RESEARCH ARTICLE

Potential Therapeutic Efficacy of Curcumin in Liver Cancer

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Abstract

Purpose: Liver cancer, one of the most common cancers in China, is reported to feature relatively high morbidity and mortality. Curcumin (Cum) is considered as a drug possessing anti-angiogenic, anti-inflammation and anti-oxidation effect. Previous research has demonstrated antitumor effects in a series of cancers. Materials and Methods: In this study the *in vitro* cytotoxicity of Cum was measured by MTT assay and pro-apoptotic effects were assessed by DAPI staining and measurement of caspase-3 activity. *In vivo* anti-hepatoma efficacy of Cum was assessed with HepG2 xenografts. Results: It is found that Cum dose-dependently inhibited cell growth in HepG2 cells with activation of apoptosis. Moreover, Cum delayed the growth of liver cancer in a dose-dependent manner in nude mice. Conclusions: Cum might be a promising phytomedicine in cancer therapy and further efforts are needed to explore this therapeutic strategy.

Keywords: Curcumin - apoptosis - liver cancer - in vitro - in vivo

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Introduction

Liver cancer, one of the most common cancers in China, is reported with relatively high morbidity and mortality. Due to its resistance to chemotherapy, the response and survival of patients with liver cancer remains poor (Di et al., 2012; Kudo et al., 2012; Li et al., 2012; Ni et al., 2013). Recent studies focus on the potential of Traditional Chinese Medicine (TCM) in treating different kinds of cancers (Yin et al., 2011; Li et al., 2012).

Curcumin (Cum) is a phenolic pigment isolated from the root of Turmeric (Curcuma Longa) (Link et al., 2013; Yin et al., 2013). It is formerly considered as a drug possessing anti-angiogenic, anti-inflammation and anti-oxidation effect (Kwon et al., 2005; Suckow et al., 2006; EI-Azab et al., 2011; Wu et al., 2013). Previous researches in 2013 have demonstrated the antitumor effect of Cum in a series of cancers (Bayet-Robert et al., 2013; Du et al., 2013; Kumaravel et al., 2013; Masuelli et al., 2013; Ono et al., 2013; Sun et al., 2013; Wei et al., 2013; Yallapu et al., 2013; Zhang et al., 2013).

In the current study, we systematically evaluated the in vitro and in vivo anticancer efficiency of Cum in liver cancer. The cell inhibition effect of Cum was measured by MTT assay. Apoptotis of liver cancer cells was measured by DAPI and the activity of Caspase-3. The growth curve of tumor volume and bodyweight of the mice were measured every two days.

Materials and Methods

Materials

Curcumin, was purchased from Sigma Chem.Co., (St. Louis, USA). All other chemicals were of analytical grade and used without further purification. Human liver cancer cell line HepG2 was obtained from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China).

Male and female nude mice (nu/nu; 6–8 weeks old and weighing 18–22 g) were purchased from Model Animal Research Center of Nanjing University (Nanjing, China). The mice were housed and maintained in the animal facility of the Animal Center of Nanjing Medical University. The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

In vitro cytotoxicity

The half maximal inhibitory concentrations (IC50s) of HepG2 cells were determined by the MTT assay. Briefly, cells were seeded in 96-well plates (1×10^4 cells per well) 24 h prior to the assay. Then cells were exposed to a series of doses of Cum. After 24, 36 and 48 hrs of incubation, 20 μl of 5 mg/mL MTT solution was added to each well and the plate was incubated for 4 h. Then, the media were removed and dimethylsulfoxide (DMSO) (150 μL) was added to each well. The optical density (OD) of each well

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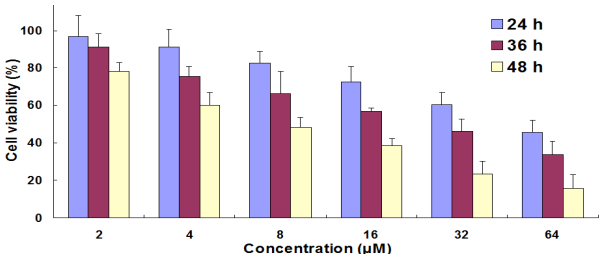

Figure 1. Effects of Cum on HepG2 Cell Proliferation. (A) HepG2 cells were treated with Cum at 2, 4, 8, 16, 32 and 64 μM for 24, 36 and 48 hours

was measured using a microplate reader at 560 nm (Bio-Rad, Hercules, USA).

Cell viability was determined by following formula:

Cell viability (%) = OD (test well)/OD (reference well) × 100% (1)

All the results obtained from MTT assays were confirmed by repeating the experiment on at least three independent occasions and testing in triplicate each time.

DAPI staining

The cells were treated with Cum at three doses (4, 8 and 16 μM) for 48 hrs, and then washed once in PBS followed by fixation in cold methonal : acetone (1:1) for 5 min. After washing thrice in PBS for 5 min, these cells were treated with 4 μg/ml DAPI for 10 min at routine temperature. The cells treated with the combination of the agents showed morphological changes of apoptosis, including a condensed and fragmented nuclear structure and decreased cell size (original magnification 200×).

