Dihydroartemisinin induces apoptosis in human leukemia cells HL60 via downregulation of transferrin receptor expression

Hui-Jun Zhou, Zeng Wang and Ao Li

Dihydroartemisinin (DHA), a water-soluble active metabolite of artemisinin derivatives, is the safest and most effective antimalarial analog of artemisinin. In the present investigation, we assessed the apoptotic effect of DHA on leukemia HL60 cells and its regulation of transferrin receptor (TfR). Cell growth inhibition was assessed by Trypan blue exclusive staining; the expression of caspase-3, Bcl-2, and Bax in HL60 cells was evaluated by Western blotting; DHA-induced apoptosis was determined by AO/EB double staining, DNA fragmentation assay, and flow cytometric analysis; the expression of TfR in HL60 cells was examined by real-time PCR assays, Western blotting, and flow cytometric analysis. DHA could specifically reduce the mRNA and protein expression of TfR in HL60 cells, and the flow cytometric analysis presented the unity tendency that the TfR content decreased progressively in a dose-dependent manner. Consequently, DHA exhibited high anticancer activity in HL60 cells; MTT assay and growth inhibition assay showed that DHA could specifically inhibit the growth of HL60 cells in a dose-dependent (0.25–8 μmol/l) and time-dependent (12–72 h) manner. DHA-induced DNA fragmentation also induced the activation of caspase-3 and influenced the expression of Bcl-2 and Bax. Taken together, these data from our study show that DHA can induce HL60 cell apoptosis via the effect of downregulation TfR expression resulting in an induction of apoptosis through the mitochondrial pathway, and it might be a potential antileukemia strategy for leukemia therapy. *Anti-Cancer Drugs* 19:247–255 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Artemisinin is a sesquiterpene lactone endoperoxide found in the traditional Chinese medicinal plant *Artemisia annua* [1,2], and its derivatives are very effective to blood schistocidal antimalarials with fewer adverse side effects than any other antimalarial drug. It contains an endoperoxide bridge that reacts with ferrous iron to generate free radicals, leading to macromolecular damage and cell death [2–4]. Recently, certain artemisinin derivatives were shown to inhibit the growth of a limited number of human cancer cell lines [5]. Of all the artemisinin derivatives, dihydroartemisinin (DHA) is the main active metabolite of and is a more water-soluble and effective antimalarial than artemisinin [6]. Several studies have shown that DHA also reveals profound cytotoxic activity against tumor cells [6,7]. The most sensitive cancer cell lines to DHA are characterized by their rapid proliferation, often accompanied by a high intracellular iron concentration to sustain continued proliferation [8,9]. Thus it has been hypothesized that iron activation of the endoperoxide bridge is an essential event in cytotoxicity. The anticancer mechanism and the role of iron in the toxicity of artemisinins toward tumor cells are, however, still unclear.

Iron is an essential element for cell proliferation and metabolism. In most tissues, transferrin receptor (TfR)-mediated endocytosis is a major pathway for cellular iron uptake [10]. TfR expression is controlled by the amount of iron required by the cell to maintain its metabolism, and therefore TfR is more abundantly expressed in rapidly dividing cells than quiescent cells. More importantly, as tumor cells grow in a highly proliferative state, TfR is expressed more abundantly in malignant tissues than in their healthy counterparts [11]. To maintain high proliferative rates, cancer cells require large amounts of iron for related proteins. This characteristic renders tumor cells more sensitive to iron depletion which is well known to cause cell cycle arrest and apoptosis [12,13].

Aoptotic cell death most commonly occurs by either one of two distinct pathways, either ligand-dependent activation of cell surface receptors (e.g. Fas ligand or tumor necrosis factor-α) or activation of the mitochondrial death pathway [14]. The barrier to apoptosis induction depends on an intact outer membrane, which sequesters a variety of apoptotic proteins in an inactive form in the mitochondrial intermembrane space. When

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the integrity of this membrane is lost, these proteins are released into the cytosol. Upon the induction of apoptosis a conserved family of cysteine aspartic acid-specific proteases, known as caspases, are activated in a cascade mechanism to bring about cell death [15].

In an attempt to find the possible mechanism of HL60 cell apoptosis induced by DHA, we investigated cell surface expression of TfR and intracellular relative proteins which are known to be critical factors in the induction of apoptosis. Our results show that DHA-mediated apoptosis seems to be initiated by a down-regulation of TfR expression, resulting in a reduction of iron uptake followed by an induction of apoptosis through the mitochondrial pathway.

Materials and methods

Materials

DHA was kindly presented by the Engineer, Liuxu of Guiling Pharmaceutical Co. (Guangxi, China). Working solutions were prepared by dissolving the compound in dimethylsulfoxide (DMSO) before experiments. The final concentration of DMSO is less than 0.1% in all experiments. Cells cultured with 0.1% DMSO were used as a negative control. Penicillin, streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMSO, ethidium bromide, and propidium iodide were purchased from Sigma (St Louis, Missouri, USA). Polyvinylidene difluoride membranes were supplied by Millipore Co. (Billerica, Massachusetts, USA). Mouse anti-Bcl-2 (C-2), mouse anti-Bax (B-9), rabbit anti-caspase-3 (H-277), mouse anti-TfR (3B8 2A1), and goat anti-actin polyclonal antibody (I-19) and all the secondary antisera were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA).

Cell culture

Cells from the human promyeloid leukemia lines HL60 were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were grown in RPMI1640 standard medium supplemented with 10% fetal calf serum and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) in humidified air at 37°C with 5% CO_2. Exponentially growing cells were used throughout the study.

