Puerarin increases the chemosensitivity of hepatocellular carcinoma cells

ZHEN WU[1], JING WU[2], PING FANG[3] and SHIFENG KAN[4]

Departments of [1]Clinical Laboratory and [2]Pharmacy, The Second Hospital of Shandong University, Jinan, Shangdong 250033; [3]Department of Blood Transfusion, Qianfoshan Hospital of Shandong, Jinan, Shangdong 250014; [4]Department of Clinical Laboratory, Qilu Hospital, Shandong University, Jinan, Shangdong 250012, P.R. China

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Abstract. The present study investigated the effect of puerarin (Pu) on the sensitivity of HepG2 human hepatocellular carcinoma (HCC) cells to chemotherapeutic drugs to determine the possible mechanism. HepG2 cells were treated with different concentrations of Pu and cisplatin (CDDP), alone or in combination. MTT assay was used to determine the inhibitory effects of the different drugs on HepG2 cells. Cell morphology was observed by inverted microscopy. The expression of B-cell lymphoma 2 (Bcl-2) and Bax protein was measured by western blot analysis. Pu and CDDP, alone or in combination, inhibited the proliferation of HepG2 cells. The inhibitory effect of CDDP combined with Pu on HepG2 cells was significantly higher than that of the single drug treatments ($p<0.01$). In addition, compared with the single drug groups, cellular morphology was significantly altered and the apoptotic rate of cells and the expression of Bax protein were significantly increased ($p<0.01$). However, the expression of Bcl-2 protein was significantly decreased ($p<0.01$) in the combined drug group. In conclusion, Pu can increase the sensitivity of HCC to chemotherapeutic drugs, enhance the inhibitory effect of chemotherapeutic drugs on cell proliferation and synergistically induce apoptosis of HepG2 cells. The mechanism is likely related to the upregulation of Bax protein and the downregulation of Bcl-2 protein.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignant tumor in the world and its incidence is increasing. It is the third most common cause of cancer-related death among malignant tumors (1), ranking second and sixth among tumor-related causes of death in males and females, respectively (2). Currently, surgery is the most common method of treatment of liver cancer. However, the clinical symptoms of liver cancer are not obvious and are often overlooked. When patients experience symptoms such as weight loss, jaundice, abdominal mass and liver pain, the vast majority have reached the advanced or terminal stage of the disease. Although radiotherapy and chemotherapy have certain effects, only 10% of patients are likely to undergo complete hepatectomy. While 90% of patients are treated with radiotherapy, chemotherapy, or radiofrequency ablation, it is difficult to achieve the expected clinical results because of low efficacy and high rate of severe side effects. Therefore, effective treatment of liver cancer is severely lacking (3,4).

In traditional Chinese medicine, Chinese herbal medicines have many advantages. Their active ingredients generally have high efficiency and low toxicity. Scientists worldwide have paid increasing attention to Chinese herbal medicines because of their irreplaceable advantages. Studies have shown that Chinese herbal medicine has significant advantages for the prolongation of survival, prevention and treatment of liver cancer and metastasis (5). In recent years, with studies on the anticarcinogenic mechanisms of Chinese herbal medicine, more active ingredients have been extracted. Puerarin (Pu) belongs to the group of isoflavone flycoside compounds and is extracted from the traditional Chinese medicine leguminous plants, *Pueraria thomsonii* Benth and *Pueraria lobata* (Willd.) Ohwi. It was reported that Pu has antitumor activity (6-8).

The aim of the present study was to investigate the effect of Pu on the sensitivity of HepG2 human HCC cells to chemotherapeutic drugs and its possible mechanism, to provide a foundation for the treatment of HCC.

