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ORIGINAL ARTICLE

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Dihydroartemisinin downregulates vascular endothelial growth factor expression and induces apoptosis in chronic myeloid leukemia K562 cells

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Abstract Dihydroartemisinin (DHA), a more water-soluble active metabolite of artemisinin derivatives, is safe and the most effective antimalarial analog of artemisinin. In the present investigation, we assessed the effect of DHA on vascular endothelial growth factor (VEGF) expression and apoptosis in chronic myeloid leukemia (CML) K562 cells. The results demonstrated that in addition to its antiproliferation effect on CML cells, DHA was also found to induce K562 cells apoptosis. The percentage of apoptotic cells was increased to 6.9 and 15.8% after being treated with 5 and 10 μmol/l DHA for 48 h, respectively ($P < 0.001$). In order to analyze the effect of DHA on VEGF expression in K562 cells, we assessed the level of VEGF expression by western blot; detected the form of VEGF mRNA by RT-PCR and examined the level of VEGF secreted in conditioned media (CM) by ELISA assay. All these experiments suggested that DHA could inhibit the VEGF expression and secretion effectively in K562 cells, even at a lower concentration (2 μmol/l, $P < 0.05$). Moreover, we further assessed the stimulating angiogenic activity of CM from K562 cells on CAM model. The angiogenic activity was decreased in response to the CM from K562 cells pretreated with DHA in a dose-dependent manner. Taken together, these results from our study together with its known low toxicity make it possible that DHA might present potential antileukemia effect as a treatment for CML therapy, or as an adjunct to standard chemotherapeutic regimens.

Keywords Dihydroartemisinin · Vascular endothelial growth factor · Apoptosis · Angiogenesis · Chronic myeloid leukemia

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Introduction

Angiogenesis, the development of new blood vessels by sprouting from pre-existing vasculature, plays a fundamental role in the neoplastic process and is essential for local progression and metastatic spread of solid tumors [13, 12]. Among many kinds of activators of angiogenesis, vascular endothelial growth factor (VEGF) is considered as one of the most potent angiogenic factors. The expression of VEGF has been suggested to be related to some fundamental features of solid tumors, such as growth rate, microvessel density, and the development of tumor metastasis [19, 8]. Recently, there are increasing evidences indicating that VEGF also plays an important role in the development and progression of chronic myeloid leukemia (CML) [1]. In addition, the VEGF concentrations in the bone marrows were found to correlate inversely with the length of survival in patients having chronic phase CML [2]. Therefore, VEGF has been expected as an emerging target for CML therapy and some corresponding strategies have been raised including decreasing the production of VEGF [15], blocking the binding of VEGF to its receptors [23], and inhibiting VEGF receptor tyrosine kinases [6]. In present study, we investigate the effect of artemisinin analogs on VEGF production in CML.

Artemisinin, a sesquiterpene lactone isolated from the plant Artemisia annua L., and its derivatives are presently used in various countries as an antimalarial drug with little toxicity to humans and have a potent effect on chloroquine-resistant malarial parasites [21, 34]. Dihydroartemisinin (DHA) is the main active metabolite of artemisinin derivatives and is more water-soluble and effective anti-malarial than artemisinin. In our previous report [5], we have shown that DHA inhibits the VEGF expression in solid tumor xenograft and exhibits the potent antiangiogenic effect in solid tumors. However, to our knowledge, the inhibitory effect of artemisinin analogs on VEGF expression in leukemic cells has not yet been reported. Here, we report that DHA could

effectively downregulate VEGF expression in K562 leukemic cells and induce K562 cells apoptosis.

Materials and methods

Materials

Dihydroartemisinin was a gift from the Engineer, Liuxu of Guiling Pharmaceutical Co. (Guangxi, China). Working solutions were prepared by dissolving the compound in dimethyl sulphoxide (DMSO) before experiments. The final concentration of DMSO is less than 0.1% in all experiments. Hoechst33342 and propidium iodide (PI) were obtained from Sigma (St. Louis, MO, USA). Rabbit anti-human VEGF polyclonal antibody (A-20), Actin polyclonal antibody (I-19) and western blotting luminol reagent (sc-2048) were bought from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Nitrocellulose membranes were supplied by Millipore Co. (Billerica, MA, USA). M-MLV and Taq DNA polymerase were purchased from MBI Fermentas Inc. (Hanover, MD, USA). Trizol was obtained from Bio Basic Inc. (Markharn, ON, Canada). Quantikine human VEGF ELISA kit was purchased from R &D Systems Inc. (Minneapolis, MN, USA).

