Anti-proliferative effects of cucurmosin on human hepatoma HepG2 cells

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Abstract. We extracted cucurmosin (CUS) from the sarcocarp of Cucurbita moschata (pumpkin). Recently, a number of studies have indicated that CUS has cytotoxic properties and induces apoptosis in a number of human tumor cells. However, the detailed mechanisms are largely unknown. The aim of this study was to confirm CUS’s anticancer activity on human hepatoma HepG2 cells in vitro and in vivo, and to elucidate the mechanism of its activity. MTT was used to detect the cytotoxic effects of CUS. Flow cytometry was used to analyze cell apoptosis and the cell cycle. Transmission electron microscopy was used to observe the morphology of apoptotic cells. Western blot analysis was performed to measure the protein expression of bax, bcl-2 and procaspase-3. The established orthotopic transplantation models of human hepatoma in NOD/SCID mice were tested for anticancer activities in vivo. The results showed that CUS inhibited the proliferation of HepG2 cells in vitro and in vivo. CUS induced apoptosis and arrested the cell cycle. In addition, CUS increased the protein expression of bax, but decreased the bcl-2 and procaspase-3 expression in HepG2 cells. Our data indicate that CUS has potential anticancer activity for human hepatoma, which can be attributed in part to its inhibition of proliferation and apoptosis, induced by decreasing the bcl-2:bax ratio and caspase-3 activation.

Introduction

Cucurmosin (CUS) is an active component extracted from the sarcocarp of Cucurbita moschata (pumpkin), a type of vegetable, which has long been used for medicinal purposes in China. The overall structure of CUS has been elucidated (Fig. 1) and has been proved to be one of the type 1 ribosome-inactivating proteins (RIPs) (1-3). For a long time, the interest in RIP has been focused on developing antitumor drugs that selectively target tumor cells.

Studies in vitro have shown that CUS inhibits the proliferation of murine melanoma B16, lung adenocarcinoma cancer A549, human chronic myelogenous leukemia K562 cells and human pancreatic cancer PANC-1 cells (3-5); induces apoptosis of human PANC-1, HL60 and K562 cells (data not shown); and induces the differentiation of B16 cells (6).

Based on its cytotoxic activity against multiple human cancer cells through the induction of apoptosis and/or differentiation, we hypothesized that CUS is a candidate agent for human hepatoma treatment and/or chemoprevention. To confirm this hypothesis, the inhibitory effects of CUS on the growth of human hepatoma cells in vitro, the growth inhibition of human hepatoma in vivo and the mechanism of its activity were investigated in this experimental study.

Materials and methods

Reagents. CUS (99% purity; molecular weight 27 kDa) was isolated and dissolved in normal saline (NS) and stored at -80°C in the laboratory (3.68 mg/ml). RPMI-1640 cell culture medium, trypsin and fetal calf serum were purchased from Invitrogen (Carlsbad, CA, USA). DMSO and MTT were purchased from Sigma (St. Louis, MO, USA). Antibodies against bax, bcl-2, procaspase-3, β-actin and the secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Annexin V/PI-FITC kit and enhanced chemiluminescence (ECL) reagents were purchased from Beyotime Institute of Biotechnology (Haimen, China).

Cells and animals. HepG2 cells were purchased from the Cell Bank of Shanghai Institute of Cell Biology (Shanghai, China), maintained in RPMI-1640 culture medium plus 10% calf serum, 100 U/ml penicillin and 75 U/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Four-week-old NOD/SCID male mice were purchased from SIBS (Shanghai, China). The maintainance, use and treatment of all animals in this study were in accordance with accepted standards of the Ethics Committee of ECNU. The experimental protocol was
approved by the China Medical Experimental Animal Care Committee.

**Cell viability assay.** Cells in the exponential phase were seeded into 96-well plates, 100 µl (1x10⁵ cells/ml) per well. Then, various concentrations of CUS in 100 µl culture medium were added and the final concentration in each well was 1, 2, 4, 8, 16, 32 and 64 µg/ml, respectively. Each treatment was tested in tetrad wells and the control group was administered culture medium containing no drug. All of the above plates were placed in a CO₂ humidified-atmosphere incubator at 37°C for 24, 36, 48, 72 and 96 h. At the end of exposure, 20 µl MTT (5 mg/ml) was added to each well and the plates were incubated at 37°C for 4 h. Then, all culture medium supernatant was removed from the wells and replaced with 200 µl DMSO. The plates were agitated for 10 min so that all of the formazan that had been produced could be dissolved. The absorbance of each well was measured by standard enzyme-linked immuno-sorbant assay at 570 nm. The cell viability was calculated based on the following formula: cell viability (%) = average A570 nm of treated group/average A570 nm of control group x 100%.

