Korean medicine as ‘Ban-Ji-Ryun,’ and has been used as an anti-inflammatory and anti-tumor agents against human uterine leiomyoma, mammalian and ovarian cancers. Although the difference between uterine smooth muscle cell (SMC) and leiomyomal SMCs has not been clearly established, the action of SB water extract was investigated using SMCs from normal myometrium and leiomyoma. The proliferation of cultured myometrial and leiomyomal SMC was inhibited by SB treatment. Flow cytometric analysis showed that the population in the G1 phase of the cell cycle increased under SB treatment. Western blotting analysis showed that markers of SMC differentiation such as α-smooth muscle actin (α-SMA), calponin h1 and cyclin-dependent kinase inhibitor p27 were induced by treatment with SB in myometrial and leiomyomal SMCs. In contrast, cell-cycle-related gene products from the G1 phase of the cell cycle, such as cyclin E and cdk2, were not affected. Taken together, these results indicate that SB inhibits the proliferation of myometrial and leiomyomals SMC through the induction of α-SMA, calponin h1 and p27. It is suggested that SB may induce differentiation in uterine SMC and may influence tissue remodeling and reconstruction during physiological and pathophysiological events.

Keywords: *Scutellaria barbata* D. Don; α-Smooth muscle actin; Smooth muscle cell; Myometrium; Leiomyoma; Cell cycle

1. Introduction

Uterine leiomyoma is reported to be most common cause of many health problems among women and is the most common benign smooth muscle cell (SMC) tumor of the myometrium [1]. Majority of patients have multiple leiomyomas, and each leiomyoma is thought to be clonal, arising independently from a single initiated smooth muscle cell [2]. These tumors grow on during the reproductive years, increase in size during pregnancy, and regress after menopause [3,4]. In many instances, the cellular phenotypic features of

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uterine SMC are locally modulated by cytokines or growth factors [5–8]. The growth factors or their receptors are differentially regulated in leiomyomas and endometrium of patients with leiomyoma-related complications [9]. However, the control of SMC proliferation and cellular phenotypic features in the uterus has not been clearly explained.

Scutellaria barbata D. Don (SB) is one of herbs belonging to perennial plants, which is mainly distributed throughout Korea and southern China. This herbal material is known in traditional Korean medicine as 'Ban-Ji-Ryun' and has long been used for inflammation, hepatitis, osteomyelitis, gynecological, lung, and rectal tumors in Korea and China. SB is known to contain a large number of alkaloids and flavones [10], however, the active principles have not been determined fully. By bioassay-guided separation, apigenin, luteolin, emodin, emodin 8-*O*-glucopyranoside, resveratrol, baicalin, berberine and resveratrol were isolated as active principles [unpublished results]. These flavonoid congeners were selectively toxic to *Staphylococcus aureus* strains [11]. It was reported to be capable of enhancing macrophage function in vitro and inhibiting tumor growth in vivo [12]. SB also had anti-mutagenic effects and inhibited the damage to DNA in lymphocytes [13–18]. However, although SB is presently being used as an anti-gynecological tumor agent, the effect of SB on human leiomyomal SMCs remains unknown.

In the present study, we have examined the ability of SB to inhibit the growth of leiomyomal SMCs in vitro. Responses of both normal uterine myometrial and leiomyomal SMCs to SB treatment have been also analyzed. In addition, cell cycle analysis was carried out 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.

2. Materials and methods

2.1. Water extraction of Scutellaria Barbata D. Don

Authentic plant material was purchased from local market and identified at the Oriental Medical Department, Dongguk University, Kyungju, South Korea, Voucher specimen [OM-S37] is present at the Oriental Medical Department, Dongguk University, Kyungju, South 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 dried extract was weighed (yield, 1.5%). The concentration of SB extract was calculated by lyophilizing the water-soluble component that passed through a 0.22-μm membrane bottle filter system (Corning, Costar, NY, USA). The concentration of lipopolysaccharide in SB (600 μg/ml) was below 3.0 pg/ml.

