Uppregulation of the MCL-1S protein variant following dihydroartemisinin treatment induces apoptosis in cholangiocarcinoma cells

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Abstract. The aim of the present study was to determine whether dihydroartemisinin (DHA) induces apoptosis in the human cholangiocarcinoma QBC939 cell line through the regulation of myeloid cell leukemia-1 (MCL-1) expression. The inhibitory rates of DHA on QBC939 cell proliferation and the effects of DHA on the cell death rates at various DHA concentrations and following various treatment times were examined. The rate of apoptosis and cell cycle changes following DHA treatment were examined and the changes in the expression of MCL-1 mRNAs and MCL-1 proteins following DHA treatment were also examined. The MTT assay and trypan blue staining demonstrated that DHA significantly inhibited the proliferation (P<0.05) and promoted the death of QBC939 cells (P<0.05). The DNA ladder assay and flow cytometry (FCM) analysis demonstrated that the rate of apoptosis in the experimental group was significantly increased following DHA treatment (P<0.01). FCM analysis also demonstrated that DHA treatment led to a reduction in the percentage of QBC939 cells in the G0/G1 and G2/M phases, and the majority of the DNA-treated cells were arrested in the S phase of the cell cycle (P<0.01). Western blot analysis demonstrated that DHA treatment significantly upregulated the expression of the pro-apoptotic MCL-1S protein. In contrast, no significant difference in the expression of the anti-apoptotic MCL-1L protein was observed following DHA treatment. DHA affected the expression of the apoptosis-associated protein MCL-1 through multiple mechanisms. DHA treatment increased the ratio of MCL-1S/MCL-1L protein, thus inducing apoptosis in cholangiocarcinoma cells.

Introduction

Cholangiocarcinoma is a malignant tumor arising from the tumorigenic transformation of cholangiocytes (1,2). Cholangiocarcinoma is rare compared with other types of cancer. However, a high incidence of cholangiocarcinoma has been reported in Eastern Asia, particularly in Thailand (3). Cholangiocarcinoma is characterized by slow growth and late-occurring metastases. However, the majority of patients with cholangiocarcinoma are diagnosed at advanced stages when curative surgery is not an option. The efficacy of chemotherapy and radiotherapy on cholangiocarcinoma is modest at best. Therefore, the prognosis of patients with cholangiocarcinoma is poor, and effective treatments are lacking (4,5). The identification of novel molecular therapeutic targets for the improvement of cholangiocarcinoma treatment efficacy is the focus of the present study.

Dihydroartemisinin (DHA) is a sesquiterpene lactone that bears a labile peroxide bridge and is extracted from the traditional Chinese medicine *Artemisia annua* (6). It has been widely used in the treatment of malaria and exhibits remarkably high efficacy against malaria. Previous studies have demonstrated that DHA and its derivatives have numerous pharmacological activities, including antibacterial sepsis, radiotherapy sensitization, antibiotic sensitization and antitumor effects (7). The antitumor activities of DHA merit further investigation. Previous studies on the mechanisms underlying the potential antitumor effects of DHA have not resulted in major breakthroughs. However, studies have demonstrated that there are similarities between the mechanisms of the antitumor and antimalarial activities of DHA and that DHA exerts an antitumor effect by promoting tumor cell apoptosis (8-11). The present study determined that DHA treatment significantly affected the expression of MCL-1 protein variants in the human cholangiocarcinoma cell line QBC939, and therefore induced apoptosis in these cells. The results provide key information for understanding the mechanisms underlying the antitumor effect of DHA.

Materials and methods

Materials. QBC939 cells were a generous gift from the Cell Center of Xiangya Medical College (Changsha, China). DHA was purchased from Shaanxi Sciphar Biotechnology Co.,
L. Ltd. (Xi’an, China). Tetrazolium salt 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), dimethyl sulfoxide (DMSO), Triton-100, and TRizol were purchased from Sigma-Aldrich China, Inc. (Shanghai, China). Fetal bovine serum (FBS) was purchased from Hangzhou Siijing Biological Engineering Materials Co., Ltd. (Hangzhou, China). RPMI-1640 medium and trypsin were purchased from Gibico Life Technologies (Beijing, China). The Apoptotic DNA Ladder Extraction kit and Bicinchoninic Acid (BCA) Protein Assay kit were purchased from Beyotime Institute of Biotechnology (Haimen, China). Primers and probes for the MCL-1 and β-actin gene were purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The rabbit polyclonal anti-human MCL-1 and β-actin antibodies were purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China).