Growth inhibition assay

In-vitro growth inhibition effect of DHA on leukemia cells was determined by modified microculture tetrazolium (MTT) assay. Briefly, aliquots (100 μl) of the cell suspension were dispensed into 96-well flat-bottomed microplates containing 100 μl of the various chemical dilutions in three replicate rows. Plates were incubated in a humidified incubator in 5% CO_2 for 48 h at 37°C; 10 μl of MTT solution [5 mg/ml MTT in phosphate-buffered saline (PBS) stored at 4°C] was then added, and the resulting solution was incubated for 4 h at 37°C in 5% CO_2. Formazan crystals were dissolved in 100 μl of DMSO. The plates were then analyzed in a 96-well format plate reader at 570 nm.

Determination of cell growth curve

HL60 cells were suspended at a final concentration of 5×10^4 cells/ml and seeded in 24-well microtiter plates. Various concentrations (0.25–1 μmol/l) of DHA were added to each well in triplicate. After incubation for the indicated times, the viable cells were counted by the trypan blue exclusion method.

Acridine-orange/ethidium bromide double staining

Apoptosis was assessed by fluorescence microscopy (DMIL; Leica, Wetzlar, Germany), after staining of the cells with a mixture of acridine orange (AO; 100 μg/ml) and ethidium bromide (EB; 100 μg/ml) in PBS. Loss of nuclear structure or fragmented nuclei were taken as indicative of apoptotic cells.

DNA fragment assay

To examine apoptosis by electrophoresis of nucleosomal fragments, a standard procedure for precipitating cytosolic nucleic acid was used [16]. Briefly, 1×10^6 DHA-treated HL60 cells were pelleted (300 *g*, 5 min) and suspended in lysis buffer (500 μl, 0.4% Triton-X, 20 mmol/l Tris, and 0.4 mmol/l EDTA) in 55°C water bath overnight. DNA in the extract was purified by phenol/chloroform extraction and precipitated with ethanol. Finally, the extracted DNA was separated on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide and visualized on an UV transilluminator and photographed.

Flow cytometry

Apoptosis quantification

To quantitate apoptosis, flow cytometric analysis was performed using propidium iodide, as described previously [17]. Briefly, cells were treated with various concentrations (0.25–1 μmol/l) of DHA for 48 h. After treatment, cells were prepared as single-cell suspension in 50 μl PBS, fixed with 70% ethanol. The fixed cells were harvested, resuspended in 500 μl PBS supplemented with 0.1% Triton X-100 and RNase A (100 mg/ml), incubated at 37°C for 30 min, and stained with 50 mg/ml propidium iodide (PI) in the dark at 4°C for 30 min. The red fluorescence of individual cells was measured with a FACSCalibur flow cytometer (Becton Dickinson, Lincoln Park, New Jersey, USA). A minimum of 30 000 events were collected per sample. The relative DNA content per cell was obtained by measuring the fluorescence of PI that bound stoichiometrically to DNA.

Intracellular staining of transferrin receptor (CD71)

Mouse monoclonal antihuman TfR antibody was used for flow cytometric analysis and mAb (1 μl) was added to

tube containing a 50-μl aliquot of 1×10^5 cells. An isotype IgG (santa cruz-2855) was used as a negative control. Cells and antibody were gently mixed and incubated at room temperature for 1 h. FITC-conjugated goat anti-mouse immunoglobulin antiserum (1 μl; santa cruz-2010) was added after washing with 1-ml PBS–0.2% BSA solution. After another 30-min incubation, cells were washed with 1-ml PBS–0.2% BSA solution. Samples were analyzed by flow cytometry (Becton Dickinson). A typical viable cell area was gated and 1×10^4 events were counted. The isotype negative control was used to define the threshold of the background staining.

