Dihydroartemisinin inhibits angiogenesis induced by multiple myeloma RPMI8226 cells under hypoxic conditions via downregulation of vascular endothelial growth factor expression and suppression of vascular endothelial growth factor secretion

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Multiple myeloma remains incurable to date; therefore, new biologically target-based therapies are urgently needed. Our previous studies have showed that the antimalarial dihydroartemisinin possessed antiangiogenic activity in solid tumors. The present study evaluated the effect of dihydroartemisinin on human multiple myeloma-induced angiogenesis under hypoxia and elucidated its mechanism of action. An in-vivo chicken chorioallantoic membrane model was used to examine the effect of dihydroartemisinin on multiple myeloma-induced angiogenesis. Compared with conditioned medium of control, conditioned medium from human multiple myeloma RPMI8226 cells pretreated with 3 μmol/l dihydroartemisinin in hypoxia was observed to reduce microvessel growth on chicken chorioallantoic membranes by approximately 28.6% ($P<0.05$). The level of vascular endothelial growth factor in conditioned medium was determined by enzyme-linked immunosorbent assay. The results confirmed that 3 μmol/l dihydroartemisinin could significantly decrease vascular endothelial growth factor secretion by RPMI8226 cells ($P<0.05$), which correlated well with the reduction of multiple myeloma-induced angiogenesis on chicken chorioallantoic membranes. Western blot and reverse transcription-polymerase chain reaction results revealed that dihydroartemisinin downregulated the expression of vascular endothelial growth factor in RPMI8226 cells in hypoxia. In addition, we demonstrated that dihydroartemisinin reduced extracellular signal-regulated kinase 1/2 activation and inhibited growth of RPMI8226 cells under hypoxic conditions. Therefore, we concluded that dihydroartemisinin, which is already used to treat malaria and is well tolerated, possesses potential as an antiangiogenic drug in multiple myeloma therapy and thereby may improve patient outcome. *Anti-Cancer Drugs* 17:839–848 © 2006 Lippincott Williams & Wilkins.

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Introduction

Multiple myeloma (MM) is characterized by clonal proliferation of malignant plasma cells distributed at multiple sites within the bone marrow (BM) compartment. Despite recent insights into the pathogenesis of MM, it remains incurable to date. Therefore, new biologically target-based therapies are urgently needed. Many investigators have shown that BM angiogenesis is increased in MM [1,2]. The increased microvessel density in BM specimens of a MM patient correlates with disease progression and poor prognosis [3–6]. Furthermore, vascular endothelial growth factor (VEGF) and VEGF receptor expression was observed on both human MM cell lines and patient myeloma cells in BM [7]. VEGF is believed to be a key mediator of angiogenesis by acting as a potent inducer of vascular permeability as well as serving as a specific endothelial cell mitogen [8]. In the context of cancer, particularly in the BM of myeloma patients, VEGF is involved in MM-induced angiogenesis, and is also an important growth, migration and survival factor of MM cells [9–11]. In addition, signaling pathways mediating VEGF effects in MM have been delineated recently. Exogenous VEGF stimulates proliferation of MM cells through an extracellular signal-regulated kinase 1/2 (ERK1/2) signaling transduction pathway, and suggesting that inhibition of VEGF secretion reduces myeloma-induced angiogenesis and inhibits MM cells growth through downregulating phospho-ERK1/2 expression [12,13].

It has also been demonstrated that under the low oxygen environment in the BM, VEGF secreted by MM cells has

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the capacity to participate in MM angiogenesis. Hypoxia, as a key regulator of VEGF expression, mediated hypoxia-initiated angiogenesis in the BM microenvironment [14–17]. Therefore, targeting hypoxia-induced VEGF production in MM is a novel therapeutic approach.

Artemisinin, a sesquiterpene lactone isolated from the traditional Chinese herb *Artemisia annua* Linn., is a representative of a new class of antimalarial drugs [18]. Artemisinin and its derivatives are currently used in various countries as antimalarial drugs and have a potent effect on chloroquine-resistant malarial parasites [19,20]. Large clinical studies with malaria patients showed that artemisinin derivatives are well tolerated with few and insignificant side-effects [21]. Dihydroartemisinin (DHA) is the main active metabolite of artemisinin, and is more water-soluble and more effective in treating malaria than artemisinin. Our previous studies revealed that DHA possess antiangiogenic activity *in vitro* [22]. Recently, we also showed that DHA inhibits the VEGF expression in solid tumor xenografts and exhibits the potent antiangiogenic effect in solid tumors *in vivo* [23]. DHA also downregulates VEGF expression and inhibits proliferation of chronic myeloid leukemia K562 cells [24]. The effect of DHA on angiogenesis induced by other hematological malignancies and, in particular, by MM under low oxygen tension, however, remains elusive.

In the present study, we investigated the effects of DHA on angiogenesis induced by human myeloma RPMI8226 cells under 3% O_2 conditions using a chicken chorioallantoic membrane (CAM) model. We assayed the direct effects of DHA on VEGF expression and secretion by RPMI8226 cells under hypoxia. We also evaluated the effect of DHA on expression and activation of ERK1/2 signaling transduction protein in the hypoxic condition. Results from our studies indicated that DHA is a potent inhibitor of MM-induced angiogenesis under hypoxic conditions.

