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Dihydroartemisinin exhibits antitumor activity toward hepatocellular carcinoma *in vitro* and *in vivo*

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ABSTRACT

Dihydroartemisinin (DHA), a semi-synthetic derivative of artemisinin isolated from the traditional Chinese herb *Artemisia annua* L., has been shown to exhibit inhibitory effects on human cancer cells. However, its antitumor ability toward hepatocellular carcinoma (HCC) has not been studied. In this study, we demonstrated that DHA significantly inhibited HCC cell growth *in vitro* and *in vivo* via inducing G2/M cell cycle arrest and apoptosis. The induction of p21 and the inhibition of cyclin B and CDC25C contributed to DHA-induced G2/M arrest. DHA-induced apoptosis was associated with mitochondrial membrane depolarization, release of cytochrome *c*, activation of caspases, and DNA fragmentation. Activation of caspase 9 and caspase 3, but not caspase 8, was detected in DHA-treated cells. Attenuation of apoptosis in cells pretreated with Z-VAD-FMK suggested the involvement of caspase cascade. Furthermore, p53 facilitated apoptosis caused by DHA. Bcl-2 family proteins were also responsible for DHA-induced apoptosis. DHA exposure decreased Mcl-1 expression but increased the levels of Noxa and active Bak. Bak was released from the Mcl-1/Bak complex due to the decline of Mcl-1. Further study revealed that Mcl-1 was rapidly degraded in DHA-treated cells and that DHA-induced apoptosis was largely inhibited by overexpression of Mcl-1 or RNAi-mediated decrease of Bak and Noxa. In a HCC-xenograft mouse model, the intraperitoneal injection of DHA resulted in significant inhibition of HCC xenograft tumors. Taken together, our data, for the first time, demonstrate the potential antitumor activity of DHA in HCC.

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1. Introduction

Hepatocellular carcinoma (HCC) is a malignancy of worldwide significance and is currently one of the most common solid tumors and the third leading cause of cancer-related death [1]. The incidence of HCC geographically varies, due to the large heterogeneity of the penetration of the risk factors within the population. About 80% of new cases occur in developing countries, but the incidence is increasing in economically developed regions, including Japan, Western Europe, and the United States [2,3]. HCC is frequently associated with liver cirrhosis and liver dysfunction, making the treatment of HCC more difficult than many other cancers [4,5]. To date, surgery is still the most effective treatment with curative potential, but only about 20% of patients with HCC are eligible for surgical intervention [6,7]. Therefore, numerous approaches have been conducted to search for other options such as efficient chemotherapeutic agents. Since apoptotic resistance is a major challenge that hampers the efficacy of anticancer treatment, accumulating attention has been paid to the development of innovative compounds that increase the death of therapy-resistant tumor cells, such as HCC cells [8].

One of the promising approaches for anti-HCC agent development is drug screening from natural products, such as components from traditional Chinese medicine. Artemisinin, the active ingredient derived from Chinese medicinal herb *Artemisia annua* L., is a safe and effective FDA-approved and WHO-recommended mainstay in treating malaria [9,10]. Recent studies have suggested that artemisinin also exerts preferentially cytotoxic effects on human cancers. Dihydroartemisinin (DHA, CAS 71939-50-9), the main active metabolite of artemisinin derivatives, has been demonstrated to exhibit antitumor effects toward various human cancers, including lung, ovarian, and pancreatic cancer [11–13]. For example, Ji et al. [14] reported that DHA inhibited proliferation of osteosarcoma cells via inducing the expression of cyclin D1 and

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Bax and inhibiting the activity of NF-κB. Handrick et al. [15] showed that DHA induced apoptosis via the intrinsic pathway in lymphoma cells. Gao et al. [16] demonstrated that DHA exhibited the anti-leukemic activity through inactivating MEK/ERK pathways. However, the detailed mechanism through which DHA inhibits cancer growth is not fully understood. Furthermore, the effect of DHA on HCC has not been studied.

In the present study, we investigated the antitumor effect of DHA, a semi-synthetic derivative of artemisinin, on HCC *in vitro* and *in vivo*. We found that DHA displayed cytotoxicities against HCC cells via inducing G2/M arrest and apoptosis. Induction of p21 and subsequent inhibition of cyclin B and CDC25C contributed to DHA-induced G2/M phase arrest. DHA-induced apoptosis was associated with the activation of caspases and PARP, mitochondrial membrane depolarization and cytochrome *c* release. Furthermore, p53 significantly facilitated DHA-induced apoptosis which required Mcl-1 degradation and Bak activation. Moreover, the *in vivo* efficacy of DHA on Hep G2-bearing nude mice revealed a significant remission of the xenograft tumor. Taken together, our data, for the first time, have revealed the potential antitumor effect of DHA on HCC.

