



Dihydroartemisinin induces autophagy and inhibits the growth of iron-loaded human myeloid leukemia K562 cells via ROS toxicity

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ARTICLE INFO

Article history:

Received 10 February 2012

Revised 15 May 2012

Accepted 16 May 2012

Keywords:

Dihydroartemisinin

ROS

Mitophagy

K562 cell

Iron

ABSTRACT

Dihydroartemisinin (DHA), an active metabolite of artemisinin derivatives, is the most remarkable anti-malarial drug and has little toxicity to humans. Recent studies have shown that DHA effectively inhibits the growth of cancer cells. In the present study, we intended to elucidate the mechanisms underlying the inhibition of growth of iron-loaded human myeloid leukemia K562 cells by DHA. Mitochondria are important regulators of both autophagy and apoptosis, and one of the triggers for mitochondrial dysfunction is the generation of reactive oxygen species (ROS). We found that the DHA-induced autophagy of leukemia K562 cells, whose intracellular organelles are primarily mitochondria, was ROS dependent. The autophagy of these cells was followed by LC3-II protein expression and caspase-3 activation. In addition, we demonstrated that inhibition of the proliferation of leukemia K562 cells by DHA is also dependent upon iron. This inhibition includes the down-regulation of Tfr expression and the induction of K562 cell growth arrest in the G₂/M phase.

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1. Introduction

Artemisinin, the active component of the Chinese plant qinghao (*Artemisia annua* L.), is a remarkable and novel anti-malarial drug with little toxicity [1]. It contains an endoperoxide bridge that reacts with ferrous iron to generate free radicals, such as reactive oxygen species (ROS), which leads to macromolecular damage and cell death. Dihydroartemisinin (DHA), its most active derivative [2], has been recently reported to possess a preferentially cytotoxic effect on cancer cells [3,4]. It has been reported that DHA kills cancer cells via an iron-mediated mechanism [5], and this was further confirmed by a later experiment, by the same research group, on human breast cancer cells [6]. More recently, Lai et al. reported that artemisinin that is tagged with holotransferrin is more effective than artemisinin alone in killing cancer cells [7]. Because the endoperoxide bridge of dihydroartemisinin is essential for its cytotoxicity, the effects of its reaction with iron and the resulting product, ROS, deserve more investigation.

Autophagy is a non-apoptotic cell death mechanisms characterized by the engulfment of the cytoplasm and organelles by double-membrane bound structures, autophagosomes, followed

by the delivery to and subsequent degradation in lysosomes [8–10]. Autophagy has been reported to play a crucial role in many diseases, such as cancer, infectious diseases, and neurodegenerative disorders [11–14]. During autophagy, microtubule-associated protein 1 light chain 3 (LC3) is cleaved at its C-terminal arginine residue to form LC3-I. LC3-I is easily activated, conjugated to phosphatidylethanolamine, and subsequently bound to the membrane to form LC3-II. LC3-II is localized in the autophagosome and has been utilized as an autophagosome marker.

The role of autophagy in tumor progression is complex. In some systems, the induction of autophagy has been shown to contribute to or enhance the apoptotic response [15].

Mitochondria are important regulators of both autophagy and apoptosis, and one of the triggers for mitochondrial dysfunction are the ROS. ROS induce damage to the membrane, DNA, protein, and organelles. Therefore, mechanisms regulating the function and quantity of mitochondria are essential for eukaryotic cell function. Autophagy contributes to the maintenance of mitochondria by their clearance [16], and this process is mediated by a selective type of autophagy termed mitophagy [17–19]. Recent studies have also highlighted the important contributions of generated ROS to this response.

Evidence is also 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, is vital to the initiation and execution of apoptosis that is induced by various stimuli. Of the several different caspases identified in mammalian cells, caspase-3 plays a primary role in the proteolytic

Abbreviations: DHA, dihydroartemisinin; PBS, phosphate buffer saline; Tfr, transferrin receptor; ROS, reactive oxygen species; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AO, acridine orange; EB, ethidium bromide; PARP, poly(ADP-ribose) polymerases

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cleavage of the cellular proteins responsible for the progression to apoptosis [20,21].

Iron is fundamental for life because it is a cofactor of enzymes, such as cytochrome c and ribonucleotide reductase, that are essential for ATP production and DNA synthesis. The uptake of iron from transferrin (Tf) is controlled by the expression of its receptor, transferrin receptor (TfR), which is modulated by intracellular iron levels [22,23]. Erythroid precursors and malignant cells, especially leukemia, are highly dependent upon iron to sustain their characteristically high proliferation rates, and the TfR is expressed at higher levels in these cells [24,25]. This characteristic renders tumor cells more sensitive to iron depletion, which is well known to cause cell apoptosis or autophagy [26,27].

In the present study, we intended to elucidate the mechanisms underlying the autophagy induced by DHA and the inhibition of growth of iron-loaded human myeloid leukemia K562 cells. We found that DHA-induced autophagy, in which vacuoles contain intracellular organelles that are primarily mitochondria, is ROS dependent. The autophagy is followed by LC3-II protein expression and caspase-3 activation. We also demonstrated that the inhibition of leukemia K562 cell proliferation by DHA is also dependent upon iron, and this inhibition includes the down-regulation of TfR expression and the induction of K562 cell growth arrest in the G₂/M phase.

2. Experimental procedures

2.1. Reagents

Dihydroartemisinin was kindly provided by Engineer Liuxu of Guiling Pharmaceutical Co. (Guangxi, China). Holotransferrin (iron-loaded) was purchased from Sigma (St. Louis, Missouri, USA). Rabbit anti-Beclin 1 polyclonal antibody, mouse anti-TfR (3B8 2A1), rabbit anti-Caspase-3 (H-277), and goat anti-actin polyclonal antibody (I-19) and all the secondary antisera were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Rabbit anti-LC3 monoclonal antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). Acridine orange (AO), ethidium bromide (EB), and propidium iodide (PI) were obtained from Sigma (St. Louis, MO, USA).

2.2. Cell culture

K562, a chronic myelogenous leukemia line, was 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% CO₂. Exponentially growing cells were used throughout the study.

2.3. Transmission electron microscopy analysis

Cells were fixed overnight in 2.5% glutaraldehyde in 0.2 M phosphate buffer before being post-fixed in 1% OsO₄ for 45 min. Subsequently, the cells were dehydrated in an ethanol series and embedded in embedding medium. Ultra-thin sections were stained with uranyl acetate and lead citrate and observed under a Philips-TECNAI10 transmission electron microscope.

2.4. Growth inhibition assay

The *in vitro* growth inhibition effect of DHA on leukemia cells was determined by a modified microculture tetrazolium (MTT) assay.