Cell culture and treatments. QBC939 cells were maintained in a humidified incubator at 37°C and 5% CO₂ according to the cell culture instructions provided by the American Type Culture Collection (Manassas, VA, USA). The cells were cultured until adherent and passaged every 2-3 days. Once the cells were ready for passage, the culture medium was aspirated. The cells were washed twice with phosphate-buffered saline (PBS), digested with 0.25% trypsin, centrifuged at 208 x g for 7 min, resuspended in fresh medium, and seeded into tissue culture flasks. Following recovery, the cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 100 U/ml penicillin/streptomycin (Sigma-Aldrich China, Inc.) at 37°C in an incubator with a fully humidified atmosphere containing 5% CO₂. ‘Normal conditions’ is used throughout to describe the above conditions, which were used in the proceeding experiments.

Analysis of the inhibitory effect of DHA on the proliferation of the QBC939 cholangiocarcinoma cells by MTT assay. QBC939 cholangiocarcinoma cells were divided into the following groups: The control group, in which the cells were maintained under normal culture conditions; the DMSO group, in which the cells were treated with 0.1% DMSO (the solvent DMSO only, containing 0 µmol/l DHA); and the DHA group, in which the cells were treated with increasing concentrations of DHA at 10, 20, 40, 80, 160 and 320 µmol/l. Exponentially growing QBC939 cells were seeded at a density of 2 x 10⁵ cells/ml into 96-well cell culture plates at a volume of 200 µl per well and cultured under normal conditions for 24 h. The cells were then grouped and treated as described above. Each group contained 6 replicate wells. Following incubation at 37°C, 5% CO₂ and 95% saturated humidity for 6, 12, 24, 48 and 72 h, the cells were mixed with the MTT reagent (20 µl/well) and further incubated for 4 h. The supernatant was removed and discarded. DMSO was added to the cells at 150 µl/well, and the crystals that formed in living cells were fully dissolved through 10-min oscillation. The absorbance values at a wavelength of 490 nm (A490) were determined using an ELISA reader, and the cell proliferation inhibitory rates were calculated.

Detection of apoptotic DNA degradation by DNA ladder assay. QBC939 cholangiocarcinoma cells were harvested, seeded uniformly at a density of 2 x 10⁵ cells/ml into 6-well cell culture plates (2 ml/well), and cultured for 24 h under normal conditions. QBC939 cells cultured in the presence of DMSO (solvent only, prior to the addition of DHA) were used as the control group. Based on the results of the MTT assay and trypan blue experiments described above, DHA was added to QBC939 cells in the experimental group at a concentration of 20 µmol/l. The cells were then incubated at 37°C, 5% CO₂ and 95% saturated humidity for 12, 24 and 48 h. Each group contained 3 replicate wells. Following incubation, QBC939 cells were digested with 0.25% trypsin solution, dispersed into suspension by gentle pipetting and centrifuged at 208 x g for 5 min. The supernatant was removed and discarded, and the cells were washed once with PBS (pH 7.4). The total cellular DNA was extracted using the Apoptotic DNA Ladder Extraction kit according to the manufacturer’s instructions. Agarose gel was prepared by mixing 1.0 g agarose with 40 ml electrophoresis buffer. The mixture was heated to boiling until the agarose was dissolved and then cooled to below 60°C. A total of 2.5 µl of 10 mg/ml ethidium bromide was added to the agarose gel solution and mixed thoroughly by swirling. The agarose gel solution was poured onto the casting tray with an inserted comb and placed at room temperature for 30-45 min. Electrophoresis was performed once the 1.5% agarose gel was solidified. Following electrophoresis at 5 V/cm for 30 min, gel images were acquired under ultraviolet light (Gel Doc 1000, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Analysis of cell cycle changes and apoptosis by flow cytometry (FCM). QBC939 cholangiocarcinoma cells were harvested, seeded uniformly at a density of 2 x 10⁵ cells/ml into 25-cm² cell culture flasks (5 ml/flask), and cultured under the normal conditions for 24 h. The QBC939 cells were then grouped as described for the DNA ladder assay. Each group contained 6 replicate wells. DHA was added to the cells in the experimental group at a concentration of 20 µmol/l. Following incubation at 37°C, 5% CO₂ and 95% saturated humidity for 12, 24 and 48 h, QBC939 cells were digested with 0.25% trypsin, dispersed into suspension by gentle pipetting, and centrifuged for 5 min at 208 x g to
collect the cells. The supernatant was aspirated. The cells were washed twice with 0.01 mol/l PBS, fixed in pre-cooled 70% ethanol at 4°C for 1 h, and centrifuged at 208 x g for 5 min. Following removal of the supernatant, the cells were washed twice with 0.01 mol/l PBS and resuspended in 1 ml of 0.01 mol/l PBS. RNase A and propidium iodide (both Sigma-Aldrich China, Inc.) were added to the cells at final concentrations of 50 and 100 µg/ml, respectively, and incubated for 30 min at 4°C in the dark. Changes in the cell cycle and apoptosis were examined by FCM (BD FACSCalibur, Becton Dickinson UK Ltd., Oxford, UK).