2.2. Determination of baicalin, berberine and resveratrol from SB stems

Baicalin, berberine and resveratrol have been isolated from SB stems (1.0 kg), as described previously [18–20]. The HPLC system, which was obtained from Shimazu, was equipped with an HPLC pump, a variable-wavelength monitor, an LC controller, column oven and a solvent conditioner. Ion-pair HPLC methods [19] and reversed-phase HPLC [20] were reported for the simultaneous determination of protoberberine-type alkaloids. Purity tests of baicalin, resveratrol and baicalein were performed by HPLC equipped with a 280-nm detector and LiChrospher 100 RP-18e column [18]. 546.4 mg of dried resveratrol was obtained from 200 g of material. The purity of all compounds was more than 99.5%.

2.3. Samples and cell culture

Portions of uterine leiomyoma were collected from premenopausal women undergoing a hysterectomy for leiomyoma. None were receiving any type of hormonal or drug therapy. The tissues were used after obtaining written consent from the patients. Endometrial dating of the experimental uteri was carried out by the method of Noyes et al. [21]. The protocol for tissue collection was approved by the Ethical Committee of Dongguk University Oriental Hospital, Kyungju, South Korea. The average age of the patients was 43.0 years (range 28–50), and all patients were in the proliferative phase of the menstrual cycle, as confirmed by the method of Noyes et al. [21]. Cultures were determined to be pure SMC cultures (>98%) by immunostaining for α-SMA (Dako, Glostrup, Denmark), which are markers for

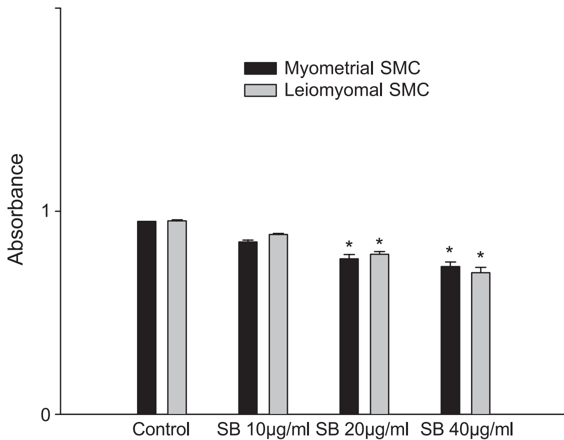


Fig. 1. Effect of SB on proliferation rate of myometrial and leiomyomal SMCs. Absorbance is plotted as a function of SB concentration ($n = 4$). Values are shown as mean ± S.E. Significantly different from control value: *$P < 0.05$.

SMC [22]. Cells used in the experiments were from passages 1 or 2.

2.4. Analysis of cell proliferation

Cell proliferation in cultures of myometrial or leiomyomal tissue was analysed by the 3,(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) colorimetric method [23]. SB was added at a concentration of 10, 20 or 40 μg/ml. The absorbance of the wells was measured using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Molecular Devices, Menlo Park, CA, USA) at a wavelength of 570 nm.

2.5. Cell cycle analysis by flow cytometry

Cultures of secondary cells from normal myometrium or leiomyoma (1.2×10^5 cells) were treated with 10, 20 and 40 μg/ml SB. The cells were analysed with a Fluorescence Activated Cell Sorter (FACS) equipped with an argon laser (488 nm; Becton Dickinson Immunocytometry System, Mountain View, CA, USA). The experiments were repeated three times for each of six cultures.

2.6. Western blotting analysis

Cultured cells were analyzed by Western blotting for α-SMA (Dako), calponin h1 (Sigma) and cell-cycle-related gene products, cyclin E, cdk2 and p27 (Santa Cruz Bio Inc., Santa Cruz, CA, USA). Bound antibody was detected with the aid of an enhanced chemiluminescence (ECL) system (Amersham). The relative expression was densitometrically analyzed using a ImmageAnalyzer Scan System (Core Biosystems, Seoul, South Korea). The intensity generated under