2.5. Morphological examination for apoptosis (AO/EB staining)

The apoptotic morphology was studied in K562 cells stained with a combination of the fluorescent DNA-binding dyes acridine orange and ethidium bromide (100 µg/ml each in phosphate buffer saline) [28]. The viable cells display nuclei with bright green fluorescence and homogeneous chromatin, whereas apoptotic cells show condensed and fragmented chromatin with bright orange fluorescence. Necrotic cells display a diffuse orange staining pattern.

After being incubated with DHA for 48 h, K562 cells were washed twice with PBS and incubated for 3–5 min with AO/EB at room temperature in the dark. The cells were then observed using an inverted fluorescence microscope (DMIL, Leica Microsystems, Wetzlar, Germany) and photographed.

2.6. Flow cytometry analysis

To quantitate intracellular staining of TfR (CD71), mouse monoclonal anti-human TfR antibody and FITC conjugated goat anti-mouse immunoglobulin antiserum (Santa Cruz-2010, Santa Cruz, California, USA) were used for flow cytometric analysis. An isotype IgG (Santa Cruz-2855, Santa Cruz, California, USA) was used as a negative control. Samples were analysed by flow cytometry (Becton Dickinson, Lincoln Park, NJ, USA). 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. All measurements were performed according to manufacturer's instructions.

To quantitate apoptosis, flow cytometric analysis was performed using propidium iodide (PI) as described previously [29]. Briefly, the cells were treated with various concentrations (0.25–1 µM) of DHA for 48 h. After treatment, the cells were prepared as a single cell suspension in 50 µl PBS, which was 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 PI in the dark at 4 °C for 30 min. The red fluorescence of individual cells was measured with a FACS Calibur flow cytometer (Becton Dickinson, Lincoln Park, NJ, USA). A minimum of 30000 events was collected per sample. The relative DNA content per cell was obtained by measuring the fluorescence of the PI that bound stoichiometrically to DNA.

To determine ROS generation within DHA-treated cells, FACS analysis was performed. Cells were exposed to a range concentrations of DHA for 48 h, stained with 5 µg/ml DCFH-DA for 20 min, subjected to flow cytometry, and analysed by Cell Quest software (Becton-Dickinson, San Jose, CA).

2.7. Atomic absorption spectrophotometric analyses

Cells were harvested by centrifugation (300 g, 5 min), washed three times with PBS, and then dried at 50 °C for 24 h. The dried cells were digested with 5 ml of 14 M nitric and 10 M perchloric acids (2:1, v/v) in a glass tube at 250 °C for 8 h. The iron contents of cell samples were determined by graphite furnace atomic absorption spectrophotometry (Thermo Electron, USA). A standard curve for iron was prepared from commercially available standards (Spectrosol; BDH). Appropriate quality controls were included as necessary.

2.8. RNA extraction and reverse transcriptase-PCR

Total RNA was isolated from the K562 cells utilizing Trizol Reagent (Bio Basic) according to manufacturer's instructions. After cDNA was amplified, PCR was carried out with a Thermal cycler (Eppendorf, 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 ethidium bromide staining. The expression intensities of optimized bands were quantified with Quantity One software. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. TFR primers: forward, 5'-CTGCCTCTTCTGTTGTTGT-3' and reverse, 5'-CTTTGGCCAAATTTGGCAGC-3' (429bp).

2.9. Western blotting

Total protein extracts from cells were obtained by lysing K562 cells that were pretreated with DHA for 483 h in cold RIPA buffer (50 mM Tris-HCl (PH8.0), 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton-X100, and 1 mM EDTA, supplemented with 1 mM 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). Subsequently, equal amounts of protein were separated by 12% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 5% non-fat milk and incubated overnight with the primary antibodies at 4 °C in PBS-T. The primary antibodies were

mouse anti-TfR (3B8 2A1), rabbit anti-Caspase-3 (H-277), rabbit anti-LC3 monoclonal antibody, rabbit anti-Beclin 1 polyclonal antibody, and goat anti-actin polyclonal antibody (I-19). Immunoreactivity was visualized with horseradish peroxidase-linked secondary antibodies and chemiluminescence; β -actin levels were analysed as controls for protein loading.

2.10. Data analysis

The statistical significance of mean values was determined by an unpaired two-tailed Student's *t*-test. The significance of the dose-response curve was determined by an ANOVA. A *P*-value <0.05 was considered to be statistically significant.

3. Results

3.1. Induction of autophagy in K562 cells

Electron microscopy experiments showed that after treating K562 cells with DHA, either with or without TF, a different morphology was observed with numerous double-walled membrane-

