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Full Length Research Paper

Effects of berberine on the proliferation and the protein expression of Bcl-2, Bax on human gastric carcinoma SGC-7901 cells

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Berberine (BBR) is widely used to treat diarrhea and inflammation, and its antitumor activity has been vastly reported recently. However, its antitumor mechanism remained unclear. In the present study, the effects of Berberine on human gastric cancer SGC-7901 cells *in vitro* and its mechanism were investigated. Cisplatin (DDP) was used as positive control. The inhibition of drugs on SGC-7901 was evaluated with MTT assay. The level of Bcl-2 and Bax in the cells treated with different concentrations of Berberine were detected with Western blotting. MTT assay indicated significantly inhibitory effect of Berberine on SGC-7901 cells in a dose- and time-dependent manner. Annexin V-FITC and propidium iodide (PI) staining showed that BBR had a positive effect on apoptosis of SGC-7901 cells. After 2.5 µg/ml of BBR treating SGC-7901 cells for 48 h, the cells apoptosis rate (24.32 ± 0.71%) had statistical significance as compared with the negative group (2.77 ± 0.39%) (P = 0.000). Berberine up-regulated the levels of Bax, but down-regulated the level of Bcl-2 protein by Western blot. These results indicated that BBR exhibited potential anticancer activity against human gastric cancer SGC-7901 cells through induction of apoptosis and the imbalance of the ratio of Bcl-2/Bax.

Key words: Human gastric carcinoma SGC-7901, berberine, Bcl-2, Bax.

INTRODUCTION

Herbal medications are currently being promoted for clinical use in cancer therapy. Many of these claims are based on anecdotes in traditional Chinese medicine (Li et al., 2000). Berberine (BBR) (Figure 1), an alkaloid extracted from *Coptis chinensis* (*Huanglian*) or *Golden cypress*, is widely used in China for treating diarrhea and anti-inflammation. Meanwhile, many other pharmacological benefits have also been reported including hypolipidemic activity, hepatoprotective effect and anticachectic effects. Recently, several studies have been conducted to investigate the antitumor mechanism of BBR (Hoshi et al., 1976; Yamagish 1962). It was reported that BBR has shown antitumor activity in many human cancer cells including promyelocytic human leukemia HL-60 (Kuo et al., 1995), human epidermoid carcinoma A431 cells (Mantena et al., 2006), human colon cancer cells (Fukuda et al., 1999; Lin et al., 1999), U937 cells (Jantova et al., 2007) and human gastric cancer cell line (SNU-5) (Lin et al., 2006) and so on. Thus, it can be assumed that BBR potently exert anticancer activity in addition to its other useful pharmacological benefits.

This study was carried out to evaluate the anti-gastric cancer activity and to investigate the possible mechanisms of proliferation inhibition elicited by BBR in cultured human gastric cancer SGC-7901 cells. Our study clearly demonstrated that BBR showed potential anti-gastric cancer activity and could inhibit proliferation of SGC-7901 cells via induction of apoptosis and the imbalance of the ratio of Bcl-2/Bax.

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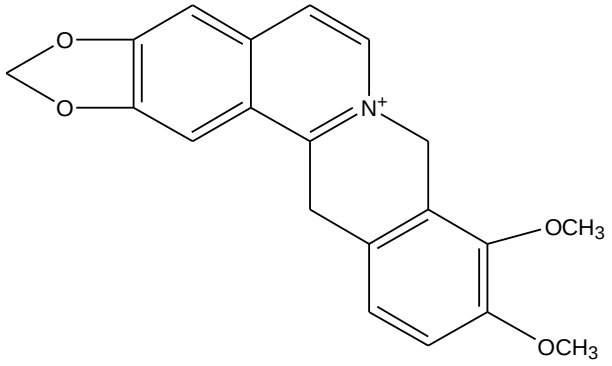

Figure 1. The chemical structure of berberine.

