Dihydroartemisinin treatment of multiple myeloma cells causes activation of c-Jun leading to cell apoptosis

YONG WANG[1,2], XIAOYUAN XU[2], XIAOJIAN WU[1], WEIBIN CHEN[1], FANGMEI HUANG[1] and XIAOMIN GUI[1]

[1]Department of Hematology, The Affiliated Hospital of Jiujiang University College of Medicine;
[2]Key Laboratory of System Bio-Medicine of Jiangxi, Jiujiang University, Jiujiang, Jiangxi 332000, P.R. China

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Abstract. The aim of the present study was to investigate the effect of dihydroartemisinin (DHA) on a multiple myeloma cell line. An MTT assay, flow cytometry and reverse transcription-polymerase chain reaction (RT-PCR) were used for the analysis of cell viability, cell cycle distribution and c-Jun N-terminal kinase (JNK) expression, respectively. Treatment of U266 cells using DHA caused a significant (P<0.05) decrease in cell viability compared with the control cells. An increase in the concentration of DHA from 1 to 100 μmol/l reduced cell viability from 87 to 35% compared with 100% in the control cultures at 48 h. A significant (P<0.05) increase was observed in the sub-G_0/G_1 phase population of the U266 cells with an increase in DHA concentration from 1 to 100 μmol/l. Treatment with 1, 3, 10, 30 and 100 μmol/l concentrations of DHA increased the sub-G_0/G_1 phase cell population to 3.13, 8.25, 24.91, 31.47 and 38.54%, respectively. RT-PCR analysis of DHA-treated or -untreated U266 cells after 48 h demonstrated a significant (P<0.01) increase in caspase-3 expression. Treatment of the cells for 48 h with DHA led to a significant increase in c-Jun expression. DHA treatment at 1, 3, 10, 30 and 100 μmol/l concentrations caused an increase in the level of c-Jun by 0.174±0.001, 0.254±0.002, 0.387±0.001, 0.502±0.003 and 0.679±0.005, respectively, compared with 0.982±0.001 in the control cells. The addition of SP600125 to the cells incubated with DHA resulted in a significant decrease in the caspase-3 and c-Jun expression levels compared with those cells incubated with DHA alone. These findings confirm that treatment with DHA increased caspase-3 and c-Jun expression in the U266 cells through activation of the JNK signaling pathway. Thus, DHA inhibited proliferation of multiple myeloma cells by interfering with the JNK signaling pathway.

Correspondence to: Dr Xiaoyuan Xu, Key Laboratory of System Bio-Medicine of Jiangxi, Jiujiang University, 57 Xunyang Donglu, Xunyang, Jiujiang, Jiangxi 332000, P.R. China
E-mail: xuxiaoyuan11@hotmail.com

Abbreviations: DHA, dihydroartemisinin; RT-PCR, reverse transcription-polymerase chain reaction; JNK, c-Jun N-terminal kinase

Key words: proliferation, myeloma, signaling, caspase-3, c-Jun

Introduction

Multiple myeloma is characterized by an increased level of light chain monoclonal immunoglobulin, as well as a simultaneous decrease in the level of normal immunoglobulin (1). In multiple myeloma, osteoclasts are activated resulting in an imbalance of the osteoclast and osteoblast equilibrium. Osteolytic lesion formation and osteoporosis is also observed at higher rate in multiple myeloma (1). Currently, there are a large number of drugs, as well as stem cell transplantation, in use for the prevention of multiple myeloma formation (2); however, development of multidrug resistance and disease relapse has been reported in patients. Thus, the development of an efficient therapeutic strategy for multiple myeloma is highly desired.

Natural products, along with their derivatives, possess the source of a large number of drugs. Studies have determined that natural products can prevent, decrease, and possibly defeat several pathologies, including cancer, diabetes, cardiovascular and neurological disorders (3-6). Artemisinin is isolated from a herbaceous plant, *Artemisia annua,* which is located in China and has been used as an anti-malarial drug for a number of years (7,8). The analogs of artemisinin, including dihydroartemisinin (DHA; Fig. 1), artesunate and artemether, also possess anti-malarial potential and are, therefore, used for the treatment of malaria (7). Screening of artemisinin and its derivatives revealed promising potential as inhibitors of malignant tumor proliferation *in vitro* (9-12). They inhibit the proliferation of breast (12) and ovarian cancer cells (7) without affecting non-malignant cells (12,13). Taking into account the anticancer potential of DHA along with its limited side effects on normal cells, the present study aimed to investigate the effect of DHA on multiple myeloma. The results obtained revealed that the DHA treatment caused the induction of apoptosis in U266 cells through c-Jun N-terminal kinase (JNK) signaling pathway and c-Jun activation.