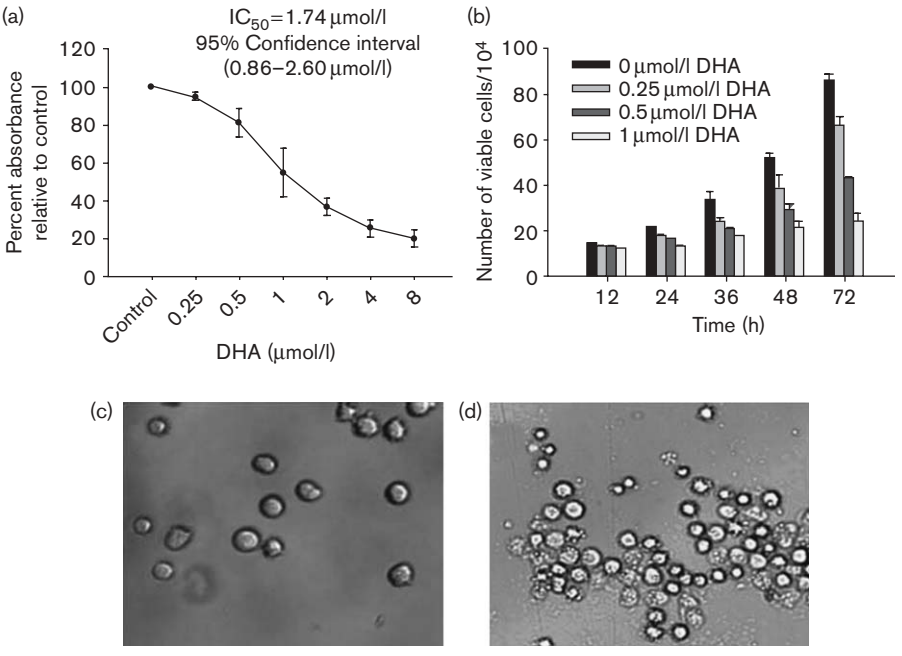
Real-time PCR analysis

Total RNA was isolated from the HL60 cells (1×10^6 cells) by using Trizol Reagent (Bio Basic, Mississauga, Ontario, Canada) according to the manufacturer's instructions. The first-strand cDNA was synthesized by M-MLV reverse transcriptase (MBI Fermentas) in a 20-μl reaction system containing 2 μg total RNA. cDNA was amplified by polymerase chain reaction (PCR) using Taq DNA polymerase (MBI Fermentas, St Leon-Rot, Germany). PCR was carried out by using a Thermal cycler (Eppendorf, Hamburg, Germany) as follows: 94°C for 5 min; 35 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s. The PCR products were separated on 1.5% agarose gel and visualized by an ethidium bromide staining. The expression intensities of optimized bands were quantified with Quantity One software. Amplification of glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. TfR primers: forward, 5′-CTGCCTCTTTCCTGTTGTTGT-3′ and reverse, 5′-CTTTGGCCAAAATTTGGCAGC-3′ (429 bp) [18]; glyceraldehyde-3-phosphate dehydrogenase primers: forward, 5′-GTCAGTGGTGGACCTGACCT-3′ and reverse, 5′-CCCTGTTGCTGTAGCCAAAT -3′ (251 bp) [19].

Protein extraction and Western blotting

Cell pellet was suspended in cold RIPA buffer [50 mmol/l Tris–HCl (PH8.0), 150 mmol/l NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton-X100, and 1 mmol/l EDTA and supplemented with 1 mmol/l PMSF and 10 μg/ml Leupeptin] and incubated on ice for 30 min. The protein concentration was determined using the bicinchoninic acid assay (Bio-Rad, Hercules, California, USA). Then equal amounts of protein were separated by 12% SDS–PAGE and transferred onto polyvinylidene difluoride membrane. Membranes were blocked with 5% nonfat milk and incubated with the primary antibodies at 4°C overnight in PBS-T. Primary antibodies were Mouse anti-TfR (3B8 2A1), mouse anti-Bcl-2 (C-2), mouse anti-Bax (B-9), rabbit anti-caspase-3 (H-277), and goat anti-actin polyclonal antibody (I-19). Immunoreactivity was visualized with horseradish peroxidase-linked secondary

Fig. 1



Effect of dihydroartemisinin on proliferation of HL60 cells. (a) HL60 cells were seeded into 96-well plates and incubated for 48 h in the presence of DHA with different concentrations. Cell proliferation was detected by MTT assay. (b) HL60 cells were seeded into 24-well plates and incubated with DHA. After incubation for the indicated time, the viable cells were counted by the Trypan blue exclusion method. The data were from three independent experiments. $\bar{x} \pm s$, $n=3$. (c) Cells treated with RPMI 1640 are specular globular, (d) whereas most of the cells treated with DHA displayed many particles. DHA, dihydroartemisinin.

antibodies and chemiluminescence, β-actin levels were analyzed as controls for protein loading.

Data analysis

The statistical significance of mean values was determined by unpaired two-tailed Student's *t*-test. Significance of dose–response curve was detected by analysis of variance.

Results

Inhibition of cell growth

The cytotoxic activities of DHA in HL60 cells were assessed by examining its effects upon cellular dehydrogenase activity using the MTT assay. DHA at 0.25, 0.5, 1, 2, 4, and 8 μmol/l for 48 h inhibited the growth of HL60 cells by 5, 19, 45.4, 63.4, 74.8, and 80.2%, respectively. The results revealed that dihydroartemisinin reduced cell survival in a dose-dependent manner. The HL60 cell lines showed low IC_{50} values (1.74 μmol/l), and its 95% confidence interval was 0.86 – 2.60 μmol/l (Fig. 1a).

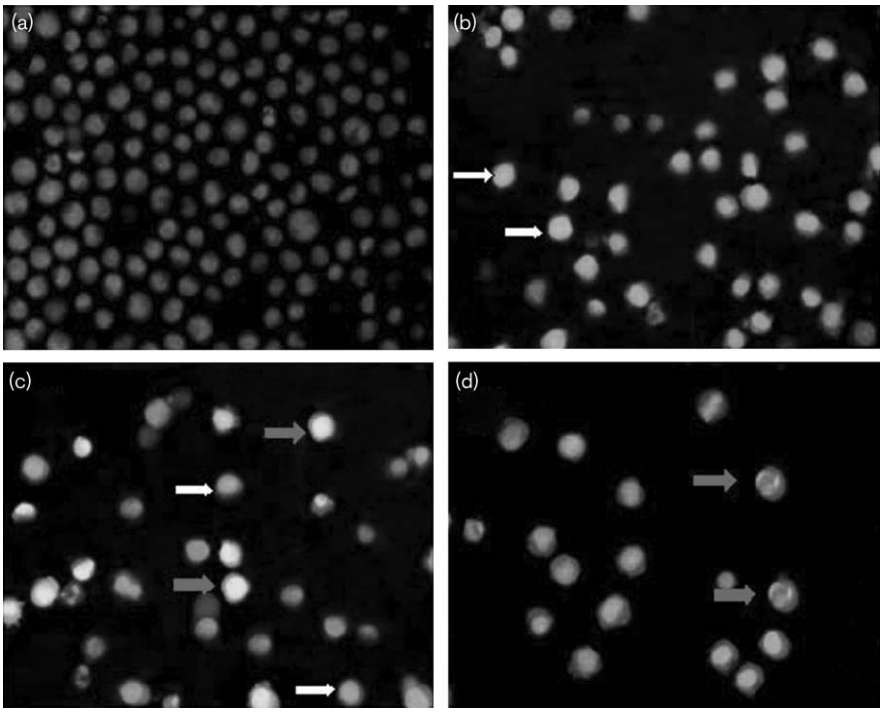
To further determine whether DHA was capable of inhibiting cell proliferation, Trypan blue exclusion assays using an inverted phase-contrast microscope (DMIL; Leica) were performed on HL60 cells. Concentrations of DHA from 0.25 to 1 μmol/l were tested and shown to suppress the growth of HL60 cells significantly in a dose-dependent and time-dependent fashion (Fig. 1b). It also revealed that the induced apoptosis of HL60 cells displayed many particles (Fig. 1d) through an inverted phase-contrast microscope, but HL60 cells of control groups with normal morphology are globular and had integrity (Fig. 1c).