MATERIALS AND METHODS

Berberine hydrochloride (MW336.37) was purchased from the National Institute of Control of Pharmaceutical and Biological Products. RPMI 1640 (GIBCO, Grand Island, NY, USA); MTT and Tris (Ameresco), 0.25% pancreatic enzymes (Gino's biomedical technology Co., Ltd), Annexin V-FITC/ PI Apoptosis Assays Kit was purchased from Nanjing Keygen Biotech. Co. , Ltd. Bcl-2 and Bax antibody (Cell Signaling) and ECL Western blot coloration kit were obtained from Pierce, HEPES and β-actin antibody (Sigma); PVDF membranes (Millipore Corporation, Bedford, MA, USA).

BBR was dissolved in dimethyl sulfoxide (DMSO) in 10 mg/ml stock solution for experimental use. This stock solution was stable and could be kept at 4°C for 2 months without loss of any effect. The final concentrations of BBR which were freshly diluted with DMSO for each experiment were 2.5, 5, 10 and 20 μg/ml, respectively.

Cell culture

Human gastric cancer SGC-7901 cells were purchased from Shanghai cell bank of the Chinese Academy of Sciences, Shanghai, China. The cells were cultured in 25 cm^2 culture flasks at 37°C in humidified 5% CO_2 and 95% air atmosphere in RPMI – 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin and 1% glutamine. The cells were passaged once they reached 70 to 80% confluence.

MTT assay measured SGC-7901 cells proliferation inhibition rate

SGC-7901 cells (1 × 10^5 cells/ml) in exponential growth stage were suspended in medium and seeded in 96-well plates at 100 μl/well. After culturing for 24 h to obtain adherent monolayer cells, the medium was discarded. Then cells were washed with PBS twice and incubated in 100 μl of the fresh medium containing a variant dose of BBR solutions (final concentrations were 2.5, 5, 10 and 20 μg/ml, respectively) for 24, 48, 72 and 96 h, respectively. Positive group was treated with DDP solution (final concentration was 70 μg/ml). Sterilized saline was used for the negative group. At the end of each time point, the drug-containing medium was replaced by fresh medium. Then, 10 μl of MTT reagent (5 mg/ml) was added to each well and incubated for 4 h at 37°C. And then, 150 μl of DMSO were added to each well, and gently shaken for 10 min at room temperature. The absorbance of each well was measured at 570 nm by a micro plate reader. Results are expressed as the percentage growth inhibition with respect to the untreated cells. The growth inhibition rate was determined using the following formula: Growth inhibition rate (%) = (negative group's OD-test group's OD)/negative group's OD × 100%.

Annexin V-FITC/PI double staining measured SGC-7901 cells apoptosis rate

SGC-7901 cells (2 × 10^5 cells/ml) in exponential growth stage were suspended in medium and seeded in 6-well plates at 2 ml/well. After cultured for 24 h to obtain adherent monolayer cells, the medium was removed. Cells were washed with phosphate buffered saline (PBS) twice, and then 2 ml of the fresh medium containing 2.5 μg/ml BBR was added. Positive group was treated with DDP solution (final concentration was 70 μg/ml). Wells that added the same volume of sterilized saline were set as negative group. After incubating for 48 h, the detached and attached cells were harvested and washed with PBS twice and centrifuged at 1000 rpm for 5 min to remove PBS. Then the cells were treated according to the Annexin V-FITC/PI apoptosis detection kit. The early apoptosis rate of cells was analysis by flow cytometer. Cells positive for Annexin V but negative for PI fluorescence were identified as apoptotic.

Western blot

Treated and non-treated cells were lysed with the lysis buffer at 4°C with 50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, 0.1% Tween 20, and 10% glycerol supplemented with the following proteinase inhibitors: 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM Na vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 10 mg/ml leupeptin. Cell lysates (50 mg) were loaded onto 10 or 13% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. The equal loading of proteins was confirmed by amido black staining (Li et al., 2000). The blot was blocked with a solution containing 5% non-fat dry milk in Tris-buffered saline (10 mM Tris, 150 mM NaCl) containing 0.05% Tween 20 for 2 h, then washed and incubated with antibodies to anti-rabbit Bcl-2, Bax and anti-mouse β-actin at 4°C overnight. After incubating with anti-mouse peroxidase-conjugated antibody, the signal was visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech). The detection of β-actin was used as an internal control in all of the data for Western blotting (Lin et al., 2008).