Cell culture

K562, a chronic myelogenous leukemia line, was obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) 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.

Cell proliferation assay

K562 cells were suspended at a final concentration of 1×10^5 cells/ml and seeded in 24-well microtiter plates. Various concentrations (2∼ 40 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. The inhibitory effects were also determined by the MTT assay in 96-well microtiter plates. After exposure to DHA for 48 h, cells were incubated with MTT (5 mg/ml) for 4 h. The formazan precipitate was dissolved in 150 μl DMSO and the optical density at 570 nm was measured with an universal microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Experiments were performed in triplicate independently.

Morphological examination for apoptosis

After being incubated with DHA, cells were washed twice with PBS and incubated for 15 min with 10 μg/ml Hoechst33342 at room temperature in the dark. The cells were then observed using an inverted fluorescence microscope (DMIL, Leica Microsystems, Wetzlar, Germany) and photographed.

Flow cytometric analysis for apoptosis

Dihydroartemisinin-induced apoptosis was also quantified by staining K562 cells with PI, and measuring fluorescence by flow cytometric assay. Briefly, K562 cells 1×10^6 were washed with PBS and fixed in cold 70% ethanol. Prior to analysis, cells were washed again and resuspended in PBS. RNase was added to the cell sample, which was then mixed with a PI solution. The PI fluorescence of individual nuclei was measured using a FACS calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Protein extraction and western blotting

Total protein extracts from cells were obtained by lysing K562 cells pretreated with DHA for 48 h in cold RIPA buffer (50 mM Tris–HCl (PH 8.0), 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton-X100, 1 mM EDTA, and supplemented with 1 mM PMSF and 10 μg/ml Leupeptin). Proteins from cell supernatants were quantified by the Bradford method. After adjusting to sample buffer, 40 μg of total protein was electrophoresed on a 12% SDS-PAGE gel along with prestained molecular weight standards (MBI Fermentas Inc.). Proteins were subsequently transferred to nitrocellulose membranes (Millipore Co.) following conventional protocols. Before being immunoblotted, membranes were blocked in 5% nonfat milk in TBST buffer (10 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween20) for 1 h at room temperature. Then rabbit polyclonal anti-VEGF (A-20: Santa Cruz Biotechnology Inc.) was used at a dilution of 1:500 for 2 h at room temperature and secondary anti-rabbit IgG–HRP was used at 1:5,000 for 1 h. Immunoreactive bands were visualized with the western blotting luminol reagent (sc-2048: Santa Cruz Biotechnology Inc.). After stripping the membranes, Actin (Goat polyclonal anti-actin: I-19, Santa Cruz Biotechnology Inc., 1:500 dilution) levels were analyzed as controls for protein loading.

RT-PCR analysis

Total RNA was extracted using Trizol (Bio Basic Inc.) according to the manufacturer's protocol and then treated with MMLV reverse transcriptase (MBI Fermentas Inc.) to form cDNA. The cDNA was amplified by PCR using Taq DNA polymerase (MBI Fermentas Inc.), which was performed by denaturation at 94°C for

50 s, annealing at 60°C for 50 s, and extension at 72°C for 70 s. This was done for 30 cycles. Then the PCR-amplified products were run on a 1.5% agarose gel and visualized by ethidium bromide staining. The expression intensities of optimized bands were quantified with Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA), and expressed as a ratio (VEGF versus GAPDH). PCR primers used to detect VEGF were: 5′-TCGGGCCTCCGAAACCATGA-3′ (forward primer) and 5′-CCTGGTGAGAGATCTGGTTC-3′ (reverse primer) [33]; and GAPDH, 5′-GTCAGTGGT GGACCTGACCT-3′ (forward primer) and 5′-CCCTGTTGCTGTAGCCAAAT-3′ (reverse primer) [16]. The VEGF primers can amplify the whole coding region of all known splicing forms of VEGF mRNA. GAPDH levels were analyzed as control.

