p53, Bcl-2 and cox-2 are involved in berberine hydrochloride-induced apoptosis of HeLa229 cells

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Abstract. The present study aimed to investigate the effects of berberine hydrochloride on the proliferation and apoptosis of HeLa229 human cervical cancer cells. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to examine the cytotoxicity of berberine hydrochloride against HeLa229 cells. The effects of berberine hydrochloride on the apoptosis of HeLa229 cells was detected by immunofluorescence and flow cytometry, and the mRNA expression levels of p53, B-cell lymphoma 2 (Bcl-2) and cyclooxygenase-2 (cox-2) were analyzed by reverse transcription-quantitative polymerase chain reaction. Berberine hydrochloride inhibited the proliferation of HeLa229 cells in a dose-dependent manner; minimum cell viability (3.61%) was detected following treatment with 215.164 μmol/l berberine hydrochloride and the half maximal inhibitory concentration value was 42.93 μmol/l following treatment for 72 h. In addition, berberine hydrochloride induced apoptosis in HeLa229 cells in a dose- and time-dependent manner. Berberine hydrochloride upregulated the mRNA expression levels of p53, and downregulated mRNA expression levels of Bcl-2 and cox-2, in a dose-dependent manner. In conclusion, berberine hydrochloride inhibited the proliferation and induced apoptosis of HeLa229 cells, potentially via the upregulation of p53 and the downregulation of Bcl-2 and cox-2 mRNA expression levels.

Introduction

Cervical cancer is one of the most prevalent female cancers (1), and is responsible for significant morbidity and mortality worldwide (2). A lack of effective treatment programs is a primary reason for this; therefore, novel therapeutic agents are required. Plants are being investigated for their use in chemotherapy, due to their availability, cost and lack of side-effects (3).

Berberine is an isoquinoline alkaloid derived from the Chinese herb Huang Lian (4), which is commonly used for the treatment of gastrointestinal complaints, diarrhea and other conditions. Previous studies have suggested that berberine exerts significant anticancer activities against various cancer cell types, including human breast cancer (1,5), lung cancer (6), colon cancer (7), uterine leiomyoma (8), multiple myeloma (9), osteosarcoma (10), prostate cancer (11,12), cervical cancer (13,14), nasopharyngeal carcinoma (15,16), hepatocellular carcinoma (17-19), gastric carcinoma (20) and murine melanoma (21).

Berberine has been reported to suppress human papilloma virus (HPV) transcription and downstream signaling to induce growth arrest and apoptosis in SiHa and HPV18-positive cervical cancer cells via the modulation of activator protein 1 activity (22). In addition, berberine may reverse epithelial-to-mesenchymal transition, and inhibit metastasis and tumor-induced angiogenesis in SiHa cells (13). In Ca Ski human cervical cancer cells, berberine has been reported to enhance apoptosis via an increase in the ratio of p53 and B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax)/Bcl-2, increased reactive oxygen species and calcium levels, disrupted mitochondrial membrane potential and increased caspase-3 activity, as mediated by GADD153 (23). Although berberine has been demonstrated to possess anticancer activities, the underlying mechanisms by which it exerts these effects remain to be fully elucidated. In addition, the effects of berberine on HeLa229 cells have not been reported. Therefore, the present study aimed to investigate the effects of berberine hydrochloride on cell proliferation, apoptosis and associated gene expression in HeLa229 human cervical cancer cells.

Materials and methods

Materials. HeLa229 human cervical carcinoma cells were obtained from the China Center for Type Culture Collection (Wuhan, China). Berberine hydrochloride was purchased from Xi'an Guanyu Bio-Tech Co., Ltd. (Xi'an, China). Fetal calf serum (FCS) was purchased from Hangzhou Sijiqing

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Key words: berberine hydrochloride, HeLa229 human cervical cancer cells, cell proliferation, apoptosis, gene expression

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Gene	Sequence (5'-3')		Product size (bp)	Reference
	Forward	Reverse		
β-actin	GTACCCTGGCATTGCCGACA	GGACTCGTCATACTCCTGCTTGCT	181	26
p53	GCCCACTTCACCGTACTAA	TGGTTTCAAGGCCAGATGT	153	25
Bcl-2	GGGAGGATTGTGGCCTTCTT	TCATCCACAGGGCGATGTT	99	27
Cox-2	CACCCATGTCAAAACCGAGG	CCGGTGTTGAGCAGTTTTCTC	103	28

Bcl-2, B-cell lymphoma 2; Cox-2, cyclooxygenase-2.

