SPANDIDOS PUBLICATIONS

Bufalin induces growth inhibition, cell cycle arrest and apoptosis in human endometrial and ovarian cancer cells

NORIYUKI TAKAI, TAMI UEDA, MASAKAZU NISHIDA, KAEI NASU and HISASHI NARAHARA

Department of Obstetrics and Gynecology, Oita University Faculty of Medicine, Oita, Japan

Received December 19, 2007; Accepted February 4, 2008

Abstract. Bufalin is a traditional Chinese medicine and it induces apoptosis in certain human tumor cell lines. We investigated the effect of bufalin on three endometrial cancer cell lines, two ovarian cancer cell lines, and on normal human endometrial epithelial cells. Endometrial and ovarian cancer cells were treated with various concentrations of bufalin, and its effect on cell growth, cell cycle, apoptosis, and related measurements was investigated. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showed that all endometrial and ovarian cancer cell lines were sensitive to the growth-inhibitory effect of bufalin, although normal endometrial epithelial cells were viable after treatment with the same doses of bufalin that induced growth inhibition in endometrial and ovarian cancer cells. Cell cycle analysis indicated that their exposure to bufalin decreased the proportion of cells in the S-phase and increased the proportion in the G0/G1 phases of the cell cycle. Induction of apoptosis was confirmed by annexin V staining of externalized phosphatidylserine and loss of the transmembrane potential of mitochondria. This induction occurred in concert with the altered expression of genes related to cell cycle and apoptosis. These results suggest that bufalin may become a useful adjuvant therapy for endometrial and ovarian cancers with minimal side effects.

Introduction

Bufalin is the major digoxin-like immunoreactive component of Chan Su, a traditional Chinese medicine obtained from the skin and parotid venom glands of the toad (1). Chan Su is the major component of such popular traditional Chinese medications as Liushenwan (2), Shexiangbaoxinwan (3), Lu-Shen-Wan and Kyusin (2). These traditional Chinese medications have long been widely applied in China, Japan, Korea, and other Asian countries, and are currently used as alternative medicines (4). The chemical structure of bufalin is shown in Fig. 1. Bufalin and other bufadienolides are cardioactive C-24 steroids that exhibit a variety of biological activities, such as cardiotonic, anaesthetic, blood pressure stimulation, respiration and antineoplastic activities (1). In terms of its anti-tumor activities, bufalin has been demonstrated to inhibit the growth of tumor cells, such as leukaemia (5-9) and prostatic cancer (10) by inducing apoptosis and the cell cycle arrest of these cells. However, the effect of bufalin on gynecologic cancer cells has not yet been thoroughly described.

This study was designed to define the biological and therapeutic effects of bufalin in treating endometrial and ovarian cancers for the first time. We examined whether this compound was able to mediate inhibition of cell growth, cell cycle arrest, apoptosis, and the expression of genes related to the malignant phenotype in endometrial and ovarian cancer cell lines.

Materials and methods

Cell lines. The Ishikawa human endometrial cancer cell line was kindly provided by Dr Masato Nishida (Tsukuba University, Ibaraki, Japan). The HHUA human endometrial cancer cell line was obtained from Riken (Ibaraki, Japan). The HEC-1B human endometrial cancer cell line and the SK-OV-3 human ovarian cancer cell line were obtained from American Type Culture Collection (Manassas, VA, USA). The OMC-3 human ovarian cancer cell line was kindly provided by Dr Masatsugu Ueda (Osaka Medical College, Osaka, Japan). The Ishikawa cells were maintained as monolayers at 37˚C in 5% CO_2 in air in Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, USA) containing 5% heat-inactivated fetal bovine serum (FBS; Omega, Tarzana, CA, USA). The other four cell lines were maintained as monolayers at 37˚C in 5% CO_2 in air in Roswell Park Memorial Institute (RPMI)-1640 (Gibco) containing 10% heat-inactivated FBS (Omega).

Normal endometrial epithelial cells. Normal endometrial specimens were obtained from ten pre-menopausal patients who had undergone hysterectomies for leiomyoma. All patients had been free of any hormonal treatments before the operation. All of the specimens were diagnosed as being

Correspondence to: Dr Noriyuki Takai, Department of Obstetrics and Gynecology, Oita University Faculty of Medicine, 1-1 Idaigaoka, Hasama-machi, Yufu-shi, Oita 879-5593, Japan
E-mail: takai@med.oita-u.ac.jp

Key words: cell cycle, apoptosis, endometrial cancer, ovarian cancer

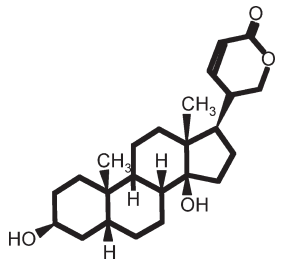

Figure 1. The chemical structure of bufalin.

from the late proliferative phase (11th to 13th day of the menstrual cycle) using a standard histological examination of endometrial tissues. This study was approved by the institutional review board of the Faculty of Medicine, Oita University, and written informed consent was obtained from all patients.

