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Dihydroartemisinin exerts cytotoxic effects and inhibits hypoxia inducible factor-1α activation in C6 glioma cells

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Abstract

Artemisinin and its analogue dihydroartemisinin exert cytotoxic effects in some kinds of cancer cell lines. Here we determined whether dihydroartemisinin inhibits the growth and induces apoptosis of rat C6 glioma cells. We found dihydroartemisinin (5–25 μM) inhibited the growth and induced apoptosis of C6 cells in a concentration- and time-dependent manner; however, it was much less toxic to rat primary astrocytes. Dihydroartemisinin (5–25 μM) also increased the generation of reactive oxygen species in C6 cells. These effects of dihydroartemisinin were enhanced by ferrous ions (12.5–100 μM) and reduced by the iron chelator deferoxamine (25–200 μM). Immunoblotting analysis revealed that dihydroartemisinin (5–25 μM) significantly reduced hypoxia- and deferoxamine-induced expression of hypoxia inducible factor-1α and its target gene protein, vascular endothelial growth factor, in C6 cells. The results showed that dihydroartemisinin exerts a selective cytotoxic effect on C6 cells by increasing the reactive oxygen species and inhibiting hypoxia inducible factor-1α activation.

Introduction

Astrocytoma and other gliomas are the most common malignant tumours in the brain. The chemotherapeutic treatment for glioma is not often effective because of the intrinsic or acquired chemoresistance of the glioma cells (Haroun et al 2002). It is necessary to discover more effective drugs or develop combination therapy to improve the life expectancy of glioma patients.

Artemisinin is extracted from *Artemesia annua* and is widely used to treat malaria with higher safety (Klayman 1985; Hien & White 1993; O'Neill & Posner 2004). Artemisinin and its analogues contain an endoperoxide bridge that can react with an iron atom to form a free radical. The formed free radicals are toxic to cells and result in death of the cells (Efferth et al 2004b; Lai et al 2005b). In addition to their anti-malarial effects, artemisinin and its analogues exert cytotoxic effects on tumour cells in-vitro and in-vivo (Woerdenbag et al 1993; Moore et al 1995; Efferth et al 1996, 2001; Dell'Eva et al 2004; Yamachika et al 2004; Berger et al 2005; Efferth 2006; Lai & Singh 2006; Paik et al 2006). Dihydroartemisinin (DHA) is the main active metabolite of artemisinins in the body. Recently, it has been reported that DHA inhibits the growth of some types of cancer cells in-vitro and in-vivo, such as leukaemia cells (Singh & Lai 2004, 2005; Lee et al 2006), fibrosarcoma cells (Moore et al 1995), ovarian cancer cells (Chen et al 2003), breast cancer cells and cervical cancer cells (Singh & Lai 2001; Disbrow et al 2005; Lai & Singh 2006). However, whether DHA is also active against glioma cells still needs to be investigated. On the other hand, angiogenesis is crucial for glioma cells' growth. Among the angiogenetic factors, vascular endothelial growth factor (VEGF) plays an important role. VEGF is induced under conditions of hypoxia and modulated by a transcriptional factor, hypoxia inducible factor-1 (HIF-1) (Jensen et al 2006; Sun et al 2006). HIF-1 is a heterodimeric protein consisting of two subunits, HIF-1α and HIF-1β. HIF-1α is known as the functional unit. Under hypoxic conditions, HIF-1α is stabilized, translocated into the nucleus where it interacts with the hypoxia responsive elements, and then activates the target genes; however, it decreases rapidly under normoxia (Iyer et al 1998). In addition to hypoxia, the iron chelator deferoxamine (DFO) can induce HIF-1 expression (Semenza et al 1994). HIF-1α induces the expression of target proteins, including erythropoietin and VEGF, which are involved in cancer cell survival, migration, invasion and angiogenesis (Wang & Semenza 1993; Damert et al 1997).

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It has been reported that artemisinin analogues, artesunate and DHA, inhibit the angiogenesis and the expression of VEGF, as well as other angiogenesis-related genes, in-vitro and in-vivo (Chen et al 2004; Anfosso et al 2006). However, it is still unclear whether DHA reduces HIF-1α expression. In this study, we investigated the effects of DHA on the growth and apoptosis of rat C6 glioma cell lines (C6 cells) with rat primarily cultured astrocytes as control cells, as well as on the generation of intracellular reactive oxygen species in C6 cells. To determine the possible mechanisms, we also observed whether DHA affected hypoxia-induced expression of HIF-1α and its target gene protein VEGF. Since iron enhances the cytotoxic effects of artemisinins (Efferth et al 2004a), and iron chelators reduce iron effects and induce HIF-1 expression (Semenza et al 1994), we used ferrous iron ($FeSO_4$) and DFO as the modulator or inducer.