Biological Engineering Materials Co., Ltd. (Hangzhou, China). Trypsin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Amresco, LLC (Cleveland, OH, USA). Penicillin, streptomycin and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). The Annexin V-Fluorescein Isothiocyanate (FITC)/Propidium Iodide (PI) Apoptosis Detection kit was from BioVision, Inc. (Milpitas, CA, USA). All other chemicals and solvents used were of the highest purity grade available.

Cell culture and treatment. Cells were cultured in RPMI-1640 medium, supplemented with 10% heat-inactivated FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2.

MTT assay. Cells in the exponential growth phase were harvested, adjusted to $2x10^4$ cells/ml and seeded in 96-well plates (200 μl/well). Following a 24-h incubation at 37˚C, the medium was removed and berberine hydrochloride was added to wells in a final concentration range of 3.362-215.168 μmol/l. The plate was incubated for a further 72 h, following which 20 μl 5 mg/ml MTT reagent was added to wells. Subsequent to a 4-h incubation at 37˚C, formazan crystals formed by live cells were dissolved with 150 μl DMSO and absorbance was measured at 490 nm using a microplate reader (DG5033A; Nanjing Huadong Electronics Group Medical Equipment Co., Ltd., Nanjing, China). Viability was determined using the following formula: % of growth = (optical density of treated cells/optical density of untreated cells) x 100. The half maximal inhibitory concentration (IC_{50}) values were calculated as the concentration of drug required to inhibit 50% proliferation compared with untreated cells.

Detection of apoptosis-microscopy. Experiments were conducted as described previously (24,25), using an Annexin V-FITC/PI Apoptosis Detection kit. Cells at a density of $1.5x10^5$ cells/ml were incubated with 26.896 or 107.584 μmol/l berberine hydrochloride at 37˚C for 48 h. Adherent and floating cells were harvested, washed twice with PBS and suspended in 500 μl of 1X Binding Buffer. Annexin V-FITC (5 μl) and 10 μl PI were added and cells were vortexed and incubated for 5 min in the dark. Cells were visualized immediately using an inverted fluorescence biological microscope XD-101 (Nanjing Jiangnan Photovoltaic Group Co., Ltd., Nanjing, China).

Detection of apoptosis-flow cytometry. Cells at a density of $1.5x10^5$ cells/ml were incubated with 42.93 or 107.584 μmol/l berberine hydrochloride at 37˚C for 24, 48 and 72 h. Apoptosis was detected using the Annexin V-FITC/PI Apoptosis Detection kit, as aforementioned. Cells were analyzed immediately by flow cytometry using an FC 500 (Beckman Coulter, Inc., Brea, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cells ($1.5x10^5$ cells/ml) were incubated with 21.465, 42.93 or 107.584 μmol/l berberine hydrochloride for 48 h. Total RNA was prepared using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol and was reverse transcribed using RevertAid™ Moloney Murine Leukemia Virus Reverse Transcriptase and oligo (dT) primers (Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). qPCR was performed on the resulting cDNA using an ABI 7900HT Fast Real Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and SYBR® Green Real Time PCR Master mix (Toyobo Co., Ltd., Osaka, Japan). The reaction mixture volume was 25 μl, including 11.2 μl PCR water, 2.5 μl SYBR® Green Real Time PCR Master mix, 0.5 μl forward primer (10 μM), 0.5 μl reverse primer (10 μM) and 0.3 μl cDNA. Primers were synthesized by Shanghai Generay Biotech Co., Ltd. (Shanghai, China), and sequences are presented in Table I. The cycling conditions were as follows: An initial denaturation step at 94˚C for 7 min, followed by 35 cycles of denaturation at 94˚C for 30 sec, annealing at 63˚C for 30 sec and extension at 72˚C for 20 sec. Results were analyzed to determine the PCR cycle number that generated the first fluorescence signal over a threshold [quantification cycle (Cq), 10 standard deviations (SDs) over the mean fluorescence generated during the baseline cycles], following which the ΔΔCq method was used to measure relative gene expression (29). Expression of the analyzed genes were normalized to the endogenous reference gene, β-actin.

Statistical analysis. All experiments were performed in triplicate. Data are expressed as the mean ± SD. Data were analyzed in SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA), using one-way analysis of variance followed by the least significant difference test to compare treatment and control groups. $P<0.05$ was considered to indicate a statistically significant difference.

