Dihydroartemisinin suppresses cell proliferation, invasion, and angiogenesis in human glioma U87 cells

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Accepted 13 August, 2012

Dihydroartemisinin (DHA), a semi-synthetic derivative of artemisinin, which is a well tolerated and effective drug for malaria treatment, has shown potent antitumor ability. This study is explored to evaluate whether DHA can inhibit glioma cell proliferation, invasion, and glioma cell mediated angiogenesis. We determined the glioma U87 cell proliferation by Cell Counting Kit-8 (CCK-8) assay, fluorescence, and flow cytometry (FCM). The invasion and migration of U87 cell were tested by wound-scratch assay and martrigel-transwell methods, while its angiogenesis tube formation assay was tested with reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. Our data suggested that DHA inhibited cell viability in a dose- and time-dependent manner, triggered a stringent G1 cell cycle arrest, and induced cell apoptosis of glioma U87 cells. Tube formation assays showed that DHA significantly decreased glioma cell tube formation in human umbilical vein endothelial cell (HUVC), and suppressed vascular endothelial growth factor (VEGF) mRNA expression and its release in U87 cells. In addition, wound-scratch assay and martrigel-transwell showed that DHA decreased U87 cell invasion and migration in vitro. These results indicated that DHA might be a valuable candidate for treatment of human glioma.

Key words: Dihydroartemisinin, glioma, proliferation, invasion, angiogenesis.

INTRODUCTION

Malignant glioma is one of the most malignant tumors with a very poor prognosis. Survival for patients with glioblastoma multiforme (GBM), the most malignant glioma (World Health Organization (WHO) grade IV), is only on average 14 months after diagnosis, despite aggressive surgery, radiation, and chemotherapies. Due to their insidious invasion and extensive neovascularization, glioblastomas are characterized by recurrence (Claes et al., 2007; Mentlein et al., 2011). The diffusing glioma cells invade into the normal brain adjacent to the tumor, which may cause treatment failure by conventional therapy. Therefore, there is an urgent need to develop effective chemotherapeutic agents for glioma.

Dihydroartemisinin (DHA), a semi-synthetic derivative of artemisinin (Figure 1), isolated from the traditional Chinese medicine, Artemisia annua, is approved for the treatment of multidrug-resistant malaria and has an excellent safety profile. More recently, growing studies suggested that artemisinin derivatives including DHA also have profound effect against human tumors, such as leukemia cells (Zhou et al., 2007), prostate cancer (He et al., 2010), ovarian cells (Jiao et al., 2007, pancreatic cancer (Du et al., 2010), lung adenocarcinoma and non-
small cell lung cancer (Lu et al., 2009; Zhao et al., 2011). However, the effect of DHA on glioma is unclear. Therefore, there is an urgent need to study the effect of DHA on glioma.

In this study, we evaluated the therapeutic potential of DHA against human glioma U87 cells in vitro, and demonstrated that DHA is a novel potential drug for the treatment of glioma via the induction of apoptosis, inhibiting tumor angiogenesis, and blocking of the cell cycle progression. We determined the glioma U87 cell proliferation by Cell Counting Kit-8 (CCK-8) assay, fluorescence, and flow cytometry (FCM). The invasion and migration of U87 cell were tested by wound-scratch assay and martrigel-transwell methods, while its angiogenesis tube formation assay was tested with reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively.

MATERIALS AND METHODS

DHA was purchased from Shanghai Yi & Yuan Ye Biotech Company in China. 284 mg of DHA was dissolved in 1 ml of dimethyl sulfoxide (DMSO) as stock solution. This stock solution of DHA (1 mmol/L) was further diluted to appropriate concentrations with cell culture medium immediately before use. Control experiments contained DMSO without DHA.

Cell culture

Human glioblastoma U87 and human umbilical vein endothelial cells (HUVEC) were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in the Central Laboratory of Zhujiang Hospital of Southern Medical University. All cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cell cultures were maintained in 25 cm² flasks and were kept in a humidified atmosphere with 5% CO₂ at 37°C.