Collection of conditioned media (CM) and ELISA assay

The preparation of CM was performed as previously described [20]. Briefly, 1×10^5 /ml K562 cells were cultured in various concentrations of DHA for 48 h before CM were generated. The cells were rinsed with RPMI three times, incubated in RPMI for 4 h, refeeded at 1×10^6 /ml with RPMI, and cultured for 24 h. Their viability, assessed by trypan blue exclusion, was >95%. Serum-free CM was collected by centrifugation sequentially at 1,200 and 12,000 rpm for 10 min under sterile condition. Levels of VEGF in the CM were quantified using the quantikine VEGF assay (R &D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. The lower detection limit of the ELISA assay is 5 pg/ml. Total protein contents of CM were measured with the Bradford method.

Chicken chorioallantoic membrane (CAM) assay

The effect on in vivo angiogenesis was evaluated using the CAM vessel development assay with slight modifications as described previously [31]. Briefly, fertilized, domestic chick embryos were incubated at 37° (humidity 55–60%). On day 6, a small hole (approximately 2 cm in diameter) was formed by removing the shell and inner shell membrane, then the exposed area was sealed with cellophane tape. The eggs were returned to the incubator and incubated with the window upright. On day 8, CM from different group of K562 cells were loaded into 1 mm^3 gelatin sponges that were implanted on top of the CAMs (25 ng protein/5 μl per embryo). Sponge loaded with RPMI-1640 medium alone was used as negative control. The sponge traps the sample, allowing slowing release of products contained in the medium. Until day 12 when the angiogenic response peaks, the embryos and their membranes were fixed in ovo using 1:1 (v/v) methanol–acetone solution. The CAMs were carefully isolated and photographed. The microvessels of CAM around the gelatin sponges were quantified using the image analysis program image-pro plus 5.0 (Media Cybernetics, MD, USA) as previously described [24].

Statistical analysis

All experiments were performed in triplicate unless otherwise noted, results were expressed as mean ± standard deviation. Dunnett's *t* test was used to compare the mean differences between samples using the statistical software SPSS version 10.0. Throughout the work, *P* values less than 0.05 were considered to be statistically significant.

Results

IC_{50} value

In the trypan blue exclusion experiment, it showed that the proliferation of K562 cell could be effectively inhibited after being incubated with 10 μmol/l of DHA for 36 h, and the number of viable cells was decreased to 71.9% compared to that of the control group. This number was continued to decrease to 48.2 and 44.8% after being incubated for 48 h and 72 h, respectively (Fig.1a). The presence of DHA at concentrations of more than 2 μmol/l for 48 h inhibited the growth of K562 cells in a concentration dependent manner as shown in the MTT assay (Fig.1b). DHA at 2, 5, 10, 20 and 40 μmol/l inhibited the growth of cells by 3.9, 18.9, 48.1, 67.5 and 82.1%, respectively. The IC_{50} value of DHA for growth inhibition of K562 cells was 13.08 μmol/l, and 95% confidence interval was 10.02–17.07 μmol/l.

Morphological changes of apoptosis in K562 cells

The Hoechst 33342, a sensitive fluorochrome to DNA, was used to assess changes in nuclear morphology following DHA treatment. The nuclei in normal cells were normal and exhibited diffused staining of the chromatin (Fig.2a). However, after exposure to 5 μmol/l DHA for 48 h, K562 cells underwent typical morphologic changes of apoptosis such as chromatin condensation, margination and shrunken nucleus (Fig.2c) while the plasma membrane remained well defined (Fig.2b, d). Therefore, these morphological changes suggested the occurrence of apoptosis in K562 cells after being treated with DHA.

Percentage of cells undergoing apoptosis

Moreover, the morphological alterations of DNA in DHA-treated K562 cell were supported by flow cytometry analysis. The dose-related increase in DHA-induced K562 cell apoptosis was initially analyzed using flow cytometry (Fig.3). After cells were treated with 2 μmol/l DHA for 48 h, there was no obvious increase of apoptotic cells by

