Mechanisms involved in the cytotoxic effects of berberine on human colon cancer HCT-8 cells

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ABSTRACT: Berberine, a constituent of some traditional Chinese medicinal plants, has been reported to have cytotoxicity effects on different human cancer cell lines. There is no available information about the effects and mechanism of action of berberine on human colon cancer cell line HCT-8. In this paper, the cytotoxicity of berberine on HCT-8 cancer cells was investigated by MTT assay, fluorescence microscopy and flow cytometry analysis. Our data revealed that berberine could significantly inhibit the growth of HCT-8 cells in a dose- and time-dependent manner. Morphology of apoptotic cells was studied with acridine orange/ethidium bromide staining. The concentrations of lactate dehydrogenase and both acid and alkaline phosphatases were significantly increased in cell supernatants after berberine treatment, suggesting cell death. Furthermore, flow cytometry analysis showed that berberine could arrest HCT-8 cells at S phase in a time-dependent manner. To further investigate the apoptotic molecular mechanism, reverse transcription-polymerase chain reaction (RT-PCR) and western blotting methods were used. The up-regulated mRNA and/or protein expressions of Fas, Fasl, TNF-α, caspase-3 and down-regulation of pro-caspase-3 suggest that the death receptor pathway may be involved in the apoptotic pathway induced by berberine. Decrease of Bcl-2 and increase of Bax in mRNA and/or protein expressions showed that the Bcl-2 family proteins were involved in berberine-induced apoptosis. We also found that berberine-induced apoptosis was associated with an up-regulated expressions of p53 and prohibitin (PHB), and decreased vimentin expression. These results suggest that berberine can suppress cell growth and reduce cell survival by arresting the cell-cycle and by inducing apoptosis of HCT-8 cells.

Introduction

Berberine is an isoquinoline alkaloid (Fig. 1A) derived from the roots, rhizome and stem bark of a number of medicinal plants including Berberis vulgaris (barberry), Berberis aquifolium (Oregon grape), and B. aristata (tree turmeric) (Tang et al., 2009; Hu et al., 2008). Antineoplastic (Hano, 1959), antiviral (Kyoko et al., 2007), antibacterial (Amin et al., 1969) and anti-inflammatory effects (Kuo et al., 2004b) of berberine have been reported. Also, this drug has been used in the treatment of intestinal infections, such as acute gastroenteritis, cholera and bacillary dysentery (Tai et al., 1981; Takase et al., 1993), as well as hypertension and arrhythmia (Huang et al., 1989; Lau et al., 2001).

Studies on drugs from plant origin have being increasing because some of them show high effectiveness
and low toxicity in cancer treatment (Panichakul et al., 2006; Prasanna et al., 2009). Numerous cancer cell lines, from either human or murine origin, have been used to examine the effects of berberine, and it has been shown effective at relatively low doses on a number of cancers arising from leucocytes, liver, lung, stomach, colon, skin, brain, bone and breast, were sensitive to berberine (Cordero et al., 2004; Iizuka et al., 2000; Peng et al., 2006; Choi et al., 2008). Berberine can induce cell-cycle arrest (Eom et al., 2008; Mantena et al., 2006a, b) and cytotoxicity mainly through caspase-3 or Fas/FasL signaling pathways (Choi et al., 2008; Mantena et al., 2006a; Hsu et al., 2007). Other researches indicated that berberine elevated the ratio of p53 and Bax/Bcl-2 proteins and increased levels of reactive oxygen species and Ca$^{2+}$ release from the endoplasmic reticulum (Meeran et al., 2008), decrease in mitochondria membrane potential and/or release of cytochrome c (Lin et al., 2007). Tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and the TNF-$\alpha$ induced cellular incasion with targeted NF-$\kappa$B signaling pathway were also involved in berberine-induced apoptosis (Kang et al., 2005).

The aim of the present paper was to investigate the mechanisms involved in the cytotoxicity of berberine on human colon cancer HCT-8 cells.

Materials and Methods

Chemicals

Berberine (>98% purity) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Hydroxycamptothecin (>98% purity) was purchased from Shenzhen Main Luck Pharmaceuticals Inc., China. A Complete Medium composed of Dulbecco’s Modified Eagle’s Medium containing 10% (v/v) fetal calf serum (FCS) was provided by GIBICO, Inc. Some other reagents including SDS and 3-(4, 5-dimethyl-thiazol-zyl)-2, 5-diphenyltetrazolium bromide (MTT) were provided by Sigma, St. Louis, MO, USA.

