

Bufalin induces apoptosis and the G0/G1 cell cycle arrest of endometriotic stromal cells: a promising agent for the treatment of endometriosis

Kaei Nasu¹, Masakazu Nishida, Tami Ueda, Noriyuki Takai, Sun Bing, Hisashi Narahara and Isao Miyakawa

Department of Obstetrics and Gynecology, Oita University, Hasama-machi, Oita, Japan

¹To whom correspondence should be addressed at: Department of Obstetrics and Gynecology, Oita University, Hasama-machi, Oita 879-5593, Japan. E-mail: nasu@med.oita-u.ac.jp

Most of the current medical treatments for endometriosis aim to down-regulate the estrogen activity. However, a high recurrence rate after medical treatments has been the most significant problem. Bufalin is a major digoxin-like immunoreactive component isolated from the skin and parotid venom glands of toad and is considered an apoptosis-inducing agent. To apply bufalin to the medical treatment of endometriosis, we investigated the effects of this agent on the cell proliferation and apoptosis of cultured ovarian endometriotic cyst stromal cells (ECSC) by a modified methylthiazolotetrazolium (MTT) assay, a 5-bromo-2'-deoxyuridine (BrdU) incorporation assay and internucleosomal DNA fragmentation assays. The effect of bufalin on the cell cycle of ECSC was also determined by flow cytometry. The expression of apoptosis- and cell cycle-related molecules was also examined in ECSC using Western blot analysis. Bufalin significantly inhibited the cell proliferation and DNA synthesis of ECSC and induced apoptosis and the G0/G1 phase cell cycle arrest of these cells. The down-regulation of the cyclin A, Bcl-2, and Bcl-X_L expression with the simultaneous up-regulation of the p21 and Bax expression, and caspase-9 activation was observed in ECSC after bufalin treatment. It is suggested that bufalin induces apoptosis of ECSC by simultaneously suppressing anti-apoptotic proteins and inducing pro-apoptotic proteins. Caspase-9-mediated cascade is involved in this mechanism. Therefore, bufalin could be used as a therapeutic agent for the treatment of endometriosis.

Key words: apoptosis/bufalin/caspase-9/cell cycle/endometriosis

Introduction

Endometriosis, a common, benign, estrogen-dependent disease affecting 3–10% of women of reproductive age, is characterized by the ectopic growth of endometrial tissue (Olive and Schwartz, 1993). It is found primarily in the peritoneum, ovary and rectovaginal septum. Women suffering from endometriosis may present with chronic pelvic pain, dysmenorrhea, dyspareunia, and subfertility. The prevalence of endometriosis in women with pelvic pain and/or subfertility is estimated to be between 20 and 90%; thus, it is one of the most frequently encountered benign gynaecological problems (Gazvani and Templeton, 2002).

Various therapies have been used for treating endometriosis, including surgical and medical strategies. Surgical intervention is the treatment of choice, because it has been shown that the ablation of the endometriotic lesions increases the pregnancy rate in infertile women (Marcoux *et al.*, 1997) and reduces pelvic pain in symptomatic patients (Sutton *et al.*, 1997). Medical therapies historically have included contraceptive steroids, progestogens and agonists of GnRH, as well as androgens and non-steroidal anti-inflammatory agents (Lessey, 2000; Rice, 2001; Valle and Sciarra, 2003; Olive *et al.*, 2004; Practice Committee of the American Society for Reproductive Medicine, 2004). Current medical treatment aims to inhibit the growth of endometriotic implants by suppressing ovarian steroids and inducing a hypoestrogenic state (Henzl *et al.*, 1988; Wong and Tang, 2004). Of these medical agents, GnRH agonists have gained predominance in

the medical treatment of endometriosis. GnRH agonists suppress the release of follicle-stimulating hormone and luteinizing hormone from the pituitary, inhibit ovarian steroidogenesis, and result in a hypoestrogenic state that is suitable for the remission of the endometriotic lesions (Bergqvist *et al.*, 1998). However, current treatments for endometriosis that aim to lower circulating estradiol (E₂) concentrations can be used only for a limited time owing to unacceptable side effects. In addition, high recurrence rates after medical treatments are the most significant problem (Bergqvist, 2000). Therefore, novel therapeutic strategies are necessary for the improved clinical management of patients with endometriosis.

Endometriosis is believed to be the result of implantation of retrogradely shed endometrium during menstruation. Endometrium that develops to endometriosis has the capacity to adhere, attach and implant ectopically (Koks *et al.*, 1999; Maas *et al.*, 2001). It has been suggested that, in healthy women, endometrial cells expelled during menstruation do not survive in ectopic locations because of programmed cell death, whereas decreased apoptosis may lead to the ectopic survival and implantation of these cells and the development of endometriosis (Harada *et al.*, 1996; Dmowski *et al.*, 1998; Gebel *et al.*, 1998; Jones *et al.*, 1998; Nishida *et al.*, 2005). Both the inability of endometrial cells to transmit a 'death' signal and the ability of endometrial cells to avoid cell death have been associated with increased expression of anti-apoptotic factors [e.g. B cell lymphoma/leukaemia-2 (Bcl-2) and Bcl-X_L] and the

decreased expression of pre-apoptotic factors (e.g. Bax) (Watanabe *et al.*, 1996; Jones *et al.*, 1998; Nishida *et al.*, 2005). These findings encouraged the authors to evaluate apoptosis-inducing agents for the treatment of endometriosis.