Normal endometrial epithelial cells were separated from stromal cells by digesting the tissue fragments with collagenase. Briefly, the tissue was minced into 2- to 3-mm pieces and incubated with collagenase (200 U/ml; Gibco) in RPMI-1640 (Gibco), and stirred for 2 h at 37˚C. The suspension was then filtered through a 150-μm wire sieve to remove mucus and undigested tissue. The filtrate was then passed through an 80-μm wire sieve, which allowed the stromal cells to pass through while the intact glands were retained. After being washed three times with serum-free RPMI-1640, normal human endometrial epithelial cells were transferred to culture flasks (Corning, New York, NY, USA) at a density of 10^6 cells/ml in RPMI-1640 supplemented with 10% heat-inactivated FBS (Omega), streptomycin (100 U/ml; Gibco), and penicillin (100 U/ml; Gibco). After 16 h, the attached cells, which were >98% pure as analyzed by immunocytochemical staining with antibodies to keratin (Dako, Copenhagen, Denmark), vimentin (V9; Dako), factor VIII (Dako), and leukocyte common antigen (2B11+PD7/26; Dako), were used for the experiments. The cultures were incubated at 37˚C in an atmosphere of 5% CO_2 in air at 100% humidity.

Chemicals. Bufalin was obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Bufalin was dissolved in anhydrous dimethyl sulfoxide (DMSO) at a concentration of 1 mg/ml, for use as a stock solution.

MTT assays. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS; 5 mg/ml) and was used to measure cellular proliferation. Cells ($1x10^3$) were incubated with 100 μl of culture medium for 48 h in 96-well plates, and 10 μl of MTT solution was added. After 4 h of incubation, solubilization solution (50 μl of 20% SDS) was added, and cells were then incubated at 37˚C for 16 h. In this assay, MTT is cleaved to an orange formazan dye by metabolically active cells. The dye was directly quantified using an enzyme-linked immunosorbent assay reader at 540 nm.

All experiments were done independently at least three times in triplicate per experimental point.

Cell cycle analysis by flow cytometry. The cell cycle was analyzed by flow cytometry after 2 days of culturing either with or without bufalin, as described (11,12). Ishikawa cells ($5x10^4$) were exposed to bufalin in 6-well, flat-bottomed plates for 48 h. Total cells, both cells in the suspension and adherent cells, were collected, washed, and suspended in cold PBS. Cells were fixed in chilled 75% methanol and stained with propidium iodine (PI). Analysis was performed immediately after staining using the CellFit program (Becton Dickinson, San Jose, CA, USA), whereby the S-phase was calculated using an RFit model.

All experiments were done independently at least three times in triplicate per experimental point.

Measurement of apoptosis (flow cytometric analysis with the annexin V/propidium iodide assay). An early step in the process of cell death (apoptosis and necrosis) is the redistribution of phosphatidylserine (PS) from the inner leaflet to the outer leaflet of the plasma membrane due to the loss of membrane asymmetry (13). The externalized PS can be readily visualized by incubating intact cells with a fluorescent derivative of the protein annexin V, a phospholipid-binding protein. Propidium iodine (PI), a fluorochrome, is used to label DNA. Unlike necrotic cells, apoptotic cells do not lose their cell membrane integrity and are thus impermeable for dyes such as PI. Therefore, the combination of annexin V and PI staining permits the simultaneous quantification of vital, apoptotic, and necrotic cells.

Cells were plated and grown overnight until they reached 80% confluence, and then treated with bufalin. After 48 h, detached cells in the medium were collected, and the remaining adherent cells were harvested by trypsinization. The cells ($1x10^5$) were washed with PBS and resuspended in 250 μl of binding buffer (AnnexinV-FITC Kit; Becton Dickinson) containing 10 μl of 20 μg/ml PI and 5 μl of annexin V-FITC, which binds to phosphatidylserine translocated to the exterior of the cell membrane early in the apoptosis pathway as well as during necrosis. After incubation for 10 min at room temperature in a light-protected area, the samples were analyzed on a FACScalibur flow cytometer (Becton Dickinson). FITC and PI emissions were detected in the FL-1 and FL-2 channels, respectively. For each sample, data from 30,000 cells were recorded in list mode on logarithmic scales. Subsequent analysis was done with CellQuest software (Becton Dickinson). We were able to discriminate intact cells (annexin$^-$/PI$^-$) from apoptotic cells (annexin$^+$/PI$^-$) and necrotic cells (annexin$^+$/PI$^+$) after treatment with bufalin.