Materials and methods

Cell culture. The cell line U266 was purchased from the Shanghai Institute of Biochemistry and Cellular Biology

Chinese Academy of Sciences (Shanghai, China). The culture medium used for the cell line was Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The medium contained 10% fetal bovine serum (HyClone, Logan, UT, USA), penicillin (100 U/ml) and streptomycin (100 U/ml). Cell culture was carried out in an incubator with 5% CO_2 and 95% air at 37˚C.

MTT assay. Effects of DHA on cell proliferation was determined using an MTT assay. The concentration of the cells was adjusted to $4x10^6$ cells/ml, and 190 μl cell suspension was added into each well of the 96-well plate. Plates were incubated for a period of 24 h using a 5% CO_2 incubator at 37˚C. Various concentrations of DHA (1, 3, 10, 30 and 100 μmol/l) were added to the plates and incubation was continued for 12, 24 and 48 h. The addition of 20 μl MTT solution (5 mg/ml) to each well of the plate was conducted at 12, 24 and 48 h. Further incubation of the plates was carried out for 4 h, followed by decantation of supernatant and the addition of 150 μl dimethyl sulfoxide to every well. Measurement of the absorbance for each well was performed in triplicate at a wavelength of 490 nm to determine the cell viability.

Flow cytometry for analysis of the cell cycle. The effect of various concentrations (1, 3, 10, 30 and 100 μmol/l) of DHA on cell proportion in various cell cycle phases were examined using flow cytometry. Exponentially proliferating cells at a concentration of $3.5x10^5$ cells/ml after 48 h of DHA treatment were collected and subsequently subjected to PBS washing. Cells were then subjected to fixing with 70% ethanol at -20˚C for 24 h followed by PBS washing and then stained with propidium iodide in the dark for 5 min at room temperature. Nuclear DNA was analyzed using a CycleTEST™ Plus kit (BD Biosciences, San Jose, CA, USA) as per the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Following treatment with DHA for a period of 48 h, cells were treated with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) to isolate the total RNA according to the manufacturer's protocol. Samples of 2 μl total RNA were used for the synthesis of cDNA, and amplification was performed using the ThermoScript RT-PCR system (Thermo Fisher Scientific, Inc.). Analysis was performed using a 2% agarose gel and a confirmation made by nucleotide sequencing. Primer design for RT-PCR was performed using GenBank sequences and Primer Express® software for Real-Time PCR (version 3; Applied Biosystems; Thermo Fisher Scientific, Inc.). The following primers were used for RT-PCR: GAPDH forward, 5'-TGAACGGGAAGCTCACTGG-3' and reverse, 5'-TCCACCACCCTGTTGCTGGA-3'; caspase-3 forward, 5'-TTTTTCAGAGGGGATCGTTG-3' and reverse, 5'-CGGCCTCCACTGGTATTTTA-3'; and c-Jun forward, 5'-CCCCAAGATCCTGAAACAGA-3' and reverse, 5'-CCGTTGCTGGACTGGATTAT-3'. Primers were supplied by Hokkaido System Science Co., Ltd. (Hokkaido, Japan).

Statistical analysis. Data are expressed as the mean ± standard deviation (SD) and were analyzed using SPSS software (version 14; SPSS, Inc., Chicago, IL, USA). The one-way analysis of variance and Dunnett's T3 post hoc analysis were used for statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference.

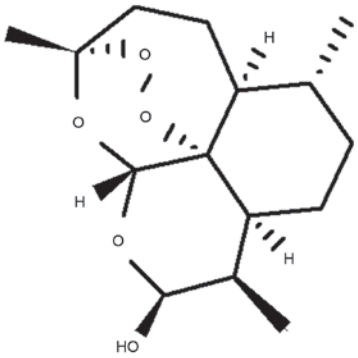

Figure 1. Chemical structure of dihydroartemisinin.