Materials and Methods

Cell culture

C6 cells were purchased from Institute of Cell Biology, Chinese Academy of Science (Shanghai, China). The cells were cultured in RMPI 1640 (Gibco, Grand Land, NY) supplemented with 10% heated inactivated fetal bovine serum, penicillin (100 000 U L^{-1}) and streptomycin (100 mg L^{-1}; Sigma-Aldrich Chemical Co., St Louis, MO) and maintained in a humidified atmosphere (5% CO_2 and 95% air) at 37°C. Rat primary astrocytes were isolated from the brains of Sprague-Dawley rats born within 24 h (Laboratory Animal Center of Zhejiang Academy of Medical Sciences, Hangzhou, China). Briefly, cortical cells were plated immediately after dissection at a density of 2×10^5 cells cm^{-2} in T-75 flasks (Falcon, Franklin Lakes, NJ). Cells were grown in high glucose DMEM supplemented with 10% heated inactivated fetal bovine serum, 2 mM glutamine, penicillin (100 000 U L^{-1}) and streptomycin (100 mg L^{-1}) in a humidified atmosphere (5% CO_2 and 95% air) at 37 °C. On day-in-vitro 13, the flasks were shaken at 260 rev min^{-1} for 24 h and then the adherent cells were trypsinized and cultured in the medium. More than 95% cells were astrocytes as confirmed by immunostaining with a mouse monoclonal antibody against glial fibrillary acidic protein (GFAP, a marker of astrocytes). Animal care was carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of School of Medicine, Zhejiang University.

Hypoxia treatment

C6 cells were subjected to hypoxia as described previously (D'Angelo et al 2003) with modifications. Briefly, C6 cells were seeded at a density of 4×10^5 cells per well into 6-well plates on the day before treatment. Then the medium was replaced with a new medium containing DHA 1–125 μM (Guilin Pharmaceutical Co., Guangxi, China) DFO (400 μM, Sigma-Aldrich), or both. The cells were placed in an anaerobic chamber filled with 94% N_2, 1% O_2 and 5% CO_2 at 37°C to induce hypoxia. The control cells were incubated in a normoxic condition containing 5% CO_2 and 95% air (about 20% O_2).

Cell growth and viability assays

C6 cells and astrocytes were seeded at a density of 2.5×10^4 or 5×10^4 cells per well into a 24-well plate. After 24-h culture, the cells were treated with DHA (1–125 μM) for 24, 48 and 72 h. After the treatments, the cells were trypsined and counted with a haemocytometer. The survival cell population was counted by trypan blue dye exclusion test.

To determine cell viability, C6 cells or astrocytes were seeded on 96-well plates at a density of 7.5×10^3 cells per well and treated with various concentrations of DHA. DFO (25, 50, 100 and 200 μM) or $FeSO_4$ (12.5, 25, 50 and 100 μM) was added to the media 1 h or 6 h before DHA, respectively, to assess the effect of DHA. After the treatments, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) was added to each well at a final concentration of 0.5 mg mL^{-1} for 4 h at 37 °C. Then, the media were removed and 100 μL dimethyl sulfoxide was added into each well. The absorbance at 490 nm was measured on a platereader (ELX800; BIO-TEK instruments Inc., USA), and the absorbance difference between the treated and untreated control groups was calculated to determine cell viability. The IC50 (the concentration that decreased cell survival by 50%) was calculated by plotting the percentages of cell growth inhibition versus the concentrations of the tested agents.

Cell apoptosis assay

C6 cells or astrocytes grown on the coverslips were stained with 10 mg L^{-1} of Hoechst 33342 and 10 mg L^{-1} of propidium iodide (PI, Sigma-Aldrich) for 10 min at 37°C. Then, the cells were photographed under a fluorescent microscope (Olympus BX51, Japan). The early apoptotic cells were determined as condensed or fragmented nuclei with strong bright Hoechst 33342 staining, and the late apoptotic cells as condensed nuclei with red PI staining (accompanied by the necrotic properties). At least 10 000 cells were counted in at least three separate fields for each coverslip, and the apoptotic or necrotic cells were reported as percentages of total cells.