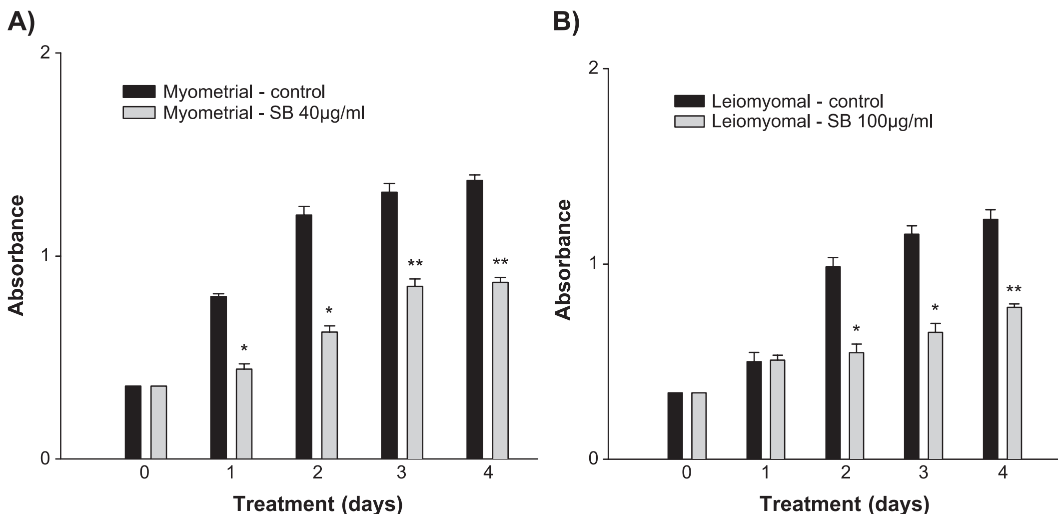


Fig. 2. Effect of SB treatment on the proliferation rate of myometrial and leiomyomal SMCs. (A) Effect of SB treatment at 40 μg/ml on proliferation rate of myometrial SMCs ($n = 4$). (B) Effect of SB treatment at 100 μg/ml on proliferation rate of leiomyomal SMCs ($n = 4$). Values are shown as mean ± SE. Significantly different from control values: *$P < 0.05$ and **$P < 0.01$.

Table 1
Analysis of cell cycle by flow cytometry

% Cell cycle	Myometrial cells		Leiomyomal cells	
	Control	SB	Control	SB
G1	65.2 ± 3.9%	82.3 ± 5.5%	70.3 ± 3.2%	79.3 ± 3.7%
S	13.7 ± 0.9	5.3 ± 0.35	7.7 ± 1.6	12.8 ± 2.6
G2/M	22.0 ± 1.5	13.1 ± 1.2	19.2 ± 0.8	12.1 ± 1.6

Cell cycle analysis was conducted for myometrial and leiomyomal SMCs treated or not treated with SB at 40 μg/ml.

SB treatment is expressed as a percentage of the corresponding untreated control value.

2.7. Statistical analysis

The data are presented as the mean ± S.E. The significance of differences was assessed by the Mann–Whitney *U*-test. Differences were considered to be significant when $P < 0.05$.

3. Results

3.1. Dose-dependent effects of SB on cell proliferation

After 3 days of SB treatment at a concentration of 10, 20 or 40 μg/ml, the MTT assay showed that the number of cultured cells per 96-well plate were reduced by SB in a dose-dependent manner (Fig. 1). The cell numbers indicated significant inhibition of cell proliferation in myometrial cells at the two highest concentrations: to 11% of control with 20 μg/ml SB and to 23% of control with 40 μg/ml SB ($P < 0.05$). The cell numbers also indicated significant inhibition in leiomyomal cells at the highest concentration: to 14% of control with 20 μg/ml SB ($P < 0.05$). There was no significant difference between myometrial and leiomyomal SMCs in terms of the inhibition of cell proliferation.

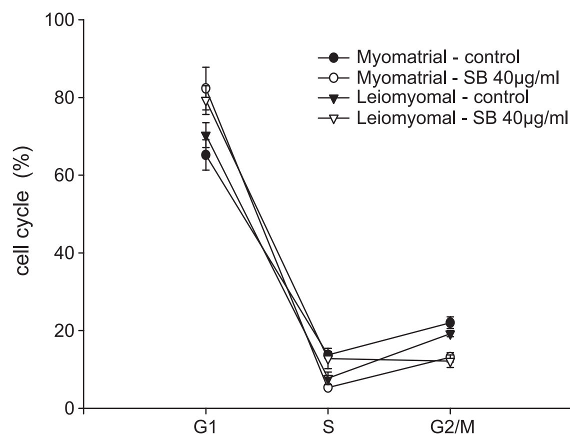
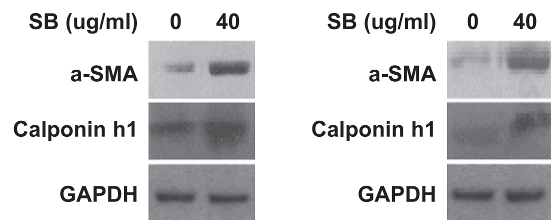


Fig. 3. Analysis of cell cycle by flow cytometry. Cell cycle analysis was conducted for myometrial and leiomyomal SMCs treated with or without SB at 40 μg/ml. Figure shows representative result.