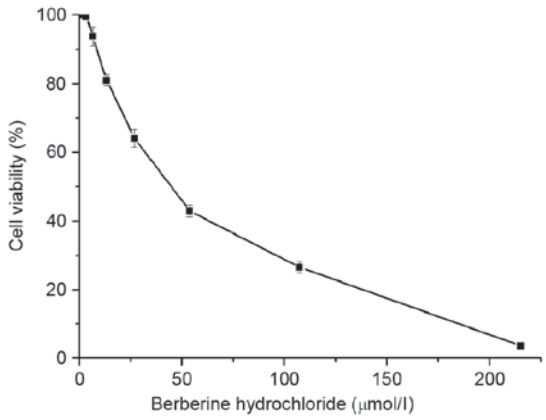

Figure 1. Cell viability of HeLa229 cells following incubation with various concentrations of berberine hydrochloride for 72 h. Berberine hydrochloride decreased HeLa229 cell viability in a dose-dependent manner. Data are presented as the mean ± standard deviation of three individual experiments.

Results

Berberine hydrochloride reduces cell viability. The effects of berberine hydrochloride on the viability of HeLa229 human cervical carcinoma cells were evaluated using an MTT assay (Fig. 1). The IC_{50} for HeLa229 cells at 72 h was 42.93 μmol/l. Berberine hydrochloride inhibited HeLa229 cells in a dose-dependent manner. Cell viability following treatment with 3.362, 6.724, 53.791 and 215.164 μmol/l berberine hydrochloride treatment was 99.56, 93.61, 42.85 and 3.61%, respectively. The results demonstrated that HeLa229 cell viability was reduced following a 72-h incubation with berberine hydrochloride.

Berberine hydrochloride induces apoptosis of HeLa229 cells. Apoptosis of HeLa229 cells was detected using the Annexin V-FITC/Propidium Iodide Apoptosis Detection kit. As presented in Fig. 2, apoptotic cells could be observed clearly by fluorescence microscopy. The cell membranes of early and late apoptotic cells were FITC-positive (green), whereas late apoptotic cells additionally had PI-positive (red) nuclei accompanied by condensed chromatin and apoptotic bodies. Increased numbers of late apoptotic cells were observed following treatment with 107.584 μmol/l, compared with 26.896 μmol/l berberine hydrochloride.

As presented in Fig. 3, no significant differences were observed in the proportions of early apoptotic cells following treatment with 42.93 or 107.584 μmol/l berberine hydrochloride for 24 or 48 h, compared with untreated control cells; or in the proportions of late apoptotic cells following treatment with 42.93 μmol/l berberine hydrochloride for 24 h, compared with control cells. Significant early apoptosis was observed following treatment with 42.93 or 107.584 μmol/l berberine hydrochloride for 72 h, at 2.37 and 8.37% of cells, respectively (all P<0.001). The percentage of late apoptotic cells treated with 42.93 μmol/l berberine hydrochloride for 48 h was greater than for cells treated with 107.584 μmol/l berberine hydrochloride; however, this was reversed at 72 h, at 16.43 and 37%, respectively. The percentage of total apoptotic cells increased markedly from 7.3% in the 42.93 μmol/l treatment group at 48 h to 45.37% in the 107.584 μmol/l treatment group for 72 h. Significant differences were observed in the proportions of intact cells (non-apoptotic live cells) at 24, 48 and 72 h between the three groups (all P<0.001). In addition, compared with control groups, significant differences were detected in the proportions of early and late apoptotic cells at 72 h [all P<0.001, except early apoptotic cells of the 42.93 μmol/l treatment group (P=0.007)], and in the percentage of total apoptotic cells at 24 (42.93 μmol/l, P=0.001; 107.584 μmol/l, P<0.001), 48 and 72 h (all P<0.001). Compared with the 42.93 μmol/l berberine hydrochloride treatment group, the 107.584 μmol/l berberine hydrochloride treatment group revealed significant