**Ultrastructure analysis using transmission electron microscopy (TEM).** Following exposure to CUS, and pre-fixing with 2.5% glutaraldehyde and 2% paraformaldehyde at 4°C for 12 h, the cells were post-fixed with 1% osmium tetroxide, dehydrated in an ethanol series and embedded in Spurr Epon. Thin sections were stained with 4% uranyl acetate and 0.2% lead citrate and examined on a JEOL 200 EX II electron microscope (JEOL, Tokyo, Japan).

**Cell cycle analysis.** Cells were collected and washed twice in cold phosphate-buffered saline (PBS). The cells were fixed in 70% ethanol, treated with 100 µg/ml RNAse at 37°C for 30 min and stained with 50 µg/ml propidium iodide (PI) (Sigma) for 30 min in the dark. Then, the percentage of cells in each phase of the cell cycle was detected and analyzed using ModFit software (Becton Dickinson, San Jose, CA, USA).

**Annexin V/PI double staining assay.** Cells were collected, washed twice in cold PBS and resuspended in 500 µl binding buffer (Sigma). Annexin V (10 µl) and PI solution (5 µl) were added to the cell preparations, and incubation was carried out for 30 min in the dark at room temperature. Binding buffer (400 µl) was then added to each tube and the samples were analyzed by FACSscan flow cytometry equipped with CellQuest software (Becton Dickinson).

**Western blot analysis.** Briefly, the HepG2 cells were plated at a density of 2x10⁵ cells/well. Following treatment, the cells were collected and lysed. The protein concentration was determined by the Lowry method using BSA as a standard. Equal amounts of protein were separated on 10% SDS-PAGE gels. The protein was then electrophoretically blotted onto polyvinylidene fluoride (PVDF) membranes. The membranes were first hybridized with primary antibodies and then with the secondary antibody. The immune blots were developed using the ECL system.

**In vivo experiments.** HepG2 cells (1x10⁵) in 0.2 ml NS were injected subcutaneously into the back of NOD/SCID mice. When the tumor reached ~2 cm in diameter, the tumor tissue was cut into 1x1x1 mm sections and implanted into the livers of 50 NOD/SCID mice by using a trocar catheter and a pair of ophthalmologic forceps. The wound was occlusively treated by medical biological glue for hemostasis and closed. On the 7th day following the establishment of the model, exploratory laparotomy was performed in all animals. The success rate of the model was 100%. The 50 models were randomized into five groups: 0.25 mg/kg CUS group (CUS-1), 0.5 mg/kg CUS group (CUS-2), 1 mg/kg CUS group (CUS-3), positive group (25 mg/kg CTX group) and negative group (NS group). Five groups were administered the drug according to the above dose groups via tail intravenous injection every other day from the 10th to the 16th day. On the 24th day, half of the animals in each group were sacrificed. The body and tumor weight of these mice were detected. The remaining animals in each group were used to observe the survival time of tumor-bearing in the following assay. The tumor inhibitory rate % = (1 - mean tumor weight of the drug group/mean tumor weight of the control group) x 100%; the prolonged survival rate % = (mean days of survival of the treatment group/mean days of survival of the control group - 1) x 100%. The survival time.
was calculated from the day from the establishment of the model to mortality.

**Statistical analysis.** The results were expressed as the means ± SD. The significance of differences in multiple comparisons was determined using the Student’s t-test. *P*<0.05 was considered to be statistically significant.