Statistical analysis

Experimental data were expressed with mean ± standard deviation ($\overline{X} \pm S$). Statistical analysis was performed with analysis of variance of factorial design and One-Way ANOVA using the statistical software SPSS 13.0. Significant level $\alpha = 0.05$, $P<0.05$ was considered statistically significant.

RESULTS

Inhibition of SGC-7901 proliferation by BBR

The growth inhibition of BBR in the cultured SGC-7901 cells was evaluated by MTT assay. It was shown that a shorter period of treatment with 2.5 μg/ml of BBR reduced growth inhibition only slightly. Further increase in

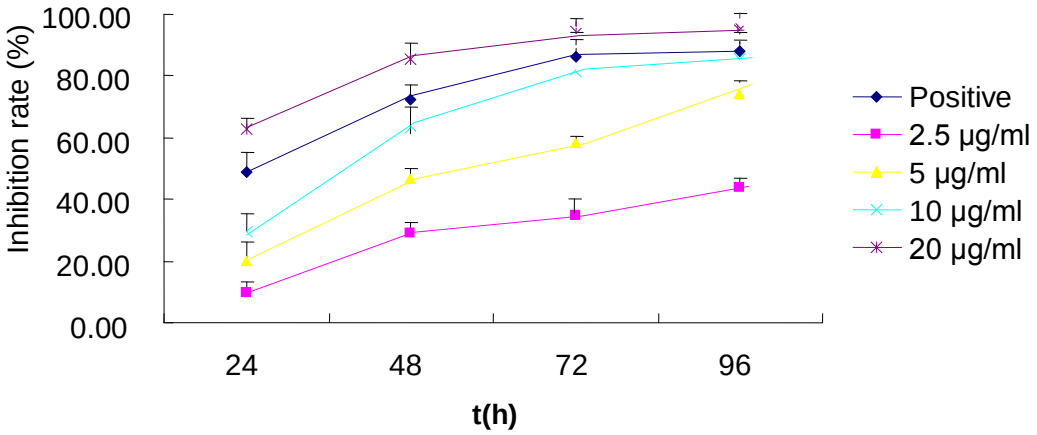

Figure 2. Inhibition of SGC-7901 cells proliferation by BBR. Cells were treated with different concentrations of BBR. The optical density of cells was determined at 24, 48, 72 and 96 h, respectively by MTT assay.70 μg/ml of DDP was used as positive control. Analysis of variance of factorial design showed that the growth of SGC-7901 cells was inhibited by BBR in a dose- and time-dependent manner ($P < 0.05$).

Table 1. Apoptosis rate of SGC-7901cells treated with BBR for 48 h.

Group	Apoptosis rate (%)
Negative	2.77 ± 0.39
Positive	1.48 ± 0.51
Test	24.32 ± 0.71[ab]

[a]P< 0.01 vs. negative group; [b]P< 0.01 VS positive group. Apoptosis rate of negative group, positive group and 2.5 μg/ml of BBR-treated cells were determined by Annexin V-FITC/PI staining and flow cytometric analysis. The values expressed were mean ± standard deviation of triplicate measurements.

the concentration of BBR resulted in greater increase in the proliferation inhibition rate of SGC-7901 cells. The growth inhibition of cells in high concentration of BBR (10 and 20 μg/ml) treatment groups was not significantly higher than that in positive group. MTT assay showed that 2.5 to 20 μg/ml of BBR could inhibit the growth of SGC-7901 cells in a time- and concentration-dependent manner (Figure 2).

Effect of BBR on SGC-7901 cells apoptosis rate

To further confirm that BBR induced cell apoptosis, SGC-7901 cells were treated with BBR to study the apoptosis by staining with Annexin V-FITC/PI and subsequently analyzed by flow cytometer. The dual parameter fluorescent dot plots showed the viable cell population in the lower left quadrant (annexin V-FITC/PI). The cells at the early apoptosis are in the lower right quadrant (annexin V-FITC/PI). As indicated in Table 1, after 2.5 μg/ml of BBR treating SGC-7901 cells for 48 h, the cells apoptosis rate (24.32±0.71%) had statistical significance compared with the negative group (2.77 ± 0.39%) and the positive group (1.48 ± 0.51) (P = 0.000). These results suggested that 2.5 μg/ml of BBR had positive effects on apoptosis of SGC-7901 cells (Figure 3).