3.2. Reaction time-dependent effects of SB on cell proliferation

Growth curves showed that a significant inhibition of myometrial cell proliferation was first obtained on



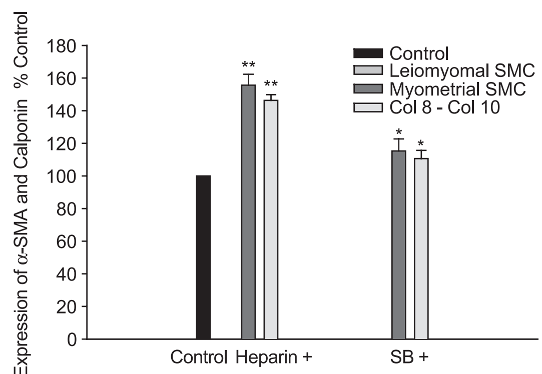


Fig. 4. Analysis of the expression of α-SMA and calponin h1. The 42-kDa band of α-SMA and the 34-kDa band of calponin h1 were increased by SB treatment in both myometrial (A) and leiomyomal (B) SMCs. (C) The relative expression of α-SMA and calponin h1 as estimated by densitometric analysis of the immunoblotting filters. The intensity generated under heparin and SB treatment is expressed as a percentage of the untreated control value. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon, CA, USA) was used as internal control. Values are shown as mean ± S.E. Significantly different from control values: $^{*}P < 0.01$ and $^{**}P < 0.001$.

day 1 ($P < 0.05$; Fig. 2A), whereas a significant inhibition of leiomyomal SMC proliferation was not obtained until day 2 ($P < 0.05$; Fig. 2B). The proliferative activity of myometrial SMC tended to be higher than that of leiomyomal SMC under control conditions. However, the growth curves showed no significant difference between myometrial and leiomyomal SMCs with respect to the inhibition of cell proliferation.

3.3. Regulation of cell cycle by SB

Representative results of the cell cycle analysis performed on myometrial and leiomyomal SMCs are shown in Table 1 and Fig. 3. A significant increase in the fraction of cells in the G1 phase of the cell cycle ($82.3 \pm 5.5\%$; $P < 0.05$) together with significant decreases in the S and G2/M phases (5.4 ± 0.35 and $13.1 \pm 1.2\%$; $P < 0.05$) occurred after SB treatment (by comparison with the corresponding values for cultured myometrial SMC without SB, which were: G1, $65.2 \pm 3.9\%$; S, $13.7 \pm 0.9\%$; G2/M, $22.0 \pm 1.5\%$). Similar results were obtained for leiomyomal SMC. The values obtained were: in the G1 phase, $79.3 \pm 3.7\%$ versus $70.3 \pm 3.2\%$ ($P < 0.05$); in the S phase, $7.7 \pm 1.6\%$ versus $12.8 \pm 2.6\%$ ($P < 0.05$); and in the G2/M phase, $12.1 \pm 1.6\%$ versus $19.2 \pm 0.8\%$ ($P < 0.05$). The results suggest that SB produces a block of the G1-S transition or G1 arrest in both myometrial and leiomyomal SMCs (Fig. 3).