Bufalin is the major digoxin-like immunoreactive component of Chan Su, a traditional Chinese medicine obtained from the skin and parotid venom glands of toad (Krenn and Kopp, 1998). Chan Su is the major component of such popular traditional Chinese medications as Liushenwan (Hong *et al.*, 1992), Shexiangbaixinwan (Song *et al.*, 2000), Lu-Shen-Wan and Kyusin (Hong *et al.*, 1992). These traditional Chinese medications have long been widely applied in China, Japan, Korea, and other Asian countries, and are currently used as alternative medicines (Morishita *et al.*, 1992). Bufalin and other bufadienolides are cardioactive C-24 steroids that exhibit a variety of biological activities, such as cardiotoxic, anaesthetic, blood pressure stimulation, respiration and antineoplastic activities (Krenn and Kopp, 1998). In terms of its anti-tumour activities, bufalin has been demonstrated to inhibit the growth of tumour cells, such as leukaemia (Numazawa *et al.*, 1994; Jing *et al.*, 1994b; Masuda *et al.*, 1995; Watabe *et al.*, 1998; Kawazoe *et al.*, 1999) and prostatic cancer (Yeh *et al.*, 2003) by inducing apoptosis and the cell cycle arrest of these cells.

In this study, we evaluated the effects of bufalin on the proliferation, cell cycle and apoptosis of cultured endometriotic cells using methods that were previously proposed to evaluate novel therapeutic agents for endometriosis (Nishida *et al.*, 2005). We also discuss new therapeutic strategies for the treatment of endometriosis.

Materials and methods

Endometriotic cyst stromal cells and normal endometrial stromal cells isolation procedure and cell culture conditions

Endometriotic cyst stromal cells (ECSC) were obtained from premenopausal patients who had undergone salpingo-oophorectomy or evisceration for ovarian endometriotic cysts ($n = 9$). Normal endometrial stromal cells (NESC) were obtained from premenopausal patients who had undergone hysterectomies for subserous leiomyoma ($n = 7$). All patients had been free of any hormonal treatments before the operation. All specimens were diagnosed as being in the mid- to late-secretory phase and considered as unaffected by the presence of leiomyoma using a standard histological examination of endometrial tissues. This study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Oita University, and written informed consent was obtained from all patients.

ECSC were isolated from ovarian endometriotic tissues by enzymatic digestion, as previously described (Nishida *et al.*, 2004). Briefly, the tissues were minced in Hank's balanced salt solution and digested with 0.5% collagenase (Gibco-BRL, Gaithersburg, MD, USA) in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) at 37°C for 40 min. The dispersed cells were filtered through a 70- μ m nylon mesh to remove the undigested tissue pieces. The filtrated fraction was separated further from the epithelial cell clumps by differential sedimentation at unit gravity as follows. The cells were resuspended in 2 ml of culture medium and layered slowly over 10 ml of the medium in a centrifuge tube. Sealed tubes were placed in an upright position at 37°C in 5% CO₂ in air for 30 min. After sedimentation, the top 8 ml of the medium was collected. Finally, the medium containing stromal cells was filtered through a 40- μ m nylon mesh. Final purification was achieved by allowing stromal cells, which attach rapidly to plates, to adhere selectively to the culture dishes for 30 min at 37°C, followed by the removal of non-adhering epithelial cells. NESC were also isolated from endometrial tissues by enzymatic digestion, as previously described (Nishida *et al.*, 2004). Isolated ECSC were cultured in DMEM supplemented with 100 IU/ml of penicillin (Gibco-BRL), 50 mg/ml of streptomycin (Gibco-BRL) and 10% heat-inactivated fetal bovine serum (FBS) (Gibco-BRL) at 37°C in 5% CO₂ in air.

ECSC and NESC in the monolayer culture after the third passage were >99% pure as analysed by immunocytochemical staining with antibodies to vimentin, CD10, cytokeratin, factor VIII, and the leukocyte common antigen, and were used for the following experiments. Each experiment was performed in triplicate and repeated at least four times.

Assessment of purity of ECSC and NESC by immunocytochemistry

ECSC and NESC were processed for indirect immunofluorescence staining as previously described (Nasu *et al.*, 2003). Subconfluent ECSC and NESC cultured on Lab-tek chamber slides (Nalge Nunc International, Naperville, IL, USA) were processed for immunofluorescence staining with antibodies to vimentin (V9; Dako, Copenhagen, Denmark), CD10 (SS2/36, Dako), cytokeratin (Dako), factor VIII (Dako) and the leukocyte common antigen (2B11+PD7/26, Dako).