Cell culture

Human colon cancer HCT-8 cells (obtained from the College of Pharmacy of Dalian Medical University) were routinely cultured at 37°C in Complete Medium, 100 U/mL of penicillin G, and 100 μg/mL of streptomycin. Cells were cultured in 95% O$_2$+5% CO$_2$ and 100% relative humidity. Cell passages were performed every 4-5 days and the cells were refed every 2 days. In all experiments, HCT-8 cells were cultured in 96-well, 24-well or 6-well plates, and they were allowed to adhere and grow for 24 h in culture medium prior to exposure to berberine.

MTT assay and fluorescence microscopy assay

The MTT assay was carried out as described by Mosmann (1983) with minor modifications. The cultured HCT-8 cells were detached by trypsinization, centrifuged at 1000 rpm for 10 min and resuspended in fresh Complete Medium at a density of 10$^4$ cells/mL. Then, 100 μL of the cells were seede onto 96-well flat bottom plates. After incubation for 24 h, solutions of berberine were added to achieve final concentrations of 0.03, 0.06, 0.12, 0.24 or 0.47 mmol/L. Hydroxycamptothecin (final concentration 0.24 mmol/L) was chosen as a positive control. After being incubated with berberine or hydroxycamptothecin for 12, 24, 48, and 72 h, the relative cell viability was measured by scanning with an ELISA Micro-plate Reader with a 570 nm filter and the inhibition rates of berberine or hydroxycamptothecin on HCT-8 cells were calculated.

Cell morphology of hydroxycamptothecin or berberine-induced apoptosis was investigated by staining the cells with a combination of the fluorescent DNA-binding dyes acridine orange (100 μg/mL in phosphate buffer-saline, PBS) and ethidium bromide (100 μg/mL in PBS). Cells were harvested and washed with PBS after being exposed to berberine (0.47 mmol/L) or hydroxycamptothecin (0.24 mmol/L) for 0, 24, 48 and 72 h, and then stained with 100 μg/mL of the fluorescent dyes for 5 min. Then cells were observed under a fluorescence microscope (Olympus) according to Pitrak et al. (1996).

Flow cytometry analysis

Flow cytometry was used to quantitatively detect the apoptotic rate (White et al., 1990). After being treated with 0.47 mmol/L berberine for different times (0, 12, 24, 48 and 72 h), cells were harvested, washed twice with PBS and centrifuged at 1000 rpm for 5 min. The cells were fixed in 70% ethanol at 4°C, and stained with 50 mg/mL propidium iodide for 30 min in a dark room. They were then washed with PBS and analyzed with an EPICS XL Flow Cytometry System with Medical MCYCLE software (Coulter, USA).
**Determination of lactate dehydrogenase, acid phosphatases, alkaline phosphatases and TNF-α**

Supernatants of berberine treated cells (0.47 mmol/L) were collected after 0, 12, 24, 48 and 72 h incubation for the determination of lactate dehydrogenase (LDH), acid phosphatases (ACP) and alkaline phosphatases (AKP) and TNF-α. The activities of LDH, ACP and AKP were measured by colorimetric assay kits (Nanjing Jiancheng Bioengineering Institute, China), while TNF-α concentration was determined by radioimmunoassay (TNF-α RIA Kit, Dongya Immunity Technical Institution, China) using a ZC-2010 g-counter (USTC Chuangxin Co., Ltd., China).

**Protein extraction and western blotting analysis**

Briefly, proteins were extracted from harvested cells after washing twice with PBS and resuspending in RIPA (radio-immunoprecipitation assay) lysis buffer containing protease inhibitors (Beyotime Institute of Biotechnology, China), and protein concentrations were determined using the bicinchoninic acid assay (Beyotime Institute of Biotechnology, China). Equal amounts of protein samples were loaded on a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride membranes using an electrobobter. Membranes were blocked in 5% non-fat milk diluted with TBS containing 0.05% Tween 20 for 3 h at room temperature. The primary antibodies were diluted (1: 500 for Bcl-2, FasL, p53, prohibitin, vimentin and procaspase-3) in blocking solution. Primary antibodies against Bcl-2, FasL, p53, prohibitin, vimentin and procaspase-3 were from rabbits, while that against GAPDH (used as internal control) was from mouse, which were purchased from Santa Cruz Biotechnology (New York, USA). Then the membrane was incubated with the antibodies overnight at 4°C. After washing with Tris-buffered saline containing 0.05% Tween 20, the membrane was incubated in horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted 1: 2000) or anti-mouse IgG (diluted 1: 2000) for 3 h at room temperature. Immunodetection was performed using ECL-plus reagents (Beyotime Institute of Biotechnology, China) and photographed by BioSpectrum Gel Imaging System (UVP, USA).