Examination of MCL-1 mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR) amplification. The ACTB gene (encoding β-actin) was used as an internal reference gene. QBC939 cholangiocarcinoma cells were grouped and treated as described for the DNA ladder assay, and the total RNA was extracted from the cells and tissues using TRizol according to the manufacturer’s instructions. cDNA was synthesized in reverse transcriptase-mediated RT reactions using mRNAs as templates and Oligo (dT) as primers. Primers and probes for the MCL-1-associated genes and the β-actin gene were designed based on the gene sequences stored in the GenBank database (www.ncbi.nlm.nih.gov/genbank/). The primer sequences for the MCL-1-associated genes were as follows: MCL1-001, F 5'-TTTGCTACGGAGA-GGGG-3' and R 5'-TTCCGAAGCATGCTTGGAAG-3' (the size of the PCR amplification product was 597 bp); MCL1-002, F 5'-CGCTTGAAGATGGAAAG-3' and R 5'-CACAACCATCCTTGGAAG-3' (the size of the PCR amplification product was 382 bp); MCL1-201, F 5'-GACTTTTGCTACGGAGATG-3' and R 5'-GACCC-GTCCGTACTGTT-3' (the size of the PCR amplification product was 163 bp). The PCR amplification conditions were as follows: 94°C for 2 min; 94°C for 20 sec, 55°C (variable) for 30 sec, and 60°C for 40 sec for a total of 45 cycles, on a FTC-300 PCR machine (Shanghai Funglyn Biotech Co., Ltd., Shanghai, China). The threshold cycle (Ct) value for each PCR sample was determined using Sequence Detection software, version 1.2.3 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The specificity of the PCR reaction was verified by melting curves and agarose gel electrophoresis. The relative quantities were calculated using the 2^ΔΔCt method.

Examination of MCL-1 protein expression by western blot analysis. QBC939 cholangiocarcinoma cells were harvested, seeded uniformly at a density of 4 x 10^5 cells/ml onto 75-cm² cell culture flasks (10 ml/flask), and cultured under the normal conditions for 24 h. QBC939 cells were then grouped and treated as described above and incubated at 37°C, 5% CO₂ and 95% saturated humidity for 12, 24 and 48 h. Following incubation, the old medium was aspirated, and the cells were washed twice with 0.01 mol/l PBS. The cells in each well were harvested in 1 ml of 0.01 mol/l PBS solution, centrifuged at 208 x g for 5 min, and lyzed using the electrophoresis sample buffer. The cell lysates were centrifuged at 4°C, 15,682 x g for 15 min. The resulting supernatant was collected, and the protein concentration in the supernatant was determined using the BCA Protein Assay kit. Equal amounts of protein preparations (20 µl) were loaded onto 1.5% agarose protein gels and subjected to separation by electrophoresis at 5 V/cm for 30 min. Following electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany). The membranes were blocked with the 5% non-fat milk at room temperature for 3 h and incubated overnight at 4°C. The membranes were then washed with PBS, probed with primary antibodies (MCL-1 and β-actin, 1:500) at 37°C for 2 h, blocked again, incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:800; Bioscience Co., Beijing, China) at 37°C for 2 h, and washed three times for 5 min each with 1X PBS with agitation. The expression of MCL-1 protein and the internal reference protein β-actin in each group of cells was visualized using an EasyBlot ECL kit (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.), imaged, and analyzed using the Quantity One software, version 4.4.0 (Bio-Rad Laboratories, Inc.).