Briefly, the chamber slides were washed with phosphate-buffered saline (PBS), fixed in cold methanol for 5 min. The slides were washed three times in PBS for 5 min and incubated for 30 min with 1% bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA) in PBS. Slides were then incubated for 1 h with a primary antibody, followed by rinsing three times in PBS for 5 min. The slides were then incubated with appropriate secondary antibodies conjugated to fluorescein (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), washed three times in PBS for 5 min and mounted with Vectorshield (Vector Laboratories, Burlingame, CA, USA). All incubations and washes were performed at room temperature. Samples were viewed with a Zeiss Axiophot Epi-fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Assessment of cell proliferation and cell viability of ECSC and NESC

Cell proliferation and the cell viability of ECSC and NESC after bufalin treatment were determined in 96-well plates by a modified methylthiazolotetrazolium (MTT) assay using WST-1 (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's protocols. About 5×10^4 cells in DMEM supplemented with 10% FBS was distributed into each well of a 96-well flat-bottomed microplate (Corning, New York, NY, USA) and incubated overnight. The medium was then removed, and the cells were incubated for 48 h with 200 μ l of experimental medium containing various concentrations of bufalin (0.001–10 ng/ml) (Biomol Research Laboratories, Plymouth Meeting, PA, USA). Thereafter, 20 μ l of WST-1 dye was added to each well and further incubated for 4 h. Cell proliferation was evaluated by measuring the absorbance at 540 nm. Data were calculated as the ratio of values obtained for the bufalin-treated cells and those for the untreated controls.

Cell proliferation of ECSC and NESC after bufalin treatment was also determined by 5-bromo-2'-deoxyuridine (BrdU) incorporation using cell proliferation ELISA (Roche Diagnostics GmbH). About 1×10^4 cells in DMEM supplemented with 10% FBS was distributed into each well of a 96-well flat-bottomed microplate and incubated overnight. The medium was then removed, and the cells were incubated for 48 h with 100 μ l of experimental medium containing various concentrations of bufalin (0.001–10 ng/ml). Thereafter, 10 μ l of BrdU (10 mM) was added to each well and further incubated for 2 h. BrdU incorporation was then evaluated according to the manufacturer's protocols. Cell proliferation was evaluated by measuring the absorbance at 450 nm. Data were calculated as the ratio of values obtained for the bufalin-treated cells and those for the untreated controls.

Assessment of internucleosomal DNA fragmentation in ECSC

Internucleosomal DNA fragmentation in ECSC after bufalin treatment was evaluated by a Quick apoptotic DNA ladder detection kit (BioVision Research Products, Mountain View, CA, USA) as previously described (Nishida *et al.*, 2005). About 1×10^7 cells of ECSC were plated on 100-mm culture dishes (Corning) in 10 ml of DMEM supplemented with 10% heat-inactivated FBS and cultured overnight. The supernatant was replaced with fresh culture medium containing recombinant human bufalin (10 ng/ml). Twenty-four hours after stimulation, the DNA was extracted from these cells according to the manufacturer's protocols. DNA fragmentation was analysed by electrophoresis on an agarose gel (1.2%). The DNA bands were visualized by staining with ethidium bromide and photographed under UV light using a transilluminator.

Assessment of bufalin-induced apoptosis in ECSC and NESC

The bufalin-induced apoptosis of ECSC and NESC was quantified by direct determination of nucleosomal DNA fragmentation with a cell death detection ELISA (Roche Diagnostics GmbH) as previously described (Nishida *et al.*, 2005). The assay used specific monoclonal antibodies directed against histones from fragmented DNA, allowing the determination of mono- and oligonucleosomes

in the cytoplasmic fraction of cell lysates. Briefly, 1×10^6 cells were plated on 24-well culture plates (Corning) in 1 ml of culture medium with 10% heat-inactivated FBS and cultured overnight. The supernatant was replaced with fresh culture medium containing various amounts of bufalin (0.001–10 ng/ml). Twenty-four hours after stimulation, the cells were lysed according to the manufacturer's manual, followed by centrifugation ($200 \times g$, 5 min). The mono- and oligonucleosomes contained in the supernatants were determined using an anti-histone-biotin antibody. The concentration of nucleosomes antibody was determined photometrically at a wavelength of 405 nm using 2,2'-azino-di(3-ethylbenzthiazolin-sulphonate) as substrate.

Analysis of cell cycle by flow cytometry

The cell cycle was analysed by flow cytometry after 2 days of culture either with or without bufalin, as previously described (Takai *et al.*, 2004). Briefly, ECSC were cultured at <60% confluence for 2 days with or without the presence of bufalin (10 ng/ml), trypsinized, washed in PBS, fixed in methanol and incubated for 30 min at 4°C in the dark with a solution of 5 µg/ml propidium iodide, 1 mg/ml RNase (Sigma) and 0.1% Nonidet P-40 (Sigma). Flow cytometrical analysis of the cell cycle was performed immediately after staining using the CELLFit program (Becton Dickinson & Co., Lincoln Park, NJ, USA), in which the S-phase was calculated using an RFit model.