Morphological changes of apoptosis in HL60 cells

After the DNA-specific fluorochromes AO and EB were used to stain HL60 cells and an inverted fluorescence microscope (DMIL; Leica) was used to observe the morphological changes of HL60 cells treated with DHA. From Fig. 2a, it can be seen that the control was morphologically normal. The nuclei of different cells were of similar sizes, regularly shaped, and evenly stained. The more deeply colored parts of the nuclei represented heterochromatin which did not take part in transcription under normal circumstances. With DHA treatment, however, the cells showed marked morphological changes.

In the groups treated with 0.25 and 0.5 μmol/l of DHA (Fig. 2b and c), the cells were wrinkled, and the chromatin was condensed and marginalized, marked by

Fig. 2



Fluorescence photomicrographs of HL60 cells stained by acridine orange/ethidium bromide (AO/EB) after being treated with dihydroartemisinin (DHA). Cells were treated with or without DHA for 48 h. Then the cells were stained by AO/EB and morphology was immediately assessed using fluorescence microscopy: (a) control cells with bright green nuclei and intact structure; (b) DHA 0.25 μmol/l; (c) DHA 0.5 μmol/l; and (d) DHA 1 μmol/l. White arrows marked the early apoptotic cells with bright green nuclei as dense green areas in nucleus. Red arrows marked late apoptotic cells with orange nucleus showing condensation of chromatin as dense orange areas.

white arrows. In the group treated with 1 μmol/l of DHA (Fig. 2d), cell orange fluorescence was significantly enhanced, representing the characteristic of late apoptotic cells, marked by red arrows.

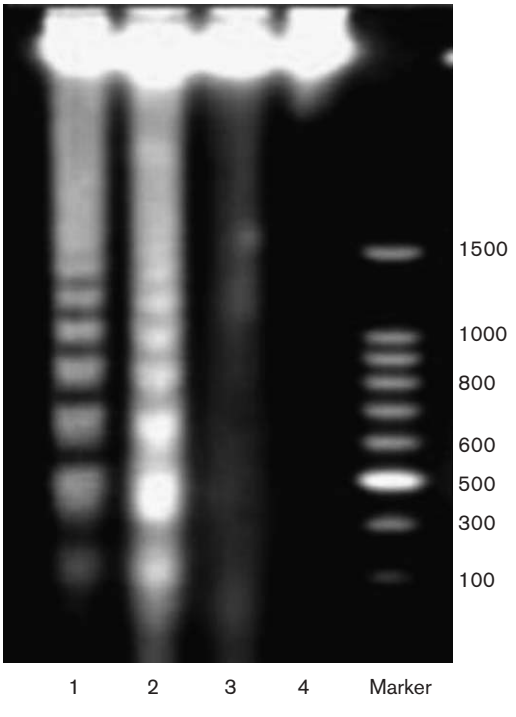
Electrophoretic analysis of DNA fragmentation

To determine the apoptotic mode of DHA in HL60 cells, we examined whether DHA could induce internucleosomal degradation of DNA, a biochemical hallmark of apoptosis. Exposure of HL60 cells to DHA (0.25, 0.5, and 1 μmol/l) for 48 h resulted in a typical characteristics of cell death. As shown in Fig. 3, a ladder pattern of DNA fragments was displayed at 0.5 or 1 μmol/l of DHA; however, no bands can be seen in control group. This indicated that DHA could induce HL60 cells apoptosis.

Percentage of cells undergoing apoptosis

To further quantitatively assess the potential of DHA inducing HL60 to apoptosis, apoptosis was determined by flow cytometry with PI staining. HL60 cells treated with 0.25, 0.5, and 1 μmol/l DHA for 48 h were stained with a combination of PI (Fig. 4A). Flow cytometry analysis of affected cells demonstrated increased surface expression of phosphatidylserine. These cells continued to exclude PI, which associates with the nuclear DNA of necrotic cells. The results of flow cytometric analysis of DNA content (Fig. 4) showed that when the cells were treated with DHA 0.25, 0.5, and 1 μmol/l DHA for 48 h, the apoptosis were 4.78, 11.88, and 32.71%, respectively. Taken together, these data clearly indicate that DHA-treated HL60 cells undergo increased levels of apoptosis.

Fig. 3



DNA fragmentation induced by dihydroartemisinin (DHA) for 48 h in HL60 cells. (1) HL60 cells were treated with DHA 1 μmol/l; (2) HL60 cells were treated with DHA 0.5 μmol/l; (3) HL60 cells were treated with DHA 0.25 μmol/l; and (4) HL60 cells were treated without DHA.