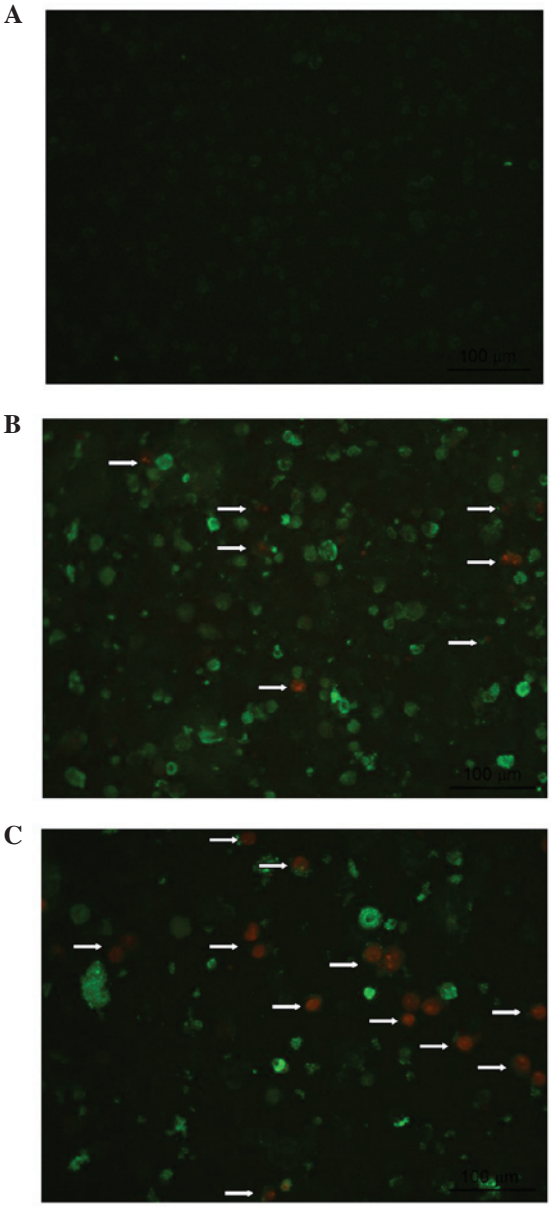

Figure 2. Apoptosis was induced in HeLa229 cells following treatment with berberine hydrochloride for 48 h. (A) Untreated control, (B) 26.896 μmol/l berberine hydrochloride-treated and (C) 107.584 μmol/l berberine hydrochloride-treated cells were stained with Annexin V-fluorescein isothiocyanate (green) and propidium iodide (red) to detect early and late apoptotic cells. The cell membranes of early and late apoptotic cells were stained green; whereas late apoptotic cells had additionally red-stained nuclei accompanied by condensed chromatin and apoptotic bodies. Magnification, x200. Arrows indicate late apoptotic cells or condensed chromatin or apoptotic bodies.