Statistical analysis. Measurement data are presented as the mean ± standard deviation. Comparison of the means between two groups was performed using Student’s t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

The inhibitory effect of DHA on QBC939 cell proliferation and the effects of DHA on the cell death rate of the QBC939 cells. The MTT assay demonstrated that the addition of a minimum of 10 µmol/l DHA significantly inhibited the proliferation of QBC939 cells (P<0.05; Fig. 1). As the concentration of DHA was increased, the inhibitory effect also increased. No statistically significant differences in cell proliferation were observed between the DMSO group and the control group. By contrast, treatment at all the examined DHA concentrations resulted in statistically significant inhibition of proliferation compared with the control group (P<0.05). Furthermore, the half maximal inhibitory concentration (IC50) of DHA decreased significantly with treatment time (P<0.01; Table I). The rate of cell death of QBC939 cells following DHA treatment was examined by trypan blue staining, and the results demonstrated similar trends to the MTT assay result. The addition of a minimum of 20 µmol/l DHA significantly increased the cell death of QBC939 cells. As the DHA concentration increased, the percentage of cell death also increased (P<0.05; Fig. 2). In addition, the proliferation-inhibiting and the death-promoting effects of DHA on QBC939 cells
gradually increased with prolonged duration of DHA treatment over the observed time points of 12, 24 and 48 h (P<0.05). Therefore, the effects of DHA on QBC939 cells exhibited an apparent time- and dose-dependence. The above experiments demonstrated that treatment with 20 µmol/l DHA for 12, 24 and 48 h resulted in significant effects on proliferation and cell death, however treatment at this concentration did not induce a high enough degree of cell death that would impede the examination of additional parameters in subsequent experiments.

Table I. Changes in IC$_{50}$ levels in DHA-treated cells over time.

<table>
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<tr>
<th>Time/h</th>
<th>IC$_{50}$/µmol/l</th>
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<tr>
<td>6</td>
<td>780.20±14.39</td>
</tr>
<tr>
<td>12</td>
<td>255.01±7.37&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>24</td>
<td>22.61±2.17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>48</td>
<td>10.12±1.10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>72</td>
<td>7.94±0.53&lt;sup&gt;a&lt;/sup&gt;</td>
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<sup>a</sup>P<0.01 vs. control group.

The effect of DHA on the apoptosis of QBC939 cells. Apoptotic DNA degradation was examined using a DNA ladder assay. Varying degrees of DNA ladder formation were detected in QBC939 cells following treatment with 20 µmol/l DHA for various time periods. In the 24- and 48-h treatment groups, DNA ladder formation was apparent (Fig. 3); however, this effect was not evident with 12-h treatment. The results indicate that DHA treatment induced apoptosis in QBC939 cells, and the apoptosis-promoting effects were apparent following at least 24 h of DHA treatment (Fig. 3).

Analysis of the effects of DHA on cell cycle and apoptosis in QBC939 cells by FCM. As presented in Fig. 4, typical G$_0$/G$_1$ sub-peaks were detected in the DHA treatment groups (Fig. 4B-D). DHA treatment resulted in a reduction in the percentage of QBC939 cells in the G$_0$/G$_1$ and G$_2$/M phases, and the number of QBC939 cells in S phase increased significantly following DHA treatment (P<0.05; Table II). These results
indicated that the QBC939 cells were arrested in the S phase of the cell cycle following DHA treatment. Thus DHA treatment induced apoptosis in a proportion of QBC939 cells. As the duration of the DHA treatment increased, the apoptotic rates of QBC939 cells also increased. Compared with the control group, the apoptotic rates in the DHA treatment groups were significantly increased at all the observed time points (Fig. 5; P<0.01).

The effect of DHA treatment on the expression of MCL-1 mRNA and protein in QBC939 cells. Fluorescence-based qPCR analysis demonstrated that the mRNA transcripts of the apoptosis-associated protein MCL-1 were expressed in QBC939 cells at various levels (Fig. 6). Compared with the control group, the expression of the MCL1-001 transcript was slightly increased in the DHA groups, whereas the expression of MCL1-002 and MCL1-201 was increased. No statistically significant differences were detected in the relative expression level (2^ΔΔCt) of MCL1-002 between the DHA group and the control group at 12 and 24 h. However, the relative expression of MCL1-002 was significantly increased following 48 h of treatment (P<0.01). The expression levels of the mRNAs MCL1-001 and MCL1-201 increased slightly following 12 h of DHA treatment, and were significantly increased following 24 and 48 h of DHA treatment (P<0.01; Fig. 6).