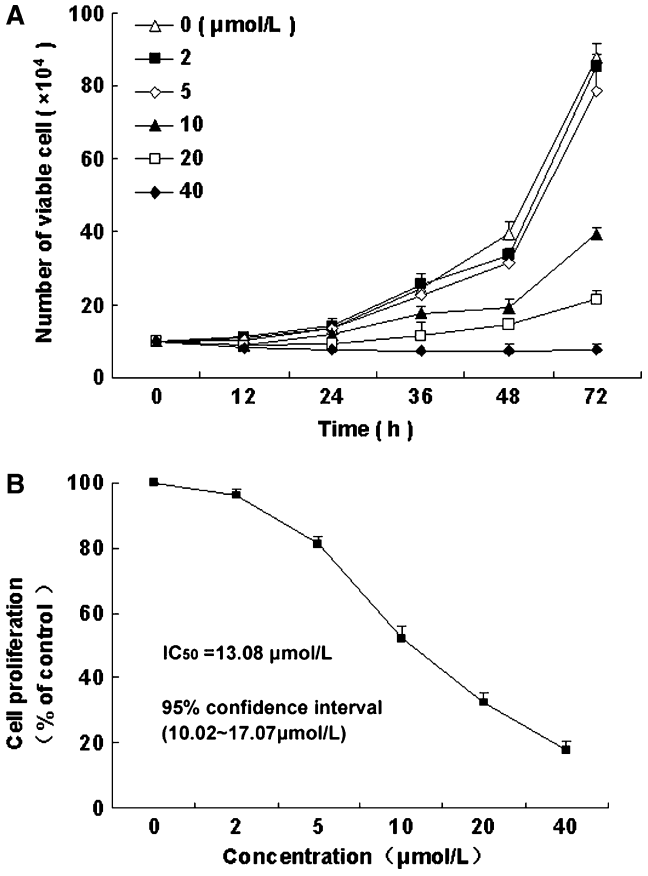

Fig. 1 Effect of DHA on proliferation of K652 cells. **a** K562 cells (1×10^5 /well) were seeded in 24-well microtiter plates and incubated with various concentrations of DHA. After incubation for the indicated times, the viable cells were counted by the trypan blue exclusion method. Values are the mean ± S.D of data from three independent experiments. **b** K562 cells (1×10^4 /well) were seeded into 96-well plates and incubated for 48 h in the presence of various concentration of DHA. Cell proliferation was detected by the MTT assay. Results of experiments in triplicate were expressed as the percentage of vehicle control (mean ± S.D)

PI-flowcytometry assay. The percentage of apoptotic cells was increased to 6.9% and 15.8% when treated with 5 and 10 μmol/l DHA for 48 h, respectively ($P<0.001$).

DHA downregulates VEGF expression in K562 cells

In order to analyze the effect of DHA on VEGF expression in K562 cells, we adopted westernblot method. We treated K562 cells in vitro with various concentrations of DHA, and found that increasing concentrations of DHA lead to a stepwise reduction in VEGF expression (Fig.4). Compared with vehicle control, the levels of VEGF in K562 cells were decreased by 24.0, 37.6 and 92.7% after being treated with 2, 5, 10 μmol/l of DHA for 48 h, respectively ($P<0.05$).

Effect of DHA on VEGF mRNA expression in K562 cells

RT-PCR analysis showed that two forms of transcripts were detected, which encoded for $VEGF_{121}$ and $VEGF_{165}$. DHA could significantly suppress VEGF mRNA expression even at very low concentration of 2 μmol/l. The levels of $VEGF_{165}$ and $VEGF_{121}$ mRNA in K562 cells pretreated with 2 μmol/l DHA were decreased by 27.1 and 11.7% respectively ($P<0.05$) and were stepwise decreased in a concentration-dependent manner (Fig.5). These results of RT-PCR correlated well with those of western blot assay.

DHA decreases the VEGF level in CM of K562 cells

ELISA analysis was performed to determine the amount of secreted VEGF protein in conditioned media. K562 cells pretreated with DHA were grown in serum-free medium for 24 h, and the secreted protein of VEGF in culture media were determined by ELISA. Compared to normal K562 cells, K562 cells pretreated with 2 μmol/l DHA showed a 25.7% decrease of the secreted VEGF level ($P<0.01$) (Fig.6).

Results of CAM assay

Macroscopically, CM-loaded sponges displayed a stimulating angiogenic response, numerous microvessels converged towards the sponges in a radial pattern (Fig.7 b, c). The angiogenic activity was decreased in response to the CM from K562 cells pretreated with DHA in a dose-dependent manner (Fig.7d). Compared with CM from normal K562 cells, the number of microvessels stimulated by CM from K562 cells pretreated with 2, 5, and 10 μmol/l DHA was decreased by 24.5, 50.5 and 65.9%, respectively. Furthermore, there was no significant difference between the microvessel number of negative control group (RPMI-1640 medium alone) and that of CM group from 10 μmol/l DHA pretreated K562 cells ($P>0.05$). It implied that DHA could effectively suppress the stimulating angiogenic ability of CM by reacting with K562 cells.