Assessment of apoptosis-related proteins and cell cycle-related proteins in ECSC

The expressions of apoptosis-related proteins (Bcl-2, Bcl-X_L, Bax, Fas, Fas ligand, caspase-3, caspase-8 and caspase-9) and cell cycle-related proteins (cyclin A, cyclin B, cyclin D3 and p21) in ECSC were investigated by Western blot analysis. Subconfluent ECSC were cultured on 100-mm dishes for 24 h with or without the presence of bufalin (10 ng/ml). The cells were then washed with PBS and whole cell extracts were prepared by lysing the cells in lysis buffer (50 mM Tris-HCl, 125 mM NaCl, 0.1% Nonidet P-40, 5 mM ethylenediamine tetraacetic acid, 50 mM NaF and 0.1% phenylmethylsulphonyl fluoride). The suspension was centrifuged at $15,000 \times g$ for 15 min at 4°C, and the supernatant was collected. The total protein concentration was quantified using the Coomassie protein assay reagent (Pierce, Rockford, IL, USA). The whole cell protein extract was resolved with sodium dodecyl sulphate-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel under reduced conditions. After transfer to an Immobilon-P transfer membrane (Millipore, Bedford, MA, USA), the protein was stained with Ponceau S (Sigma) to verify uniform loading and transfer. The membranes were blocked with 5% skim milk (Becton Dickinson & Co.) in Tris-buffered saline with Tween 20 (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4) (TBS-T) overnight and subsequently incubated with primary antibodies [Bcl-2, Bcl-X_L, Bax, Fas, Fas ligand, cyclin A, cyclin B, cyclin D3, p21 (BD Biosciences, San Jose, CA, USA), cleaved caspase-3, cleaved caspase-8, cleaved caspase-9 (Cell Signaling, Beverly, MA, USA) and glyceraldehyde-3-phosphatedehydrogenase (GAPDH) (Ambion, Austin, TX, USA)] at an appropriate dilution for 1 h at room temperature. The membrane was washed three times with TBS-T and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Subsequently, the membrane was washed three times with TBS-T and analysed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Chicago, IL, USA).

Statistical analysis

Data were calculated as percentages relative to the untreated controls, presented as the means ± SD, and were appropriately analysed by the Bonferroni/Dunn test and Student's *t*-test with StatView 4.5 (Abacus Concepts, Berkeley, CA, USA). *P* < 0.05 was accepted as statistically significant.

Results

Purity of ECSC and NESC determined by immunocytochemistry

As shown in Figure 1A, most of the cells in ECSC culture were positively stained with anti-vimentin antibody (>99%). Most of the cells in ECSC were also positively stained with anti-CD10 antibody (Figure 1B). However, these vimentin-positive cells were not stained with antibodies for cytokeratin, factor VIII or leukocyte common antigen (data not

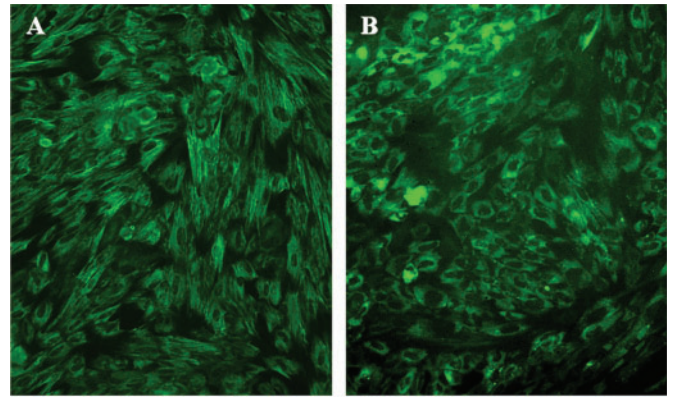


Figure 1. Indirect immunofluorescence staining for (A) vimentin and (B) CD10 in endometriotic cyst stromal cells (ECSC) culture. Most of the cells in ECSC culture were positively stained with antibodies against (A) vimentin and (B) CD10. (original magnification ×400).

shown). The results of immunocytochemical staining in NESC culture were similar to those in ECSC culture.

Effects of bufalin on the cell proliferation and cell viability of ECSC and NESC

The effects of bufalin on the cell proliferation and cell viability of ECSC and NESC were investigated by modified MTT assay. As shown in Figure 2, the number of viable ECSC was significantly decreased by the addition of increasing amounts of bufalin (61.8% decrease at a concentration of 10 ng/ml). Whereas, bufalin showed a marginal inhibitory effect on the cell viability of NESC (13.4% decrease at a concentration of 10 ng/ml). Similar results were obtained in all repeated experiments.

To further assess the effects of bufalin on the cell proliferation, the DNA synthesis of ECSC and NESC after bufalin treatment was evaluated by the BrdU incorporation assay. As shown in Figure 3, bufalin treatment showed significant inhibition of the BrdU incorporation of ECSC in a dose-dependent manner (67.2% decrease at a concentration of 10 ng/ml). Whereas, bufalin showed a weak inhibitory effect on the