SDS–PAGE analysis of target protein

After the treatment of HL60 cells with 0.25, 0.5, and 1 μmol/l DHA for 48 h, the expression of caspase-3 was measured by Western blot assay. To further assess the main pathway of DHA inducing HL60 cells to apoptosis, we also detected the Bcl-2 and Bax expression in HL60 cells. As a result, we found that Bcl-2 protein expression was significantly decreased, but caspase-3 and Bax protein expression were increased dramatically in a dose-dependent manner ($P < 0.01$), compared to control cells (Fig. 5).

Downregulate the expression of transferrin receptor in HL60 cells

Posttranscriptional regulation of transferrin receptor

To analyze the mechanism underlying the DHA-induced reduction of TfR expression, the level of mRNA for TfR was tested using a real-time (RT)-PCR assay. HL60 cells were exposed to DHA for 48 h, then total RNA was isolated and semiquantitative RT-PCR performed to measure TfR mRNA. DHA significantly decreased the level of TfR mRNA in HL60 cells by 29 ± 2.9% (0.5 μmol/l, $P < 0.05$) and 72 ± 1.1% (1 μmol/l, $P < 0.001$), compared to control cells (Fig. 6).

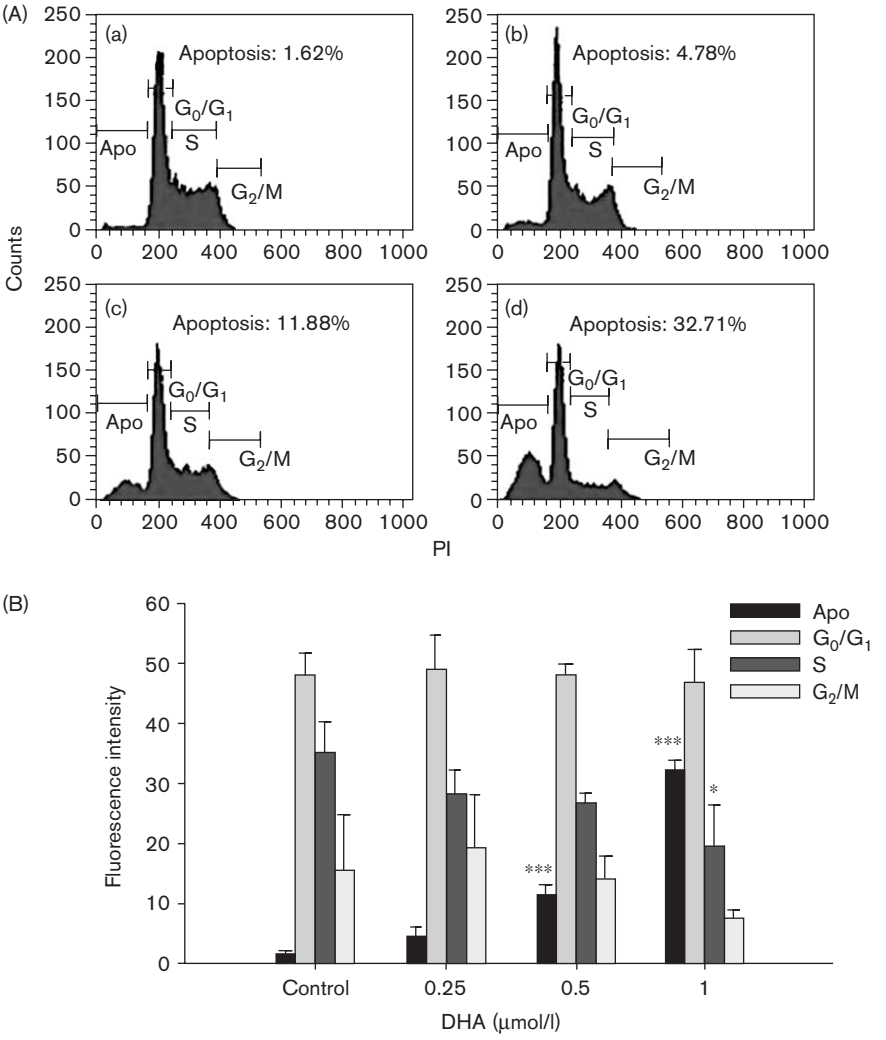
Result of transferrin receptor Western blot assay

To further investigate whether DHA effect was exerted at the protein level, after the treatment of HL60 cells with 0.25, 0.5, and 1 μmol/l DHA for 48 h, TfR expression was analyzed by western blot. As shown in Fig. 7, untreated cells expressed high levels of TfR protein, whereas in cells treated with DHA, the expression of TfR protein is downregulated in a dose-dependent manner. Furthermore, when treated with 1 μmol/l DHA for 6, 12, 24, and 48 h, the expression of TfR protein in HL60 cells was also downregulated in a time-dependent manner (Fig. 7).

Reduction of fluorescence intensity levels of transferrin receptor

To examine whether DHA interferes with TfR surface expression, we also detected the cell expression of TfR by using flow cytometry. The number of TfR positive cells and the fluorescence intensity (FI) reflect the intensity of TfR surface expression. As can be seen in Fig. 8, expression of TfR was considerably reduced. In detail, after treatment with various concentrations of DHA (0.25–1 μmol/l) for 48 h, the FI levels of TfR in HL60 cells were reduced by 12 ± 1.0% (0. 5 μmol/l, $P < 0.05$) and 33.2 ± 1.5% (1 μmol/l, $P < 0.01$), versus the control group (Fig. 8).