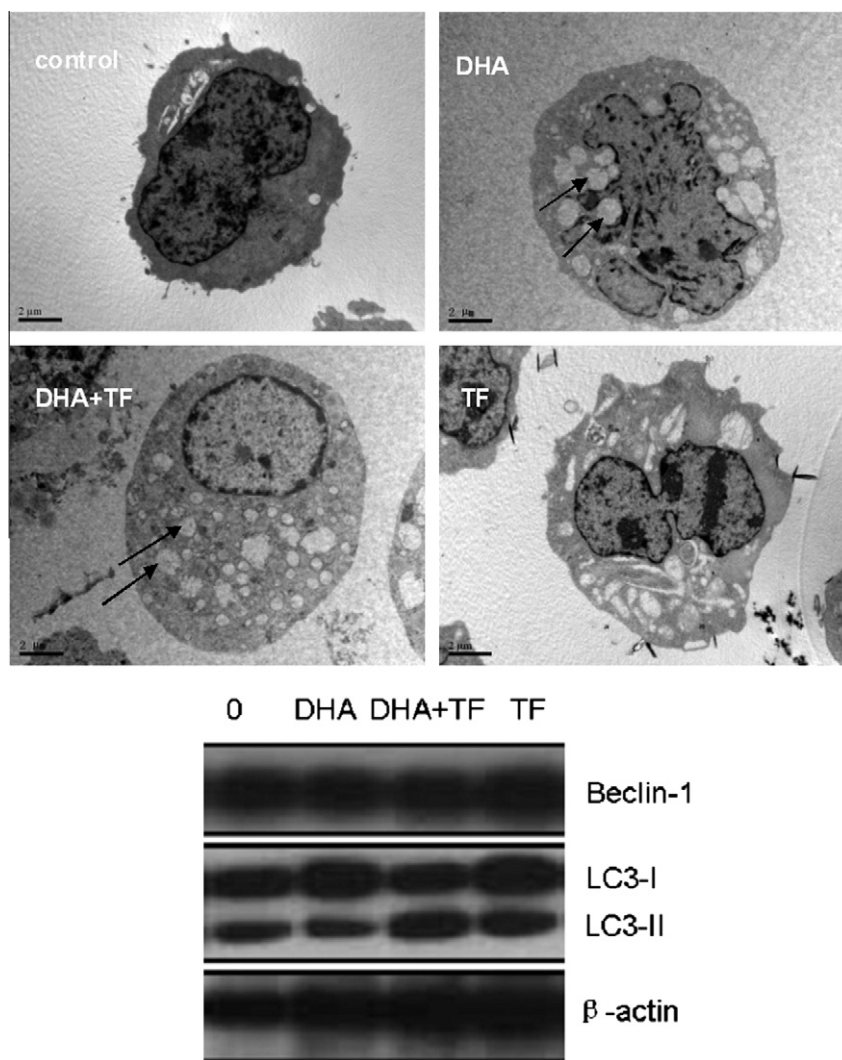


Fig. 1. DHA induced autophagy. Control group: K562 cells were incubated in RPMI1640 standard medium for 48 h. DHA group: K562 cells were incubated with 10 µM DHA for 48 h. DHA + TF group: K562 cells were incubated with 20 nM holotransferrin (TF) for 1 h and subsequently exposed to 10 µM DHA for 48 h. TF group: K562 cells were incubated with 20 nM holotransferrin (TF) for 48 h. DHA induced autophagy was analysed by transmission electron microscopy ($\times 3700$), and the autophagy pathway proteins LC3-I and LC3-II were detected by Western blotting. The 2 µm upon the scale bar stands for magnifying 3700 folds. Black arrows referred to the autophagosome which contained mitochondria.

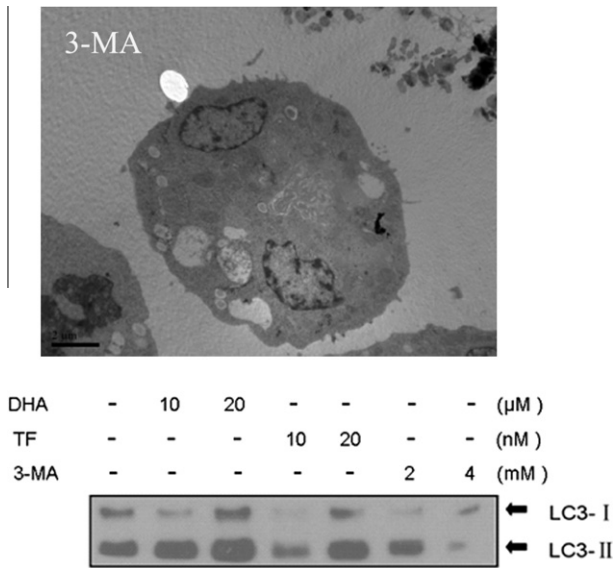


Fig. 2. Effect of autophagy blocker 3-MA. 3-MA group: K562 cells were incubated with 4 mM 3-MA. Effect of autophagy blocker 3-MA was analysed by transmission electron microscopy, and the autophagy pathway proteins LC3-I and LC3-II were detected by Western blotting. The autophagy blocker 3-MA was used to define the functional relevance of the induction of autophagy in the overall toxicity of DHA + holotransferrin. The 2 μm upon the scale bar stands for magnifying 3700 folds.

enclosed cytoplasmic vacuoles. These vacuoles contained more intracellular organelles, primarily mitochondria, than untreated cells (Fig. 1). Autophagy is also associated with the expression of autophagic proteins. Therefore, we evaluated the effect of DHA on the expression of LC3-I and LC3-II proteins, which are related to the autophagic process in K562 cells. LC3-II is localized in the autophagosome and has been used as an autophagosome marker. LC3-I is formed by the proteolytic cleavage of cytosolic microtubule-associated protein 1 light chain 3 (LC3) and eventually conjugates with phosphatidyl ethanolamine to form LC3-II. After K562 cells are treated with DHA under different conditions with iron, we found that 10 μM DHA exerted a more powerful stimulation of LC3-II protein expression (Fig. 1) when compared to K562 cells with normal iron levels. Furthermore, we used the autophagy blocker (3-methyladenine, 3-MA) to define the functional relevance of the induction of autophagy in the overall toxicity of DHA + holotransferrin. We found that when K562 cells were treated with 2 mM 3-MA, the expression of LC3-II protein was similar to the control group. When K562 cells were treated with 4 mM 3-MA, the expression of LC3-II protein was strikingly decreased. More, in the transmission electron microscopy analysis on K562 cells treated with 4 mM 3-MA, it showed little double-walled membrane-enclosed cytoplasmic vacuoles (Fig. 2). These results indicate that DHA-induced autophagy may contribute to cell death in K562 cells, not simply a non-functional consequence of the overall stress response.

Besides, in Fig. 2, it showed that 10 μM DHA could stimulate the expression LC3-II protein, and the effect of 20 μM DHA on LC3-II protein stimulation was even more severe. Though 10 nM holotransferrin had little effect on LC3-II protein stimulation, 20 nM holotransferrin could stimulate the expression LC3-II protein. In Fig. 1, the combination of 10 μM DHA and 20 nM holotransferrin exerted a more powerful stimulation of LC3-II protein expression.

3.2. Up-regulation of intracellular ROS levels in K562 cells

After clearly establishing the ability of DHA to induce autophagy in K562 cells, we assessed its effect on intracellular ROS