Caspase-3 activity analysis

HepG2 cells were treated with a series of doses of Cum for 48h. Determination of caspase-3 activity was performed by the caspase colorimetric protease assay kit (Keygen Biotech, Nanjing, China) by following the manufacturer's instruction. The optical density was measure at 405 nm. The obtained values were expressed as folds of controls.

In vivo antitumor efficacy

Nude mice implanted with HepG2 cell line were used to qualify the antitumor efficacy of Cum through intravenous administration. The mice were raised under specific pathogen-free (SPF) circumstances and all of the animal experiments were performed in full compliance with guidelines approved by the Animal Care Committee of Nanjing Medical University. The mice were subcutaneously injected at the left axillary space with 0.1 ml of cell suspension containing 4–6×106 HepG2 cells. Treatments were started after 7-8 days of implantation. The mice whose tumor reached a tumor volume of 100 mm^3 were selected and this day was designated as "Day 0".

On Day 0, the mice were randomly divided into four groups, with each group being composed of 6 mice. The mice were treated intravenously with saline and a series doses of Cum, respectively. Cum was administered at a equivalent dose of 20, 40, and 60 mg/kg. All mice were tagged, and tumors were measured every other day with calipers during the period of study. The tumor volume was calculated by the formula (W^2×L)/2, where W is the tumor measurement at the widest point, and L is the tumor dimension at the longest point.

Table 1. IC50s of Cum Against HepG2 Cells at Different Incubation Time

IC50s (μM)	24 h	48 h	72 h
Cum	45.7±3.2	23.9±1.7	8.0±0.5

Each animal was weighed at the time of treatment so that dosages could be adjusted to achieve the mg/kg amounts reported. Animals also were weighed every other day throughout the experiments. After 15 days of injections, the mice were sacrificed for the detection of peripheral blood parameters as well as liver and kidney functions.

Statistical analysis and research experience

Results were presented as Mean±SD. Statistical comparisons were made by t test or ANOVA analysis. The accepted level of significance was P value < 0.05.

We have enough experience in conducting medical researches, including clinical researches, and have published some results elsewhere (Huang et al., 2004; Zhou et al., 2009; Jiang et al., 2010; Yan et al., 2010; Gao et al., 2011; Huang et al., 2011; Li et al., 2011; Li et al., 2011; Li et al., 2011; Xu et al., 2011; Xu et al., 2011; Xu et al., 2011; Yan et al., 2011; Zhang et al., 2011; Gong et al., 2012; Gong et al., 2012; Gu et al., 2012; Li et al., 2012; Yu et al., 2012; Zhan et al., 2012; Zhan et al., 2012; Deng et al., 2013; Huang et al., 2013; Liu et al., 2013; Liu et al., 2013; Lu et al., 2013; Wu et al., 2013; Yin et al., 2013; Yin et al., 2013).

Results and Discussion

In vitro cytotoxicity of Cum against HepG2 cells

It is shown in Figure 1 that Cum inhibits the proliferation of HepG2 cells in a dose and time dependant manner. The cell viability decreases steadily when the dose of Cum increases as well as the incubation time prolongs. As indicated in Table 1, the IC50 value of Cum against HepG2 cells for 24h is 45.7±3.2 μM, while the IC50 of Cum for 36h is nearly a half (23.9±1.7 μM). Moreover, the longest incubation time of 48h produces the strongest inhibition effect of Cum with an IC50 value of 8.0±0.5 μM.

Apoptotic staining

It is indicated in Figure 2 that escalated doses of Cum leads to the increase of the apoptosis of HepG2 cells. As shown in Figure 2A-D, cells in control group have rounded and intact nuclei with diffused 4', 6-diamidine-2'-phenylindole dihydrochloride (DAPI) staining, while cells exposed to Cum have smaller and brighter stained nuclei with condensed chromatin forming crescent-shaped profiles around the periphery of the nucleus or separate globular structures (apoptotic bodies). Quantitative analysis demonstrates the dose-dependent pro-apoptotic effect in HepG2 cells (Figure 2E). Each dose of Cum led to significantly higher cellular apoptosis rate than that in control group (p<0.05). In detail, there was more

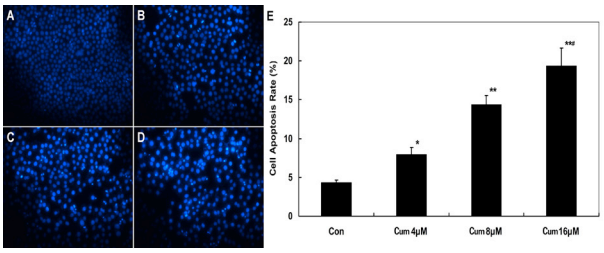

Figure 2. Apoptosis of HepG2 Cells Detected by DAPI Staining. (A) The non-treated cells. (B) Cells were treated with 4μM Cum. (C) Cells were treated with 8 μM Cum. (D) Cells were treated with 16 μM Cum. E: Quantitative analysis of apoptotic rate of cells exposed to different agents. Values represents Mean±SD (n = 3). *means $p<0.05$ vs control group. **means $p<0.01$ vs control group. #means $p<0.05$ vs 8 μM Cum