Cell proliferation assay

To measure cell viability, cells were seeded into 96-well plates at a density of 1×10⁴ per well. After overnight incubation, cells were rinsed with phosphate buffered saline (PBS) and incubated with different concentrations of DHA (0, 5, 10, 20, 40, 80, and 160 μmol/L, respectively) in complete medium. Then, CCK-8 assay was performed to test the proliferation. The absorption of solubilized formazan was measured at the wavelength of 450 nm by an ELISA plate reader (EL340 microplate reader; Bio-Tek Instruments, Winooske, VT). The cells were incubated for 6, 12, 24, 48, and 72 h after treated with different concentrations of DHA to measure the dose- and time-dependent effects, CCK-8 assay was performed at each time point in triplicate.

Determination of apoptosis

Cells were plated in triplicate and treated with 40 μmol/L DHA for 48 h. Apoptotic cell death was examined by two parameters: cell and nuclear morphology by fluorescence microscope (Axiovert-200M microscope, Zeiss) after intravitral staining with 1 μmol/ml Hoechst 33258 for 20 min at room temperature (RT) in the dark.

Cell cycle analysis

Cells (1×10⁶) were treated with DHA as described earlier for 48 h. The harvested cells were resuspended in 200 μl of cold PBS, to which cold ethanol (600 μl) was added, and the mixture was then incubated for 2 h at 4°C. After centrifugation, the pellet was washed...
with cold PBS, suspended in 500 μl PBS, and incubated with 50 μl RNase (20 μg/ml final concentration) for 30 min. The cells were incubated with propidium iodide (50 μg/ml final concentration) for 30 min in the dark. The cell cycle distribution was then elaborated by a FACS Aria instrument (BD Biosciences). The experiment was repeated thrice under the same conditions (Riganti et al., 2009).

Cell invasion and migration assays

BD BioCoat Matrigel invasion chambers were used to examine the ability of U87 cells to penetrate the extracellular matrix (ECM). Cells (5×10^4 ml^-1), after being treated with DHA for 24 h, were resuspended in 100 μl of serum-free medium and added to the upper chamber, while the lower chamber was filled with 600 μl of complete medium containing FBS, which served as a chemo-attractant. Cells were then incubated for 24 h at 37°C. After removal of cells on the upper surface of the membrane, cells on the lower surface of the membrane were fixed in 4% of formaldehyde and then stained with 0.1% of crystal violet for five min. Four fields of cells were counted randomly in each well under a fluorescent microscope at 200× magnification. Data was expressed as the percentage of invasive cells as compared to the control.

Wound scratch assay was also used to evaluate cell migration ability. In brief, cells were seeded at a density of 2×10^5 per well in 6-well plates in complete medium. After cell growth to about 80% confluence, the medium was changed into a serum-free medium for 6 h. Then, the monolayers were scratched with a 10 μl plastic pipette tip to create a uniform wound and 3 ml of complete medium containing 10% of FBS with various concentrations of DHA (25, 50, 75, and 100 μmol/L) were added to the wells. The wound area was then examined after 24 h of incubation under a phase-contrast microscope at 100× magnification. Photographs of three random fields were taken and the cell migration ability was expressed by the closure of gap distance.

Tube formation assay

Matrigel (Becton Dickinson) was added to each well of a 24-well plate and then incubated for 30 min at 37°C to allow the Matrigel solution to form a gel. After treatment with indicated concentrations of DHA for 24 h, 2×10^5 HUVEs were resuspended in 1 ml of complete medium, seeded onto the solidified Matrigel gel, and incubated for 8 h. The endothelial tubes of five random fields were examined under a phase-contrast microscope at 100× magnification. Single peak indicated that a single DNA sequence was amplified during RT-PCR. Each sample was tested in triplicate with RT-PCR, and samples obtained from three independent experiments were used for analysis of relative gene expression. The following primers for RT-PCR were designed using Premier 5 software. VEGF: forward 5'- CAGCTACTGCCATCAATC-3'; reverse 5'- CAATGCTTTCTCCGCTGCT-3'; product size: 313 bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward 5'- TCCACCCAC CCTGTGTGCTGTA-3'; reverse 5'- ACCACAGTCATGCCATCAG-3'; product size: 450 bp, GAPDH was used as the internal control.