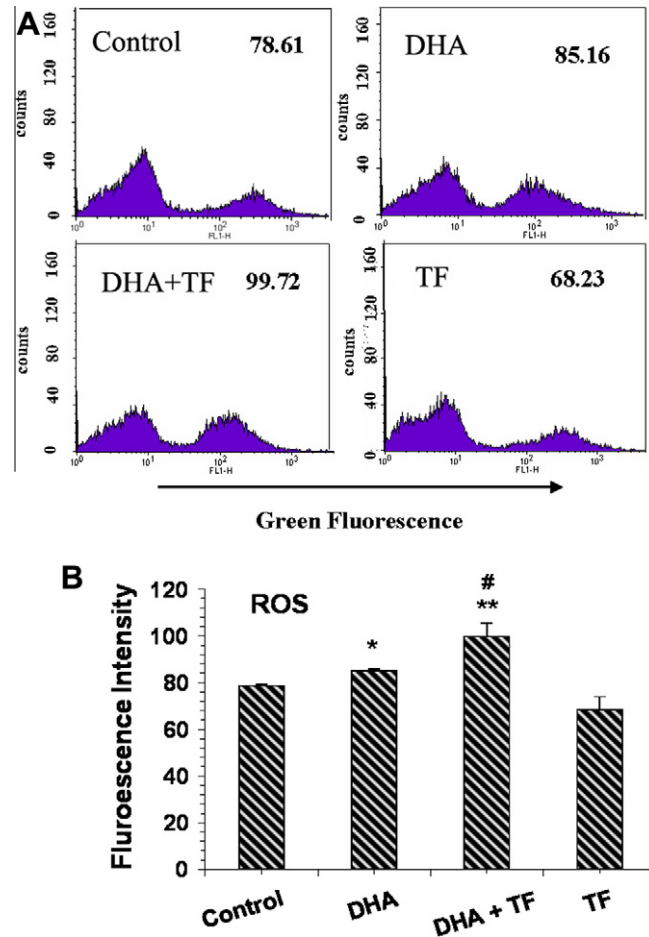


Fig. 3. Effect of DHA on intracellular ROS generation. (A) ROS generation within DHA-treated cells was determined by FACS analysis. Numbers indicate the median fluorescence intensity values. Control group: K562 cells were incubated in RPMI1640 standard medium for 48 h. DHA group: K562 cells were incubated with 10 μM DHA for 48 h. DHA + TF group: K562 cells were incubated with 20 nM holotransferrin (TF) for 1 h and subsequently exposed to 10 μM DHA for 48 h. TF group: K562 cells were incubated with 20 nM holotransferrin (TF) for 48 h. (B) Estimating the number of TFR surface antigenic sites in K562. Cells are expressed as arbitrary units of fluorescence. * $P < 0.05$; ** $P < 0.01$, compared with control group, # $P < 0.05$ compared to 10 μM DHA.

production using fluorescent flow cytometry analysis (Fig. 3). The exposure of cells to DHA resulted in a significant increase in intracellular ROS production. Moreover, after pretreatment of the K562 cells with 20 nM holotransferrin, an iron-loaded transferrin (plasma iron carrying protein), the intracellular ROS level was higher than that observed in the DHA group ($P < 0.05$).

3.3. Caspase cascade in DHA-induced apoptosis

We also studied the activation of caspase-3 and PARP by DHA in iron-overloaded K562 cells. All groups expressed pro-caspase-3 (inactive form, a 32 kD band) as observed by Western blot analysis. However, the cleaved form of caspase-3 (active), a 17 kD band, could only be detected in the 10 μM DHA group both with and without 20 nM holotransferrin. Meanwhile, the expression of poly(ADP-ribose) polymerases (PARP, 112 kD) was also observed to be down-regulated by 10 μM DHA with a corresponding up-regulation of cleaved PARP (active form, 85 kD) (Fig. 4). In addition, cells pretreated with 20 nM holotransferrin 1 h prior to the administration of 10 μM DHA showed significant up-regulation of cleaved PARP in K562 cells when compared to iron-normal cells

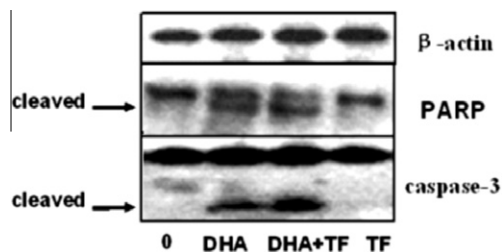


Fig. 4. Cleavage of caspase-3 and PARP. Control group: K562 cells were incubated in RPMI1640 standard medium for 48 h. DHA group: K562 cells were incubated with 10 μ M DHA for 48 h. DHA + TF group: K562 cells were incubated with 20 nM holotransferrin (TF) for 1 h and subsequently exposed to 10 μ M DHA for 48 h. TF group: K562 cells were incubated with 20 nM holotransferrin (TF) for 48 h. Caspase-3, PARP and beta-actin expressions were analysed by Western blot.

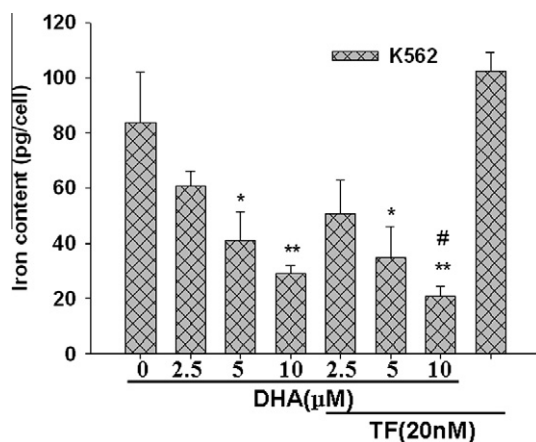


Fig. 5. Effect of DHA on iron content in iron-normal and iron-overload K562 cells. K562 cells were either incubated with 20 nM holotransferrin for 1 h and subsequently exposed to 2.5, 5, or 10 μ M DHA or only exposed to 2.5, 5, or 10 μ M DHA for 48 h. TF group: K562 cells were incubated with 20 nM holotransferrin (TF) for 48 h. The quantitative iron content was determined with an atomic absorption spectrophotometer. * $P < 0.05$; ** $P < 0.01$, compared with control group, # $P < 0.05$ compared to 10 μ M DHA.

treated with DHA at the same concentration. These data indicate that caspase-3 and PARP were activated in apoptotic K562 cells during treatment with DHA, especially when overloaded with iron.

3.4. Decrease of iron content in K562 cells

When the intracellular iron contents of K562 cells were measured by atomic absorption spectrometry, we found that the iron content of iron-normal K562 cells was diminished by DHA in a dose-dependent manner (Fig. 5). However, after pretreatment of K562 cells with 20 nM holotransferrin, DHA exerted a more potent effect than that observed under iron-normal conditions. Specifically, the iron content of iron-overloaded K562 cells decreased $27.7 \pm 2.0\%$ ($P < 0.05$) with 10 μ M DHA when compared with the iron-normal group. These results suggest that the decrease in cellular iron content is partially caused by the consumption of iron through its reaction with DHA.