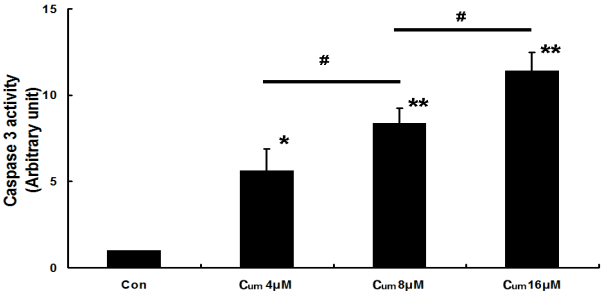

Figure 3. Analysis of caspase-3 activity in Cells Exposed to a Series of Doses of Cum. Values represents Mean ± SD. * represents $p<0.05$ vs control, **represents $p<0.01$ vs control, #represents $p<0.05$

than 8% cells exhibiting apoptosis in cells exposed to the low dose of Cum (4 μM), while less than 5% cells underwent apoptosis in control group. Most importantly, there is significant increase in apoptosis rate among groups exposed to different doses of Cum ($p<0.05$).

Caspase-3 activation

It is reported that the Caspase family is crucial in apoptosis. Caspase-3, the key member of Caspase family, is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins (Szymczyk et al., 2006). Therefore, caspase-3 is essential for certain processes associated with the dismantling of the cell and the formation of apoptotic bodies. In the current study, the same doses of Cum produced similar tendency as in DAPI staining (Figure 3). Increasing the dose of Cum caused the elevation of Caspase-3 activity with a remarkable difference, which was more significant than in control group. Results from DAPI staining and Caspase-3 activity demonstrate the potential of Cum in promoting the apoptosis of HepG2 cells.

In vivo antitumor evaluation of Cum in HepG2 xenograft mice

The therapeutic efficiency of Cum was evaluated in HepG2 human liver cancer xenografts in nude mice. As shown in Figure 4, Cum dose-dependently delayed tumor growth in HepG2 xenografts. All the three doses of Cum significantly inhibited the growth of liver cancer since Day 5 ($p<0.05$ vs control). Moreover, the group that received 60 mg/kg Cum was observed to maintain the greatest amount

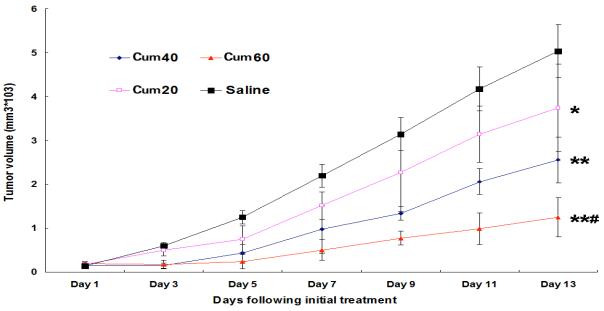

Figure 4. Antitumor Effect of Cum in HepG2 Xenograft Models. (A) Tumor volume of established HepG2 xenografts in nude mice during therapy under different treatments. Mice were treated with different protocols on Day 0 as showed in the figure. Saline: vehicle; Cum was administered at the doses of 20, 40 and 60 mg/kg. Different agents were delivered through intravenous pathway when tumor volume measured 100 mm3. Data are presented as mean±SD (n = 6). The difference between tumor volumes in the group of saline and Cum is significant (*means $P<0.05$, * means $P<0.01$). Significant difference (#means $P<0.05$) also is observed between the group receiving 60 mg/kg Cum and 40 mg/kg Cum

of anti-tumor activity among all the four groups (Figure 4). In detail, The tumor volumes of the group received low dose of Cum (20 mg/kg) is nearly 3740 mm^3 at the end of treatment, while that of the group received high dose of Cum (60 mg/kg) is around 1300 mm^3, which is the lowest among all the groups indicating the strongest tumor inhibition. Statistical analysis reveals the significant differences between the group receiving Cum and control group. It is also noted that the high dose of Cum inhibited the growth of tumor more significantly than the other two doses of Cum.

In conclusion, the current study demonstrates the antitumor effect of Cum in the treatment of liver cancer. In vitro cytotoxicity evaluation indicates that Cum possesses a dose-dependent cell inhibition effect against HepG2 cells with the activation of Caspase-3. In vivo evaluation shows that Cum effectively inhibits the growth of liver cancer in a dose-dependent manner in nude mice. Therefore, Cum might be a promising phytomedicine in cancer therapy and further efforts are needed to explore this therapeutic strategy.

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