Life Sciences xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Dihydroartemisinin induced caspase-dependent apoptosis through inhibiting the specificity protein 1 pathway in hepatocellular carcinoma SK-Hep-1 cells

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ARTICLE INFO

Keywords:
Dihydroartemisinin
Sp1
Caspase
Apoptosis
SK-Hep-1 cells

ABSTRACT

Aims: Dihydroartemisinin (DHA) is a semi-synthetic derivative of artemisinin, well known for a safe and effective first-line antimalarial agent. This study investigated whether and how DHA induces apoptosis focusing on the specificity protein 1 (Sp1) pathway in hepatocellular carcinoma (HCC) SK-Hep-1 cells.

Main methods: The cell viability was evaluated by MTT assay. Cell cycle analysis was performed after PI staining by flow cytometry system. Apoptosis was confirmed by DAPI staining and by detecting cytoplasmic histone-associated-DNA-fragments using a cell death detection ELISA^{PLUS} kit. The expression of proteins involved in apoptosis was evaluated by Western blot. The nuclear localization of Sp1 was evaluated by immunofluorescence assay.

Key findings: DHA exerted potent cytotoxicity against HCC SK-Hep-1 cells compared with normal hepatocyte AML12 cells. The sub-G_1 DNA content and apoptosis index were increased by DHA, which was accompanied by nuclei condensation and fragmentation. DHA activated caspase 3, caspase 8, and caspase 9 and cleaved poly (ADP-ribose) polymerase (PARP). DHA-induced apoptotic cell death, activation of caspases and cleavage of PARP were dramatically inhibited by pan caspase inhibitor *Z*-VAD-FMK. DHA down-regulated protein expression and nuclear localization of Sp1, which in turn decreased Sp1 downstream target protein, X-linked inhibitor of apoptosis. Decreased Sp1 protein expression by DHA was restored by proteasome inhibitor MG132. DHA led to a down-regulation of phospho-ERK, -p38 and -JNK without affecting their total forms.

Significance: These results demonstrate that DHA induces caspase-dependent apoptosis in HCC SK-Hep-1 cells by proteasome-dependent degradation of Sp1, which is involved in mitogen-activate protein kinase pathway.

1. Introduction

Apoptosis, also known as programmed cell death, is an important physiological process and plays critical roles in development, morphogenesis, regulation of the immune system, and deletion of damaged and dangerous cells [1]. Tissue homeostasis are maintained by removal of superfluous cells through apoptotic events [1,2]. Insufficient apoptosis leads to the pathogenesis such as autoimmune diseases, viral infections and cancer [3]. Resistance to apoptosis participates in expansion of a population of cancer cells and defects in the apoptotic pathway can increase not only tumor mass but also cancer cells resistant to therapy, resulting in clinical problem [4]. Thus, improvement of apoptosis sensitivity may be an attractive therapeutic strategy for cancer patients.

The transcription factor specificity protein 1 (Sp1) controls cell proliferation, differentiation, the DNA damage response, apoptosis, senescence, angiogenesis and inflammation through controlling expression of downstream target genes. Sp1 regulates a large number of essential key factors in cancer cells including growth factors, growth factor receptors, telomerase, anti-apoptotic factors and pro-invasive factors [5]. The Sp1 level is elevated in several human cancers including HCC [6] and the high level of Sp1 is correlated with tumor stage, metastasis and poor prognosis [5]. Several studies reported that natural products have an apoptotic effect by inhibiting Sp1 and its target genes [6–8]. Therefore, targeting Sp1 may contribute to the therapeutic approach for treatment of cancers.

Artemisinin is a naturally occurring sesquiterpene lactone isolated from *Artemisia annua* L. in 1972 [9,10] and is a safe and effective World

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https://doi.org/10.1016/j.lfs.2017.11.008
Received 8 August 2017; Received in revised form 2 November 2017; Accepted 7 November 2017

Health Organization-recommended the first-line antimalarial agent [11]. Dihydroartemisinin (DHA) is a semi-synthetic derivative of artemisinin and has at least three times more effective antimalarial activity than artemisinin [12]. Previous studies demonstrated that pharmacokinetic parameters including maximum serum concentration (Cmax) and area under the plasma level-time curve (AUC) for DHA are higher and the bioavailability of oral DHA is significantly higher in patients with malaria (88%) than in healthy volunteers (45%) [13–15]. In addition, DHA has been reported to have no serious side effects [16]. DHA is known to have an anti-cancer activity by inducing apoptotic cell death in several cancer cells through the mitochondria-dependent pathway [12], Noxa-mediated pathway [17], activation of JNK1/2 and p38 MAPK signaling pathways [18], Bim-mediated intrinsic pathway [19], and increasing the intracellular production of reactive oxygen species [20]. However, the molecular mechanism underlying the effect of DHA on apoptosis is not yet well defined. In addition, there is no evidence of a link between DHA and Sp1 in apoptosis. Thus, this study focused on the Sp1 pathway to investigate molecular mechanism by which DHA induces apoptosis in hepatocellular carcinoma SK-Hep-1 cells.