Measurement of intracellular reactive oxygen species

The measurement of intracellular reactive oxygen species (ROS) was based on the oxidation of 2′,7′-dichloro-dihydrofluorescein diacetate (H_2DCFDA, Sigma-Aldrich) to yield an intracellular fluorescent compound. Briefly, C6 cells seeded on 96-well plates or coverslips were washed with Hank's solution and incubated with 50 μM H_2DCFDA in Hank's solution for 30 min. DHA, $FeSO_4$ and DFO were added into the media 2 h before reaction with H_2DCFDA. After the cells were washed twice with Hank's solution, the fluorescence was measured at excitation of 485 nm and emission of 530 nm, or observed under a fluorescence microscope (Olympus BX51, Japan).

Western blotting analysis

The extraction and Western blotting analysis of the protein were performed as described previously (D'Angelo et al 2003) with modifications. Briefly, after C6 cells were treated with

hypoxia and DFO, the cells were washed in ice-cold phosphate-buffered saline, and lysed in lysis buffer (50 mM NaCl, 5 mM EDTA, 0.5% SDS, 0.1 mM sodium orthovanadate, 50 mg L^{-1} aprotinin, 1 mM phenylmethysulfonyl fluoride, and 10 mM Tris-HCl, pH 7.4) at 4°C for 30 min with continuous rocking. The lysates were then centrifuged at 12 000 *g* for 30 min at 4°C and the supernatants were collected as the protein samples. The protein samples (80 μg) were electrophoresed in 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Then the membranes were incubated at 4°C overnight with monoclonal antibodies against HIF-1α clone 1α 67 (1:500; Novus Biological, Littleton, USA), VEGF (1:1000; Zhongshan Biotechnology Co., Beijing, China) and GAPDH (1:4000; Kangcheng Biological Co., Shanghai, China). The membranes were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse antibodies (1:2000; Zhongshan Biotechnology Co.) for 2 h at room temperature, and reacted with enhanced chemiluminescence reagent (Kangcheng Biological Co.), then finally exposed to X-ray films. The expressions of HIF-1α and VEGF were reported as the ratios of HIF-1α/GAPDH and VEGF/GAPDH.

Statistical analysis

Data are expressed as mean ± s.d. Statistical significance of the differences between groups was analysed by one-way analysis of variance followed by Bonferroni's multiple comparisons tests using SPSS 10.0 for windows. $P < 0.05$ was considered statistically significant.

Results

Effect of DHA on the cell growth, viability and apoptosis of C6 glioma cells and astrocytes

DHA inhibited the growth of C6 cells in a concentration- and time-dependent manner. The toxic concentrations of DHA ranged from 5 to 125 μM. The inhibition of C6 cell growth was noted at 24 h and became more obvious at 48 and 72 h after DHA treatment (Figure 1A). In addition, MTT reduction assay also showed the same effect on the C6 cell viability at 48 h after DHA treatment (Figure 1C) with an IC50 value of 23.4 (95% CI 20.1–26.6) μM. However, DHA at 5 and 25 μM did not significantly affect the growth and viability of astrocytes at 24 and