BBR down-regulated the expression of Bcl-2, but up-regulated Bax expression

To investigate the anti-tumor mechanism of BBR, SGC-7901 cells were treated with BBR in a dosage of 0, 2.5, 5, 10 and 20 μg/ml for 24 h, respectively. And, the expression of Bcl-2 and Bax was determined by Western blot. The data suggested that the presence of BBR might decrease the expression of Bcl-2, but increase the expression of Bax. Also, we found that the effect of BBR on the Bcl-2 and Bax expression in SGC-7901 cells was dose-dependent (Figure 4).

DISCUSSION

Globally, gastric cancer remains the fourth commonest cancer, and the second commonest cause of cancer death (Ford, 2011). Chemopreventive strategies to reduce the incidence of gastric cancer are required. But side effects and resistance to chemo-drugs are of crucial to be overcome in the treatment of cancer. Recently, interests in applying plant materials for treating various human diseases including cancer are mounting. Berberine, an alkaloid which is extracted from *Coptis chinensis* (*Huanglian*) or *Golden cypress*, was widely

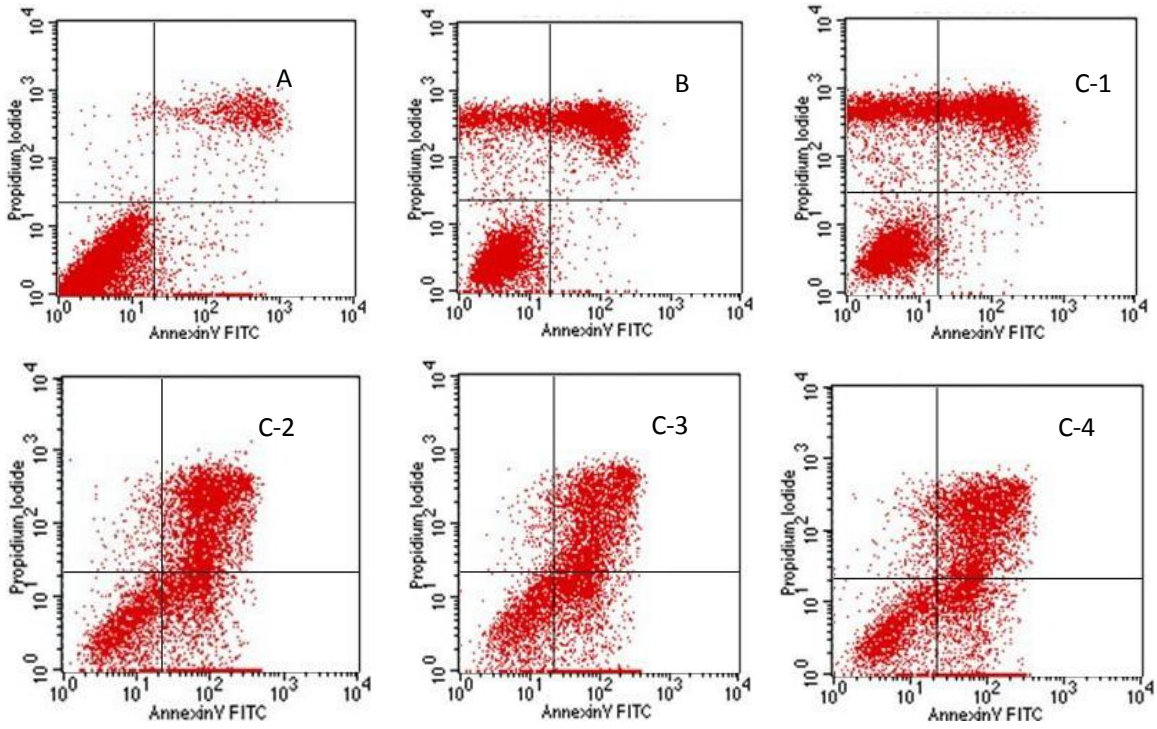

Figure 3. The apoptosis of SGC-7901 cells after treated with BBR for 48 h. (A) Blank; (B) DDP; (C-1 to C-4) cells treated with 2.5, 5, 10 and 20 μg/ml of BBR, respectively.