Statistical analysis

The Statistical Program for the Social Sciences software for Windows 16.0 (SPSS, Chicago, IL, USA) was used for all statistical analyses. All experiments were performed in triplicate unless otherwise noted; results were expressed as mean ± standard deviation (SD). Significance between the two groups was examined by a two tailed. When two groups were compared, the unpaired Student’s t-test was used. When multiple groups were evaluated, one-way analysis of variance (ANOVA) was used. P ≤ 0.05 was considered statistically significant.

RESULTS

DHA inhibits proliferation of glioma cells

CCK-8 was used to determine anti-proliferative effect of DHA on glioma U87 cells. The proliferation of the cells was inhibited in a dose-dependent manner at 48 h after treatment with different concentration (0, 10, 20, 40, 80, and 160 μmol/L) of DHA (Figure 2A). Treatment with 40 μmol/L of DHA for different times (0, 6, 12, 24, 48, and 72 h) demonstrated that the cells growth was inhibited in a time-dependent manner (Figure 2B). These results showed that the proliferation of the cells could be significantly inhibited by DHA, even at a very low treating-dose (5 μmol/L) and a very short treating-time (6 h) after treatment with indicated concentrations of DHA.

DHA reduces glioma cell migration and invasion

Gliomas are characterized by insidious invasion to
adjacent normal brain tissue (Claes et al., 2007; Mentlein et al., 2011). To investigate the effect of DHA on glioma cell U87 migration and invasion ability, a wound-scratch assay and martrigel-transwell assay were used. As compared to the control group, the gap distance was significantly reduced in U87 cells by DHA at the concentration of 20 and 40 μmol/L for 24 h (P < 0.01 or P < 0.05). DHA dramatically inhibited cell migration to 71.82 and 36.12% of the control in U87 cells, respectively (P < 0.01 or P < 0.05) (Figure 3A). Meanwhile, as shown in Figure 3B, DHA caused a significant reduction of invasiveness to approximately 76.09 and 39.80% of the control at a concentration of 20 and 40 μmol/L (P < 0.01 or P < 0.05).

**DHA induce G1-phase cell cycle arrest in human glioma cells**

As there is a significant growth-inhibitory effect of DHA on glioma cells, we investigated whether DHA had any inhibitory effect on cell cycle progression. After incubation for 48 h in the presence of DHA, cells were permeabilized and assessed for the cell cycle phases by fluorescence activated cell sorting (FACS) analysis. DHA (40 μmol/L) lowered the percentage of cells entering the S-phase (39.30%), relative to the untreated cells (20.90%, P < 0.05). G1-phase arrest was also observed when the effects of DHA on cell cycle progression of U87 were analyzed (P < 0.01). The percentage of cells entering the G1-phase increased from 26.50% in untreated cells to 67.19%. These results suggest that DHA showed stronger inhibitory effects on cell cycle progression and was associated with the induction of G1 arrest (Figure 4).

**Morphological changes of DHA-induced apoptosis**

After exposure to 40 μmol/L DHA for 48 h, cells underwent typical morphologic changes of apoptosis such as cell shrinkage, membrane frill, blebbing, and ovalisation, while the cell membrane remained well defined (Figure 5B). The Hoechst 33258, a sensitive fluorochrome to DNA, was used to assess changes in nuclear morphology following DHA treatment. The nuclei in normal cells exhibited diffused staining of the chromatin (Figure 5C). However, DHA significantly induced chromatin condensation, margination and the boundary between nucleus and cytoplasm became blurred (Figure 5D).