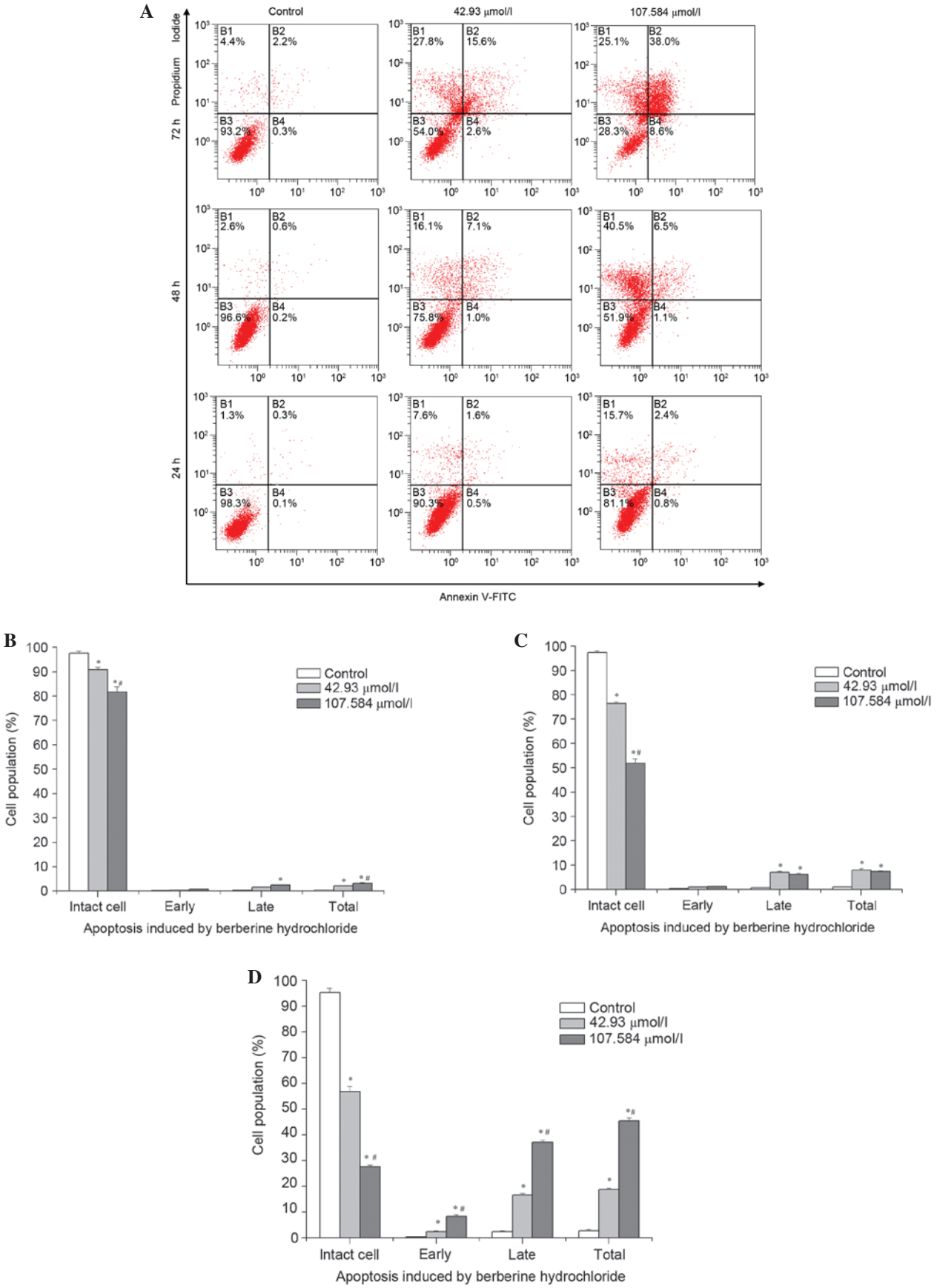

Figure 3. Apoptosis of HeLa229 cells treated with 42.93 or 107.584 μmol/l berberine hydrochloride for 24, 48 or 72 h. (A) Flow cytometry was performed on cells stained with Annexin V-FITC and PI. The intact (live) cells are negative for Annexin V-FITC and PI (lower left quadrant). Early apoptotic cells are defined as Annexin V-FITC-positive and PI-negative (lower right quadrant) and late apoptotic cells are defined as Annexin V-FITC-positive and PI-positive (upper right quadrant). The proportion of intact, and early, late, and total apoptotic cells was determined at (B) 24, (C) 48 and (D) 72 h. Berberine hydrochloride induced apoptosis of HeLa229 cells in a dose- and time-dependent manner. Data are presented as the mean ± standard deviation of three determinations. *P<0.05 vs. control; $^{\#}$P<0.05 vs. 42.93 μmol/l berberine hydrochloride. FITC, fluorescein isothiocyanate; PI, propidium iodide.

differences (P<0.001) in the percentage of intact cells at all time points and early and late apoptotic cells at 72 h, and in the percentage of total apoptotic cells at 24 h (P=0.014) and 72 h (P<0.001). These results suggest that berberine hydrochloride induced apoptosis of HeLa229 cells in a dose- and time-dependent manner.

mRNA expression levels in berberine hydrochloride-treated cells. p53, Bcl-2 and cox-2 mRNA expression levels in HeLa229 cells were assessed by RT-qPCR, following treatment with 21.465, 42.93 or 107.584 μmol/l berberine hydrochloride for 48 h (Fig. 4). Berberine hydrochloride upregulated mRNA expression levels of p53, whereas mRNA expression levels of Bcl-2 and cox-2 were downregulated in a dose-dependent manner. mRNA expression levels of p53 increased from 1.287- to 2.57-fold relative to control, whereas mRNA expression levels of cox-2 decreased from 0.856-to 0.545-fold, and Bcl-2 decreased from 0.962- to 0.775-fold. Significant differences were observed in p53 mRNA expression levels between treated (21.465 μmol/l, P=0.025; 42.93 μmol/l, P<0.001; 107.584 μmol/l, P<0.001) and untreated control cells, and in cox-2 mRNA expression levels between cells treated with 42.93 (P=0.039) or 107.584 (P=0.002) μmol/l berberine hydrochloride and control cells.