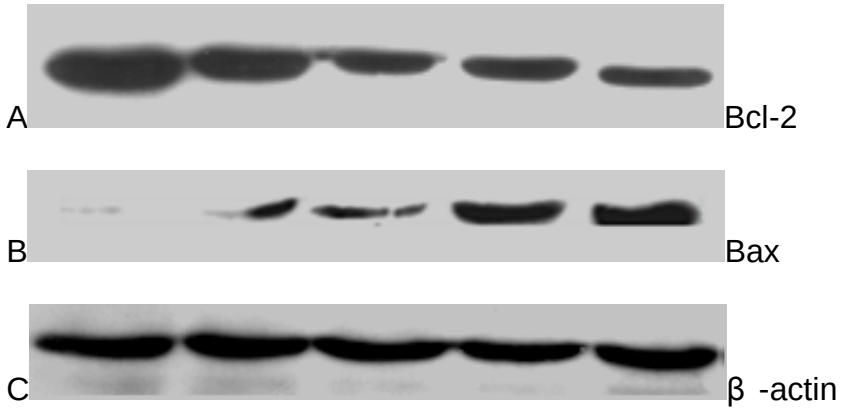

Figure 4. Effects of BBR on the expression of Bcl-2 (A) and Bax (B) in SGC-7901 cells after exposure to BBR. SGC-cells were treated with 0, 2.5, 5, 10 and 20 μg/ml of BBR for 24 h, respectively.

used in China for treating diarrhea and anti-inflammation. Recently, BBR showed antitumor activity in many cancer cells, suggesting a positive benefit of anticancer like colon cancer and gastric cancer (Lizuka et al., 2000; Zhang et al., 2005). Nevertheless, the underlying mechanism of anti-cancer remains unclear. Thus, the present study investigated the effect of BBR on the proliferation, apoptosis or cell cycle of cultured SGC-7901 cells.

Apoptosis has now been widely accepted as a prominent tumour-suppression mechanism. The basic apoptotic pathway includes death of receptor pathway and mitochondrial pathway. Furthermore, Bcl-2 plays an important role in the mitochondrial pathway, which was

considered that overexpression of Bcl-2 will inhibit cell death. The intrinsic pathway—also called the Bcl-2-regulated or mitochondrial pathway is activated by various developmental cues or cytotoxic insults, such as viral infection, DNA damage and growth-factor deprivation, and is strictly controlled by the Bcl-2 family of proteins. While Bax, belonging to the pro-apoptotic family members, is crucial for inducing permeabilization of the outer mitochondrial membrane and the subsequent release of apoptogenic molecules, which leads to caspase activation. Bcl-2 interacts with Bax as homodimers, which exist in the cells, leading to the tumor development. Therefore, the ratio of Bcl-2/Bax and Bax/Bax are determined by the levels of Bcl-2 and Bax, which determine the susceptibility of cells to the apoptosis signal and regulate the balance between cell apoptosis and survival (Heath-Engel et al., 2008; Claudia et al 2009; Youle and Strasser, 2008).

In the present study, the MTT assay showed that BBR could inhibit the proliferation of SGC-7901 cells in a dose- and time-dependent manner. The cellular morphology which progressively changed with increasing duration of exposure to BBR had also shown it had potent cytotoxicity on the SGC-7901 cells. Moreover, AnnexinV-FITC and PI staining showed that after 2.5 μg/ml of BBR treating SGC-7901 cells for 48 h, the cells apoptosis rate (24.32 ± 0.71%) had statistical significance as compared with the negative group (2.77 ± 0.39%). This finding suggested that it may have potential as a cancer-prevention agent. Cancer is characterised by the imbalance between cell proliferation and apoptosis. Inhibiting cell proliferation and increasing apoptosis in tumors are effective tactics for preventing tumor growth.

Conclusion

In this paper, we have demonstrated that BBR could inhibit the proliferation of the SGC-7901 cells and induce apoptosis through down-regulating the Bcl-2 expression and up-regulating the Bax expression. These results suggested that the BBR had a positive anticancer activity *in vitro*. It could be considered as an effective and safe material for anticancer drug discovery. However, more works should be done to further investigate its anticancer mechanism *in vivo*, that is the specific molecular signaling pathways for induction of apoptosis remained to be identified.

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