All experiments were done independently at least three times in triplicate per experimental point.

Mitochondrial transmembrane potential. The electron gradient across the mitochondrial membrane space during normal respiration is called the mitochondrial transmembrane potential (MTP). Disruption of the MTP is one of the earliest intracellular events following the induction of apoptosis. The MitoCapture Apoptosis Detection Kit (Biovision Research Products, Palo Alto, CA, USA) provides a simple, fluorescent-based method for distinguishing between healthy and apoptotic cells by detecting the changes in MTP. The kit utilizes MitoCapture, a cationic dye that fluoresces differently in

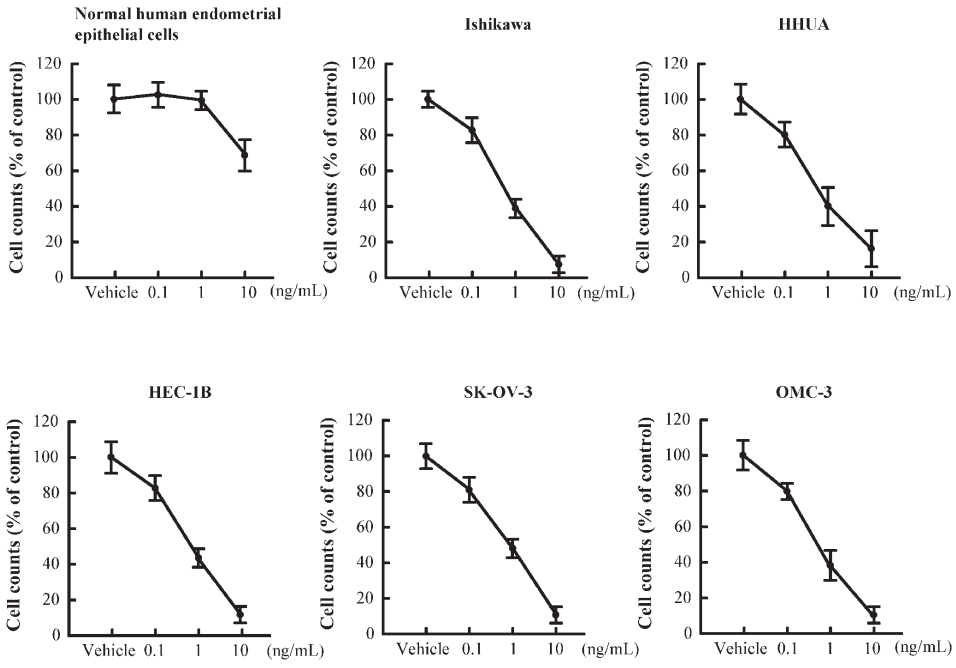

Figure 2. Effect of bufalin on the growth of endometrial and ovarian cancer cell lines, and normal human endometrial epithelial cells (NHEEC) *in vitro*. Ishikawa, HHUA, and HEC-1B endometrial cancer cells, SK-OV-3 and OMC-3 ovarian cancer cells, and normal human endometrial epithelial cells were treated with either bufalin at various concentrations (0.1-10 ng/ml) or the dilutant (control) for 48 h, and growth (% of control) was measured using an MTT assay. Results represent means ± SD of three independent experiments with triplicate dishes.

healthy and apoptotic cells. In healthy cells, MitoCapture accumulates and aggregates in the mitochondria, giving off a bright red fluorescence. In apoptotic cells, MitoCapture cannot aggregate in the mitochondria due to the altered mitochondrial transmembrane potential, and it therefore remains in the cytoplasm in its monomer form, fluorescing green. The fluorescent signals can be easily detected by fluorescence microscopy using a band pass filter (detects FITC and rhodamine) or analyzed by flow cytometry using the FITC channel for green monomers (525 nm) and the PI channel for red aggregates (575 nm).

Cells were prepared for FACS as described above and stained using the Mitocapture Apoptosis Detection kit obtained from Biovision with a fluorescent lipophilic cationic reagent that assesses mitochondrial membrane permeability, according to the manufacturer's recommendation. Briefly, cells were incubated with the MitoCapture reagent at 37˚C in a 5% CO_2 incubator for 15 min. This assay uses an intramitochondrial dye, which forms aggregates in healthy cells, leading to increased 575 fluorescence (PI channel, FL2) and indicating a normal MTP. In apoptotic cells, however, the dye is not included in the mitochondria, leading to a loss of 575 fluorescence (aggregate) and an increase in 525 fluorescence (FITC channel, FL1; monomer). Data were converted to density plots using System 2 software. In some experiments, FACS data were confirmed by fluorescent microscopy using a wide band pass filter. Cells with intact mitochondria exhibited focal red cytosolic fluorescence, whereas cells with permeabilized mitochondria exhibited diffuse green cytosolic fluorescence. Cells lacking red fluorescence and having green fluorescence were scored positive.