Discussion

VEGF is a multifunctional cytokine that acts as both a potent inducer of vascular permeability and a specific endothelial cell mitogen. There are five human isoforms derived from alternative splicing (VEGF121, 145, 165, 189, 206), [17] of which $VEGF_{121}$ and $VEGF_{165}$ are soluble ones. VEGF is commonly expressed in a wide variety of human tumor cells and has been associated with angiogenesis, growth, metastasis, and poor outcome in solid tumors. Recently, it was also shown that VEGF expression was higher in the bone marrow of

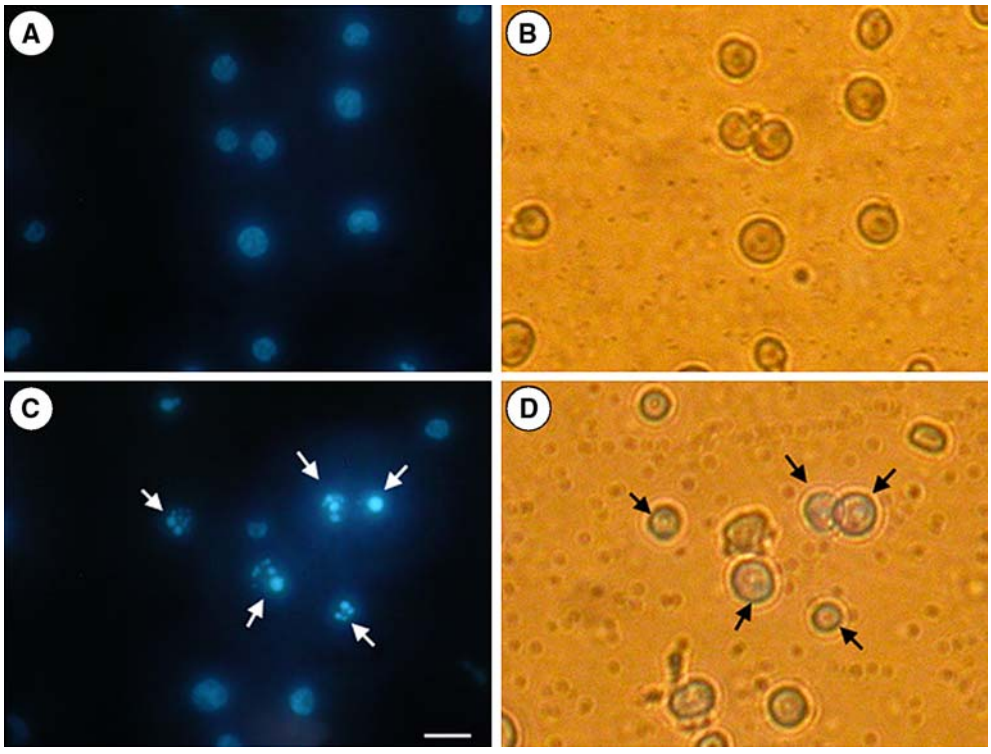

Fig. 2 Fluorescence photomicrographs of K562 cells stained by Hoechst 33342 after being treated with DHA. Cells were incubated with DHA for 48 h. Then the cells were stained by Hoechst 33342 and morphology was immediately assessed using fluorescence microscopy. **a** control: viable cells with dispersive light blue nuclei and intact structure; **c** Five micro mol/l DHA treated: cell shrinkage, chromatin condensation and margination in the nucleus (*arrow*); (b and d) the same cells as panel a and c in a brighter visual field showing that the plasma membrane remained well defined (*bar* = 25 μm)

patients with CML than in that of normal individual [1]. And the mean VEGF concentration in plasma from untreated CML patients was also higher than that from CML patients post allograft bone marrow transplantation [25]. In addition to express VEGF, CML cells was also reported to express the VEGF-related receptors KDR and Flt-1.[3, 32] Consequently, CML cells release VEGF, which can either bind to the receptors on their own surface and stimulate CML cell proliferation (autocrine loop) [26] or bind to the receptors of endothelial cells and activate various functions of the cell including stimulation of growth, migration of endothelial cells and angiogenesis in the bone marrow (paracrine loop) [18]. VEGF has been recognized to play an important role in the pathogenesis of CML by autocrine and paracrine regulation, and several research groups