**Reverse transcription-polymerase chain reaction (RT-PCR)**

After treated with berberine, HCT-8 cells were washed twice with PBS. The total RNA was purified with a Trizol reagent (Beyotime Institute of Biotechnology, China), following its standard protocol. The extracted RNA was stored at -70°C. cDNA synthesis and PCR amplification were performed as previously described (Kuo et al., 2004a). For reverse transcription, PCRs were done by mixing 500 ng total RNA with 20 μL reaction containing 4 μL dNTPs (2.5 mM), 2.5 μL Oligo dT (10 pmol/μL) and RTase (200 U/μL), and the reaction was performed at 42°C for 30 min. Afterwards, 5 μL of cDNA product was used as templates in PCR amplifications together with appropriate primers (sequence of Bcl-2, forward: 5’-ATGTGTGTGGAGAGCTAACCC-3’; reverse: Bcl-2, 5’-TGAGCAGAGTCTTTAGGGG-3’; sequence of Bax, forward: 5’-CTTTTCGCTCTCG GCCAC-3’; reverse: 5’-ACATGGTCATGTTTCA-3’; reverse: 5’-GATGGTCAGGGTCT GCCAC-3’; sequence of Fas, forward: 5’-ATGCTGGG CATCTGGGCCT-3’; reverse: 5’-CTTAGACCAG CTTTGAGTTTC-3’; sequence of FasL, forward: 5’- CCTCCAGGCACAGTCTTTCC-3’; reverse: 5’-ATC TGGCTGGTAGACTCTCG-3’; sequence of caspase-3, forward: 5’-ACATGGTCGTCAAAAATCC-3’; reverse: 5’-CACAAAGCGACTGGATGAC-3’; sequence of vimentin, forward: 5’-AGGAAATGGCTCGTCACC TCTCGTGAATA-3’; reverse: 5’-GGAGTGGCTGGG TTTAGAACCTAGAGC-3’). The PCR products were analyzed by horizontal gel electrophoresis in 2% agarose gels with TAE buffer supplemented with 0.005% ethidium bromide.

**Statistical analysis**

Data were analyzed with the SPSS11.5 software. Results are presented as the mean ± SD of three independent experiments. The two groups were evaluated by one way ANOVA test followed by the post-hoc Tukey’s test.

**Results**

In order to investigate the cytotoxic effect of berberine, HCT-8 cells were treated with various concentrations of berberine (0.03 mmol/L, 0.06 mmol/L, 0.12 mmol/L, 0.24 mmol/L and 0.47 mmol/L) for 12, 24, 48 and 72 h, respectively. Meanwhile, growth inhibition by hydroxyacamptothecin (0.24 mmol/L) was chosen as a positive control. According to the inhibitory rates, it was found that the cell inhibition ratio was increased in dose- and times-dependent manners (shown in Fig. 1B), and the maximum inhibition ratio (64.77%) was obtained at 0.47 mmol/L of berberine for 72 h.
In addition, HCT-8 cells were treated with berberine (0.47 mmol/L) or hydroxycamptothecin (0.24 mmol/L) for different times, and the results of microscopy assay are shown in Fig. 1. As shown in Fig. 1C, chromatin of living cells was green and that of necrotic cells was orange. Chromatin of Apoptotic cells’ chromatin was always condensed, but that of non-viable cells was orange, while those of still viable apoptotic cells were green. The number of necrotic cells and non-viable apoptotic cells increased with time in berberine-treated cells.

To determine whether berberine exerted its inhibitory effect by arresting the cell cycle we examined the distribution of cell cycle phases after berberine treat-
ment by flow cytometry. As shown in Fig. 2, after incubation with 0, 12, 24, 48 and 72 h, the percentage of hypodiploid cells were 7.23 ± 1.02%, 11.78 ± 1.33%, 25.62 ± 3.21%, 42.04 ± 2.38% and 45.34 ± 4.37%, respectively. The percentages of cell in G1 phase were decreased, compare with control cells, from 49.19% to 21.71%, while the percentage of cells was markedly increased at 72 h from 47.43% to 78.30% in S phase. The results suggested that berberine arrested cells at S phase in a time-dependent manner.

TNF-α concentration was increased in cell supernatants after berberine treatment (0.47 mmol/L), at both 48 h and 72 h (Fig. 3A). Also, the activities of AKP, ACP and LDH in the supernatants of berberine-treated cells increased after 72 h (Fig. 3B, C and D).

Also, the expression of Bcl-2, procaspase-3 and vimentin in HCT-8 cells was consistently decreased as compared with untreated cells. On the other hand, protein levels of FasL, p53 and PHB were significantly increased in a time-dependent manner when cells were treated with 0.47 mmol/L berberine (Fig. 4).