All experiments were done independently at least three times in triplicate per experimental point.

Table I. Cell cycle changes mediated by bufalin in endometrial cancer and ovarian cancer cell lines.

	Vehicle	Bufalin (1 ng/ml)
Ishikawa cells		
G0/G1 (%)	50±5	70±11
S (%)	27±7	12±4
G2/M (%)	23±6	18±6
HHUA cells		
G0/G1 (%)	47±5	68±7
S (%)	39±6	24±3
G2/M (%)	14±3	8±3
HEC-1B cells		
G0/G1 (%)	45±8	70±10
S (%)	34±7	19±4
G2/M (%)	21±6	11±3
SK-OV-3 cells		
G0/G1 (%)	50±7	75±11
S (%)	33±6	15±5
G2/M (%)	17±4	10±3
OMC-3 cells		
G0/G1 (%)	49±9	70±8
S (%)	28±6	14±6
G2/M (%)	23±4	16±5

Endometrial and ovarian cancer cells were plated in triplicate wells and grown in the presence or absence (control) of bufalin for 2 days at the indicated concentration, and cell cycle distribution was measured. Means ± SD.

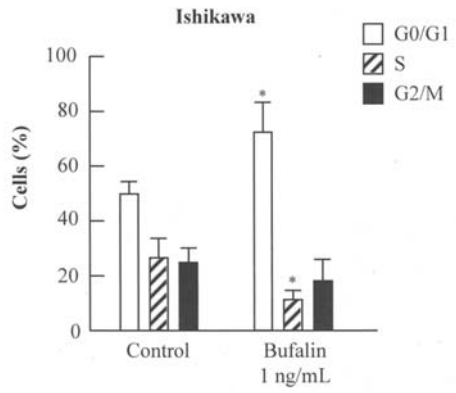

Figure 3. Cell cycle analysis of Ishikawa cells by flow cytometry. Ishikawa cells were cultured with bufalin for 48 h, harvested, and stained with propidium iodine (PI). Control cells were treated with vehicle alone. Cell cycle analysis was performed by flow cytometry (see Materials and methods). Results represent means ± SD of three independent experiments. *p<0.05 vs. the control group (Student's t-test).

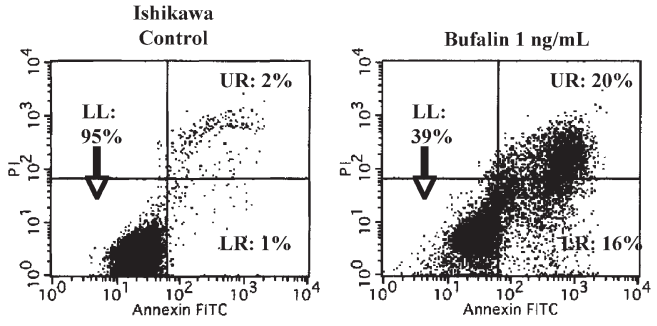

Figure 4. Cell death measured by annexin V and PI staining detected by flow cytometry. Ishikawa cells were treated with 1 ng/ml of bufalin for 48 h. Cells were then stained with annexin V and PI. The positive cells were detected by flow cytometry. The viable cells were negative for both annexin V and PI staining (the lower left quadrant of the cytograms, LL), apoptotic cells were positive for annexin V staining but negative for PI staining (the lower right quadrant, LR), and necrotic cells were positive for both annexin V and PI staining (the upper right quadrant, UR). Each experiment was repeated three times. Two typical flow cytometric results are shown. Histograms for untreated and treated cells at 48 h are shown.

Western blot analysis. Cells were washed twice in PBS, suspended in lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, phenylmethylsulfonyl fluoride at 100 μg/ml, aprotinin at 2 μg/ml, pepstatin at 1 μg/ml, and leupeptin at 10 μg/ml], and placed on ice for 30 min. After centrifugation at 15,000 x g for 15 min at 4˚C, the suspension was collected. Protein concentrations were quantified using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's recommendations. Whole cell lysates (40 μg) were resolved by SDS-PAGE in a 4-15% gel, transferred to a polyvinylidene difluoride membrane (Immobilon; Amersham, Arlington Heights, IL, USA), and probed sequentially with antibodies against $p21^{WAF1}$ (Ab-1, Oncogene, San Diego, CA, USA), cyclin A, cyclin D3, bcl-2, bcl-xL (BD Biosciences, San Jose, CA, USA), cleaved caspase-9 (Cell Signaling, Beverly, MA, USA) and glyceraldehyde-3-phosphatedehydrogenase (GAPDH) (Research Diagnostics, Flanders, NJ, USA). The blots were developed using an enhanced chemiluminescent (ECL) kit (Amersham).