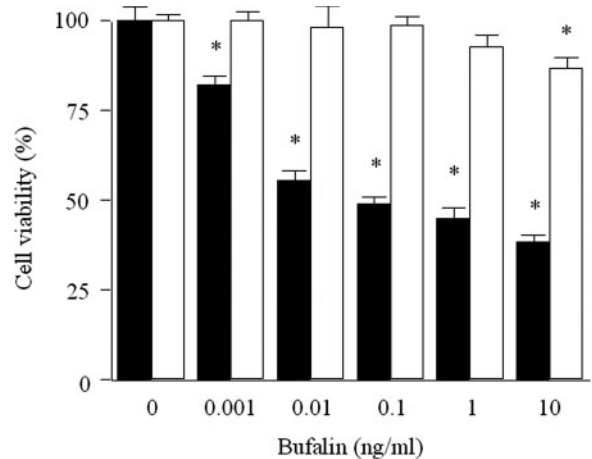


Figure 2. The effects of bufalin on the cell viability of endometriotic cyst stromal cells (ECSC) (closed bars) and normal endometrial stromal cells (NESC) (open bars) as assessed by WST-1 assay. ECSC and NESC were treated with bufalin (0.001–10 ng/ml) for 48 h. The data are presented as the percentages relative to the untreated controls. Representative results are shown. **P* < 0.0001 versus untreated controls (Bonferroni/Dunn test).

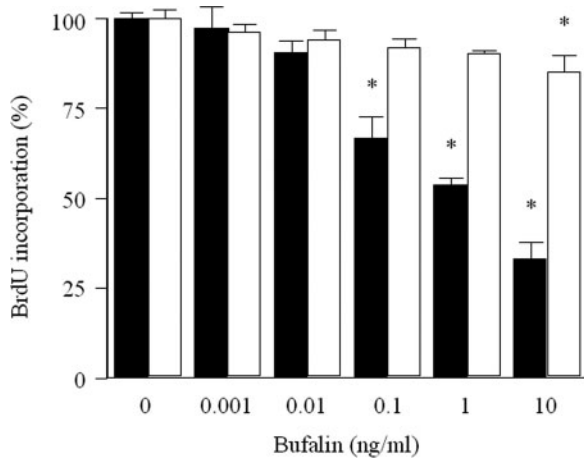


Figure 3. The effects of bufalin on the BrdU incorporation of endometriotic cyst stromal cells (ECSC) (closed bars) and normal endometrial stromal cells (NESC) (open bars). ECSC and NESC were treated with bufalin (0.001–10 ng/ml) for 48 h. The data are presented as the percentages relative to the untreated controls. Representative results are shown. * $P < 0.0001$ versus untreated controls (Bonferroni/Dunn test).

BrdU incorporation of NESC (14.9% decrease at a concentration of 10 ng/ml). Similar results were obtained in all repeated experiments.

Effects of bufalin on apoptosis of ECSC and NESC

The apoptotic effects of bufalin on ECSC and NESC were assessed by evaluating the presence of internucleosomal DNA fragmentation. Bufalin induced the fragmentation of internucleosomal DNA in ECSC (Figures 4 and 5). DNA ladder was detected by electrophoresis in the bufalin-treated ECSC, suggesting the presence of apoptotic cells (Figure 4). The apoptosis of ECSC was significantly induced by the addition of increasing amounts of bufalin (221.0% increase at a concentration of 10 ng/ml) (Figure 5). Whereas, bufalin showed a weak effect on the induction of apoptosis of NESC (63.7% increase at a

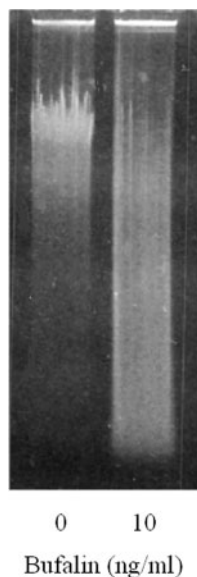


Figure 4. The effects of bufalin on the internucleosomal DNA fragmentation of endometriotic cyst stromal cells (ECSC). DNA was extracted from ECSC cultured for 24 h with or without bufalin (10 ng/ml) and analysed on agarose gel electrophoresis. DNA ladder was detected in the bufalin-treated ECSC, suggesting the presence of apoptotic cells. Representative results are shown.

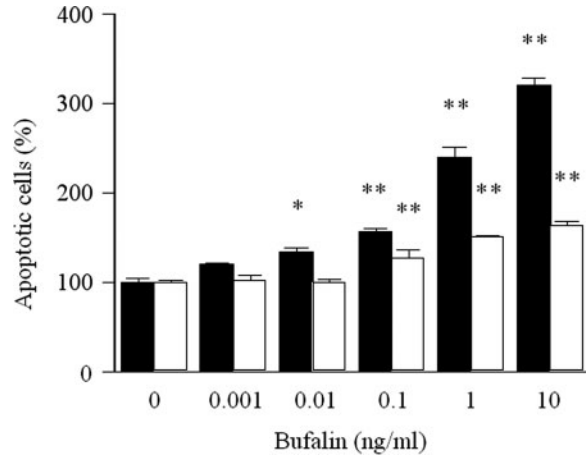


Figure 5. The effects of bufalin on the apoptosis of endometriotic cyst stromal cells (ECSC) (closed bars) and normal endometrial stromal cells (NESC) (open bars) as accessed by cell death detection ELISA. The assay used specific monoclonal antibodies directed against histones from fragmented DNA, allowing the determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. ECSC and NESC were treated with bufalin (0.001–10 ng/ml) for 24 h. The data are presented as the percentages relative to the untreated controls. Representative results are shown. * $P < 0.0005$, ** $P < 0.0001$ versus untreated controls (Bonferroni/Dunn test).

concentration of 10 ng/ml) (Figure 5). Similar results were obtained in all repeated experiments.