3.5. Down-regulation of TfR expression in K562 cells

TfR expression is controlled by the amount of iron that is required by the cell to maintain its metabolism. Therefore, tumor cells in a state of high proliferation possess a higher density of transferrin receptors [25]. We specifically raised the iron concentration in the cell cultures with holotransferrin. The results of

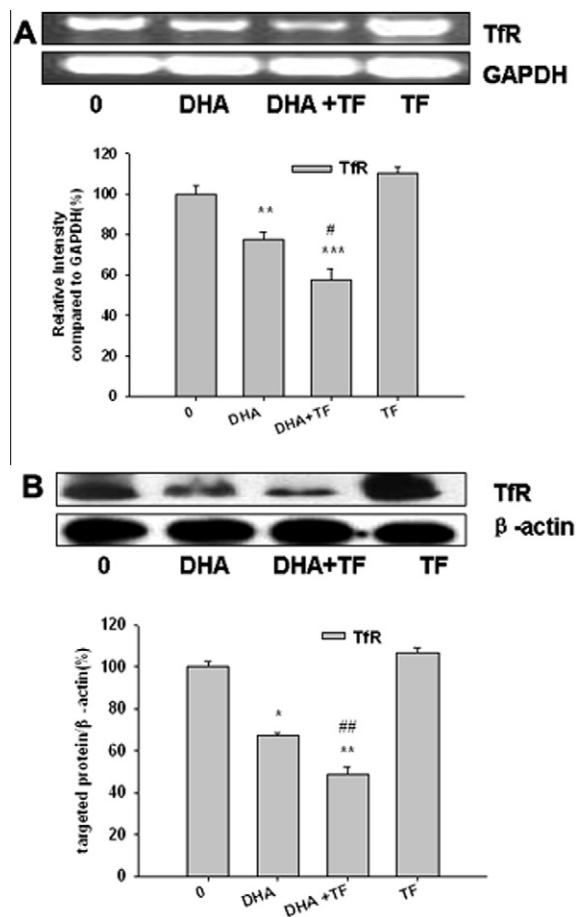


Fig. 6. Effect of DHA on expression of TfR mRNA and protein. K562 cells were incubated with 20 nM holotransferrin for 1 h and subsequently exposed to 10 μ M DHA for 48 h or only exposed to 10 μ M DHA for 48 h. The GAPDH and beta-actin were used as controls. The levels of TfR mRNA (A) and protein (B) were detected. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with control group, # $P < 0.05$; ## $P < 0.01$ compared to 10 μ M DHA.

Western blot analysis and RT-PCR assay (Fig. 6) show that 10 μ M DHA inhibited the expression of TfR mRNA and TfR protein to a greater extent under iron-overload conditions than under iron-normal conditions in K562 cells ($P < 0.05$). These results indicate that DHA may have a direct effect on TfR expression and that the effect is further enhanced by iron. However, the mechanism still needs more definitive study. Based on these results, it seems that TfR expression in K562 is regulated at both the protein and post-transcriptional level.

In addition, to determine if DHA interferes with the TfR surface expression, we observed the expression of TfR in the cell using flow cytometry (Fig. 7). The number of TfR positive cells and the fluorescence intensity (FI) reflect the intensity of the TfR surface expression. As shown in Fig. 7, K562 cells pretreated with 20 nM holotransferrin 1 h prior to the administration of 10 μ M DHA showed a significant reduction in the fluorescence intensity levels of TfR. The levels decreased by $30.2 \pm 0.5\%$ ($P < 0.05$) when compared with iron-normal cells that were treated with DHA at the same concentration. This is further supported by the results shown above. According to the results of atomic absorption spectrophotometric analyses and flow cytometry analysis, the decrease of transferrin receptor might be related to the consumption of iron through its reaction with DHA. In this study, we found that the iron content in TfR high expression K562 cells was diminished by DHA in a dose-dependent manner. “Iron is important for mitochondrial

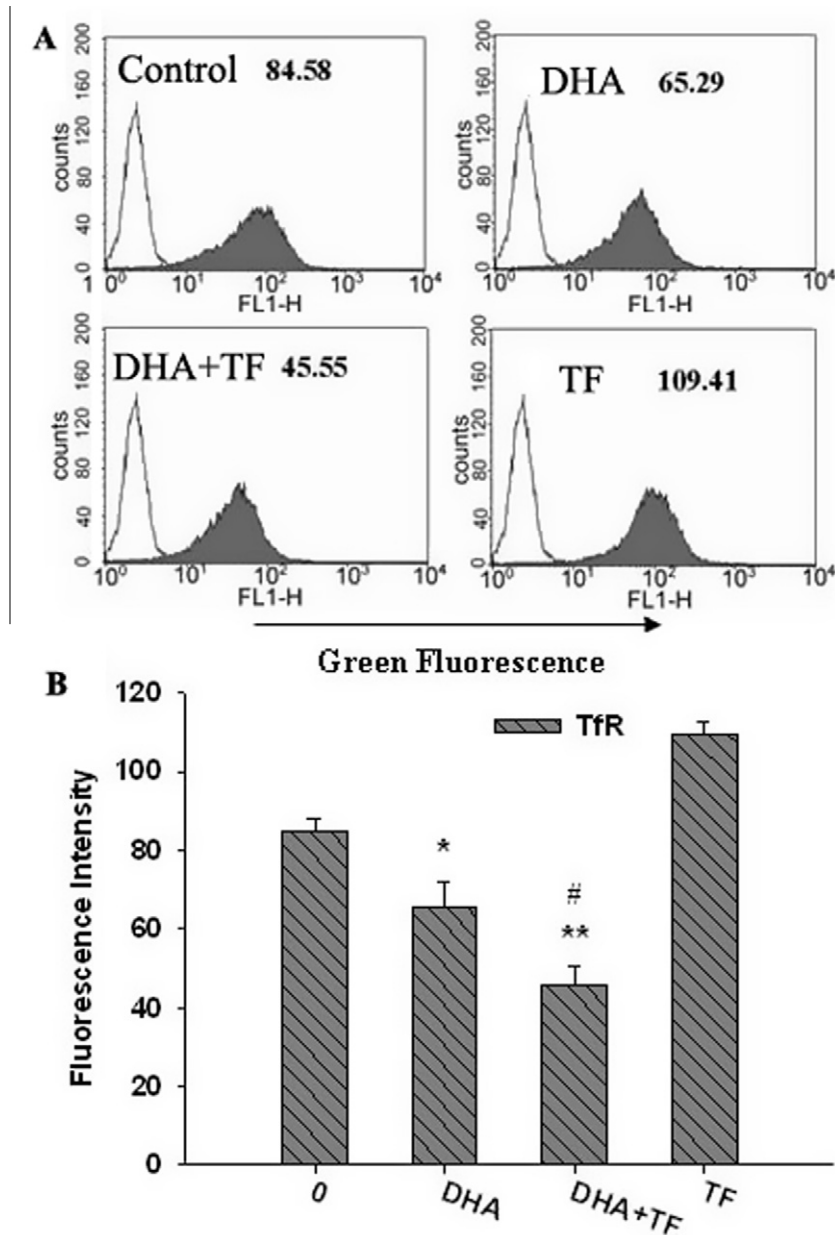


Fig. 7. Effect of DHA on TfR content. Flow cytometry analysis was performed after K562 cells were stained with monoclonal antibodies (closed histograms) against Tfr and with control isotype (open histograms). Numbers indicate the median fluorescence intensity values. (A) Control group: K562 cells were incubated in RPMI1640 standard medium for 48 h. DHA group: K562 cells were incubated with 10 μ M DHA for 48 h. DHA + TF group: K562 cells were incubated with 20 nM holotransferrin (TF) for 1 h and subsequently exposed to 10 μ M DHA for 48 h. TF group: K562 cells were incubated with 20 nM holotransferrin (TF) for 48 h. (B) Estimating the number of TfR surface antigenic sites in K562. Cells are expressed as arbitrary units of fluorescence. * $P < 0.05$; ** $P < 0.01$, compared with control group, # $P < 0.01$ compared to 10 μ M DHA.