Fig. 4



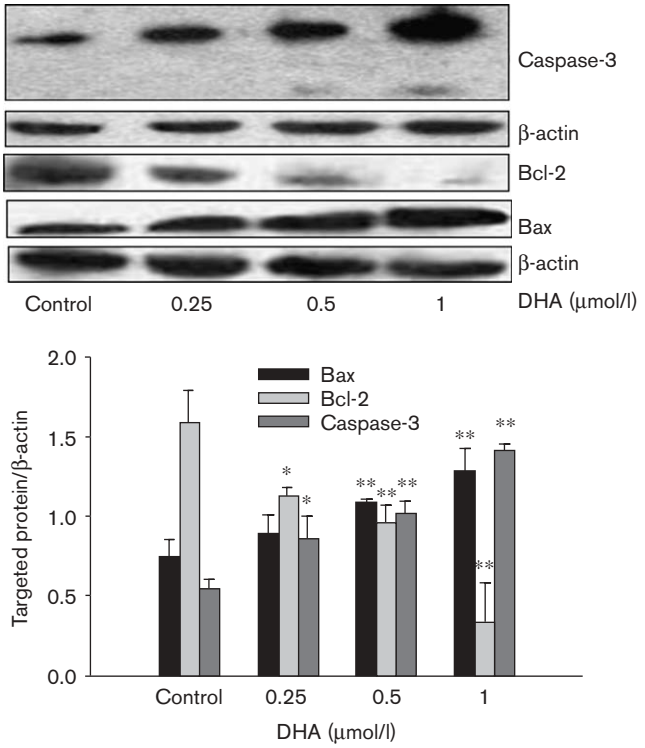
Flow cytometric analysis of DNA content in HL60 cells treated with DMSO or DHA. (A) Cells were incubated with or without DHA for 48 h and analyzed after the addition of PI solution for staining, and cell cycle profiles were determined by flow cytometry: (a) control; (b) DHA 0.25 μmol/l; (c) DHA 0.5 μmol/l; and (d) DHA 1 μmol/l. (B) Cells in sub-G_1 (apoptosis), S, G_0/G_1, and G_2/M phases of the cell cycle fractions were expressed as percentages of the total cell population. $\bar{x} \pm s$, $n=3$. *$P<0.05$; ***$P<0.001$, compared with control group. DHA, dihydroartemisinin; DMSO, dimethylsulfoxide; PI, propidium iodide.

Discussion

In our earlier studies, we observed that cancer cell growth was inhibited in a dose-dependent manner by DHA [20,21]. In this study, we have analyzed the antiproliferative and apoptotic effects of DHA and its regulation of TfR in a human acute promyeloid leukemia cell line, HL60. From the results of this study, it now appears that DHA may decrease TfR expression, then upregulate proapoptotic protein Bax and downregulate antiapoptotic protein Bcl-2, downstream activate caspase-3, finally leading to cell apoptosis.

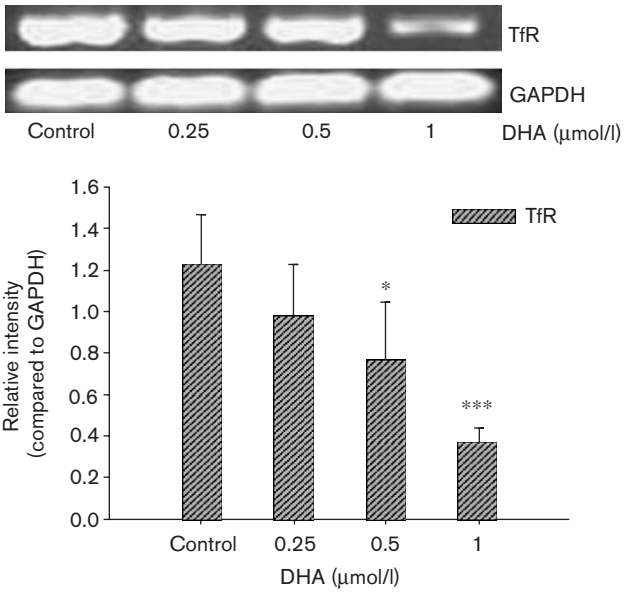
It has been reported that the antimalarial activity of DHA is dependent on high TfR protein content [22]. As DHA has an endoperoxide bridge whose cleavage by reacting with ferrous iron results in the generation of reactive oxygen species and/or artemisinin carbon-centered free radicals [23]. Such free radicals, when formed intracellularly, can cause macromolecular damage and subsequent cell death. Hence, iron is required for the drug-induced formation of reactive oxygen species, the activation of caspases, and consequent apoptosis. Whereas TfR is closely related to iron, so the protein TfR which expresses high level in the majority of cancer cell lines could be a good target. Increased TfR expression was detected on peripheral blood mononuclear cells from lymphoma, myeloma, or leukemic tumor patients compared with those taken from normal patients [24].

Fig. 5



Protein expression of caspase-3, Bcl-2 and Bax. HL60 cells were treated with DHA for 48 h, levels of Bax, BCL-2, and caspase-3 proteins were detected by Western blot. Over expressions of Bax and caspase-3 and reduction of Bcl-2 expression by DHA in a dose–response pattern were observed. β-Actin (43 kDa) levels were analyzed as internal controls. Relative expression levels of Bax, Bcl-2, and caspase-3 were expressed as the relative intensity compared with β-actin. $\bar{x} \pm s$, $n=3$. *$P<0.05$; **$P<0.01$, compared with control group. DHA, dihydroartemisinin.