3.4. Western blotting analysis on the expression of α-SMA and calponin h1

Protein extracts from cells treated for 2 days with 40 μg/ml of SB were analysed by Western blotting to detect the expression of α-SMA and calponin h1,

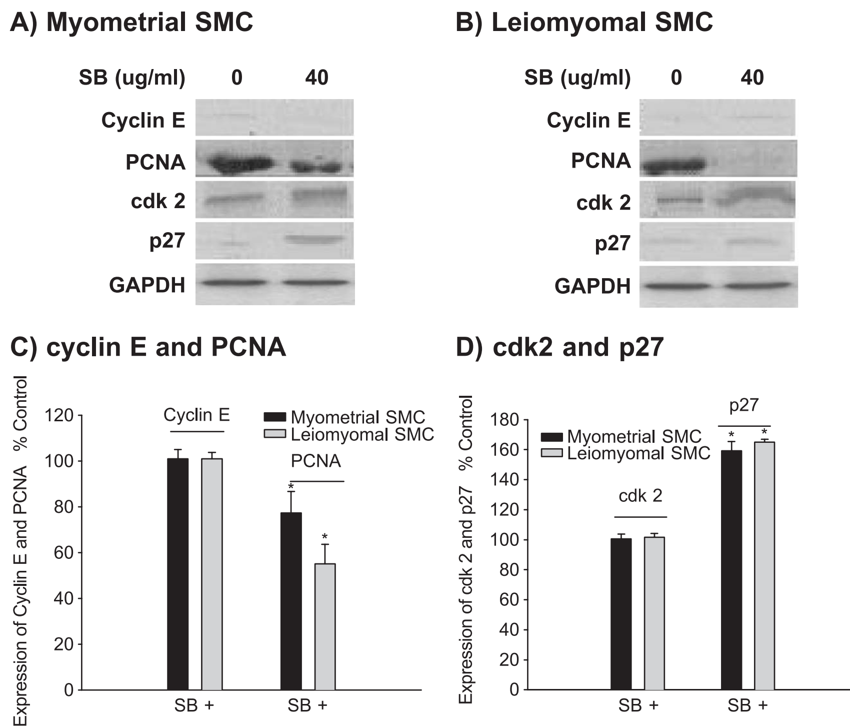


Fig. 5. Analysis of the expression of the G1 phase-related gene products cyclin E, cdk2, PCNA and p27 after SB treatment. (A) The 50-kDa band of cyclin E and the 33-kDa band of cdk2 were not changed by SB treatment in either myometrial or leiomyomal SMCs. (B) In contrast, the 27-kDa band of cdk inhibitor p27 was increased by SB treatment in both myometrial and leiomyomal SMCs. (C) The relative expressions of cyclin E and PCNA. (D) The relative expressions of cdk2 and p27. The relative expressions were estimated by densitometric analysis of the immunoblotting filters. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon) was used as internal control. The intensity generated under SB treatment is expressed as a percentage of the untreated control values. Values are shown as mean ± S.E. Significantly different from control values: *$P < 0.01$.

which are markers of the differentiation of SMC. The results are shown in Fig. 4A and B. The 42-kDa band of α-SMA and the 34-kDa band of calponin h1 were both increased by treatment with SB in myometrial and leiomyomal SMCs. For myometrial SMC, the ratio between the values obtained for the expression of α-SMA with or without SB treatment was 1.59 ($P<0.01$). For leiomyomal SMC, the values obtained for this ratio were 1.48 ($P<0.01$) (Fig. 4C). The corresponding ratios for the expression of calponin h1 were: 1.17 in myometrial SMC ($P<0.05$), 1.17 in leiomyomal SMC ($P<0.01$) (Fig. 4C). As reported for heparin [9], the heparin treatment also increased the α-SMA and calponin h1 amounts both in leiomyomal and myometrial SMCs.

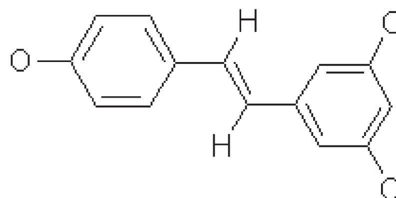
3.5. Western blotting analysis of the expression of the G1 phase-related gene products cyclin E, cdk2, proliferating cell nuclear antigen (PCNA) and p27 after SB treatment

To confirm that SB induced a block of the G1-S transition or G1 arrest in these SMC, we analysed the expression of the G1-phase-related gene products cyclin E, cdk2 and p27 (Fig. 5A, B). Neither the 50-kDa band of cyclin E nor the 33-kDa band of cdk2 was changed by SB treatment in myometrial or leiomyomal SMCs (Fig. 5C,D). In contrast, the 27-kDa band of p27, which is a cdk inhibitor, was increased by treatment with SB in both myometrial and leiomyomal SMCs (Fig. 5A,B). The ratio between the values obtained for the expression of p27 in myometrial SMC with or without SB treatment was 1.67 ($P<0.01$). The values obtained for this ratio in leiomyomal SMC was 1.71 ($P<0.01$) (Fig. 5D). In contrast, suppression of PCNA, which is a cofactor of DNA polymerase, was observed after SB treatment, the ratios being 0.79 for myometrial SMC ($P<0.01$) and 0.51 for leiomyomal SMC ($P<0.01$) (Fig. 5C).