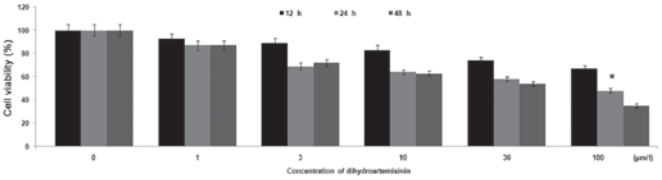

Figure 2. DHA treatment of U266 cells caused a reduction in cell proliferation in a concentration- and time-dependent manner. Cells following incubation had the addition of 1, 3, 10, 30 and 100 μmol/l concentrations of DHA for 12, 24 and 48 h and were analyzed for viability. The presented data represents the mean ± standard deviation of experiments performed in triplicate. *$P<0.05$ vs. control cells. DHA, dihydroartemisinin.

Results

DHA decreases in viability of U266 cells. The effect of different concentrations of DHA (1, 3, 10, 30 and 100 μmol/l) for various time durations (12, 24 and 48 h) on U266 cell proliferation was analyzed using an MTT assay. Treatment of U266 cells with DHA caused a significant ($P<0.05$) decrease in cell viability compared with control cells. Increasing the concentration of DHA from 1 to 100 μmol/l reduced cell viability from 87 to 35%, compared with 100% in the control cultures (Fig. 2). Treatment of U266 cells with 100 μmol/l DHA for 12, 24 and 48 h caused a reduction in cell proliferation to 67, 48 and 35%, respectively (Fig. 2).

DHA causes cell cycle arrest in the sub-G_0/G_1 phase. U266 cells were treated with 1, 3, 10, 30 and 100 μmol/l DHA for 48 h and then examined by flow cytometry. A significant ($P<0.05$) enhancement was observed in the U266 cell population in sub-G_0/G_1 phase with an increase in DHA concentration from 1 to 100 μmol/l. Treatment with 1, 3, 10, 30 and 100 μmol/l DHA increased the sub-G_0/G_1 phase cell population to 33.13, 38.25, 54.91, 74.47 and 88.54%, respectively (Fig. 3).

DHA causes an increase in c-Jun expression in U266 cells. The expression of c-Jun in DHA treated or untreated U266 cells was examined using RT-PCR analysis. Treatment of

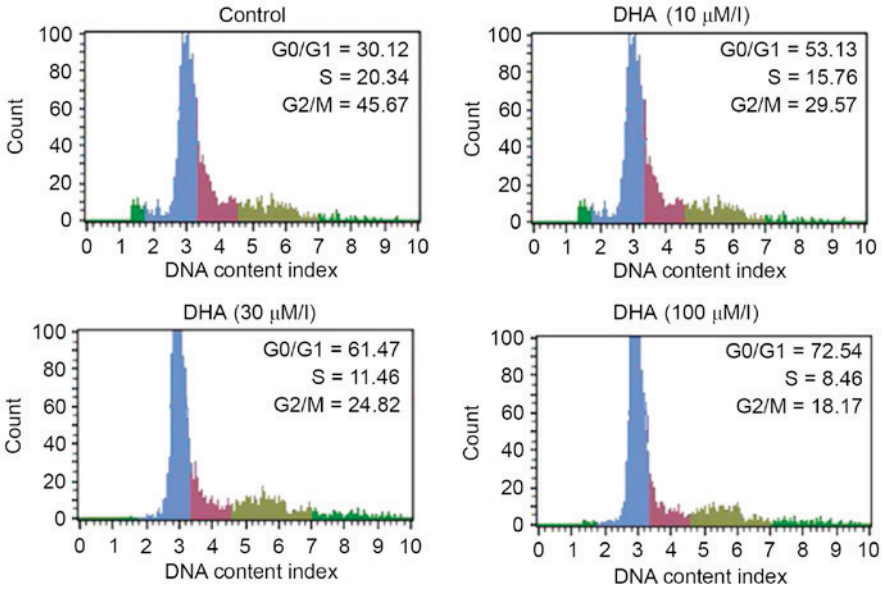

Figure 3. DHA treatment of U266 cells caused an increase in the sub-G_0/G_1 cell population in a concentration-dependent manner. U266 cells were incubated with 1, 3, 10, 30 and 100 μmol/l DHA for 48 h and were then analyzed for distribution in various phases of the cell cycle. The presented data are the mean and the standard deviation of triplicate experiments performed independently. *P<0.05 vs. control cell culture. DHA, dihydroartemisinin.