Table II. Cell death measured by annexin V and PI staining detected by flow cytometry in endometrial and ovarian cancer cells.

	Vehicle	Bufalin (1 ng/ml)
Ishikawa cells		
Viable (LL) (%)	95±4	39±11
Apoptosis (LR) (%)	1±1	16±14
Necrosis (UR) (%)	2±1	20±4
HHUA cells		
Viable (LL) (%)	90±2	48±9
Apoptosis (LR) (%)	4±1	37±8
Necrosis (UR) (%)	4±1	9±2
HEC-1B cells		
Viable (LL) (%)	92±2	59±10
Apoptosis (LR) (%)	2±1	22±8
Necrosis (UR) (%)	2±1	10±4
SK-OV-3 cells		
Viable (LL) (%)	94±3	55±9
Apoptosis (LR) (%)	3±1	27±11
Necrosis (UR) (%)	1±1	12±4
OMC-3 cells		
Viable (LL) (%)	92±3	62±8
Apoptosis (LR) (%)	4±1	23±9
Necrosis (UR) (%)	3±1	12±6

For further information, see Fig. 4. Each experiment was repeated three times. Means ± SD.

Statistical analysis. All numerical data were expressed as means ± SD. Significance was determined by conducting a paired Student's t-test.

Results

Effects of bufalin on the proliferation and viability of normal human endometrial epithelial cells, endometrial cancer cell lines, and ovarian cancer cell lines in vitro. We examined the anti-tumor effects of bufalin on the normal human endometrial epithelial cells, three endometrial cancer cell lines, and two ovarian cancer cell lines *in vitro*, using an MTT assay with a 2-day exposure to the bufalin (Fig. 2). Ishikawa endometrial cancer cells, HHUA endometrial cancer cells, HEC-1B endometrial cancer cells, SK-OV-3 ovarian cancer cells and OMC-3 ovarian cancer cells showed significant sensitivity to bufalin with 0.7, 0.5, 0.6, 1.0, and 0.6 ng/ml, respectively, which caused a 50% inhibition (ED50) in their growth. On the other hand, the normal human endometrial epithelial cells showed little sensitivity to bufalin from 0.1 to 10 ng/ml.

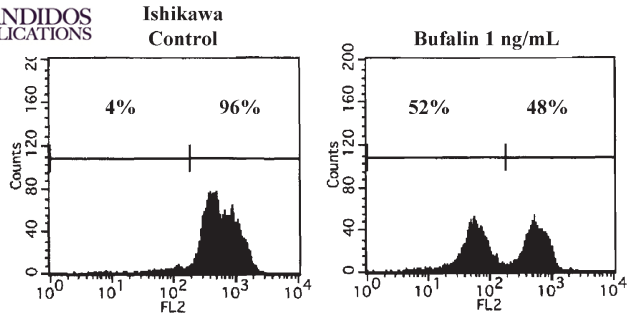

Figure 5. Effect of bufalin treatment on MTP analyzed using the MitoCapture assay. Ishikawa cells were treated with bufalin for 48 h and harvested for flow cytometry. Each histogram profiles the number of cells relative to their fluorescent intensity. In healthy cells, intramitochondrial dye forms aggregates, leading to increased FL2 fluorescence. In apoptotic cells, the dye is not included in the mitochondria, resulting in loss of FL2 fluorescence.

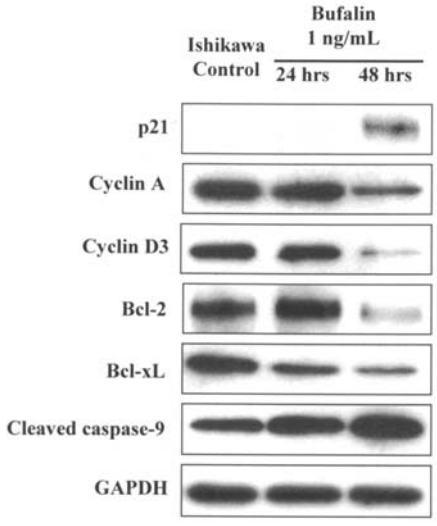

Figure 6. Cell cycle- and apoptosis-related protein expression in Ishikawa cells measured by Western blot analysis. Ishikawa cells were treated with 1 ng/ml bufalin, and cell lysates were harvested after 24 and 48 h. Western blot analysis was performed with a series of antibodies. Control cells were treated with vehicle alone. The amount of protein was normalized by comparison to GAPDH levels.