2. Materials and methods

2.1. Reagents

Dihydroartemisinin (DHA) (purity: ≥ 97% as determined by TLC, Fig. 1A), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), RNase A, DAPI, cell death detection ELISA^{PLUS} and the primary antibody for β-actin (A5316) were purchased from Sigma-Aldrich (St Louis, MO, USA). The primary antibodies for cleaved-Caspase 8 (9496, 1:1000), cleaved-Caspase 9 (7237, 1:1000), cleaved-Caspase 3 (9664, 1:1000), cleaved-PARP (5625, 1:2000), XIAP (2045, 1:1000), pERK1/2 (4370, 1:1000), ERK1/2 (4695, 1:2000), pp38 (4511, 1:1000), p38 (8690, 1:1000), pJNK (4668, 1:500), JNK (9252, 1:1000) and goat anti-rabbit (1:3000) or mouse (1:3000) IgG-HRP secondary antibodies were obtained from Cell Signaling (Beverly, MA, USA). Sp1 (sc-420, western blot-1:500, immunofluorescence-1:50), survivin (sc-17779, 1:1000) and m-IgGκ BP-PE secondary antibodies (sc-516141, immunofluorescence-1:50) were obtained from Santa Cruz Biotechnologies (Danvers, MA, USA). *Z*-VAD-FMK was from Adooq BioScience (Irvine, CA, USA). Mithramycin A was from Enzo Life Sciences (Farmingdale, NY, USA). MG132 was from Calbiochem (San Diego, CA, USA). RIPA buffer, phosphatase inhibitor cocktail and protease inhibitor cocktail were from Thermo Scientific (Rockford, IL, USA). All other chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA).

2.2. Cell culture

The human HCC SK-Hep-1 cells and mouse normal hepatocyte AML12 cells were purchased from Korean Cell Line Bank (KCLB, Seoul) and ATCC (Manassas, VA), respectively, and maintained in DMEM supplemented with 10% fetal bovine serum, 10 mg/L streptomycin, and 10,000 U/L penicillin in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Cell viability assay

The effect of DHA on the viability of SK-Hep-1 cells and AML12 cells was evaluated by the MTT assay as described previously [21,22]. Briefly, cells were seeded at a density of 2×10^4 cells per well in a 96-well plate, and treated with various concentrations (20, 40, 60 and 80 μM) of DHA in a serum-free culture medium. After incubation at 37 °C for 24 h, 20 μl of MTT solution (1 mg/ml) was added for 2 h, And then the production of formazan was dissolved with DMSO, and optical density (OD) was measured by microplate reader (Sunrise, TECAN, Austria) at 570 nm. The cell viability was calculated by the following equation: Cell viability (%) = [optical density (OD) (DHA) − OD (blank)] / [OD (control) − OD (blank)] × 100.

2.4. Cell cycle analysis

The cells treated with DHA were harvested, washed twice with cold PBS and fixed in 70% ethanol at − 20 °C. After washing twice with cold PBS, the fixed cells were resuspended in 200 μl of RNase A (1 mg/ml) in PBS and incubated for 1 h at 37 °C. And then the cells were stained with 1 ml of PI solution (50 μg/ml) in PBS for 30 min at room temperature

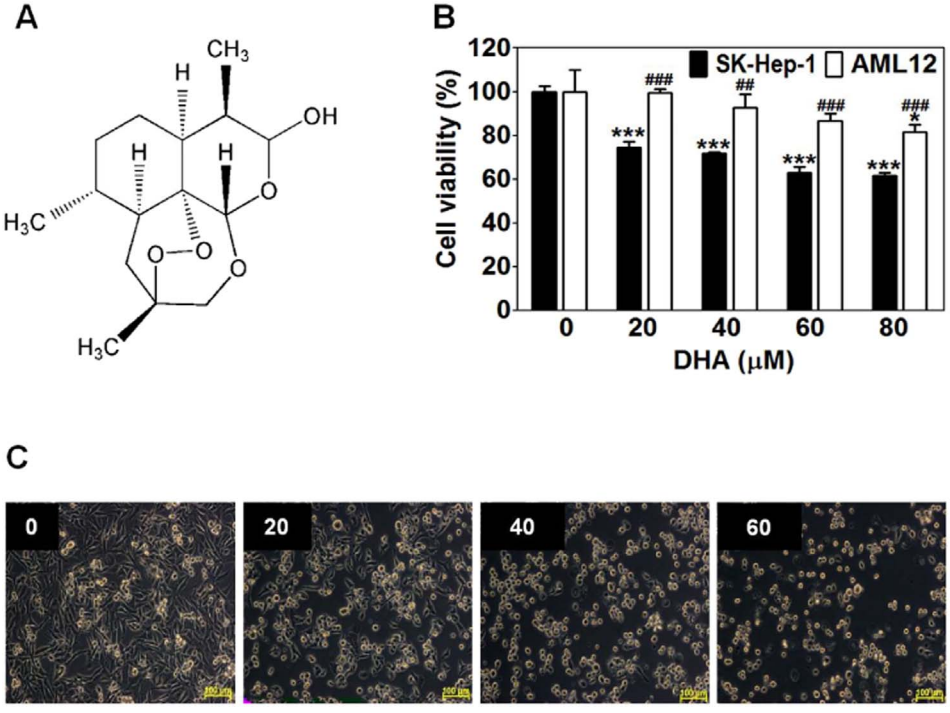

Fig. 1. Dihydroartemisinin (DHA) decreases the viability of SK-Hep-1 cells. (A) Chemical structure of DHA. (B) Cells were treated with various concentrations (0, 20, 40, 60 and 80 μM) of DHA for 24 h and cell viability was evaluated by MTT assay. Data are reported as mean ± SD. Statistical significance was analyzed by Student's *t*-test. $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. untreated control. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. SK-Hep-1 cells. (C) Cells were treated with various concentrations (0, 20, 40 and 60 μM) of DHA for 24 h and cell morphology was photographed by phase contrast microscopy at 100 ×. Scale bar = 100 μm.