heme and iron sulfur cluster synthesis"; it is likely that iron chelation will also affect basic mitochondrial metabolism and function. Furthermore, the expression of TfR was influenced by the iron status [30].

3.6. Enhancement of the cytotoxicity of DHA toward iron-overloaded K562 cells

The cytotoxic activities of DHA in K562 cells were assessed by examining its effects on cellular dehydrogenase activity with an MTT assay. The results revealed that DHA reduced cell survival in a dose-dependent manner. The K562 cell lines showed low IC50 values (11.33 μ M), and the 95% confidence interval was 9.38–13.68 μ M (Fig. 8B).

Since DHA becomes more cytotoxic in the presence of ferrous iron, we investigated the effect of holotransferrin on the proliferation of K562 cells. The result shows that holotransferrin maximally promoted cell growth at a concentration of approximately 20 nM, but the effect was not dose-dependent (Fig. 8A).

We subsequently examined the effect of holotransferrin on the sensitization of K562 cells to DHA. As shown in Fig. 8B, cells pretreated with 20 nM holotransferrin 1 h prior to administration of DHA showed a significantly reduced level of cell survival when compared to cells that were not treated with holotransferrin. The iron-overloaded K562 cell lines showed even lower IC50 values (3.67 μ M), and the 95% confidence interval was 3.20–4.21 μ M. The combination of holotransferrin and DHA resulted in 3.1-fold increase in cytotoxicity when compared with the result from

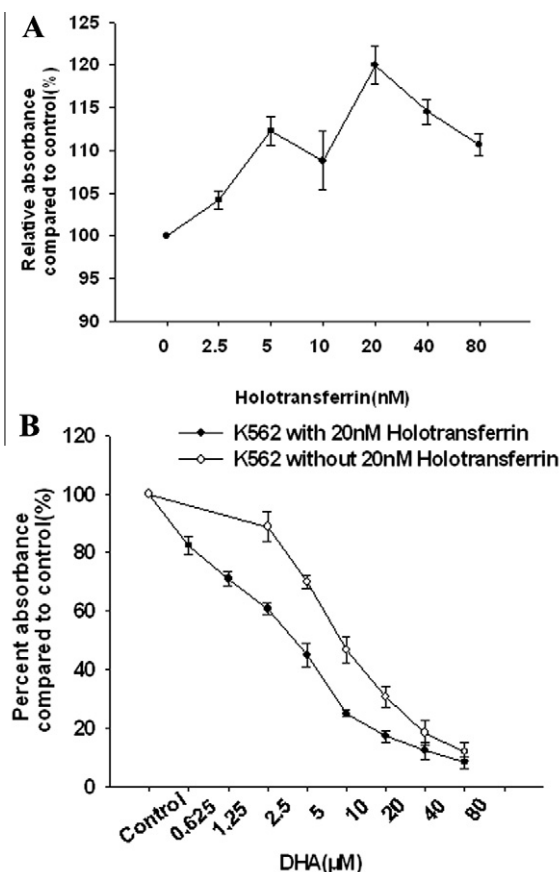


Fig. 8. Effect of DHA on proliferation of iron-normal and iron-overload K562 cells. (A) K562 cells were seeded into 96-well plates and incubated with holotransferrin at various concentrations for 48 h. (B) Open circle – K562 cells were seeded into 96-well plates and incubated for 48 h with DHA at various concentrations; solid circle – K562 cells were seeded into 96-well plates and incubated with 20 nM holotransferrin for 1 h, then various concentrations of DHA were added and incubation was continued for 48 h. Subsequently, the growth inhibition effect of DHA on K562 cells was determined by modified microculture tetrazolium (MTT) assay.

DHA alone. According to our previous research [31], a similar effect was also observed in HL60 cells when DHA was administered with or without holotransferrin (Table 1), but the degree of modulation of the IC₅₀ is lower than that in K562 cells that were administered DHA with or without holotransferrin.

3.7. Growth of K562 cells in iron-normal and iron-overloaded conditions arrested by DHA

To provide more direct evidence for the effect of DHA-induced cytotoxicity in K562 cells, we raised the iron concentration in the cell cultures with holotransferrin. The K562 cells were incubated with 20 nM holotransferrin for 1 h, and then the cells were treated with DHA for 48 h. Fig. 9 shows the comparison of morphological apoptotic alterations after 48 h of treatment with DHA between the early and late apoptotic cells as shown by their membrane integrities. The normal morphologies of the cells are shown in Fig. 9A. When treated with 2.5–10 μM DHA for 48 h, the cells exhibited cell shrinkage, membrane blebbing, chromatin condensation, and the formation of apoptotic bodies (Fig. 9B–D). All of these are characteristic morphological changes associated with apoptosis. Therefore, these morphological changes suggest that apoptosis occurs in iron-overloaded K562 cells after treatment with DHA.

Furthermore, we investigated the effects of DHA on the cell growth cycle of K562 cells by flow cytometry analysis (Fig. 10).

Table 1

Anti-tumor activity of DHA alone and in combination with holotransferrin in K562 and HL60 cells.

Cell line	DHA IC ₅₀ (μM)	DHA + TF IC ₅₀ (μM)	Degree of modulation
K562	11.33	3.67	3.1
HL60	1.74	0.91	1.9

DHA IC₅₀ (μM): K562 cells and HL60 cells were seeded into 96-well plates and incubated for 48 h with DHA at various concentrations. DHA + TF IC₅₀ (μM): K562 cells were seeded into 96-well plates and incubated with 20 nM holotransferrin for 1 h, then various concentrations of DHA were added and incubation was continued for 48 h. Subsequently, the growth inhibition effect of DHA on K562 cells was determined by modified microculture tetrazolium (MTT) assay.