Table III. Apoptotic cells measured by MTP in endometrial and ovarian cancer cells

	Vehicle	Bufalin (1 ng/ml)
Ishikawa cells		
Viable (%)	96±2	48±8
Apoptosis (%)	4±1	52±6
HHUA cells		
Viable (%)	94±2	37±5
Apoptosis (%)	6±1	63±7
HEC-1B cells		
Viable (%)	97±2	49±6
Apoptosis (%)	3±1	51±9
SK-OV-3 cells		
Viable (%)	95±4	47±6
Apoptosis (%)	5±2	53±9
OMC-3 cells		
Viable (%)	96±3	49±9
Apoptosis (%)	4±1	51±8

Cell cycle analysis of endometrial cancer cells and ovarian cancer cells after exposure to bufalin. Endometrial and ovarian cancer cells cultured for 2 days in the presence of bufalin accumulated in the G0/G1 phase (1 ng/ml of bufalin) of the cell cycle, with a concomitant decrease in the proportion of those in the S phase (Table I). Of the untreated Ishikawa cells, 50±5% (compared with 70±11% of cells cultured with 1 ng/ml bufalin) were in the G0/G1 phase and 27±7% (compared with 12±4% of cells cultured with 1 ng/ml bufalin) were in the S phase (Fig. 3). This was representative of all the cell lines tested (Table I).

Apoptotic changes in bufalin-treated endometrial cancer cells and ovarian cancer cells. To assess the capability of endometrial cancer cells and ovarian cancer cells to undergo apoptosis in response to drug exposure and to help distinguish between different types of cell death, we double-stained bufalin-treated cells with annexin V and PI and analyzed the results using flow cytometry. Annexin V binding combined with PI labeling was performed for the distinction of early apoptotic (annexin V^+/propidium $iodide^-$) and necrotic (annexin V^+/propidium $iodide^+$) cells. At increasing doses of bufalin, we also detected a simultaneous increase in both the annexin V^+/propidium $iodide^-$ fraction (early apoptotic) and annexin V^+/propidium $iodide^+$ (regarded as necrotic) subpopulations (Fig. 4). After incubations with death stimuli (1 ng/ml of bufalin) for 48 h, 16±14% of Ishikawa cells were annexin V^+/propidium $iodide^-$. However, a similar pattern of labeling was detectable in approximately 1% of Ishikawa cells incubated under normal conditions. The percentage of annexin V^+/propidium $iodide^+$ Ishikawa cells was 20±4% in cultures exposed to noxious stimuli (1 ng/ml of bufalin) for 48 h, but 2% in Ishikawa cells incubated under normal conditions. A typical cytogram obtained by flow cytometry and CellQuest software is shown in Fig. 4, and the results (means ± SD in triplicate) of all of the cell lines are shown in Table II.

Loss of MTP in response to bufalin. Loss of MTP has been shown to occur prior to nuclear condensation and caspase activation and is linked to cytochrome *c* release in many, but not all, apoptotic cells (14,15). Using MitoCapture staining and flow cytometry, we analyzed MTP in three endometrial and two ovarian cancer cell lines treated with bufalin. Intracellular fluorescence was assayed by FACS after loading cells with an intramitochondrial dye. High fluorescence at 575 nm (FL2) corresponds to the aggregated form of the dye and is proportional to an intact MTP, whereas loss of MTP leads to a loss of 575 fluorescence and an increase in fluorescence at 525 nm (FL1; monomeric form of the dye). As seen in Fig. 5, untreated cells exhibit high 575 fluorescence, indicating normal MTP. Treatment of cells with bufalin results in loss of 575 fluorescence and an increase in fluorescence at 525, indicating loss of MTP (Fig. 5, Table III).

Effect of bufalin on the expression of cell-cycle and apoptosis-related proteins. $p21^{WAF1}$ is a cyclin-dependent kinase inhibitor (CDKI) that binds to cyclin-dependent kinase complexes and decreases kinase activity, and it may act as a key regulator of G0/G1 accumulation [reviewed in (11,12)]. We examined the effect of bufalin on the expression of $p21^{WAF1}$ in three endometrial and two ovarian cancer cell lines by Western blot analysis (Ishikawa, Fig. 6; HHUA, HEC-1B, SK-OV-3 and OMC-3, data not shown). Bufalin markedly up-regulated the levels of $p21^{WAF1}$ protein, which were expressed at negligible levels in the three untreated endometrial and two untreated ovarian cancer cell lines. Bufalin decreased cyclin A levels by 15%, cyclin D3 levels by 5%, bcl-2 levels by 7% and bcl-xL levels by 10% in the Ishikawa cells. Furthermore, bufalin caused an increase in cleaved caspase-9 expression in the Ishikawa cells (Fig. 6). These results were representative of all the cell lines tested.

Discussion

In this study, we demonstrated for the first time that bufalin inhibited cell proliferation by the G0/G1-arrest of the cell cycle and by inducing the apoptosis of endometrial cancer and ovarian cancer cells *in vitro*. In contrast, bufalin has little effect on normal human endometrial epithelial cells, suggesting that the effects of bufalin might be cell-type specific and could be weaker on the normal endometrium.