Effects of bufalin on the cell cycle of ECSC

The effect of bufalin on the cell cycle of the ECSC was determined by flow cytometry. As shown in Figure 6, ECSC cultured for 2 days in the presence of bufalin (10 ng/ml) showed an accumulation of these cells in the G₀/G₁ phase of the cell cycle, with a concomitant decrease in the proportion of those in the S phase. Similar results were obtained in all repeated experiments.

Effects of bufalin on the expression of apoptosis-related and cell cycle-related proteins in ECSC

To analyse the underlying mechanisms of the above findings, we evaluated the expression of apoptosis-related and cell cycle-related proteins in ECSC. As shown in Figure 7, bufalin down-regulated the expression of cyclin A, Bcl-2, and the Bcl-X_L proteins and up-regulated the expression of the Bax, p21 and cleaved caspase-9 protein in ECSC, whereas the levels of cyclin B, cyclin D3, Fas, Fas ligand, cleaved caspase-3 and cleaved caspase-8 protein in ECSC were unchanged. Similar results were obtained in all repeated experiments.

Discussion

It has been suggested that decreased apoptosis in endometriotic cells plays an essential role in the development of endometriosis (Harada *et al.*, 1996; Dmowski *et al.*, 1998; Gebel *et al.*, 1998; Jones *et al.*, 1998; Nishida *et al.*, 2005). Recently, we have demonstrated that endometriotic cells are resistant to cytokine-induced apoptosis in comparison with eutopic endometrial stromal cells (Nishida *et al.*, 2005). In this study, we demonstrated for the first time that bufalin inhibited the cell proliferation by inducing apoptosis and the G₀/G₁-arrest of the cell cycle of endometriotic stromal cells *in vitro*. Western blot analysis showed the down-regulation of the expression of cyclin A, Bcl-2 and Bcl-X_L, and the simultaneous up-regulation of the p21, Bax and activated caspase-9 expression in endometriotic stromal cells. It is suggested, from these results, that bufalin induces apoptosis

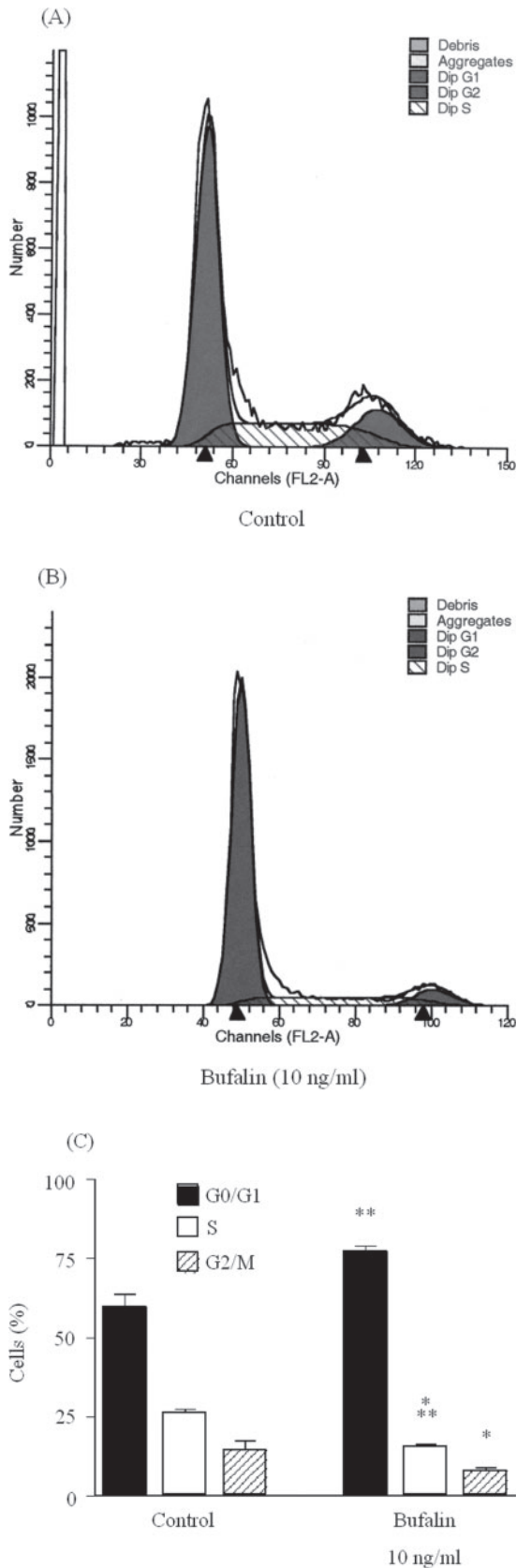


Figure 6. Cell cycle analysis of endometriotic cyst stromal cells (ECSC) by flow cytometry. ECSC were cultured with or without bufalin (10 ng/ml) for 48 h, harvested and stained with propidium iodide. (A) Representative result of untreated controls. (B) Representative result of bufalin-treated cells. (C) Distribution of cells in the G0/G1, S and G2/M phases of the cell cycle. * $P < 0.025$, ** $P < 0.005$, *** $P < 0.0005$ versus controls (Student's *t*-test).