Materials and methods

Reagents. Pu (Aladdin Reagent Co. Ltd., Shanghai, China); HepG2 cell line (Cell Bank of Chinese Academy of Sciences, Shanghai, China); Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT, USA); primary rabbit polyclonal GAPDH antibody (dilution, 1:1,000; cat. no. 10494-1-AP), rabbit polyclonal

Correspondence to: Dr Shifeng Kan, Department of Clinical Laboratory, Qilu Hospital, Shandong University, 115 West Wenhua Road, Jinan, Shangdong 250012, P.R. China
E-mail: kjs2sn@163.com

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Bax antibody (dilution, 1:500; cat. no. 50599-2-Ig), rabbit polyclonal Bcl-2 antibody (dilution, 1:500; cat. no. 12789-1-AP) and mouse monoclonal HRP-conjugated secondary antibody (dilution, 1:2,000; cat. no. HRP-66008) were all purchased from Sanying Biotechnology Co. Ltd. (Wuhan, Hubei, China). Cell lysis buffer, BCA protein concentration quantification kit, Annexin V-FITC apoptosis detection kit (all from Biyuntian Biotechnology Research Institute, Nantong, China); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA).

Cell culture. HepG2 cells were cultured in an incubator at 37˚C and 5% CO_2 in DMEM medium containing 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FBS. When cells were in the logarithmic phase of growth, they were digested with trypsin and single cell suspensions were prepared. Cells were grouped and seeded in different culture plates or Petri dishes according to experimental needs.

MTT assay. Cells were divided into the experimental and control groups. HepG2 cells in the logarithmic growth phase were collected and seeded in 96-well plates at a concentration of $1x10^4$/ml, with 200 μl per well. After 24 h, the experimental group was divided into 12 subgroups according to different treatments: Four groups treated with Pu alone (final concentrations of 25, 50, 100 and 125 μM, respectively), four groups treated with cisplatin (CDDP) alone (final concentrations of 2.5, 5, 10, and 20 μM, respectively) and four groups treated with the combined drugs (final concentrations of 25 μM Pu + 2.5 μg/ml CDDP, 50 μM Pu + 5 μg/ml CDDP, 100 μM Pu + 10 μg/ml CDDP and 125 μM Pu + 20 μg/ml CDDP, respectively). Each condition was repeated five times. Cells in the control group were cultured in the same culture medium without treatment. After 48 h, the culture medium was discarded and cells were washed three times with phosphate-buffered saline (PBS). The cells were then incubated with 100 μl of MTT (5 mg/ml) for 4 h and 100 μl of DMSO was added to each well with shaking in the dark for 10 min. The absorbance value (OD) at 570 nm was measured using a microplate reader (Thermo Fisher Scientific, New York, NY, USA). The rate of inhibition was calculated according to the following formula: Inhibitory rate (%) = (OD value of the control group - OD value of the experimental group/OD value of the control group) x 100%.

Morphological observation. The cells were divided into four groups: The control group, Pu group (100 μM), CDDP group (10 μg/ml) and Pu (100 μM) + CDDP (10 μg/ml) group. After the cells were treated accordingly, morphological changes were observed and recorded with an inverted microscope (AZ100; Nikon, Tokyo, Japan).

Analysis of apoptosis. According to the results of the MTT assay, cells were divided into four groups: The control group, Pu group (100 μM), CDDP group (10 μg/ml) and Pu (100 μM) + CDDP (10 μg/ml) group. In the logarithmic growth phase, HepG2 cells were seeded in 6-well plates. After cells adhered, they were treated accordingly for 24 h, washed three times with PBS, digested with trypsin and centrifuged.

Table I. The effects of Pu and CDDP on HepG2 cell proliferation (mean ± SD).

Concentration		
Pu (μM)	CDDP (μg/ml)	Inhibitory rate (%)
0	0	0
25	0	13.20[a]
50	0	27.95[a]
100	0	31.87[a]
125	0	46.91[a]
0	2.5	7.85[a]
0	5	17.35[a]
0	10	28.71[a]
0	20	39.29[a]
25	2.5	27.08[a,b]
50	5	47.25[a,b]
100	10	60.61[a,b]
125	20	90.29[a,b]

[a]p<0.01 vs. the control group; [b]p<0.01 vs. the same dose of single drug group. Pu, puerarin; CDDP, cisplatin.

All protocols were followed according to the instructions of the kit. Cells were resuspended in 0.3 ml of binding buffer, followed by addition of 5 μl of Annexin V and 5 μl of PI. After incubation at room temperature for 15 min in the dark, 0.2 ml of binding buffer was added to each sample. Apoptosis was detected by flow cytometry (Becton Dickinson, New York, NY, USA).