Fig. 6

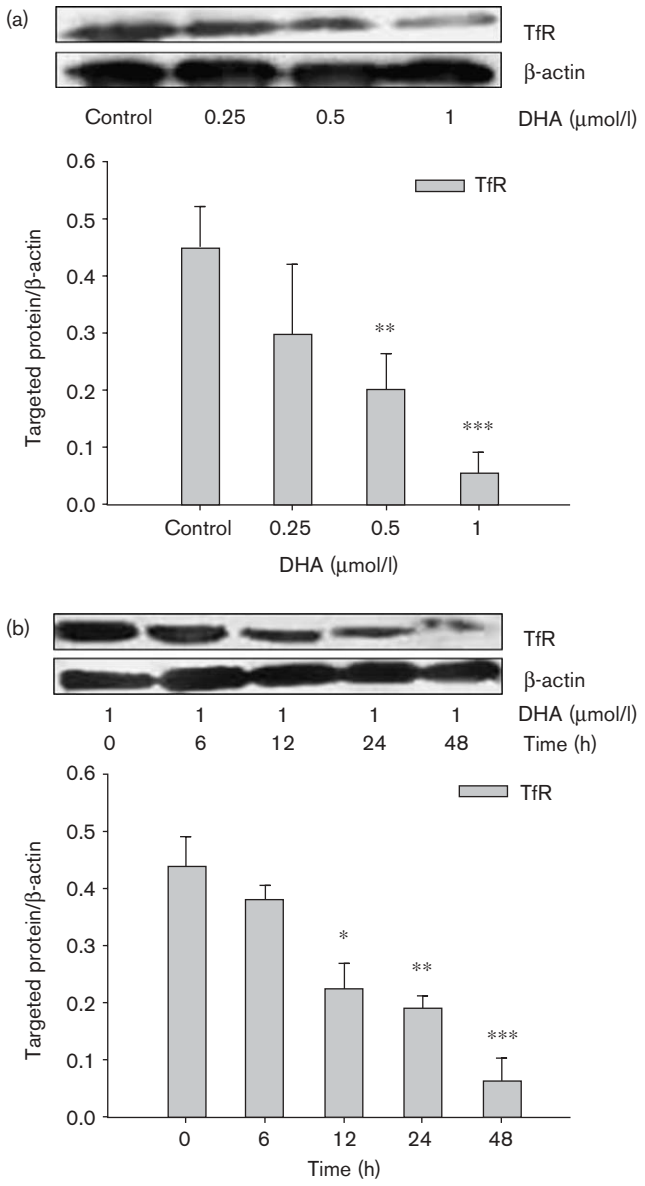


RT-PCR analysis of TfR mRNA levels after DHA exposure. HL60 cells were exposed under different concentrations of DHA during 48 h. RT-PCR analysis was performed using specific primers for TfR and GAPDH. The PCR products were analyzed by electrophoresis and photographed. Results shown are representative of three separate experiments. $\bar{x} \pm s$, $n=3$. *$P<0.05$; **$P<0.01$, ***$P<0.001$, compared with control group. DHA, dihydroartemisinin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TfR, transferrin receptor; real-time.

Bladder transitional cell carcinomas, breast cancer, gliomas, lung adenocarcinoma, chronic lymphocytic leukemia, and non-Hodgkin's lymphoma also showed increased TfR expression that correlated with tumor grade and stage or prognosis [25]. So according to the characteristic that the HL60 cell line expresses high levels of TfR on its cell surface [26], it is used for our investigation to examined whether DHA could interfere with TfR expression. The RT-PCR analysis showed that the decreased TfR mRNA level might be attributed to the operation of posttranscriptional mechanisms in the control of TfR gene expression in HL60 cells. This is further supported by the finding of the Western blot assay. The expression of TfR protein was decreased – as well as flow cytometry analysis. The FI of TfR was diminished after being exposed to DHA for 48 h. Level of TfR protein was also decreased by DHA in a time (12–48 h)-dependent manner. Particularly, at the early time (12 h) of treatment by DHA, the TfR expression level was significantly decreased by $48.8 \pm 9.3\%$ ($P < 0.05$). These results suggest that the quantity of TfR protein molecules on the surface of HL60 cells is proportional to the quantity of TfR mRNA transcripts to some extent. On the basis of the above findings, it would seem that TfR expression in HL60 is regulated both at the posttranscriptional level and protein level.