Western blot analysis demonstrated that MCL-1L and MCL-1S protein variants were expressed in the cholangiocarcinoma cell line QBC939 (Fig. 7). The expression of MCL-1L protein increased following DHA treatment (P<0.05). The expression of MCL-1S protein was markedly increased following DHA treatment for 12 h, and significantly increased following 24 and 48 h treatment (P<0.01; Fig. 7).

Discussion

The present study demonstrated that the treatment of QBC939 cells with various concentrations of DHA resulted in varying degrees of proliferative activity impairment. As the
concentration of DHA increased, the proliferation inhibitory rate increased correspondingly. In addition, the proliferative activity of QBC939 cells was negatively correlated with the duration of DHA treatment. DHA exhibited a significant inhibitory effect on the proliferation of QBC939 cholangiocarcinoma cells cultured in vitro, and the IC50 changed significantly with treatment time. Therefore the experimental results demonstrated that DHA treatment significantly inhibited the proliferation of QBC939 cells and that the inhibitory effects of DHA on cell proliferation were dose- and time-dependent.

In cells undergoing apoptosis, DNA is cleaved by endogenous endonucleases at the internucleosomal linker sites between the nucleosomes into oligonucleotide fragments of integer multiples of 180-200 bp (12). Agarose gel electrophoresis demonstrated the DNA ladder pattern characteristic of apoptosis following DHA treatment of QBC939 cells for a duration of at least 24 h. DNA ladder formation is closely associated with apoptosis and has been used as one of the most important criteria for the detection of apoptosis. The detection of DNA laddering by agarose gel electrophoresis demonstrated that DHA treatment inhibited QBC939 cell proliferation through the induction of apoptosis. In addition, FCM analysis demonstrated that prolonged duration of DHA treatment was associated with an increased percentage of apoptotic QBC939 cells. The FCM results further confirmed the apoptosis-promoting effect of DHA on QBC939 cells and indicated that the effect was dose-dependent. FCM analysis also demonstrated that the majority of DHA-treated cells arrested in the S phase of the cell cycle. The results indicated that DHA blocked genomic DNA synthesis, thereby inhibiting cell division and proliferation and promoting apoptosis.

The anti-apoptotic gene MCL-1 is a member of the B-cell lymphoma 2 (BCL-2) family of apoptosis-regulating genes. The MCL-1 protein shares sequence and functional similarities with the BCL-2 protein and is important in the process of apoptosis. MCL-1 pre-mRNA undergoes alternative splicing to produce the short splice isoform MCL-1S. The unspliced, longest gene product of MCL-1 is referred to as MCL-1L. A total of 4 transcript variants of MCL-1 gene have been identified, which are referred to as MCLI-001, -002, -003 and -201. MCLI-003 does not encode any protein. MCLI-001 encodes the MCL-1L protein. MCLI-002 and MCLI-201 encode the MCL-1S protein. In tumor cells cultured under normal conditions, MCL-1L protein is expressed at a much higher level than the MCL-1S protein. MCL-1L is usually referred to as MCL-1. Although the MCL-1L and MCL-1S proteins are encoded by the same gene, the two proteins exhibit opposite activities. MCL-1L inhibits apoptosis, whereas MCL-1S promotes apoptosis. Therefore, the ratio of MCL-1L/MCL-1S in cells expressing the MCL-1 gene determines the fate of the cells (13-15). Cholangiocarcinoma cells express high levels of the anti-apoptotic protein MCL-1L, indicating that cholangiocarcinoma tumorigenesis is closely associated with the dysregulation of MCL-1 expression. In the present study, it was demonstrated that DHA inhibited the proliferation and promoted apoptosis of QBC939 cholangiocarcinoma cells. Following DHA treatment, QBC939 cells exhibited significantly increased apoptotic activity; therefore DHA may induce apoptosis in QBC939 cells through the regulation of MCL-1 protein expression. Western blot analysis was performed to examine the expression of MCL-1 proteins. The results demonstrated that compared with the control group, DHA treatment significantly increased the expression of MCL-1S protein and increased the ratio of MCL-1S/MCL-1L in QBC939 cells in the experimental groups.

In summary, the present study demonstrated that DHA promoted apoptosis in QBC939 cholangiocarcinoma cells. In addition, the present study demonstrated that DHA induced apoptosis in QBC939 cells through the upregulation of the expression of the pro-apoptotic protein MCL-1S. These results provide a basis for the development of effective treatments for cholangiocarcinoma.

References