Western blot analysis. Cells were treated in the same manner as for morphological observation, harvested and lysed with lysis buffer. The supernatant was collected after centrifugation at high speed for 15 min. The protein concentration was determined using a BCA kit. A total of 40 μg of protein was used for SDS-PAGE and the wet transfer method was used for protein transfer to membranes. Membranes were blocked in 10% skim milk, and incubated with primary antibodies against Bcl-2, Bad and GAPDH (1:1,000) overnight at 4˚C. Next, secondary antibody (1:2,000) was added and membranes were incubated at room temperature for 2 h. ECL was added to membranes, blots were developed in a dark room and images were scanned and recorded.

Statistical analysis. Data are presented as mean ± standard deviation. Data were analyzed by SPSS 17.0 (IBM Corp., New York, NY, USA) using one-way ANOVA. $p<0.05$ was considered statistically significant.

Results

The effect of Pu combined with CDDP on the proliferation of HepG2 cells. MTT assay was used to determine the effects of Pu and CDDP, alone or in combination, on the proliferation of HepG2 cells (Table I). Pu and CDDP alone inhibited the proliferation of HepG2 cells in a concentration-dependent

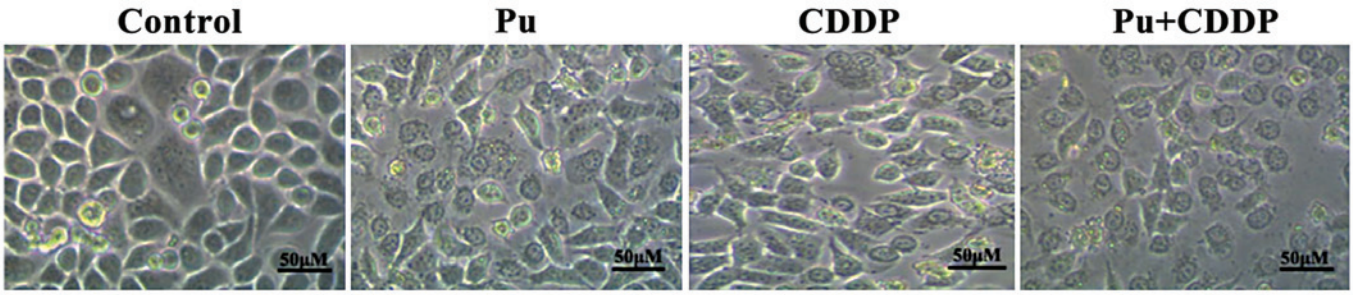

Figure 1. The effect of Pu combined with CDDP on the morphology of HepG2 cells. The morphological changes were observed after treatment with Pu, CDDP and Pu + CDDP for 48 h. Cell morphology in the treatment groups changed markedly, especially in the combined drug group. Morphological changes included cell shrinkage, reduction of cell adherence, decrease in cell number, and increase in the number of dead cells. Pu, puerarin; CDDP, cisplatin.

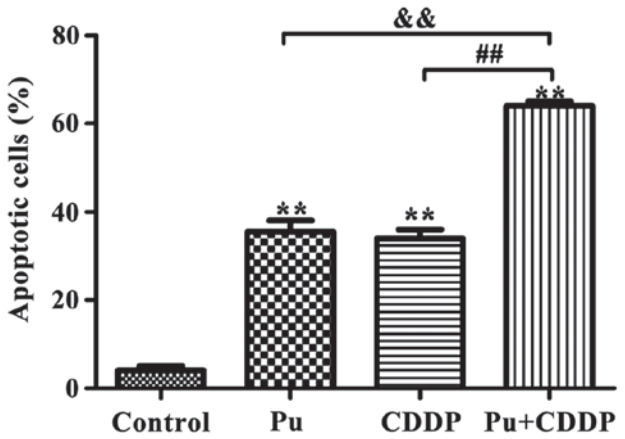

Figure 2. Detection of cell apoptosis in different treatment groups by flow cytometry. **p<0.01 vs. the control group; ##p<0.01 the combined treatment group vs. the CDDP alone group; &&p<0.01 the combined treatment group vs. the Pu alone group. CDDP, cisplatin; Pu, puerarin.

manner. The inhibitory effect increased with increasing drug concentration. The inhibitory effect of Pu and CDDP in combination on the proliferation of HepG2 cells was significantly higher than that of the corresponding single drug treatments ($p<0.01$). The doses of 100 μM Pu and 10 μg/ml CDDP and 100 μM Pu + 10 μg/ml CDDP were used in the single drug groups and combined drug group, respectively, for subsequent experiments.