**Results**

**Cell viability.** As shown in Fig. 2, the viable cell percentages relative to the control were 90.12±3.93, 82.26±5.14, 71.94±6.27, 63.17±4.79, 50.73±3.99, 46.17±5.24 and 44.43±6.11%, respectively, when treated with various concentrations of CUS (1, 2, 4, 8, 16, 32 and 64 µg/ml) for 24 h. The viable cell percentages relative to the control were 80.12±3.42, 68.14±3.58, 60.19±2.05, 57.74±4.29, 45.51±2.62, 33.13±2.19 and 25.45±2.77%, respectively, when treated with various concentrations of CUS (1, 2, 4, 8, 16, 32 and 64 µg/ml) for 48 h. The viable cell percentages relative to the control were 70.78±4.46, 69.74±3.58, 51.33±3.95, 45.53±3.49, 35.43±4.69, 31.53±2.89 and 21.95±4.71%, respectively, when treated with various concentrations of CUS (1, 2, 4, 8, 16, 32 and 64 µg/ml) for 72 h. The viable cell percentages relative to the control were 51.36±1.28, 42.41±1.71, 32.63±0.29, 21.73±1.17, 14.23±0.79, 11.15±1.21 and 9.61±2.11%, respectively, when cultured with various concentrations of CUS (1, 2, 4, 8, 16, 32 and 64 µg/ml) for 96 h. The IC₅₀ were 18.24±4.55, 13.17±3.31, 6.34±2.19 and 1.27±0.37 µg/ml, respectively, when HepG2 cells were treated with CUS for 24, 48, 72 and 96 h. The proliferation of HepG2 cells was markedly inhibited by CUS in a dose- and time-dependent manner.

**Cell cycle analysis.** To analyze the effect of CUS on HepG2 cell growth and apoptosis, the cell cycle distribution was determined by flow cytometry. As shown in Table I, when HepG2 cells were treated with CUS at 0, 2.5, 10 and 40 µg/ml for 48 h, the percentage of cells in the sub-G1 phase increased from 1.79±0.38 to 9.45±1.43, 11.23±2.72 and 17.85±3.29%, respectively. This increase was accompanied by an increase in the number of G0/G1 phase cells and a decrease in the cell population in the S phase.

**Expression of apoptosis-related proteins.** Western blotting was used to detect the levels of bcl-2 and bax during the CUS-induced apoptosis. As shown in Fig. 5, up-regulation of bax and down-regulation of bcl-2 in cells treated with CUS was observed. Next, we examined whether caspase-3 was activated during the induction of apoptosis by CUS. The level of procaspase-3 (32 kDa precursor) was significantly reduced in CUS-treated cells compared to the level of the control cells, which suggest that the activation of caspase-3 occurs in CUS-treated cells.

**Tumor growth in vivo.** To investigate the antitumor activities of CUS in vivo, NOD/SCID mice were used to establish...
XIE et al.: ANTI-PROLIFERATIVE EFFECTS OF CUCURMOSIN ON HEPG2 CELLS

As shown in Table II, there was no significant difference in the weight of mice among these five groups prior to the establishment of the model (p>0.05), indicating that the mice of the five groups were comparable in vivo. Compared to the NS group, the weights of the tumors of the CUS-1, CUS-2, CUS-3 and CTX groups were reduced significantly (p<0.01), particularly in the CUS-3 group, with a tumor inhibitory rate of 78.4%. The tumor inhibitory rate of the CTX, CUS-2 and CUS-1 groups was 69.8, 66.3 and 53.7%, respectively (Table II and Fig. 6). There was no significant difference in the weight of mice among all CUS groups and the NS group (p>0.05). Compared to the NS groups, the weight of mice in the CTX group decreased significantly (p<0.05).

**Mean survival and life prolonging rate.** Tumor-bearing survival of the CUS-1-3 groups was prolonged, and the difference was significant among all the CUS groups and the NS and CTX groups (p<0.05), particularly in the CUS-3 group. Survival of the CTX group was not significantly prolonged compared to the NS group (p>0.05; Table III). There was no evident change in weight, appetite and behavior following administration of CUS at all concentrations, and there was no treatment-related mortality.

**Discussion**

Our previous study indicated that CUS exerted antitumor activities by inducing apoptosis in numerous tumor cell lines (data not shown). However, there have been no studies on the anticancer activity of CUS in human hepatoma cells in vitro and in vivo. The cell line was valuable for the rapid screening of potential anticancer agents and the elucidation of the mechanism of their activity (7). In the present study, the HepG2 cell line was used as a cellular model of hepatoma for drug screening. We confirmed that CUS exhibited strong dose- and time-dependent anticancer activity in human hepatoma HepG2 cells in vitro.

Next, the results of flow cytometry showed that CUS induced significant cell cycle arrest at the G0/G1 interface,
structure homology and studies have demonstrated that CUS is a promising agent in inhibiting the growth and relatively low toxicity in hepatoma HepG2 cells. It may affect hepatoma progression as a result of its effects on cell cycle progression and apoptosis by reducing the ratio of bcl-2/bax and the activation of caspase-3. However, determination of the optimal dosage in vivo and other related mechanisms is necessary in order to establish the scientific basis for the possible application of CUS in the treatment of hepatoma.

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**References**


