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N-Methylcantharidinimidum Induces Apoptosis in Human Hepatocellular Carcinoma Cell HepG2

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors and the prognosis of patients was poor. Cantharidin is an effective anti-tumor component of Chinese traditional medicine. N-methylcantharidinimidum is a novel cantharidin derivative with better curative effect and less side effect. In this study, the effects of N-methylcantharidinimidum on proliferation, apoptosis and cell cycle of human hepatocellular carcinoma cell line HepG2 were examined. The results demonstrated that N-methylcantharidinimidum significantly induced apoptotic cell death in a dose and time-dependent manner and arrested cell cycle in HepG2 cells. N-methylcantharidinimidum also suppressed cyclin D1. This study suggests that N-methylcantharidinimidum may serves as a potential chemopreventive agent for live cancer.

Keywords

N-Methylcantharidinimidum, HepG2, Apoptosis, HCC

1. Introduction

Liver cancer is the third leading cause of cancer-related death worldwide. Although young patients have an improved prognosis, older patients have an increased mortality rate after being diagnosed with liver cancer. Cantharides is a kind of arthropod insect and cantharidin is a sesquiterpenoid derivative extracted from cantharides. Cantharidin is an effective anti-tumor component of Chinese traditional medicine [1]. It has been shown to effectively inhibit cell proliferation in numerous cancer cell lines including colorectal, hepatic, bladder, pancreatic and breast cancers [2] [3] [4]. The clinical application of can-

tharidin is restricted due to its severe irritant to the digestive and urinary systems. N-methylcantharidinimidum is a novel cantharidin derivative. In xenograft model, the survival time of liver cancer mice was prolonged by N-methylcantharidinimidum at the concentration of 16 - 18.5 mg/ml. The clinical trial was used to treat primary liver cancer, and its curative effect was better than cantharidin and did not have apparent toxic side effect on digestive and urinary systems. In this study, HepG2 cells were used to observe the anti-tumor effect and its mechanism of N-methylcantharidinimidum.

2 Materials and Methods

2.1. Cell Culture

N-methylcantharidinimidum was a generous gift from Chongqing Institute of Chinese Materia Medica. Hepatocellular carcinoma cell HepG2 was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured with complete medium MEM/EBSS (HyClone, UT, USA) containing 10% fetal bovine serum (Gibco BRL, MD, USA), 2 mM glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin. Cells were maintained at 37˚C in a humidified atmosphere of 5% CO_2.

2.2. Cell Proliferation Assay

The effect of N-methylcantharidinimidum on cell proliferation was assessed by MTT assay. HepG2 cells were seeded at a density of 1×10^4 cells per well in triplicate in 96-well plates and cultured for 24 h to allow the cells for attachment. N-methylcantharidinimidum was dissolved in DMSO and diluted in MEM/EBSS. The cells were treated with 5, 6, 7 and 8 mg/ml of N-methylcantharidinimidum for 24, 48 and 72 h. After treatment, 20 μl of 5 mg/ml MTT solution was added to each well and incubated for 4 h at 37˚C. The supernatant was removed and 200 μl of DMSO was added to each well. The absorbance was quantified using enzyme-linked immunosorbent assay (ELISA) reader at 570 nm.

2.3. Morphological Analysis

According to the results of MTT assay, HepG2 cells (5×10^4/ml) were inoculated in 24-well plates with presetted slides, the cells were treated with 5, 6 and 7 mg/ml of N-methylcantharidinimidum for 1 to 6 days. Then the slide were removed and stained with Wright's stain, and observed under light microscope.