Western blot analysis showed the down-regulation of the expression of cyclin A, bcl-2 and bcl-xL, and the simultaneous up-regulation of $p21^{WAF1}$ and activated caspase-9 expression in endometrial cancer and ovarian cancer cells. $p21^{WAF1}$ is a cyclin-dependent kinase inhibitor that has an important role in blocking the cell cycle in the G1 phase (16). Protein levels of both $p21^{WAF1}$ and caspase-9 increased following treatment of endometrial and ovarian cancer cells with bufalin, supporting their contribution as a possible mechanism by which these agents inhibit endometrial and ovarian cancer growth. Cyclins are defined as key proteins in the control of cell proliferation, and cyclin A acts from the late G1 phase through the M phase of the cell cycle, and forms a complex with cdk2 in the late G1-S phase and with cdc2 in the G2/M phase (17). Cyclin A expression is involved in the progression to malignancy of the endometrium and is correlated with proliferative activity and prognostic features (18). Bufalin decreased expression of cyclin A, with this effect modulating the activity of the downstream pRb/E2F axis, thereby triggering cell cycle arrest, especially in the G1 phase at a higher concentration.

We demonstrated that treatment with bufalin dramatically and significantly increased the number of apoptotic cells in endometrial and ovarian cancer cell lines. This effect was associated with a decrease in the levels of the anti-apoptotic protein bcl-2. After bufalin treatment, the apoptotic signal may probably be induced by the down-regulation of bcl-2 expression. 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. Notably, bufalin showed weak effects on the cell proliferation and apoptosis of normal human endometrial epithelial cells in comparison with those of endometrial and ovarian cancer cells, suggesting that bufalin has cell-specific effects on the growth of these cells. These results suggested that bufalin could be usefully investigated as a novel therapeutic agent for the medical treatment of endometrial and ovarian cancer cells with few side effects.

The mechanisms of bufalin-induced apoptosis have been exclusively examined in human leukaemic cells (8,9). It has been demonstrated that bufalin can induce the apoptosis of these cells by the activation of AP-1 (8), the c-Jun N-terminal protein kinase (8,9), Rac1 (9), cdc2 kinase and casein kinase II (5,6), as well as by the induction of Tiam1 expression (9), the induction of bcl-2 and c-myc expression (7), and by the inhibition of protein kinase A and protein kinase C (5,6). In contrast to our present results, bufalin has been reported to induce cell cycle arrest in the G2/M phase of leukaemic cells (5,6). Interestingly, Jing *et al* (19) 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 endometrial cancer and ovarian cancer, although normal endometrial epithelial cells were viable after treatment with the same doses of bufalin that induced growth inhibition in endometrial and ovarian cancer cells.

Toxin-induced cell killing or cell apoptosis are processes provoked by a sustained elevation of cytosolic Ca^{2+} (20,21). 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 (22).

There could be several benefits to using bufalin for cancers. Chan Su, a traditional Chinese herbal medicine, has been used for a long time in Asian countries (2,23). 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 (23). The structural similarity between bufadienolides and digoxin accounts for the toxic effects. As demonstrated in this study, bufalin can induce the apoptosis of endometrial cancer and ovarian cancer cells by simultaneously suppressing anti-apoptotic proteins in these cells. Therefore, it is considered that the apoptosis-inducing mechanism of bufalin is suitable for the treatment of cancers. Clinically, bufalin can be applied to the treatment of endometrial cancer and ovarian cancer under the following circumstances. Firstly, bufalin may be applicable as a supplementary drug in combination with the current anti-cancer drugs. 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. Secondly, 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. Thirdly, bufalin may also be used to prevent the development of endometrial cancer and ovarian cancer for high-risk populations.

In summary, this is the first report showing that bufalin can induce G0/G1-phase cell cycle arrest and the apoptosis of

SPANDIDOS PUBLICATIONS

ial and ovarian cancer cells. The down-regulation of cyclin D3, bcl-2 and bcl-xL expression with the simultaneous up-regulation of $p21^{WAF1}$ and cleaved caspase-9 expression was induced by bufalin treatment. These findings suggest that bufalin may be applicable for the medical treatment of endometrial and ovarian cancers. As the present study involves preliminary *in vitro* experiments, an animal model is necessary to confirm the benefit of this agent for the treatment of these cancers.