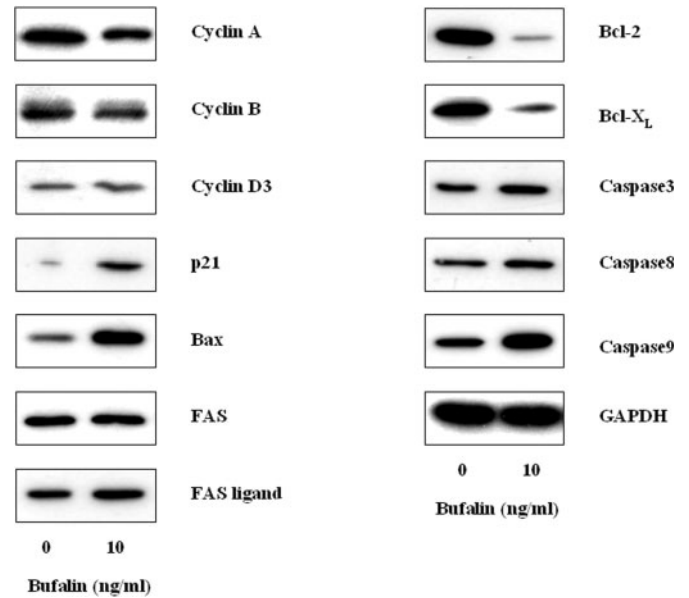


Figure 7. Western blot analysis for the expression of cycle-related proteins (cyclin A, cyclin B, cyclin D3 and p21), apoptosis-related proteins (Bcl-2, Bcl-X_L, Bax, Fas, Fas ligand, cleaved caspase-3, cleaved caspase-8 and cleaved caspase-9) and the glyceraldehyde-3-phosphatedehydrogenase (GAPDH) protein in endometriotic cyst stromal cells (ECSC). ECSC were cultured with or without bufalin (10 ng/ml) for 24 h, and the total protein was extracted and subjected to Western blotting. Bufalin suppressed the expression of cyclin A, Bcl-2, and the Bcl-X_L proteins, and simultaneously induced the expression of the Bax, p21 and cleaved caspase-9 protein in ECSC. Whereas, the levels of cyclin B, cyclin D3, Fas, Fas ligand, cleaved caspase-3 and cleaved caspase-8 protein in ECSC were unchanged. Representative results are shown.

of ECSC by suppressing anti-apoptotic proteins (e.g. Bcl-2 and Bcl-X_L) and inducing pro-apoptotic protein (e.g. Bax). Activation of caspase-9, an initiator caspase closely coupled to pro-apoptotic signals, was observed after bufalin treatment, suggesting that caspase-9-mediated cascade is involved in the mechanism of bufalin-induced apoptosis (Otsuki, 2001). Activation of caspase-3, a downstream effector caspase, was not detected. Interestingly, bufalin showed marginal effects on NESC, suggesting the cell-specific effects of bufalin on ECSC. These results indicated that bufalin could be used as a novel therapeutic agent for the medical treatment and/or prevention of endometriosis.

The mechanisms of bufalin-induced apoptosis have been exclusively examined in human leukaemic cells (Watabe *et al.*, 1998; Kawazoe *et al.*, 1999). It has been demonstrated that bufalin can induce apoptosis of these cells by the activation of AP-1 (Watabe *et al.*, 1998), the c-Jun N-terminal protein kinase (Watabe *et al.*, 1998; Kawazoe *et al.*, 1999), Rac1 (Kawazoe *et al.*, 1999), cdc2 kinase and casein kinase II (Numazawa *et al.*, 1994; Jing *et al.*, 1994b), as well as by the induction of Tiam1 expression (Kawazoe *et al.*, 1999), the induction of the bcl-2 and c-myc expression (Masuda *et al.*, 1995), and by the inhibition of protein kinase A and protein kinase C (Numazawa *et al.*, 1994; Jing *et al.*, 1994b). In contrast to our present results, bufalin has been reported to induce cell cycle arrest in the G2/M phase of leukaemic cells (Numazawa *et al.*, 1994; Jing *et al.*, 1994b). Interestingly, Jing *et al.* (1994a) demonstrated that apoptosis was not induced by bufalin in normal mononuclear and polymorphonuclear cells, suggesting that the effects of bufalin may be cell-type specific. In this study, bufalin significantly induced the apoptosis of ECSC. In contrast, only marginal effects were observed in NESC. Therefore,

further investigations are needed to fully elucidate the apoptosis-inducing mechanism of bufalin on endometriotic cells.