The effect of Pu combined with CDDP on the morphology of HepG2 cells. Except for the control group, cells in the treatment groups were treated with Pu (100 μM), CDDP (10 μg/ml) and Pu (100 μM) + CDDP (10 μg/ml), respectively (Fig. 1). After 48 h, cell morphology in the treatment groups changed significantly compared with the control group, especially in the combined treatment group. These changes included cell shrinkage, reduction of cell adherence, decrease in cell number and increase in the number of dead cells.

The effect of Pu combined with CDDP on apoptotic rate of HepG2 cells. According to the results of the MTT assay, 100 μM Pu and 10 μg/ml CDDP were selected to investigate whether Pu enhanced the sensitivity of HepG2 cells to the chemotherapeutic drug, CDDP. As shown in Fig. 2, the apoptotic rates in the control, Pu, CDDP and combined drug groups were 3.92±0.42, 35.74±4.18, 32.93±3.68 and 62.03±7.65%, respectively. Compared with Pu or CDDP alone, the apoptotic rate of the combination group was significantly increased ($p<0.01$).

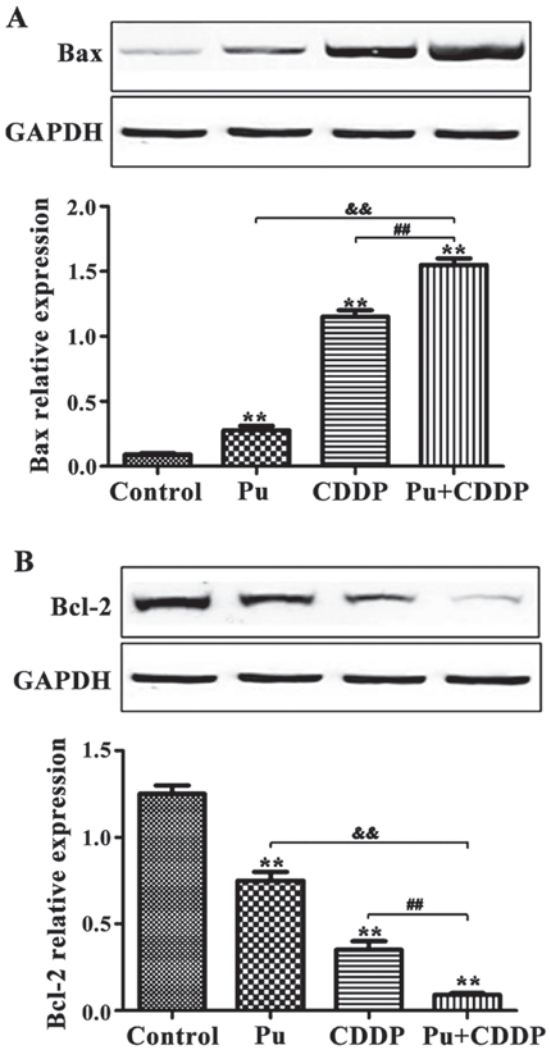

Figure 3. Determination of apoptosis-related protein expression by western blot analysis. (A) Bax protein expression; (B) Bcl-2 protein expression; **p<0.01 vs. the control group; ##p<0.01 the combined treatment group vs. the CDDP alone group; &&p<0.01 the combined treatment group vs. the Pu alone group. CDDP, cisplatin, Pu, puerarin.