Acknowledgements

The study was supported by a Grant-in-Aid (No. 16790961 to NT) for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

References

1. Krenn L and Kopp B: Bufadienolides from animal and plant sources. Phytochemistry 48: 1-29, 1998.
2. Hong Z, Chen K and Yeung HW: Simultaneous determination of bufadienolides in the traditional Chinese medicine preparation, Liu Shen Wan, by liquid chromatography. J Pharm Pharmacol 44: 1023-1026, 1992.
3. Song H, Guo T, Bi K, Wang H and Zhang R: Determination of resibufogenin and cinobufagin in heart-protecting musk pill by HPLC. Biomed Chromatogr 14: 130-132, 2000.
4. Morishita S, Shoji M and Oguni Y: Pharmacological actions of 'Kyusin', a drug containing toad venom: cardiotonic and arrhythmogenic effects. Am J Chin Med 20: 245-246, 1992.
5. Numazawa S, Shinoki M, Ito H, Yoshida T and Kuroiwa Y: Involvement of Na+,K+-ATPase inhibition in K562 cell differentiation induced by bufalin. J Cell Physiol 160: 113-120, 1994.
6. Jing Y, Watabe M, Hashimoto S, Nakajo S and Nakaya K: Cell cycle arrest and protein kinase modulating effect of bufalin on human leukemia ML1 cells. Anticancer Res 14: 1193-1198, 1994.
7. Masuda Y, Kawazoe N, Nakajo S, Yoshida T, Kuroiwa Y and Nakaya K: Bufalin induces apoptosis and influences the expression of apoptosis-related genes in human leukemia cells. Leuk Res 19: 549-556, 1995.
8. Watabe M, Ito K, Masuda Y, Nakajo S and Nakaya K: Activation of AP-1 is required for bufalin-induced apoptosis in human leukemia U937 cells. Oncogene 16: 779-787, 1998.
9. Kawazoe N, Watabe M, Masuda Y, Nakajo S and Nakaya K: Tiam1 is involved in the regulation of bufalin-induced apoptosis in human leukemia cells. Oncogene 18: 2413-2421, 1999.
10. Yeh J-Y, Huang WJ, Kan S-F and Wang PS: Effects of bufalin and cinobufagin on the proliferation of androgen dependent and independent prostate cancer cells. Prostate 54: 112-124, 2003.
11. Takai N, Desmond JC, Kumagai T, Gui D, Said JW, Whittaker S, Miyakawa I and Koeffler HP: Histone deacetylase inhibitors have a profound anti-growth activity in endometrial cancer cells. Clin Cancer Res 10: 1141-1149, 2004.
12. Takai N, Kawamata N, Gui D, Said JW, Miyakawa I and Koeffler HP: Human ovarian carcinoma cells: histone deacetylase inhibitors exhibit antiproliferative activity and potently induce apoptosis. Cancer 101: 2760-2770, 2004.
13. Rimon G, Bazenet CE, Philpott KL and Rubin LL: Increased surface phosphatidylserine is an early marker of neuronal apoptosis. J Neurosci Res 48: 563-570, 1997.
14. Chen Y, Kramer DL, Diegelman P, Vujcic S and Porter CW: Apoptotic signaling in polyamine analogue-treated SK-MEL-28 human melanoma cells. Cancer Res 61: 6437-6444, 2001.
15. Sandberg EM and Sayeski PP: Jak2 tyrosine kinase mediates oxidative stress-induced apoptosis in vascular smooth muscle cells. J Biol Chem 279: 34547-34552, 2004.
16. Johnson DG and Walker CL: Cyclins and cell cycle checkpoints. Annu Rev Pharmacol Toxicol 39: 295-312, 1999.
17. Sherr CJ: G1 phase progression: cyclin on cue. Cell 79: 551-555, 1994.
18. Kyushima N, Watanabe J, Hata H, Jobo T, Kameya T and Kuramoto H: Expression of cyclin A in endometrial adenocarcinoma and its correlation with proliferative activity and clinicopathological variables. J Cancer Res Clin Oncol 128: 307-312, 2002.
19. Jing Y, Ohizumi H, Kawazoe N, Hashimoto S, Masuda Y, Nakajo S, Yoshida T, Kuroiwa Y and Nakaya K: Selective inhibitory effect of bufalin on growth of human tumor cells *in vitro*: association with the induction of apoptosis in leukemia HL-60 cells. Jpn J Cancer Res 85: 645-651, 1994.
20. Jones DP, McConkey DJ, Nicotera P and Orrenius S: Calcium-activated DNA fragmentation in rat liver nuclei. J Biol Chem 264: 6398-3403, 1989.
21. Trump BF and Berezesky IK: Calcium mediated cell injury and cell death. FASEB J 9: 219-228, 1995.
22. Furaya Y, Lundmo P, Short AD, Gill DL and Isaacs JY: The role of calcium, pH, and cell proliferation in the programmed (apoptotic) death of androgen-independent prostatic cancer cells induced by thapsigargin. Cancer Res 44: 3522-3529, 1984.
23. Panesar NS: Bufalin and unidentified substance(s) in traditional Chinese medicine cross-react in commercial digoxin assay. Clin Chem 38: 2155-2156, 1992.