Although endometriosis is considered a benign disorder, endometriotic cells have been shown to exhibit various neoplastic potentials, such as anti-apoptotic, angiogenic, invasive and metastatic abilities (Gaetje *et al.*, 1995; Jimbo *et al.*, 1997; Nishida *et al.*, 2005). Apoptosis-inducing agents specific for tumour cells might be expected to be ideal anti-tumour drugs, because apoptotic cell death does not induce an inflammatory response. Chemotherapeutic agents such as cisplatin (Kaufmann, 1989), paclitaxel (Bhalla *et al.*, 1993), camptothecin (Kaufmann, 1989), VP16 (Kaufmann, 1989), etoposide (Kaufmann, 1989) and all-trans-retinoic acid (Martin *et al.*, 1990) have been shown to induce apoptosis in tumour and normal cells. However, most of these anti-cancer drugs have severe side effects and thus cannot be used in the medical treatment of benign diseases such as endometriosis, especially for the prevention of the disease. The use of bufalin for the treatment and/or prevention of endometriosis may be superior to other apoptosis-inducing anti-cancer drugs on these points. Toxin-induced cell killing or cell apoptosis are processes provoked by a sustained elevation of cytosolic Ca^{2+} (Jones *et al.*, 1989; Trump and Berezsky, 1995). Therefore, manipulations aimed at increasing the concentration of intracellular Ca^{2+} may induce necrosis or apoptosis, or enhance the concentration of intracellular Ca^{2+} . Thus, it is logical to treat neoplasms with cardioactive steroids (Furaya *et al.*, 1984).

Current and standard medical treatments for endometriosis include GnRH agonists, contraceptive steroids, progestogens and androgens (Lessey, 2000; Rice, 2001; Valle and Sciarra, 2003; Olive *et al.*, 2004; Practice Committee of the American Society for Reproductive Medicine, 2004), all of which aim to lower circulating E_2 concentrations. Of these agents, GnRH agonists appear to be the most effective, but they are expensive, and long-term treatment is not possible because of the loss of bone mineral density. Progestogens have the best clinical profile and a good cost-effectiveness balance; however, most studies found that they were not as effective as GnRH agonists. Oral contraceptives are only effective during treatment and have a high relapse rate after therapy is completed. Markedly high recurrence rates of up to 45% after current medical or surgical therapy have been reported (Bergqvist, 2000). Therefore, changes or additions of medications for endometriosis are needed.

The novel medical treatment of endometriosis is an important clinical problem in routine practice. New findings on the genetics, the possible roles of the environment and the immune system, and intrinsic abnormalities in the endometrium of affected women and secreted products of endometriotic lesions have given insight into the pathogenesis of this disorder, and serve as the background for new treatments (Nishida *et al.*, 2004, 2005). These novel therapeutic approaches are likely to be based on many of the molecular targets, including progesterone receptors, aromatase, angiogenic factors, metalloproteinases, cytokines and chemokines, haptoglobin, peroxisome proliferator-activated receptor- γ and antioxidants. New therapeutic agents, including aromatase inhibitors, selective estrogen receptor modulators, antiprogestins, selective progesterone receptor modulators, GnRH antagonists, cyclooxygenase-2 inhibitors and angiostatic agents have been evaluated and found to be useful for the treatment of endometriosis (Chwalisz *et al.*, 2001; Nap *et al.*, 2004; Schroder *et al.*, 2004).

There could be several benefits to using bufalin for endometriosis. Chan Su, a traditional Chinese herbal medicine, has been used for a long time in Asian countries (Hong *et al.*, 1992; Panesar, 1992). Bufalin can be safely used for long periods without severe side effects. At high dosages, however, cardioactive steroids cause cardiac arrhythmia, breathlessness, seizure and coma (Panesar, 1992). The structural similarity between bufadienolides and digoxin account for the toxic

effects. We have demonstrated that endometriotic cells are resistant to cytokine-induced apoptosis (Nishida *et al.*, 2005). Enhanced expression of anti-apoptotic molecules (Bcl-2 and Bcl- X_L) was considered to be involved in this phenomenon. As demonstrated in this study, bufalin can induce apoptosis of endometriotic cells by simultaneously suppressing anti-apoptotic proteins and inducing pro-apoptotic proteins in these cells. Therefore, it is considered that the apoptosis-inducing mechanism of bufalin is suitable for the treatment of endometriosis.

Clinically, bufalin can be applied to the treatment of endometriosis under the following circumstances. First, bufalin may be applicable as a supplementary drug in combination with the current medical treatment GnRH agonist. Although we did not evaluate the synergistic effects of bufalin with other agents, the action mechanism of bufalin as an apoptosis-inducing agent may suggest the advantages of a combination therapy with this drug. Second, because bufalin can be used for long periods without severe side effects, medical treatment with this agent would be a good option for avoiding relapse of the disease after the initial surgical and/or medical therapy. Third, bufalin may also be used to prevent the development of endometriosis for high-risk populations.

In summary, we demonstrated that bufalin could induce apoptosis and the G0/G1 phase cell cycle arrest of ECSC. The down-regulation of the cyclin A, Bcl-2 and Bcl- X_L expression with the simultaneous up-regulation of the p21, Bax and cleaved caspase-9 expression was induced by bufalin treatment. These findings suggest that bufalin may be applicable for the medical treatment of endometriosis. In addition, further studies with other apoptosis-inducing agents on the cell proliferation and apoptosis of endometriotic cells may contribute to the establishment of more sophisticated treatment strategies for endometriosis.

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