Degree of modulation = DHA IC₅₀ (μM)/(DHA + TF)IC₅₀ (μM).

K562 cells exposed to 10 μM DHA in an iron-overloaded condition (Fig. 10, DHA + TF) showed a significant decrease of cells in the S phase (53.82%) and a corresponding increase of cells in the G₂/M phase (100.06%) and the sub-G₁ phase (610.29%) when compared to the control group. When K562 cells were exposed to 10 μM DHA in an iron-normal condition (Fig. 10, DHA), the decrease of cells in the S phase was just 17.55%, and the increase of G₂/M phase and sub-G₁ phase was 62.37% and 237.45% when compared to the control group. Compared to K562 cells in iron-normal conditions, DHA more effectively induced the arrest of iron-overloaded K562 cell growth in the G₂/M phase and partially arrested in the apoptotic sub-G₁ phase. These results were directly correlated with the inhibition of cell proliferation.

4. Discussion

In our previous study [31], we found that DHA-mediated apoptosis in HL60 cells was associated with the down-regulation of TfR expression, reduction of iron uptake, and regulation of the mitochondrial pathway protein. In the present study, we further investigated the anti-proliferation and ROS toxicity induced by DHA and its possible mechanism in myeloid leukemia K562 cells when pre-treated with holotransferrin. We found that DHA inhibited the growth of K562 cells more effectively when the iron concentration was raised. The mechanism may be related to the autophagic effect induced by ROS toxicity, which includes the activation of caspases, down-regulation of TfR expression, reduction in iron uptake, and arrest of the cell cycle by DHA.

Autophagy, which is designated as type II programmed cell death, has also been reported to be an anticancer mechanism of ROS, which results in the inhibition of the growth of a variety of malignant cells, such as chronic myeloid leukemia cells, malignant glioma cells, and esophageal cancer cells. In this report, we demonstrate an increase in the autophagic flux in K562 cells that are treated with DHA. This could be partially caused by the toxicity of ROS that are generated by DHA. In addition, DHA-induced autophagy was monitored by measuring LC3-I and LC3-II protein expression. LC3-I (18 kDa) is cleaved to produce LC3-II (16 kDa), which is localized exclusively in autophagosomal membranes [32]. These results showed that DHA increased LC3-II protein expression. When compared to iron-normal K562 cells, 10 μM DHA showed a more powerful stimulation of expression of LC3-II protein under iron-overloaded conditions. In order to define the effect of functional relevance of the induction of autophagy, we treated K562 cells with the autophagy blocker (3-methyladenine, 3-MA). According to the results, we suggest that DHA activates the autophagic pathway.

It has been reported that DHA displays cytotoxic activity in actively proliferating mammalian cells [2], and it has also been suggested that this toxicity is related to a high intracellular iron concentration and the generation of ROS [3]. When formed intracellularly, ROS can cause macromolecular damage followed by cell

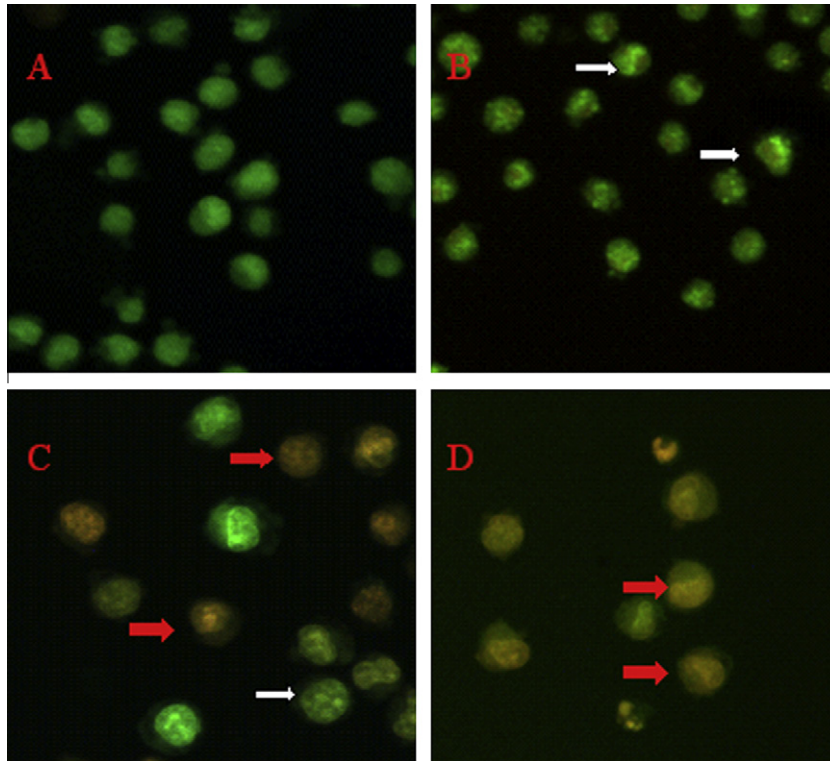


Fig. 9. Morphological analysis performed with fluorescence microscopy. K562 cells were incubated with 20 nM holotransferrin for 1 h, then treated with DHA or without for 48 h. The cells were stained by AO/EB, and the morphology was immediately assessed using fluorescence microscopy. (A) Control cells with bright green nuclei and intact structure; (B) DHA 2.5 μM ; (C) DHA 5 μM ; (D) DHA 10 μM . White arrows mark the early apoptotic cells with bright green nuclei as dense green areas in the nucleus. Red arrows mark late apoptotic cells with an orange nucleus showing the condensation of chromatin as dense orange areas and reduced cell size.

death. In several settings, autophagic signaling initiates apoptosis, but under other conditions, inhibition of autophagy triggers an apoptotic cell death program [33,34]. Our data show that DHA significantly increased intracellular ROS production. Moreover, after pretreatment of K562 cells with 20 nM holotransferrin, the intracellular ROS level was much higher than that observed with the DHA group ($P < 0.05$). This suggests that the regulation of the intracellular ROS level by DHA might be related to DHA-induced autophagy, its detail effect needed our further investigation.