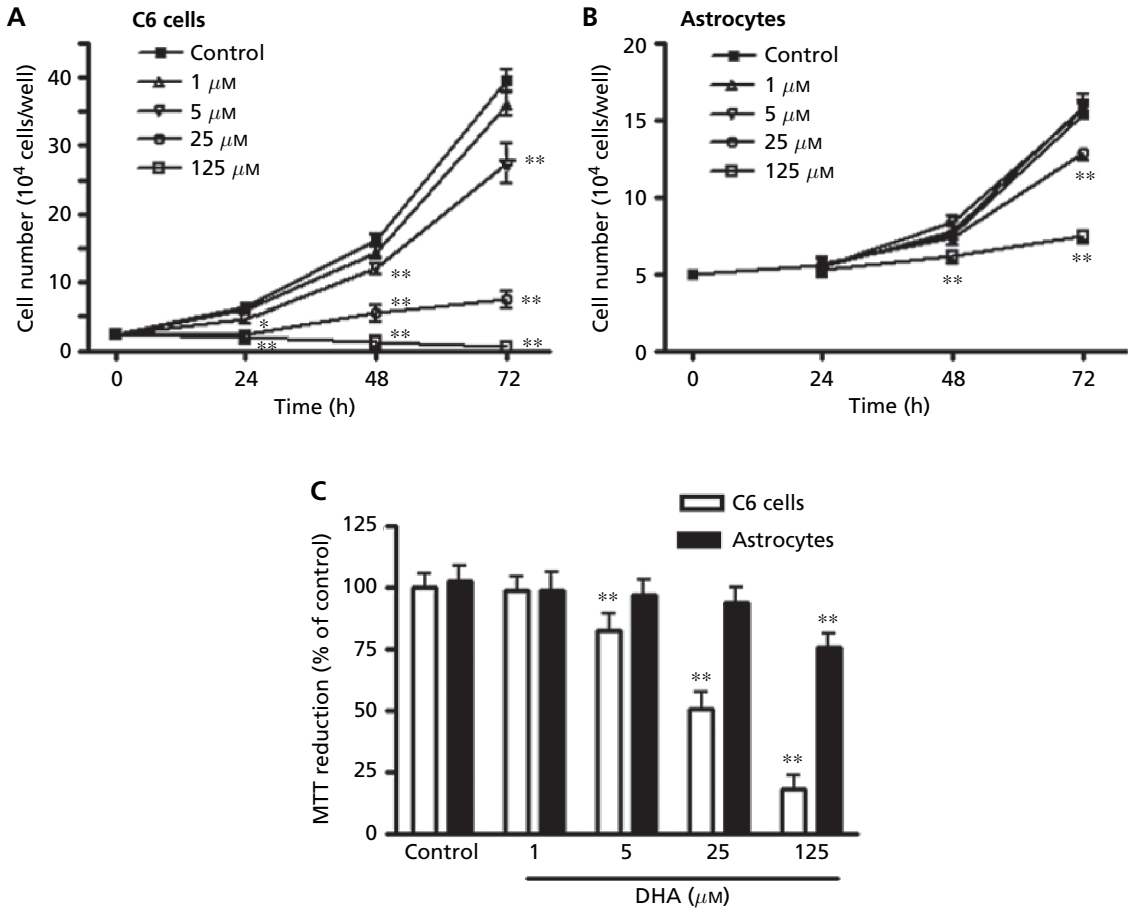

Figure 1 Effect of DHA on the growth and viability of C6 cells and astrocytes. C6 cells and astrocytes were treated with DHA (1–125 μM) for 24, 48 and 72 h, and cell numbers were counted (A, B). Cell viability (48 h) was determined by MTT reduction assay (C). Data are expressed as means ± s.d., n = 4 wells for cell counting, n = 8 wells for MTT assay. *$P < 0.05$ and **$P < 0.01$, compared with control, one-way analysis of variance.

48 h; it only reduced astrocyte number and viability at 25 μM (72 h) or 125 μM (48 and 72 h, Figure 1B, C).

In a preliminary experiment, we found that DHA induced apoptosis, not necrosis, of C6 cells. Therefore, we determined DHA-induced apoptosis by double fluorescent staining with Hoechst 33342 and PI. At 24 h after treatment, DHA induced early and late apoptosis of C6 cells at 5 and 25 μM during 72 h (Figure 2, Table 1). However, DHA did not significantly induce apoptosis of astrocytes, except that 125 μM induced apoptosis mildly at 72 h (Table 1).

Effect of deferoxamine and ferrous sulfate on DHA-produced ROS in C6 cells

DHA treatment for 2 h increased ROS in C6 cells at 5 (Figure 3, upper panels) and 25 μM. DFO (50 and 100 μM) ameliorated DHA (5 and 25 μM)-produced ROS, but $FeSO_4$ (25 and 50 μM) enhanced DHA (5 μM)-produced ROS (Figure 3, lower panel). Similarly, DFO (25–200 μM) ameliorated, but $FeSO_4$ (12.5–100 μM) enhanced the effects of DHA (1–125 μM) on C6 cell viability (Table 2) and growth (data not shown).

Effect of DHA on the expression of HIF-1α and VEGF

Western blotting analysis showed that C6 cells expressed HIF-1α weakly and VEGF moderately under normoxic conditions, whereas 8-h and 24-h hypoxia or 5-h DFO (400 μM) treatment significantly enhanced HIF-1α expression (Figure 4A), and 24-h and 48-h hypoxia or 24-h DFO