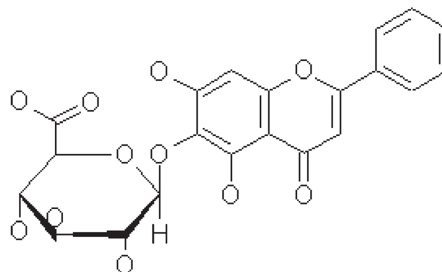
3.6. Identification of the three chemical ingredients from SB stem

For SB preparation, the raw materials were extracted. The chemical structures of resveratrol, baicalin, and berberine are shown in Fig. 6. 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.

A) Resveratrol



B) Baicalin



C) Berberine

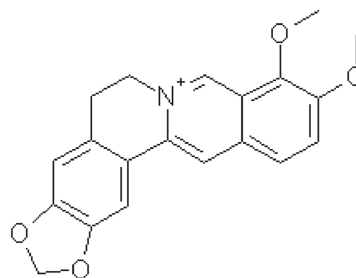


Fig. 6. Structures of resveratrol, baicalin, and berberine.

4. Discussion

From natural plants, several polyphenolic compounds showed their biological activities such as anti-inflammatory [24] and anticarcinogenic activities [25]. Medicinal plants have been used as traditional remedies for hundreds of years. Among these herbs, *S. barbata* D. Don, which has been traditionally used for hepatitis, inflammation, allergic diseases, osteomyelitis

and gynecological diseases in South Korea and China, was found to show potent growth inhibitory activity against leiomyomal SMCs [25]. 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 three flavonoids were isolated and the chemical structures of resveratrol, baicalin, and berberine were determined. The effects of these compounds on the above parameters have not been significantly evidenced, indicating that some other different molecules are mixed in the crude extracts of the SB.

The present results demonstrated that SB inhibits the proliferation of SMCs from the human myometrium and leiomyoma, both effects occurring in dose-dependent manners. With respect to this inhibitory action, there was no significant difference between myometrial and leiomyomal SMCs. However, leiomyomal SMC inhibition began later than that in the normal myometrium. This inhibitory difference between normal myomatrial and leiomyomal SMCs would be explained by the following two reasons: first, the proliferative rate of myometrial SMC tended to be higher than that of leiomyomal SMC under control conditions; second, the leiomyomal SMC were in some way more resistant to SB treatment than myometrial SMC.

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 human myometrium and leiomyoma SMCs 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 [26]. 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. Since α-SMA and calponin h1 are expressed in differentiated SMC, our data suggest that the anti-proliferative effect of SB is closely associated with an up-regulation of p27 and an induced differentiation of SMC.

With respect to the effect of SB on myometrial and leiomyomal SMCs, our results suggest that: (i) SB inhibits SMC proliferation; (ii) SB increases the expression of α-SMA; and (iii) SB most likely acts in the G1 phase of the cell cycle. Thus, this is showing SB inhibition of myometrial and leiomyomal SMC. These results show that the effect of SB is associated with an up-regulation of p27 and an induced differentiation of SMC. Thus, it is suggested that SB may be of therapeutic or preventative value in cases of leiomyoma. SB may potentially be of use to regulate angiogenesis in women with leiomyoma or abnormal bleeding.

In conclusion, we have demonstrated an inhibitory effect of SB on the proliferation of myometrial and leiomyomal SMC. A marked increase in the fraction of cells in the G1 phase of the cell cycle was observed in both cells after SB treatment. This study also showed that SB induced cytoskeletal muscle proteins such as α-SMA, calponin h1 and cell cycle inhibitor p27. These results clearly suggested that SB inhibits the proliferation of uterine SMC and may induce differentiation of SMC, and that the interaction between SMC and SB's constituent molecules may influence tissue remodeling and reconstruction during pathophysiological events.

Acknowledgements

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