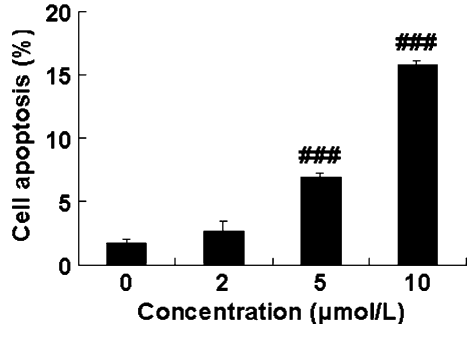

Fig. 3 The apoptotic effect of DHA on K562 cells. Cells were incubated with or without DHA for 48 h and analyzed by flow cytometry after the addition of propidium iodide (PI) solution for staining. The percentage of apoptotic cells was in a dose-dependent manner. Values are the mean ± SD of data from three independent experiments. ###, $P < 0.001$ versus the vehicle control group (Dunnett's *t* test)

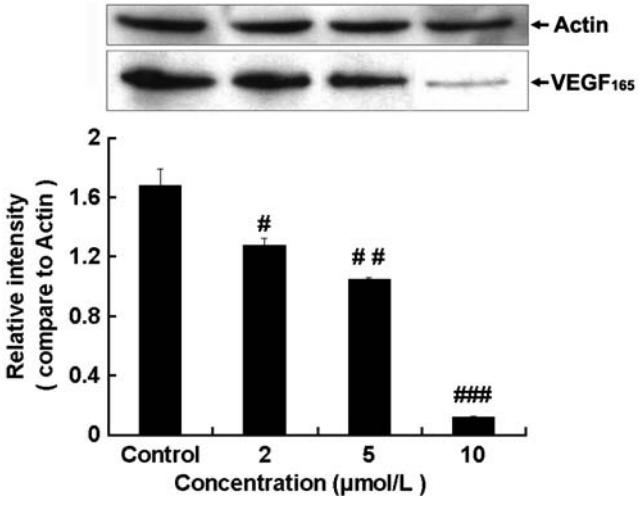

Fig. 4 Western blotting analysis for $VEGF_{165}$ (46 kDa) in K562 cells. Total protein (40 μg) of K562 cells pretreated with various concentrations of DHA for 48 h was electrophoresed on a 12% SDS-PAGE gel and probed on a nitrocellulose membrane with anti-human VEGF antibody. Immune complexes were visualized by the enhanced chemiluminescence method using the Western blotting luminol reagent. Actin (43 kDa) levels were analyzed as controls for protein loading. Relative expression levels of $VEGF_{165}$ were expressed as the relative intensity compared with Actin. Values are the mean ± SD of data from three independent experiments. #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ versus the vehicle control group (Dunnett's *t* test)