**DHA inhibits tube formation**

Glioma induces extensive neovascularization in adjacent tissue (Kesari, 2011; Corsini et al., 2012). Therefore, we investigated whether DHA had the ability to inhibit tube formation in HUVECs treated for 24 h. Control groups treated with medium only were composed of multiple cells gathered together and that adhered to each other. However, DHA significantly decreased tube formation by 69.50 and 73.97% of the control group, at all concentrations of 20 and 40 μmol/L, respectively (Figure 6A and B).
Concentration of DHA ($\mu$mol/L) 

DHA inhibits U87 cells migration and invasiveness. (A) The effect of DHA on migration was measured by wound healing assay. The wound line was prepared using the tip of 10 µl pipett, and the cells were then treated with various concentration of DHA. Photographs were taken and the width of the wound line was measured at 24 h. Results were representative of three independent experiments (magnification, ×50). Bar graphs revealed that DHA dramatically inhibited cell migration to 71.82 and 36.12% of the control in U87 cells at the concentrations of 20 and 40 µmol/L, respectively. (B) DHA inhibits U87 cells invasiveness using matrigel-transwell assay. Cells were starved for 6 h and suspended in serum free DMEM containing various concentration of DHA. 5×10^4 cells were added to the upper chamber. Twenty-four hours in vitro invasion assay revealed that the invasiveness of U87 cells were inhibited by DHA. Bar graphs showed that DHA inhibited cell invasion to 76.09 and 39.80% of the control at the concentrations of 20 and 40 µmol/L, respectively. *P < 0.05, **P < 0.01, compared with control group.

DHA decreases VEGF mRNA expression and its release in U87 cells

The level of VEGF mRNA expression was tested by using a RT-PCR assay. U87 cells were exposed to different concentrations of DHA for 48 h, then total RNA was isolated and RT-PCR was performed to measure the cells. DHA significantly decreased the level of VEGF mRNA in U87 cells from 68.3 to 45.0% and from 68.3 to 28.7%, as compared to GAPDH (Figure 6C).

VEGF protein showed the same trend as mRNA level in all experimental groups. ELISA analysis was performed to determine the amount of secreted VEGF protein. Cells pretreated with DHA were grown in serum-free medium for 24 h, and the secreted protein of VEGF in culture media was determined by ELISA. The levels of VEGF in U87 cells decreased by 21.4 and 43.1% after being treated with 20 and 40 µmol/L of DHA for 48 h, when compared with the control group (P < 0.05) (Figure 6D).

DISCUSSION

Lack of effective long term treatments for glioma highlights the necessity to identify new potent anti-cancer
Figure 4. Effects of on glioma cells cycle progression of human glioma cells U87. Cells were exposed to 40 µmol/L DHA for 48 h followed by cell cycle distribution assay. All assays were done in triplicate. DHA lowered the percentage of cells entering the S-phase (A, 39.30%), relative to the untreated cells (B, 20.90%, \( P < 0.05 \)). However, the percentage of cells entering the G1-phase increased from 26.50% in untreated cells to 67.19% (\( P < 0.01 \)).