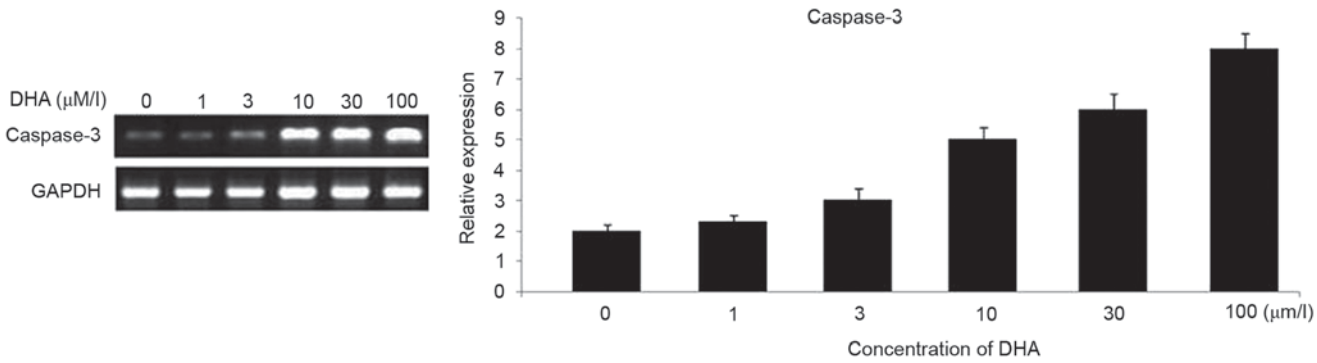

Figure 4. DHA treatment of U266 cells increased caspase-3 expression in a dosage-based manner. The cells were treated with 1, 3, 10, 30 and 100 μmol/l DHA for 48 h, and the expression of caspase-3 was examined by reverse transcription polymerase chain reaction analysis. The experiments were performed in triplicate. GAPDH was used as the internal control. DHA, dihydroartemisinin.

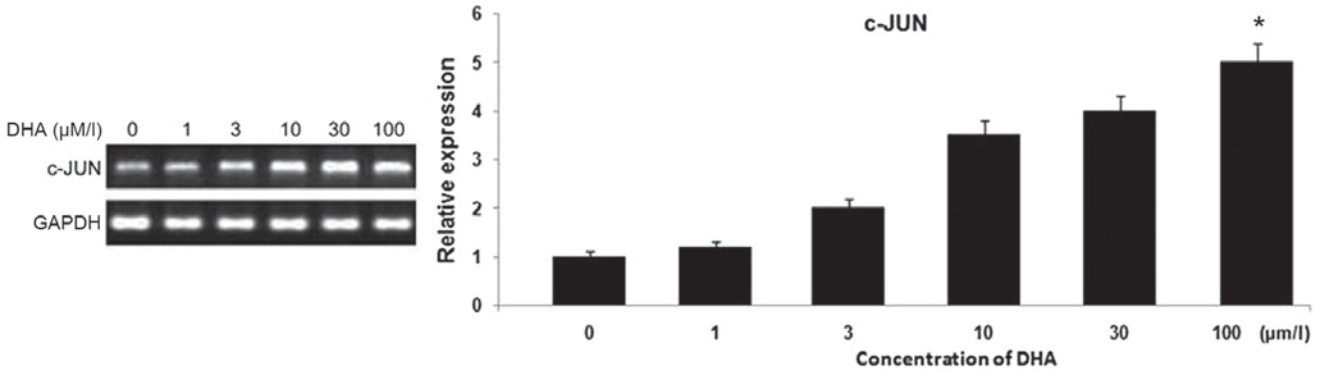

Figure 5. DHA treatment of U266 cells increased c-Jun expression in a concentration-dependent manner. Cells were treated with 1, 3, 10, 30 and 100 μmol/l doses of DHA for 48 h, and the expression of c-Jun was examined using reverse transcription polymerase chain reaction analysis. The experiments were performed in triplicates. *P<0.05 vs. control cell culture. GAPDH was used as the internal control. DHA, dihydroartemisinin.

the cells for 48 h with DHA caused a significant increase in c-Jun expression (Fig. 5). DHA treatment at 1, 3, 10, 30 and 100 μmol/l caused enhancements in the level of c-Jun to 0.174±0.001, 0.254±0.002, 0.387±0.001, 0.502±0.003 and 0.679±0.005 respectively, compared with 0.982±0.001 in the control cells.