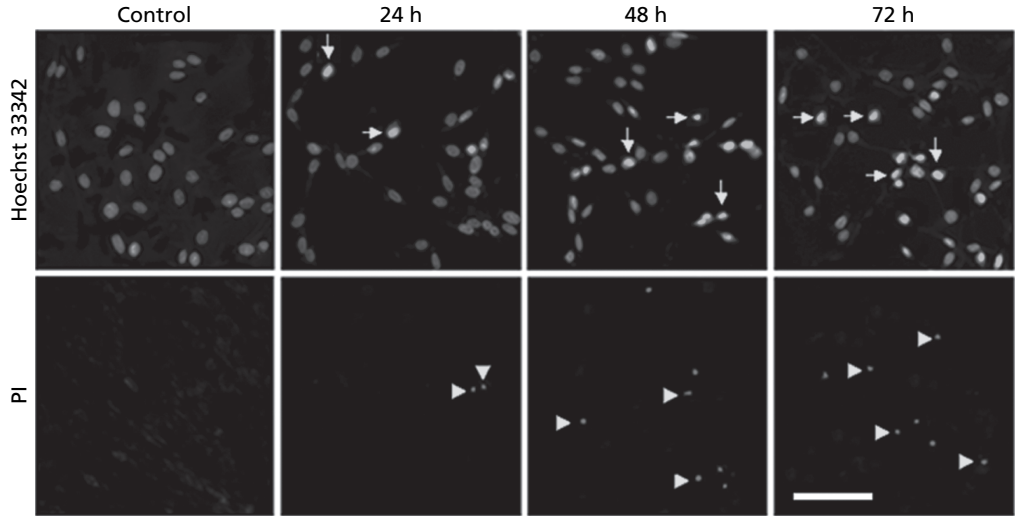

Figure 2 Effect of DHA on apoptosis of C6 cells. After DHA treatment for 24, 48 and 72 h, C6 cells were stained with Hochest 33342 (10 mg L^{-1}) and PI (10 mg L^{-1}) for detecting apoptotic cells. Representative microphotographs show the early (arrow, upper panels) and late apoptotic C6 cells (arrowhead, lower panels) after DHA (5 μM) treatment. Scale bar = 50 μm

Table 1 Effect of DHA on apoptosis of C6 cells and astrocytes

Time (h)	DHA (μM)	C6 cell apoptosis		Astrocyte apoptosis	
		Early (%)	Late (%)	Early (%)	Late (%)
24	0 (control)	3.4 ± 1.7	n.d.	3.8 ± 1.3	n.d.
	1	6.4 ± 0.9	3.6 ± 1.9	2.6 ± 1.1	n.d.
	5	16.7 ± 5.7**##	7.8 ± 2.2#	4.3 ± 1.5	n.d.
	25	25.3 ± 6.2**##	13.1 ± 3.4##	3.9 ± 1.3	n.d.
48	0 (control)	4.7 ± 1.2	n.d.	3.0 ± 1.0	n.d.
	1	6.5 ± 1.1	4.4 ± 2.4	3.7 ± 1.3	n.d.
	5	33.7 ± 8.2**##	12.8 ± 2.9##	4.3 ± 1.7	n.d.
	25	45.0 ± 6.3**##	22.1 ± 3.7##	3.0 ± 1.5	n.d.
72	0 (control)	2.7 ± 0.8	n.d	4.0 ± 1.3	n.d
	1	7.3 ± 4.0	4.3 ± 2.2	3.1 ± 1.6	n.d.
	5	36.0 ± 6.8**##	15.2 ± 2.7##	5.5 ± 1.9	n.d.
	25	48.5 ± 11.2**##	30.1 ± 5.2##	14.6 ± 2.0*#	3.9 ± 1.2

Data are expressed as mean ± s.d., n = 6. **$P < 0.01$ compared with DHA 0 μM (control); #$P < 0.05$, ##$P < 0.01$ compared with DHA 1 μM, one-way analysis of variance. n.d., not detectable.