The effect of Pu combined with CDDP on the expression of apoptosis-related proteins in HepG2 cells. Western blot

analysis results are shown in Fig. 3. Compared with the control group, 100 μM Pu or 10 μg/ml CDDP alone induced Bax protein expression in HepG2 cells. In contrast, Pu and CDDP in combination significantly increased Bax protein expression ($p<0.01$). The expression of Bcl-2 protein in HepG2 cells was downregulated by 100 μM Pu or 10 μg/ml CDDP alone and the inhibitory effect was more significant when Pu was combined with CDDP ($p<0.01$).

Discussion

HCC is a common malignant tumor and has a high mortality rate because of the lack of effective early diagnosis and therapy (9). Liver cancer has various causes, among which chronic liver inflammation caused by excessive drinking, viral hepatitis and non-alcoholic liver fatty degeneration is a key factor (10). As a method of discovering new drugs, extracting natural compounds from Chinese herbal medicines has been increasingly accepted and employed. This not only results in acquiring better active components, but also better control of the quality of Chinese medicines. Therefore, extracting chemically active substances from traditional Chinese medicine has been an area of strong interest in antitumor studies. Pu belongs to the group of isoflavone flycoside compounds and is one of the main active ingredients of leguminous plants. It was approved for clinical use by the Ministry of Health in 1993. Initially, it was primarily used for the treatment of cerebrovascular disease and as the result of further study, Pu was found to have a certain effect on cancer treatment (11).

Bcl-2 has been shown to inhibit apoptosis (12-14) and it plays an important role in the mechanism of apoptosis. It can protect cells from various causes of death, and improve cell survival, thereby increasing the number of cells. In some tumor cells, when Bcl-2 gene expression is upregulated, tumor cells are prevented from dying, or have their survival prolonged (15), suggesting that the Bcl-2 gene is closely related to the tumor. In contrast, Bax can promote apoptosis. Bcl-2 and Bax belong to the same gene family, and Bax binds the Bcl-2 protein, which can not only inhibit Bcl-2-mediated apoptosis, but also directly promote cell apoptosis (16,17). The BH1 and BH2 domains in the coding region of the Bax gene are highly homologous to the Bcl-2 gene, which are important components involved in the regulation of apoptosis. When Bcl-2 forms a homodimer, it plays a role in inhibiting apoptosis. When Bax protein expression increases and aggregates with Bcl-2 forming a dimer, or Bax protein itself homodimerizes, it plays a role in promoting apoptosis (11).

In this study, we found that Pu or CDDP alone inhibited HepG2 cell proliferation and induced apoptosis. The inhibitory effect on HepG2 proliferation was significantly greater in the Pu and CDDP combination group compared with the same doses in the single drug groups. The morphological changes such as shrinkage, decreased cell adherence, reduced cell number and increased cell death number were more obvious in the combined drug group. The results of flow cytometry using Annexin V-PI double staining showed that the combination of the two drugs resulted in a significantly higher rate of apoptosis compared with the single drug groups. Western blot analysis showed that compared with the control group, Pu or CDDP alone induced the expression of Bax protein in HepG2 cells. However, the expression of Bax protein was more significant when Pu and CDDP were used in combination. Pu or CDDP alone downregulated the expression of Bcl-2 protein in HepG2 cells. The inhibitory effect on the expression of Bcl-2 protein was significant in the Pu and CDDP combination group compared with the same doses in the single drug groups. The study by Xi *et al* (18) indicated that some active ingredients of traditional Chinese medicines can upregulate Bax expression by downregulating Bcl-2 expression in tumor cells, thereby inducing apoptosis of tumor cells. For example, carnosol was shown to upregulate Bax expression and downregulate Bcl-2 expression by 34-53%. Neri *et al* (19) showed that the expression of Bax protein was upregulated and the expression of Bcl-2 was downregulated when epithelial tumor cells of the digestive tract were abnormal, which was likely related to the formation of digestive tract tumors. Their results were similar to those of our study, further confirming that Pu increases the apoptosis of liver cancer cells, likely by upregulating Bax and downregulating Bcl-2 protein expression.

In conclusion, this study demonstrated that Pu can enhance the sensitivity of HepG2 cells to chemotherapeutic drugs and induce the apoptosis of HepG2 cells. The mechanism is likely related to the upregulation of Bax and downregulation of Bcl-2 protein expression.

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