2.4. Flow Cytometric Analysis

HepG2 cells at 5×10^5 cells/ml were inoculated into 6-well culture plate and incubated at 37˚C. The next day, after the medium was removed, 2 ml of MEM/EBSS complete medium with 6 mg/ml N-methylcantharidinimidum was added to each well. After cultured for 3, 4 and 5 d, cells were harvested by trypsinization, washed three times with PBS, and suspended in 500 μl binding buffer. PI (50

mg/ml, 5 ml) was added and followed by incubation at room temperature in dark for 30 min. The apoptosis rate was immediately measured by FACSCalibur (BD Biosciences, USA). To evaluate the effect of N-methylcantharidinimidum on the cell cycle, the harvested cells were washed twice with ice-cold PBS, fixed with ice-cold 70% ethanol and maintained overnight at −20˚C. DNA was stained with 100 μg/ml propidium iodide (PI) solution. The cell cycle distribution was analyzed by flow cytometry.

2.5. TUNEL Assay

In situ cell death, detection kit-POD (Boehringer Mannheim, German) was used to detect the late-stage apoptosis. Cells were seeded in 96-well plates and treated with 6 mg/ml of N-methylcantharidinimidum. Cells were fixed with 4% paraformaldehyde. Fixed cells were penetrated with 0.5% Triton X-100 for 2 min on the ice and then incubated with TUNEL reaction mixture for 1 h at 37˚C. All the slides were stained by DAB coupling and counterstained with hematoxylin. The cells were then examined using a light microscope.

2.6. Western Blot Analysis

HepG2 cells were treated with 6 mg/ml N-methylcantharidinimidum for 3 d. Total protein of cells was resolved by SDS-PAGE and transferred onto a PVDF membrane. After blocking with 5% non-fat milk in tris-buffered saline with 0.1% Tween 20 (TBST), membranes were incubated with respective primary antibodies directed against CyclinD1 (1:1000) and β-actin (1:3000) at 4˚C overnight. Membranes were washed and then incubated with HRP-conjugated rabbit anti-IgG (1:5000) for 1 h at room temperature. Protein bands were assessed by enhanced chemiluminescence system (ECL, KeyGEN, China).

2.7. Statistical Analysis

All statistical analyses were evaluated using SPSS 17.0 software. The significance of difference between the groups was analyzed with two-way ANOVA test or two-tailed unpaired Student's t-test. P-values < 0.05 were considered as statistically significant.

3. Results

3.1. N-Methylcantharidinimidum Inhibits Cell Proliferation

The effect of N-methylcantharidinimidum on HepG2 cells was investigated by MTT assay. The results showed that N-methylcantharidinimidum inhibited the proliferation of HepG2 cells in dose- and time-dependent manners (**Table 1**). The cytostatic does of 6 mg/ml was taken for further study of N-methylcantharidinimidum on HepG2 cells.

3.2. N-Methylcantharidinimidum Induces Cell Apoptosis

1) The morphological changes of cells were observed by light microscope.

Table 1. Inhibitory Effect of N-methylcantharidinimidum on the growth of HepG2 cell.

N-methylcantharidinimidum (mg/ml)	Inhibitory rate (72 h)
5	24.3%
6	40.4%
7	67.2%
8	89.6%

After treated with N-methylcantharidinimidum, vacuoles appeared in the cytoplasm. Some cells were shrunken, with condensed nuclear chromatin and cytoplasm. Condensed nuclei were frequently fragmented. Apoptosis was induced after 3 days of treatment, and the number of apoptotic cells increased gradually with the extension of treat time.

2) Apoptosis was detected by flow cytometric analysis. 6 mg/ml N-methylcantharidinimidum promoted HepG2 cells apoptosis after an exposure of 3, 4 and 5 days (22.17%, 23.87% and 33.79% of cells, respectively). The result also showed that N-methylcantharidinimidum induced cell cycle arrest at G2/M phase.

3) In situ determination of apoptosis by TUNEL. The TUNEL assay was used to detect DNA fragmentation characteristic for apoptosis, seen as nuclei stained dark brown. A number of TUNEL positive cells substantially increased after 6 mg/ml N-methylcantharidinimidum treated for 4 days, dark brown granules were seen in the nucleus, indicated that DNA strand breaks had occurred.