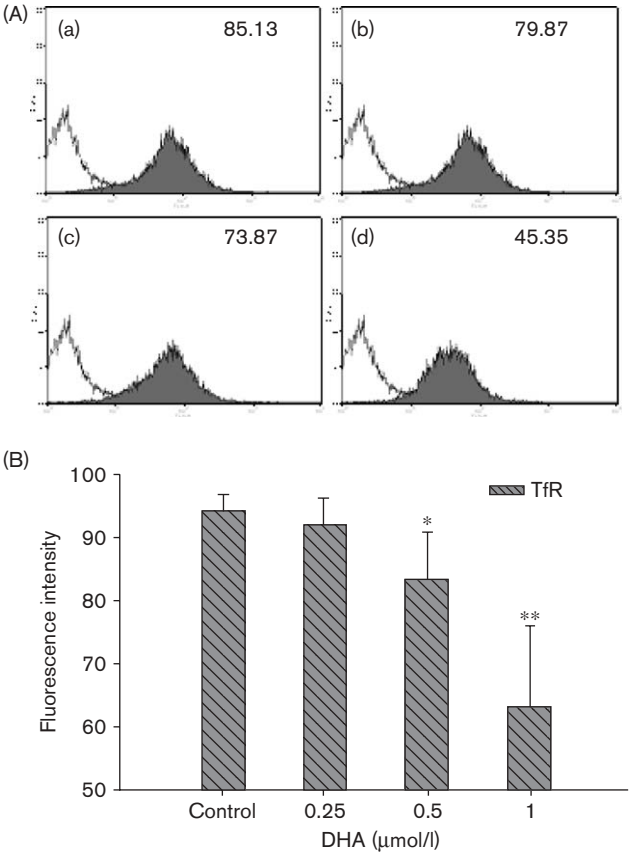
As discussed above, cancer cells require large amounts of iron for related proteins to maintain high proliferative rates. Hence, DHA could induce HL60 cell apoptosis by disturbing TfR expression and then result in iron deficiency. Apoptosis is a fundamental process essential for both development and maintenance of tissue homeostasis and this process was first recognized by Kerr *et al.* [27]. Apoptosis includes cell shrinkage and loss of contact with neighboring cells, formation of cytoplasmic vacuoles, plasma and nuclear membrane blebbing, chromatin condensation, and formation of apoptotic bodies [28]. After HL60 cells were treated with DHA for 48 h, the phase-contrast microscopic and fluorescence microscopic observations demonstrated these apoptotic characteristics. We have also noted DNA fragment ladder formation, a characteristic gel electrophoretic band pattern associated with apoptosis. DNA ladder, the biochemical hallmark of apoptosis, is degradation of DNA by endogenous DNase, which cuts the internucleosomal regions into double-stranded DNA fragments of 180–200 bp [29].

Fig. 7



Protein expression of TfR. (a) HL60 cells were treated with various concentrations of DHA for 48 h, levels of TfR (95 kDa) were analyzed by Western blot. β-Actin (43 kDa) was analyzed as internal control. (b) HL60 cells were treated with 1 μmol/l DHA for 0, 6, 12, 24, and 48 h, levels of TfR were analyzed by Western blot. Relative expression levels of TfR were expressed as the relative intensity compared with β-actin. Reduction of TfR expression by DHA in a dose–response and time–response pattern were observed. $\bar{x} \pm s$, $n=3$. *$P<0.05$; **$P<0.01$; ***$P<0.001$, compared with control group. DHA, dihydroartemisinin; TfR, transferrin receptor.

Fig. 8



Determination of TfR (CD71) content by flow cytometry. HL60 cells were exposed to DHA for 48 h. Then, flow cytometry analysis was performed after HL60 cells were stained with monoclonal antibodies (closed histograms) against TfR and with control isotype (open histograms). Numbers indicate the median fluorescence intensity values (A). The amount of TfR surface antigenic sites is expressed as arbitrary units of fluorescence (B). $\bar{x} \pm s$, $n=3$. *$P<0.05$; **$P<0.01$, compared with control group. DHA, dihydroartemisinin; TfR, transferrin receptor.

In addition, the results of flow cytometric analysis of DNA content (Fig. 4) further shows that DHA induced 1.62–32.71% increases in the DNA fragments when HL60 cells were treated with DHA (0.25–1 μmol/l) for 48 h.

Evidence is emerging that mitochondria play a key role in the activation or amplification of the caspase cascade. The activation of a family of intracellular cysteine proteases, called caspases, plays a key role in the initiation and execution of apoptosis induced by various stimuli. Among the several different members of caspases identified in mammalian cells, caspase-3 plays a direct role in the proteolytic cleavage of the cellular proteins responsible for progression to apoptosis [15,30]. Apparently, in our study, the mitochondrial apoptotic pathway is activated preferentially when cells are treated with DHA. The induction of caspase-3 activates downstream effector caspases, which ultimately leads to the cleavage of cytoskeletal and nuclear proteins. Woong *et al.* [31] also reported that artemisinin and its derivatives can induce caspase-3 activation.

Several apoptosis inducing agents are known to trigger mitochondrial uncoupling, leading to the rupture of outer membrane. Proteins such as Bcl-2 and Bcl-X_L prevent the release of apoptogenic proteins from mitochondria and

therefore protect against outer membrane permeabilization while proapoptotic Bcl-2 family members, such as Bax and Bak, induce outer membrane permeabilization and cause the release of proapoptotic factors from mitochondria [32,33]. Bcl-2 can form heterodimers with Bax protein – a Bcl-2 associated protein which antagonizes Bcl-2 function and induces cell apoptosis [34]. In this study, we found that both Bcl-2 and Bax were expressed in HL60 cells. The levels of Bcl-2 in HL60 cells decreased with increasing concentrations of DHA. At the concentrations of 0.5 and 1 μmol/l, DHA potently downregulated Bcl-2 expression. In contrast to the decreased Bcl-2 levels, the expression of Bax was upregulated after treatment with DHA at various concentrations. The resulting net effect could thus lead to a lowered ratio of Bcl-2/Bax, which might be responsible for the DHA-induced apoptosis in HL60 cells.

Overall, we have demonstrated that DHA induces HL60 cell apoptosis via the TfR interfering mechanism, consequently inhibiting cell iron uptake and resulting in upregulation of the Bcl-2/Bax ratio and downstream activation of caspase-3, followed by an induction of apoptosis through the mitochondrial pathway. Collectively, these results suggest that DHA is a good candidate for future leukemia treatment strategies.

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