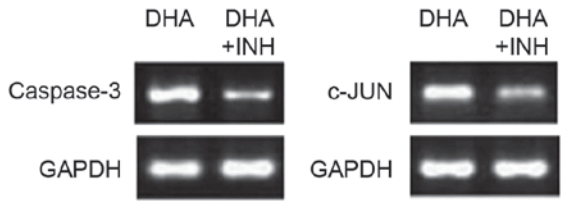

Figure 6. DHA treatment of U266 cells causes c-Jun N-terminal kinase signaling pathway activation. Following treatment with 100 μmol/l DHA for 48 h, cells were incubated with SP600125 (INH) and examined using reverse transcription polymerase chain reaction analysis. All experiments were performed in triplicate. The internal control used was GAPDH. DHA, dihydroartemisinin; INH, inhibitor.

DHA causes activation of the JNK signaling pathway in multiple myeloma cells. In order to confirm whether the DHA-mediated increase in caspase-3 and c-Jun expression in U266 cells is associated with JNK signaling, cells were treated with SP600125 (JNK signaling pathway inhibitor; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Treatment with SP600125 (2 mg/ml) following incubation with DHA caused a significant decrease in caspase-3 and c-Jun expression in U266 cells compared with cultures incubated with DHA alone (Fig. 6). These findings confirmed that DHA treatment increases the level of caspase-3 and c-Jun in the cells through activation of the JNK signaling pathway.

Discussion

DHA and its various derivatives have the potential of being malignant tumor proliferation inhibitors *in vitro* (3-6). They inhibit the proliferation of breast (6) and ovarian cancer cells (7) without affecting non-malignant cells (6,7). Apoptosis serves an important function in the maintenance of homeostasis in the body by removing unwanted cells (6). Any disturbance in the process of apoptosis results in the onset of various diseases, including cancer and autoimmune disease (7). In the bone marrow, increased levels of malignant plasma cells result in the development of multiple myeloma, which prevents cancer cell apoptosis and causes an alteration in the rate of cell proliferation. The present study was performed to determine the influence of DHA on a multiple myeloma human cell line. The results revealed that DHA caused a significant reduction in the viability of multiple myeloma cells compared with the control cells. Increasing the concentration of DHA from 1 to 100 μmol/l reduced cell viability from 87 to 35%, compared with 100% in the control cultures. Flow cytometric analysis revealed a significant increase in the population of U266 cells in the sub-G_0/G_1 phase when the concentration of DHA was increased from 1 to 100 μmol/l. Treatment with 1, 3, 10, 30 and 100 μmol/l DHA increased the sub-G_0/G_1 phase cell population to 3.13, 8.25, 24.91, 31.47 and 38.54%, respectively.

Transduction of signals and the expression of proteins are mediated by the mitogen-activated protein kinase (MAPK) (14). Various members of the MAPK family, including the extracellular signal regulated kinase, JNK/stress-activated protein kinase (SAPK) and p38, are in dynamic equilibrium with one another and serve a vital function in maintaining cell survival and apoptosis (15,16). JNK is a serine/threonine protein kinase and, due to its interaction with c-Jun as well as its ability to phosphorylate, it is called the c-Jun N-terminal kinase (15). The center of transcription factor-activated protein-1 is c-Jun, which on combining with Fos causes activation of transforming growth factor [activating transcription factor (ATF)-2, liver regeneration factor-1/ATF-3 and Jun dimerization protein-1]. The activation of c-Jun and ATF-2 is followed by the activation of a transcription factor, which in turn complexes with Fas via caspase-8 activation (17). Studies have indicated that JNK causes cell apoptosis through B-cell lymphoma (Bcl)-2 and Bcl-extra-large activation (18). The mitochondrial pathway of cell apoptosis involves cytochrome *c* release and caspase-9 activation (19). It is reported that in multiple myeloma cells, anti-Fas monoclonal antibodies induce apoptosis through the expression of JNK/SAPK and transcription factor c-Jun (20,21). Activation of c-Jun leads to the apoptosis signal transduction pathway resulting in cell apoptosis (22). Results from the present study revealed that DHA treatment resulted in the elevation of the expression levels of c-Jun (JNK pathway member) and caspase-3 in multiple myeloma cells. The addition of SP600125, an inhibitor of JNK, to the cell culture medium resulted in the reduction of c-Jun and caspase-3 expression. These findings suggest that DHA induces apoptosis in multiple myeloma cells by activating the JNK signaling pathway through the activation of c-Jun.

Thus, the present study demonstrated that DHA caused an inhibition of proliferation for multiple myeloma cells through JNK signaling pathway activation. Therefore, DHA can be used for the treatment of multiple myeloma.

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