2. Materials and methods

2.1. Cell culture and transfection

Human HCC cell lines (Hep G2, PLC/PRF/5 and Hep3B) were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD) containing 10% fetal bovine serum (FBS), 100 mg/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cells were seeded in 6-well plate for 24 h, and then transfected with pCDNA 3.1, pCDNA 3.1-Mcl-1, pCDNA 3.1-p53, according to the manufacture's instruction of Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

2.2. Antibodies and reagents

Primary antibodies for cyclin A, cyclin B, cyclin E, Beclin 1, Mcl-1, Bcl-xL, Bid, p53, PARP, Bak, β-Actin, Actin, COX-VI and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for CDC25C, ATG-5, LC3, active Bak, caspase 8, caspase 9, caspase 3, p21, Bcl-2, cytochrome *c*, Bax, and Noxa were provided by Cell Signaling (Danvers, MA). Dihydroartemisinin (dissolved in DMSO), Cycloheximide (CHX, dissolved in DMSO) and the caspase inhibitor (Z-VAD-FMK, dissolved in DMSO) were purchased from Sigma (St. Louis, MO).

2.3. MTT

Cell viability was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazo-lium bromide (MTT) assay. Briefly, 8×10^3 of cells were seeded into 96-well plates for 24 h, followed by incubation with various doses of DHA for indicated time. After adding 100 μl/well of MTT solution, the cells were incubated for another 2 h. Supernatants were then removed and the formazan crystals were dissolved in 100 μl/well DMSO. The absorbance at 570/630 nm of each sample was measured using multilabel plate reader (PerkinElmer). Three independent experiments were performed.

2.4. Colony formation

One hundred of cells were seeded into 6-well plates, and cultured for 5 d. And then the medium was replaced by fresh one containing DHA. After being incubated for another 5 or 10 d, colony formed by HCC cells was stained with 0.05% crystal violet (Sigma, St. Louis, MO) for 8 min. The number of colony was then quantified.

2.5. Cell cycle analysis

Following DHA treatment for indicated periods, HCC cells were washed with ice-cold PBS, and fixed with 70% ethanol at 4 °C for overnight. Ethanol was removed. Cells were re-suspended in PBS containing PI (50 μg/ml, Sigma) and RNase A (50 μg/ml, Sigma) for 30 min in dark. And then cells were subjected to flow cytometry (FACS Vantage, Becton Dickinson, Franklin Lakes, NJ). The percentage of cells at each cell cycle phase was analyzed.

2.6. Western blot

Cell lysates were boiled with 6× sodium dodecyl sulfate (SDS) loading buffer and then fractionated by SDS-PAGE. The proteins were transferred to PVDF membrane which was then incubated with a primary specific antibody in 5% of non-fat milk, followed by a horse radish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit second antibodies. ECL detection reagent (Amersham Life Science, Piscataway, NJ) was used to demonstrate the results.

2.7. Morphological analysis

To evaluate the apoptotic activity of DHA, we performed nuclear staining, using the DNA-binding dye Hoechst-33342. Cells were plated and exposed to DMSO, or 40 μM DHA for 24 h. Cells were then fixed with 4% PFA in PBS for 10 min. Fixed cells were washed with PBS, incubated with Hoechst-33342 (10 μg/ml, Sigma, St. Louis, MO) for 15 min in dark. Apoptotic cells were identified by condensation of chromatin and fragmentation of nuclei under a fluorescent microscope. Images were taken, using a camera Qimaging (Burnaby, BC, Canada).

2.8. TUNEL assay

Apoptosis assay was performed using Apo-Direct TUNEL Assay kit (Millipore, Billerica, MA). Cells were harvested and fixed in 4% PFA for 60 min at 4 °C, followed by a second fixation in 70% (v/v) ethanol overnight at −20 °C. Cells were then treated with various reagents for a designed period according to the manufacture's instruction. Finally, cells were analyzed by flow cytometry using FACS Vantage machine (Becton Dickinson). The Cell Quest software (Verity Software House) was used to analyze the data.

2.9. In situ cell death detection

Labeling of fragmented DNA to assess apoptosis was performed with TUNEL staining (green fluorescence), using *In Situ* Cell Death Detection Kit (Roche, Indianapolis, IN), as described in our previous study [17].

2.10. Mitochondrial membrane depolarization

Changes of mitochondrial transmembrane potential (lower $\Delta\Psi_m$) in HCC cells induced by DHA were measured using the same procedure as our previous study with JC-1 (Sigma, St. Louis, MO) staining at a dose of 2.5 μg/ml.

2.11. RNA interference

Small interfering RNAs (Invitrogen, Carlsbad, CA) for p53 (si-p53), Mcl-1 (si-Mcl-1), Bak (si-Bak), and Noxa (si-Noxa) were synthesized as 5′-GACUCCAGUGGUAAU CUAC-3′, 5′-GCATCGAACCATTAGCAGA-3′, 5′-GUACGAAGAUUCUUCAAA U-3′, and 5′-GGAGAUUUGGAGA-CAAA CU-3′, respectively. Nonspecific siRNA (si-ctrl) was also designed. For RNA interference, 2×10^5 of cells were seeded into 6-well Plates 24 h prior to transfection. For each well, 30 nM siRNA was

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