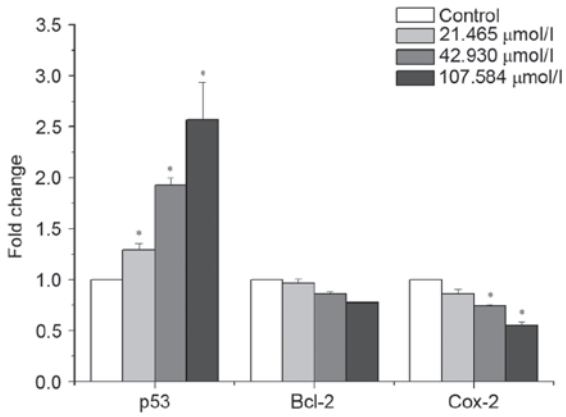

Figure 4. Berberine hydrochloride alters mRNA expression levels in HeLa229 cells. Cells were stimulated with 21.465, 42.93 or 107.584 μmol/l berberine hydrochloride for 48 h, and RNA was extracted for reverse transcription-quantitative polymerase chain reaction analysis of p53, Bcl-2 and cox-2. Berberine hydrochloride upregulated mRNA expression levels of p53, whereas mRNA expression levels of Bcl-2 and cox-2 were downregulated in a dose-dependent manner. Each sample was run in triplicate, and the relative amount of mRNA was normalized to β-actin. Fold-changes compared with control are presented. Data are presented as the mean ± standard deviation of three determinations. *P<0.05 vs. control. Bcl-2, B-cell lymphoma 2; cox-2, cyclooxygenase-2.

Discussion

Berberine is a naturally-occurring isoquinoline alkaloid, which exerts antitumor effects on numerous cancer types (5,30-38) and is non-toxic to normal cells (22). However, the effects of berberine on the HeLa229 human cervical carcinoma cell line remain unclear.

The results of the present study suggested that treatment with berberine hydrochloride for 72 h significantly decreased the viability of HeLa229 cells. Annexin V and PI staining demonstrated that berberine hydrochloride treatment resulted in apoptosis of HeLa229 cells. Apoptosis is tightly regulated by anti- and proapoptotic effector molecules (39) and is caused by the activation of caspases. Two separate pathways (extrinsic and intrinsic) of caspase activation have been described (40). p53 is a critical regulator of apoptosis (41), initiating the intrinsic pathway via the transcriptional activation of Bcl-2 family members (42). The Bcl-2 family consists of three major groups, which differ in regions of Bcl-2 homology (BH domains) and function: Multidomain anti-apoptotic (including Bcl-2), multidomain proapoptotic and BH3-only proapoptotic (43). Berberine hydrochloride may upregulate the expression levels of p53, triggering the intrinsic pathway of apoptosis via downregulation of Bcl-2 expression levels. This would result in release of cytochrome *c* in the mitochondrial membrane and activation of caspase-9, resulting in apoptosis (44). Cox-2 is a target for anticancer therapy (45), which is involved in the extrinsic pathway. Its expression increases as cells become cancerous (46), and it is associated with the stimulation of angiogenesis, and tumor growth, invasion and metastasis (47-49). In the present study, treatment with berberine hydrochloride increased the expression of p53 and decreased the expression of Bcl-2 and cox-2, in a dose-dependent manner. These results are consistent with a previous study, which demonstrated that berberine induced apoptosis via a significant decrease in the Bcl-2/Bax ratio, and the upregulation of Fas, Fas ligand, tumor necrosis factor (TNF)-α, TNF receptor-associated factor 1 and p53 in HeLa cells (14).

In conclusion, the results of the present study suggested that berberine hydrochloride may exhibit significant cytotoxicity against HeLa229 cells. At the lowest concentration assessed (3.362 μmol/l), the inhibition of HeLa229 cells by berberine hydrochloride was <1% (0.44%); however, inhibition increased to >96% (96.39%) at the maximum concentration examined (215.168 μmol/l). Berberine hydrochloride induced typical characteristics of apoptosis in HeLa229 cells, including nuclear condensation, nuclear fragmentation and the formation of apoptotic bodies. In addition, 42.93 and 107.584 μmol/l berberine hydrochloride induced apoptosis in a time-dependent manner. Berberine hydrochloride induced apoptosis in HeLa229 cells via the activation of the extrinsic and intrinsic pathways, involving the upregulation of p53 mRNA expression levels and the downregulation of Bcl-2 and cox-2 mRNA expression levels. Therefore, berberine appears to be a potential therapeutic agent for the treatment of cervical cancer.

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