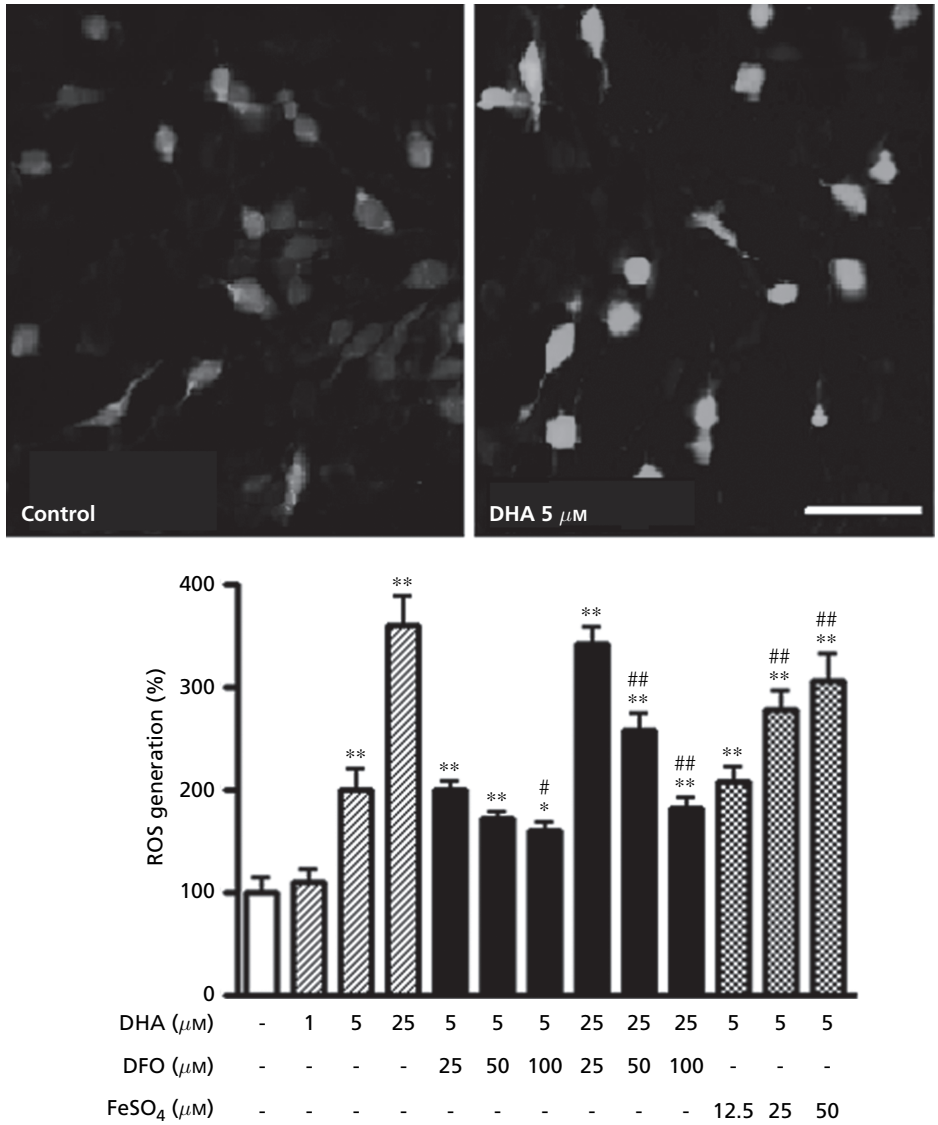

Figure 3 Effect of DHA on intracellular reactive oxygen species (ROS) and the modulation by deferoxamine (DFO) or ferrous sulfate ($FeSO_4$) in C6 cells. ROS were determined by H_2DCFDA oxidation-based fluorescence after 2-h exposure to 5 μM DHA. DHA-increased ROS was enhanced by $FeSO_4$ (25 and 50 μM) but attenuated by DFO (50 and 100 μM). Data in the lower panel are expressed as mean ± s.d., n = 8 wells. *$P < 0.05$ and **$P < 0.01$, compared with control (without DHA, DFO and $FeSO_4$); #$P < 0.05$ and ##$P < 0.01$, compared with DHA (5 or 25 μM) alone, one-way analysis of variance. Scale bar = 50 μm.

(400 μM) treatment enhanced VEGF expression (Figure 4B). DHA (5–125 μM) concentration-dependently reduced hypoxia- and DFO-enhanced expression of HIF-1α and VEGF. DHA (25 and 125 μM) also reduced VEGF expression under normoxic conditions (Figure 4, Table 3).

Discussion

In this study, we found that DHA is toxic to rat C6 glioma cells but much less toxic to astrocytes. DHA inhibited the growth and viability and induced apoptosis of C6 cells at 5–125 μM, but did not affect those of astrocytes at concentrations less than 125 μM within 48-h treatments (Figures 1, 2). This finding is consistent with the observations that artemisinin and its analogues are selectively toxic to tumour cells (Singh & Lai 2001; Disbrow et al 2005; Singh & Lai 2005; Efferth 2006) and that DHA possesses in-vitro anti-tumour activity (Singh & Lai 2001; Disbrow et al 2005; Lee et al 2006). Because different culture media were used in C6 cell and astrocyte cultures, whether the culture media might cause different responses could not be explained. However, the media did not contain major pharmacologically active substances, thus there may be not much likelihood that the different media led to different responses.