3.3. N-Methylcantharidinimidum Decreased the Expression of Cyclin D1

The protein level of cyclin D1 was determined by immunoblotting. As shown in **Figure 1**, expression of cyclin D1 decreased in HepG2 cell after treated with 6 mg/ml N-methylcantharidinimidum for 3 days. β-actin served as an internal control.

4. Discussion

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors and the third leading cause of cancer-related mortality worldwide [5] [6]. Chemotherapy is one of the therapeutic methods for HCC treatment [7], but the effects are poor due to the relapse of disease and resistance to chemotherapeutics [8]. Recent years, many natural products with stronger antitumor activity were studied and some components of traditional Chinese medicine have been shown to have anti-tumor effects. Cantharidin, a type of terpenoid obtained from the blister beetle, is used as a traditional Chinese medicine with antibiotic, antiviral, anticancer and immune-regulating activities. Studies have shown that cantharidin and its derivatives exert cytotoxic effects against cancer cells through cell growth inhibition and cell apoptosis induction *in vitro* and *in vivo*. These

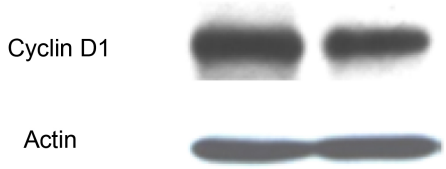

Figure 1. Expression of cell cycle-controlling protein Cyclin D1 in HepG2 cell.

compounds inhibit cell proliferation in numerous cancer cell lines such as hepatic, bladder, pancreatic, colorectal, leukemic, oral, and breast cancers [9] [10] [11]. In order to improve pharmacokinetic properties, increase efficacy and reduce toxic side effects, some new cantharidin derivatives were synthesized and be studied. Cantharidin showed inhibitory effects on murine ascites hepatoma and ascites reticulum cell sarcoma [12]. Cantharidin sodium and Shenmai injection combined with chemotherapy significantly reduced the incidence of side effects in postoperative breast cancer patients in clinical trial [13]. Norcantharidin, a demethylated analogue of cantharidin, induced cell apoptosis in human oral cancer cells through a mitochondria-mediated pathway [14]. Here, we investigated the anti-tumor effect and its mechanism of N-methylcantharidinimidum, a novel cantharidin derivative, in hepatocellular carcinoma cell line HepG2.

In this study, N-methylcantharidinimidum was shown to exert strong cytotoxicity against human hepatocellular carcinoma cell line HepG2, inhibiting cell proliferation *in vitro* through inducing apoptosis. The result of the cell proliferation assay showed that N-methylcantharidinimidum exerted a potent cytotoxic effect on HepG2 in a dose and time-dependent manner. The inhibition of proliferation in HepG2 cells was a result of apoptosis induction and cell cycle arrest. Apoptosis is an important homeostatic mechanism that is characterized by unique morphological and biochemical features and is used to maintain the appropriate numbers of cells in the body. In current study, we demonstrated that N-methylcantharidinimidum treated cells express an apoptotic reaction. Cyclin D1 is an oncoprotein that plays a key role in the development of tumor. High expression of cyclin D1 is considered to be indicative of a poor prognosis and related to an unfavorable therapeutic outcome. Overexpression of cyclin D1 protein leads to increased cell proliferation, which gives neoplastic cells a growth advantage and may also favor the occurrence of additional genetic lesions with potential oncogenic effects. Cyclin D1 is an important regulator of G1 phase progression in many different cell types. In this study, N-methylcantharidinimidum treatment decreased the level of cyclin D1 in HepG2 cells, which is correlated with the cell cycle analysis showing G2/M phase arrest.

In conclusion, our study demonstrated that N-methylcantharidinimidum treatment inhibited cell proliferation in HepG2. N-methylcantharidinimidum inhibited HepG2 cells mainly through apoptosis and cell cycle arrest. This work provides a novel insight that N-methylcantharidinimidum may serve as a potential candidate for chemoprevention of hepatocellular carcinoma in the future.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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