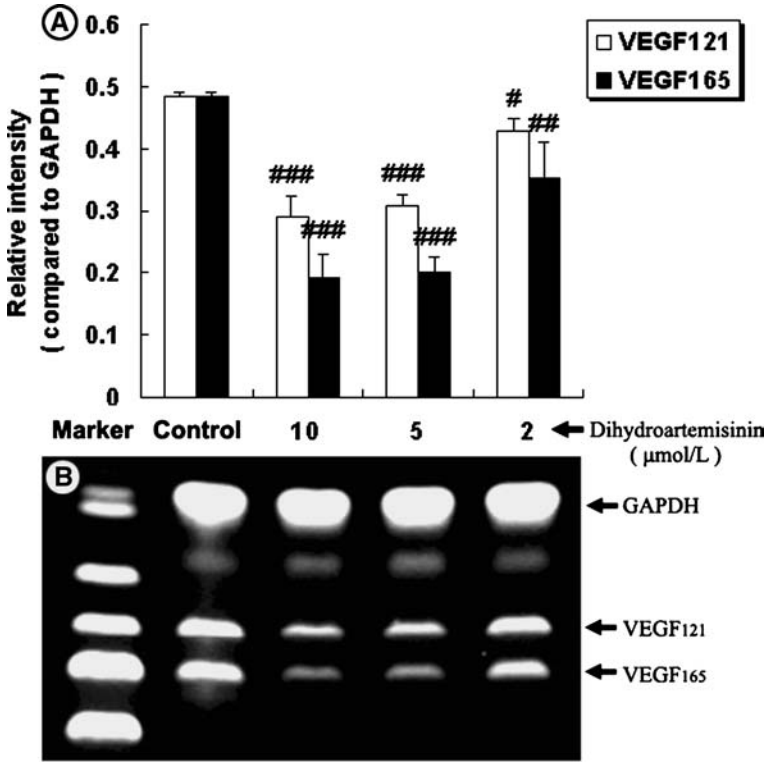

Fig. 5 Effect of DHA on VEGF mRNA expression in K562 cells. Total RNA was isolated from K562 cells pretreated with vehicle or dihydroartemisinin. RT-PCR analysis was performed as described in materials and methods. **a** Relative expression levels of $VEGF_{121}$ and $VEGF_{165}$ mRNA were expressed as the relative intensity compared with GAPHD. Values are the mean ± SD of data from three independent experiments. #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ versus the vehicle control group (Dunnett's *t* test). **b** The PCR products of $VEGF_{121}$ (516 bp), $VEGF_{165}$(648 bp) and GAPDH (251 bp) were electrophoresed on 1.5% agarose gel and visualized with ethidium bromide staining

have displayed the antileukemia effect by downregulating VEGF expression in CML cells [15].

In present study, we assessed the effect of DHA on VEGF expression in human CML cells. K562 cell line was used for our investigation because this cell line expresses high levels of VEGF mRNA, secretes the VEGF protein and expresses the VEGF receptors [14]. We first investigated the anti-proliferation effect of DHA in K562 cells in vitro. The results indicated that DHA could effectively inhibit the proliferation of K562 cells, and the inhibitory effect of DHA on the proliferation of K562 cells was in a dose and incubation time-dependent manner. Artemisinin derivatives in the range of 20–180 μmol/l have been reported to inhibit cancer cell proliferation in vitro [10, 29]. Our study also showed that the IC_{50} value of DHA on K562 cells is 13.08 μmol/l. In order to analyze the effect of DHA on VEGF expression in K562 cells, we assessed the level of VEGF expression by Western blot; detected the form of VEGF mRNA by RT-PCR and examined the level of VEGF secreted in CM by ELISA assay. All these experiments suggested that DHA could inhibit the VEGF expression and secretion effectively in K562 cells, even at a lower concentration (2 μmol/l, $P < 0.05$).

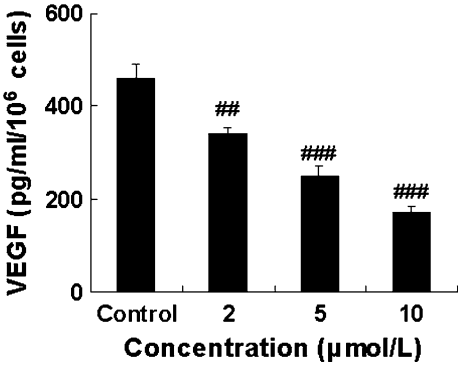

Fig. 6 ELISA assay for VEGF concentrations in CM from K562 cells pretreated with various concentrations of DHA. Serum-free CM were collected by incubate different group of K562 cells in RPMI for 24 h. Levels of VEGF in the CM were quantified using the quantikine VEGF assay according to the manufacturer's protocol. Values are the mean ± SD of data from three independent experiments. ##, $P < 0.01$; ###, $P < 0.001$ versus the vehicle control group (Dunnett's *t* test)

Artemisinin is a sesquiterpene lactone peroxide containing an endoperoxide moiety. Previous studies have reported that in addition to its antimalarial effect, artemisinin and derivatives also showed antitumor activity both in vitro and in vivo [9, 22] The mechanism of its anti tumor effect was its focus on the endoperoxide bridge structure, which react with a ferrous iron atom to form free radical and lead to cellular destruction directly [27, 11]. In present study, we reported that DHA could effectively inhibit the proliferation and downregulate VEGF expression in K562 leukemic cells. Ruan et al. [26] have reported that VEGF play an important role on the abnormal proliferation of CML cells through autocrine mechanism and suppression of the VEGF expression will inhibit the CML cell proliferation. This suggested that the inhibitory effect of DHA on VEGF expression might also contribute to its antiproliferation effect in CML cells. In addition to its antiproliferation effect