The toxic effect of DHA is enhanced by iron and ameliorated by the iron chelator DFO (Figure 3C–F). In the cytotoxicity experiments, the enhancement by ferrous sulfate

Table 2 Effect of DHA on cell viability (A_{490} per well) and the modulation by deferoxamine (DFO) or ferrous sulfate ($FeSO_4$) in C6 cells

	0 (control)	DHA (μM)			
		1	5	25	125
DFO (μM)					
0	0.549 ± 0.026	0.550 ± 0.020	0.414 ± 0.010**	0.405 ± 0.010**	0.236 ± 0.010**
25	0.533 ± 0.020	0.547 ± 0.027	0.411 ± 0.012	0.410 ± 0.011	0.297 ± 0.013##
50	0.554 ± 0.019	0.538 ± 0.022	0.444 ± 0.021	0.454 ± 0.021#	0.313 ± 0.009##
100	0.535 ± 0.010	0.537 ± 0.018	0.451 ± 0.017	0.484 ± 0.015##	0.342 ± 0.023##
200	0.545 ± 0.013	0.551 ± 0.015	0.490 ± 0.011##	0.500 ± 0.018##	0.389 ± 0.014##
FeSO4 (μM)					
0	0.528 ± 0.019	0.522 ± 0.024	0.427 ± 0.018*	0.229 ± 0.023**	0.129 ± 0.004**
12.5	0.529 ± 0.019	0.521 ± 0.023	0.450 ± 0.020	0.201 ± 0.011#	0.074 ± 0.041##
25	0.506 ± 0.015	0.415 ± 0.016##	0.326 ± 0.027#	0.191 ± 0.009##	0.067 ± 0.022##
50	0.529 ± 0.022	0.398 ± 0.016##	0.294 ± 0.024##	0.163 ± 0.014##	0.060 ± 0.015##
100	0.517 ± 0.017	0.286 ± 0.011##	0.230 ± 0.020#	0.158 ± 0.017##	0.058 ± 0.009##

Data are expressed as mean ± s.d., n = 8 wells. *$P < 0.05$, **$P < 0.01$ compared with control (DHA, DFO or $FeSO_4$); #$P < 0.05$, ##$P < 0.01$ compared with DHA alone, one-way analysis of variance.

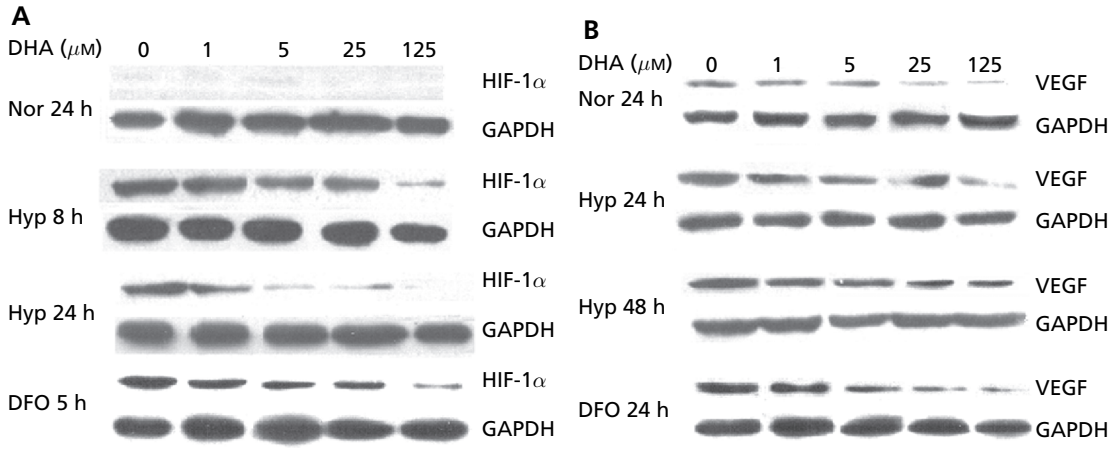

Figure 4 Western blotting analysis for the effect of DHA on hypoxia- or deferoxamine (DFO)-induced HIF-1α and VEGF expression in C6 cells. C6 cells were treated with hypoxia (1% O_2, Hyp) or cultured under normoxia (Nor) in the absence or presence of DFO (100 μM). DHA (1–125 μM) concentration-dependently inhibited hypoxia- and DFO-induced HIF-1α (A) and VEGF expression (B).