Accumulating data indicate that there is a link between apoptosis and autophagy. In some systems, the induction of autophagy has been shown to contribute to or enhance the apoptotic response. When treated with 2.5–10 μM DHA for 48 h, K562 cells that were pretreated with holotransferrin exhibited cell shrinkage, membrane blebbing, chromatin condensation, and the formation of apoptotic bodies, which suggests the occurrence of apoptosis in iron-overloaded K562 cells after treatment with DHA. In most cases, the activation of the caspase cascade accompanies apoptosis. Of the several different members of caspases identified in mammalian cells, caspase-3 plays a direct role in the proteolytic cleavage of cellular proteins that are responsible for the progression to apoptosis. The observation of DHA-mediated activation of pro-caspase-3 and the subsequent cleavage of its substrate PARP suggest that the mitochondrial pathway plays an important role in DHA-induced apoptosis, and the effect of DHA was more significant under conditions of iron overload. The results of cell cycle analysis in our study also demonstrate that the DHA-induced arrest of K562 cell growth in the G_2/M phase, and partially in the apoptotic sub- G_1 phase, is directly correlated with the inhibition of cell proliferation, which may also relate to iron levels and ROS toxicity.

Because iron is required for the drug-induced formation of ROS, the activation of caspases, and consequent apoptosis, the intracellular iron content of K562 cells was measured by atomic absorption

spectrometry. We found that the iron content in iron-normal K562 cells was decreased by DHA in a dose-dependent manner. It has been reported that iron(II)-glycine sulfate (Ferrosanol) and transferrin have increased the cytotoxicity of free artesunate, artesunate microencapsulated in maltosyl-h-cyclodextrin, and artemisinin toward CCRF-CEM leukemia and U373 astrocytoma cells 1.5- to 10.3-fold when compared with that result from artemisinins applied without iron [35]. Therefore, to provide more direct evidence for the effect of DHA-induced cytotoxicity in K562 cells, we raised the iron concentration in the cell cultures with holotransferrin. The iron content of iron-overloaded K562 cells was decreased $27.7 \pm 2.0\%$ ($P < 0.05$) by 10 μM DHA when compared to the results from the iron-normal group.

Because TfR expression is controlled by the amount of iron required to maintain the metabolism of the cell, tumor cells in a state of high proliferation have a high density of transferrin receptors [36]. Therefore, we examined whether DHA could interfere with TfR expression, which would imply an effect on the proliferation of cancer cells. The results showed that 10 μM DHA inhibited the expression of TfR mRNA and protein in iron-overloaded conditions to a greater extent than in iron-normal K562 cells ($P < 0.05$). This is further supported by the results from flow cytometry analysis. According to the results of atomic absorption spectrophotometric analyses and flow cytometry analysis, the decrease in cellular iron content is not only partially linked to the decrease in transferrin receptor expression, but also partially caused by the consumption of iron through its reaction with DHA. DHA inhibited the proliferation of iron-normal K562 cells in a dose- and time-dependent manner, and the IC₅₀ was 11.33 μM . Moreover, DHA suppressed the proliferation of iron-overloaded K562 cells to a greater extent, and the IC₅₀ was 3.67 μM , which represents a 3.1-fold increase in the cytotoxicity of DHA when compared to the result from iron-normal cells. This result is consistent with the data published by

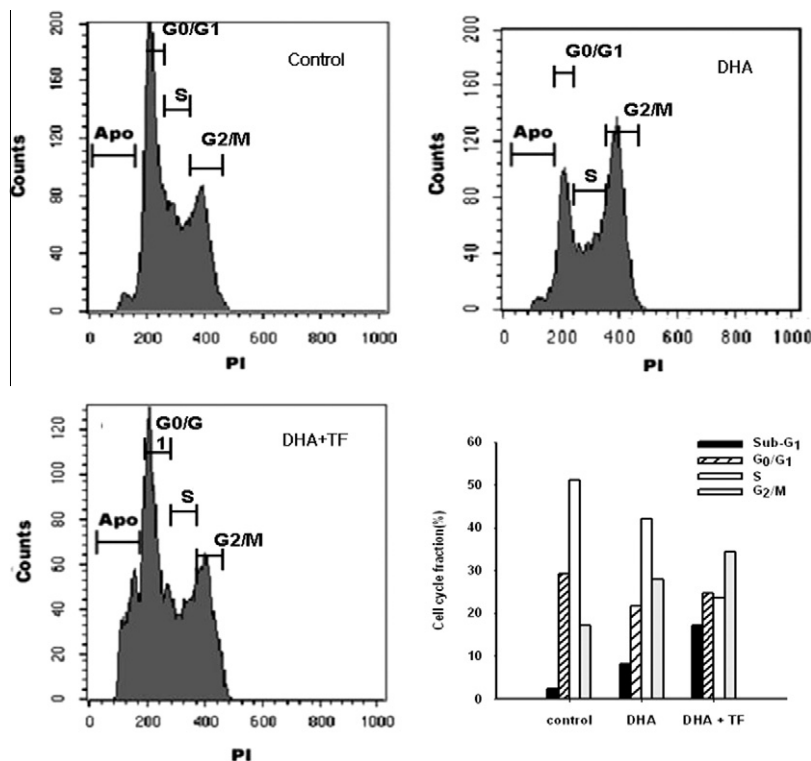


Fig. 10. Flow cytometric analysis of propidium iodide-stained and iron-overloaded K562 cells. The growth of K562 cells arrested by dihydroartemisinin (DHA). Control group: K562 cells were incubated in RPMI1640 standard medium for 48 h. DHA group: K562 cells were incubated with 10 μ M DHA for 48 h. DHA + TF group: K562 cells were incubated with 20 nM holotransferrin (TF) for 1 h and subsequently exposed to 10 μ M DHA for 48 h. The cells were next stained with propidium iodide (PI), and the cell cycle profiles were determined by flow cytometry. Cells in sub-G₁, G₀/G₁, S, and G₂/M phases of the cell cycle fractions are expressed as percentages of the total cell population. The graph is representative of three separate experiments.

Lai et al. [7] and Efferth et al. [4]. However, in our previous research, we found that treatment with holotransferrin made little difference in the effect of DHA on HL60 cells. In addition, a recent report on 36 human tumor cell lines showed that an increase in the cytotoxicity of another artemisinin derivative, artesunate, was observed in only two-thirds of the cell lines, and the addition of iron did not enhance the cytotoxicity of artesunate in the other cell lines [37]. This may indicate that the iron dependency of dihydroartemisinin-mediated cytotoxicity may differ from one cell line to another, and further investigations are still needed.

In summary, DHA induces autophagy and inhibits the growth of iron-loaded human myeloid leukemia K562 cells via ROS toxicity, which includes the down-regulation of TfR expression and induction of apoptosis through the mitochondrial pathway. However, we suggest that the mechanism of inhibition by DHA on K562 cell proliferation *in vitro* is still not clear, and further studies are needed to gain a full understanding of the *in vivo* effects.

Acknowledgments

The study was supported by research grants from the National Natural Science Foundation of China (No. 81072659) and by a grant from the Zhejiang Provincial Science and Technology Program (No. 2008C23067), China.

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