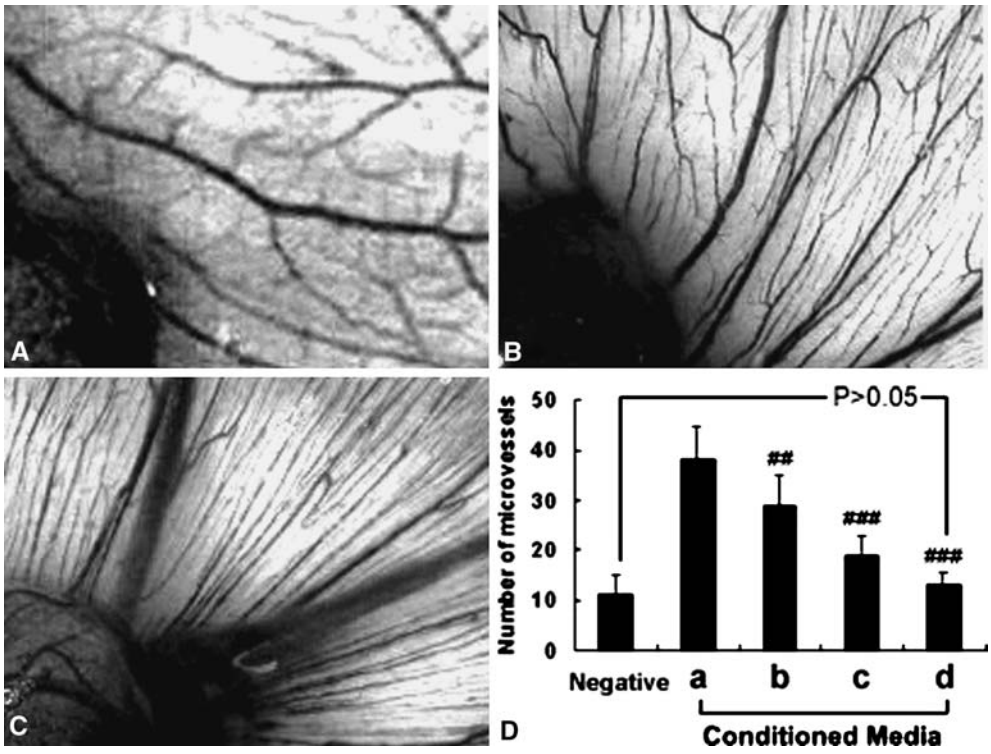

Fig. 7 Angigoenic activity of CM from K562 cells on chicken chorioallantoic membrane (CAM). The CAM of a 12-day-old chick embryo incubated for 4 days with a gelatin sponge loaded with **a** RPMI-1640 medium alone as negative control; **b** CM from K562 cells pretreated with 5 μmol/l of DHA for 48 h; or **c** CM from normal K562 cells (25 ng protein/5 μl per embryo). **d** The CAM was carefully isolated and photographed, and the microvessels of CAM around the gelatin sponges were quantified using the image analysis program image-pro plus 5.0. **a** CM from normal K562 cells; **b, c**and **d** CM from K562 cells pretreated for 48 h with 2, 5 and 10 μmol/l DHA, respectively. Results are expressed as the mean ± SD (n = 12). $^{\#\#}$, $P < 0.01$; $^{\#\#\#}$, $P < 0.001$ versus (a) the normal CM group (Dunnett's *t* test)

in CML cells, DHA was also found to induce K562 cells apoptosis from our data. This was consistent with previous reports [28] which indicated that the anti tumor effect of DHA was ascribed to the rapid induction of apoptosis in cancer cells after treatment with DHA. It has been shown that elevated circulating VEGF level will confer $VEGFR^+$ expressing tumor cells with great survival potential and resistance to apoptosis in an autocrine fashion [7]. So, it further confirmed that the antiproliferation and inducing apoptosis effect of DHA in K562 cells maybe also be partly related to the downregulation of VEGF expression.

Besides stimulating CML cell proliferation through autocrine mechanism, VEGF could also bind to the receptors on the surface of vascular endothelial cells and stimulate angiogenesis in bone marrow through paracrine mechanism. So we further assessed the stimulating angiogenic activity of CM from K562 cells on CAM model. The result indicated that compared with CM from normal K562 cells, the ability of stimulating angiogenesis of CM from K562 cells pretreated with DHA was decreased. Combinded with the results from ELISA assay, it implied that DHA could also reduce the CML angiogenesis partly by downregulating the VEGF expression in CML cells.

In summary, in addition to its antiproliferation and inducing apoptosis effects in K562 cells, we have demonstrated that DHA could also downregulate the expression of VEGF and reduce the angiogenic activity of CM from K562 cells. These data further confirmed the role of VEGF in CML through autocrine and paracrine mechanism. The results from our study together with its known low toxicity [30, 4] make it possible that DHA might present potential antileukemia effect as a treatment for CML therapy, or as an adjunct to standard chemotherapeutic regimens.

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