confirms that the effects of DHA are iron-dependent. Several studies have demonstrated the involvement of iron in toxicity of artemisinins in tumour cells. For example, iron-loaded transferrin (holotransferrin) enhances the cytotoxicity of DHA in human leukaemia cells (Lai & Singh 1995), iron glycine sulfate increases the cytotoxicity of artesunate on leukaemia cells (Efferth et al 2004a), and combined DHA and ferrous sulfate retard fibrosarcoma growth in-vivo (Moore et al 1995). In addition, the iron chelator DFO reverses the DHA cytotoxicity in a concentration-dependent manner, indicating an essential role for iron as well. Iron is involved in a variety of biochemical processes in the division and proliferation of cells; uptake of extracellular irons is important for cells. Tumour cells take in more iron due to their growth being more rapid than that of normal cells (Lai et al 2005a, b), and glioma cells often over-express the transferrin receptor (TfR), which can bind with transferrin and then transfer iron into cells (Weaver & Laske 2003). DHA reacts with intracellular iron and then generates much more ROS in cancer cells than in normal cells; therefore DHA is selectively toxic to cancer cells. The interaction between iron and ROS is confirmed by the finding that DHA increased ROS in C6 cells that were enhanced by iron and ameliorated by DFO (Figure 3B). Since oxidative stress results in the destruction of intracellular macromolecules, such as DNA and membrane proteins, and then leads to cell death (Chandra et al 2003), ROS generation may be essential for DHA to exert the cytotoxic activity.

An important finding in this study is that DHA concentration-dependently inhibits hypoxia- and DFO-induced expression of HIF-1α and VEGF in C6 cells. We assessed the expression of HIF-1α and VEGF because they play important roles in anti-angiogenic therapy in solid tumours. The inhibition by DHA of VEGF expression in tumour tissues has been reported (Lee et al 2006), while our results also show the inhibition of the up-stream step, HIF-1α activation. HIF-1α is crucial for the hypoxic induction of the target protein expression,

Table 3 Effect of DHA on hypoxia- or deferoxamine (DFO)-induced HIF-1α and VEGF expression in C6 cells treated by hypoxia (1% O_2, Hyp) or cultured under normoxia (Nor) in the absence or presence of DFO (100 μM)

Treatment	DHA (μM)				
	0 (control)	1	5	25	125
HIF-1α					
Nor24 h	0.13 ± 0.06	0.15 ± 0.06	0.12 ± 0.09	0.13 ± 0.06	0.10 ± 0.07
Hyp 8 h	0.54 ± 0.10**	0.47 ± 0.15	0.41 ± 0.09#	0.28 ± 0.11##	0.26 ± 0.11##
Hyp 24 h	0.60 ± 0.14**	0.48 ± 0.19	0.41 ± 0.11#	0.35 ± 0.10##	0.31 ± 0.12##
DFO 5 h	0.52 ± 0.11**	0.43 ± 0.22	0.37 ± 0.12#	0.30 ± 0.16##	0.24 ± 0.13##
VEGF					
Nor 24 h	0.36 ± 0.13	0.37 ± 0.17	0.30 ± 0.17	0.20 ± 0.11##	0.18 ± 0.14##
Hyp 24 h	0.67 ± 0.12**	0.53 ± 0.15	0.48 ± 0.09#	0.42 ± 0.12##	0.31 ± 0.17##
Hyp 48 h	0.64 ± 0.11**	0.52 ± 0.10#	0.51 ± 0.11#	0.46 ± 0.11##	0.45 ± 0.10##
DFO 24 h	0.55 ± 0.08**	0.53 ± 0.12	0.40 ± 0.10##	0.31 ± 0.09##	0.28 ± 0.09##

HIF-1α and VEGF expressions are expressed as the ratios of HIF-1α/GAPDH or VEGF/GAPDH. Mean ± s.d., n = 4; $^{**}P < 0.01$, compared with normoxia control (DHA 0 μM); $\#P < 0.05$ and $\#\#P < 0.01$, compared with corresponding controls (DHA 0 μM), one-way analysis of variance

such as VEGF and platelet derived growth factor-B in glioma cells in-vitro (Yoshida et al 2006). The expressed proteins are important for the survival and proliferation of glioma cells. HIF-1α over-expression in tumour tissue is considered as a poor prognostic indicator (Sobhanifar et al 2005). HIF-1α is also important for glycolysis and pH regulation in tumour cells (Maxwell et al 1997; Wykoff et al 2000). Therefore, the inhibition of the expression of HIF-1α by DHA may have a beneficial effect on the survival and invasion of glioma cells (Blum et al 2005). In addition, DHA at higher concentrations (25 and 125 μM) inhibits VEGF expression under normoxic conditions, indicating that it also affects other activating pathway(s) for VEGF expression.

In summary, we found that DHA inhibits the proliferation and induces the apoptosis of C6 cells. One possible mechanism may be that DHA directly kills C6 cells by increasing the intracellular ROS; another may be that DHA inhibits HIF-1α and VEGF expression. Since artemisinins exhibit higher safety (Moore et al 1995; Berger et al 2005; Lai & Singh 2006), DHA may be represent a new type of effective and well-tolerated therapeutic agent for the treatment of astrocytoma.

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