Figure 5. Apoptosis induced by DHA in U87 cells. The cells were treated with 40 µmol/L of DHA for 48 h. (A) Control cells; (B) treatment of U87 cells with 40 µmol/l DHA, cell shrinkage, membrane frill, blebbing, and ovalisation, while the cell membrane remained well defined (magnification, \( \times 200 \)). Nuclear morphology was detected by Hoechst 33258 staining and examined by fluorescence microscope. (C) Control cells with diffused staining of the chromatin in normal cells. (D) With 40 µmol/L of DHA treated, chromatin condensation, margination and the boundary between nucleus and cytoplasm became blurred (the arrows point to cells displaying nuclear condensation) (magnification, \( \times 200 \)).
Figure 6. DHA inhibits tube formation in human umbilical vein endothelial cells. (A) Representative photomicrographs during the tube formation of human umbilical vein endothelial cells pretreated with indicated concentrations of DHA for 24 h. (B) The ability to form tubes was expressed as ratios of length of formed tubes per picture field. DHA significantly decreased tube formation by 69.50 and 73.97% of the control group, at all concentrations of 20 and 40 µmol/L, respectively when compared to the control group. (C) DHA significantly decreased VEGF mRNA expression from 68.3 to 45.0% and 68.3 to 28.7%, respectively after 48 h treatment. (D) Secreted VEGF release was also significantly inhibited by DHA in cell culture supernatants. With 20 and 40 µmol/L of DHA treated, the secreted protein of VEGF in culture media was decreased by 21.4 and 43.1% when compared with the control group, *P < 0.05, **P < 0.01, compared with control group.

Compounds. Naturally occurring plant compounds represent a possible source of molecules that may have anti-proliferative effects on a variety of cancers. Although, it has been reported that artemisinin and several of its derivatives inhibit the growth of various cancer cell types, it is still little known whether artemisinin and its derivatives work as growth inhibitors in glioma cells. Our results in this study suggest that DHA inhibits growth of human glioma U87 cells by doses at micromolar levels (Figure 2), and these doses of DHA exhibit little or no significant cytotoxicity and neuropathologic toxicity in normal neuronal cells (Meshnick, 2002; Houle, 2011).

Cell proliferation is highly regulated in the G1 phase of the cell cycle in most normal and tumorigenic mammalian systems, because cancer cells are highly proliferative (Willoughby et al., 2009). Cellular apoptosis is characterized morphologically by cell shrinkage, nuclear pyknosis, chromatin condensation, and blebbing of the plasma membrane (Lu et al., 2009). DHA results from dose-dependent G2 arrest, although, the involved mechanisms is unclear. Thus, cell cycle arrest and apoptosis might be responsible, at least in part, to DHA inhibition of cell growth in glioma.

Angiogenesis, the growth of new capillaries in response to pro-angiogenic factors secreted by glioma cells due to a lack of oxygen and nutrients, plays a crucial role in tumor growth (Kesari, 2011; Corsini et al., 2012). Among various activators of angiogenesis, VEGF is considered as one of the most potent angiogenic factors (Arbab, 2012). The expression of VEGF was closely associated with angiogenesis, growth, metastasis, and poor outcome in solid tumors (Holmes et al., 2007; Kaiser et al., 2011). In order to analyze the effect of DHA on VEGF secretion and expression in U87 cells, we detected the VEGF mRNA expression by RT-PCR and the secreted VEGF level in supernatant by ELISA assay. These results suggested that DHA could inhibit VEGF expression and secretion effectively in U87 cells, similar to previous studies (Chen et al., 2004; Lee et al., 2006). Moreover, we selected an in vitro tube formation assay for the
angiogenesis study (Figure 6). DHA dramatically decreases the tube formation rate in HUVECs, which means DHA might be useful for targeting against angiogenesis.

Invasiveness and migration are important characteristics of glioma cells and targets of anti-cancer agent development (Claes et al., 2007; Mentlein et al., 2011). In this study, we used the wound scratch assay to assess the motility of U87 cells and the Matrigel invasion assay to assess the ability of U87 cells to penetrate the ECM. The data show that the motility as well as the invasion potential of U87 cells were significantly reduced by DHA, but further clarification of the underlying mechanisms is required.

**Conclusion**

Conclusively, our results demonstrate that DHA significantly inhibits human glioma U87 cells viability, proliferation, invasion, and tube formation. Furthermore, our results provide little evidence of potential implications for the rational application of DHA as a potential anticancer drug against human glioma, although, much studies are needed.

**REFERENCES**