Finally (Fig. 5), levels of mRNA corresponding to Fas, FasL, Bax and caspase-3 were significantly increased, while mRNA levels of Bcl-2 and vimentin were decreased, after berberine exposure (0.47 mmol/L) for different times (12, 24 and 48 h).
FIGURE 5. Cytotoxicity of berberine on mRNA levels of: (A) Bcl-2 and Bax, (B) Fas and FasL, (C) caspase-3 and vimentin in HCT-8 cells. RT-PCR analysis followed agarose gel electrophoresis was performed using GAPDH as an internal control. The relative level of mRNA was expressed as a percentage of 0 h in HCT-8 cells treated with berberine for 0, 12, 24, and 48 h. Data are presented as mean ± SD of three independent experiments. Stars indicate statistically significant differences from control: *p<0.05; **p<0.01.

Discussion

In the present study, the results of MTT assay showed that berberine was a potent anti-proliferative and cyto-toxic agent on human colon cancer HCT-8 cells in both time- and dose-dependent manners. The results of acridine orange/ethidium bromide staining, the time-dependent increase in the number of hypodiploid cells and of those arrested at S phase all of them supported the view that apoptosis of HCT-8 cells is induced by berberine.

In the current study, LDH, ACP and AKP levels of activity were increased in cell supernatants after berberine treatment, which would suggest that berberine promoted an increase in cell death.

Apoptosis is a physiologically programmed mechanism of cell death that may be activated by drugs. Two major pathways mediating berberine-induced apoptosis will be discussed: a mitochondria-dependent pathway (involving Bax and Bcl-2, and PBH) and a death receptor-dependent pathway (involving Fas and FasL, TNF-α, and perhaps p53 and PHB). Bax and Bcl-2 have been identified as major regulators to regulate the release of mitochondrial cytochrome c (Kluck et al., 1997; Cory and Adams, 2005). Bax translocates to the mitochondria, and inserts into the outer mitochondrial membrane, allowing the release of cytochrome c. In contrast, Bcl-2 blocks cytochrome c efflux by binding to the outer mitochondrial membrane. After HCT-8 cells were treated with berberine, the mRNA level of Bax was significantly increased while both the mRNA and protein expressions of Bcl-2 were down-regulated. The increased Bax/Bcl-2 ratio suggests that mitochondrial permeability may be involved in the induction of apoptosis by berberine.

Fas is a member of the tumor necrosis factor family, and is expressed by a wide variety of normal and abnormal cell types, including many tumor cells (Houston and O’Connell, 2004; Reed et al., 2005). Fas ligand (FasL), was first found on the surface of activated T and NK cells, and was also found to be expressed by some non-lymphoid and tumor cells (Nagata, 1996; Peter and Krammer, 2003; Tanaka et al., 1995; Walker et al., 1997). Fas/FasL binding is known to induce apoptosis (Nagata, 1996). In this study, the elevated mRNA and/or protein expression levels of Fas and FasL also suggest the involvement of the Fas/FasL pathway in berberine-induced apoptosis of HCT-8 cells.

Also, TNF-α is another important member of the tumor necrosis factor family. It binds by tumor necrosis factor receptor (TNFR) to play a role as an antineoplastic agent by promoting proliferation and differentiation of immune cells. The elevated concentrations of...
TNF-α in HCT-8 cells supernatant showed that berberine can trigger the TNF pathway through up-regulated expression of TNF-α (Lejeune et al., 2006; Pennica et al., 1984).

The accumulation of tumor suppressor protein p53 upon stress signals, may induce two different sets of genes related with cell growth and apoptosis through cell cycle arrest which due to an increase in levels of p21 and Bax (Stoffel and Levine, 2004; Lee et al., 2008). Also, PHB is a potential tumor-suppressor gene that has been shown to interact with p53, which enhance the p53 promoter with DNA binding capacity. PHB inhibits cell proliferation by regulating p53 pathway (Mishra et al., 2006; Fusaro et al., 2003). In HCT-8 cells, we have observed that berberine increases the expression of p53 and PHB, and hence these proteins may be involved in berberine-induced apoptosis.

We have also shown that the levels of caspase-3 and its precursor procaspase-3, as the downstream executioner at apoptotic process, were also changed after berberine treatment. In fact, down-regulation of procaspase-3 and up-regulation of caspase-3 mRNA expression suggest that berberine can induce cell death through activation of caspase-3. In turn, either caspase-3 or caspase activated proteases may affect vimentin, a major structural protein serving as an organizer of critical proteins involved in attachment, migration, and cell signaling. We have found, however, that vimentin expression, both at the mRNA and protein levels, was decreased by berberine, thus suggesting that berberine may be also affecting vimentin through a caspase independent pathway.

We conclude that caspase-3 and the Fas/FasL pathway, p53 and PHB, activation of caspase-3 and vimentin down-regulation and cleavage.

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References