Materials and methods

Reagents and antibodies

DHA, a gift from Engineer Liuxu (Guiling Pharmaceutical, Guangxi, China), was freshly dissolved in dimethylsulfoxide (DMSO) just before experiments. The final concentration of DMSO was less than 0.1%. Cells cultured with 0.1% DMSO were used as a negative control. Penicillin, streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMSO, ethidium bromide and propidium iodide were purchased from Sigma (St Louis, Missouri, USA). M-MLV and Taq DNA polymerase were from MBI Fermentas (Hanover, Maryland, USA). TRIzol was obtained from Bio Basic (Markharn, Ontario, Canada). Quantikine human VEGF enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems (Minneapolis, Minnesota, USA). Nitrocellulose membrane was bought from Millipore (Billerica, Massachusetts, USA). Rabbit anti-human VEGF polyclonal antibody (pAb) (A-20), goat anti-actin pAb (I-19), rabbit anti-ERK2 pAb and mouse anti-phospho-ERK1/2 monoclonal antibody (mAb) and all the secondary antisera were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA).

Cell culture conditions and treatment

Human MM cell line RPMI8226, originally obtained from American Type Culture Collection (Rockville, Maryland, USA), was maintained in RPMI1640 medium (Gibco/BRL, Merelbeke, Belgium), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, and cultured at 37°C under a humidified atmosphere of 5% CO_2/95% air (normoxia). For the condition of hypoxia, cells were incubated in a hypoxic incubator with a humidified atmosphere of 5% CO_2/3% O_2 balanced with N_2 (hypoxia) at 37°C. Exponentially growing cells were treated with DHA throughout the study. For hypoxic experiments, cells were first incubated in hypoxia for 24 h before being treated with DHA. Their viability, measured by trypan blue exclusion, was above 99%.

Preparation of conditioned medium

Conditioned medium (CM) was prepared as previously described [25]. Briefly, 1×10^5/ml RPMI8226 cells were cultured in various concentrations of DHA for 48 h under hypoxic conditions. After treatment, cells were rinsed with RPMI1640 3 times and then incubated in RPMI1640 for 4 h. Cells were recollected and resuspended at 1×10^6 cells/ml with serum-free medium and cultured in six-well plates for another 24 h under hypoxic conditions. Cell viability, examined by trypan blue exclusion, was above 95%. After 24 h, CM was collected, and centrifuged at 1200 and 12 000 r.p.m. for 10 min under sterile condition. CM was either used immediately or stored at −80°C. Protein content in CM was determined with the Bradford method.

Chicken chorioallantoic membrane assay

The effect on in-vivo angiogenesis was evaluated using a CAM vessel development assay as described previously [26] with slight modifications. Fertilized, domestic chicken eggs were incubated at 37°C at constant humidity. On day 6, a small square window (approximately 1.5 cm × 1.5 cm) was opened in the shell and then the exposed area was sealed with a cellophane tape. The eggs were returned to the incubator. On day 8, CM from RPMI8226 cells treated with different doses of DHA or serum-free RPMI1640 medium were loaded into 1-mm^3 gelatin sponges that were implanted on top of the CAM (200 ng total protein/10 μl per egg). A sponge loaded with RPMI1640 medium alone was used as a negative control. The sponge traps the sample, allowing slow release of products secreted by cells. The CAM was examined daily

until day 12 when the angiogenic response peaked [27]. The CAM was fixed *in ovo* with 1:1 (v/v) methanol/acetone for 15 min, and isolated carefully and photographed. The microvessels of CAM around the gelatin sponges were quantified using the image analysis program Image-Pro Plus 5.0. (Media Cybernetics, Maryland, USA) [28].

Enzyme-linked immunosorbent assay

RPMI8226 CM, pretreated with DHA, was collected as described above. VEGF concentrations in the CM were quantified following the manufacturer's protocol of a commercially available human VEGF assay kit (R&D Systems). The lower detection limit of the kit is 30 pg/ml for VEGF.

Reverse transcription-polymerase chain reaction analysis

RPMI8226 cells (2×10^6cells) were incubated with different concentrations of DHA in a hypoxic incubator for 48 h. Total RNA was extracted from pretreated cells following the manufacturer's protocol of TRIzol reagent (Bio Basic). The first-strand cDNA was synthesized by M-MLV reverse transcriptase (MBI Fermentas) in a 50-μl reaction system containing 2 μg total RNA. cDNA was amplified by polymerase chain reaction (PCR) using Taq DNA polymerase (MBI Fermentas). The thermal cycle profile consisted of denaturing at 94°C for 50 s, annealing at 60°C for 50 s and extension at 72°C for 70 s [24]. The samples were amplified for 30 cycles; 20 pmol primers were used. PCR products were electrophoresed on a 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 an internal control. VEGF primers: 5′-TCG GGC CTC CGA AAC CAT GA-3′ (forward) and 5′-CCT GGT GAG AGA TCT GGT TC-3′ (reverse) [29]; GAPDH primers: 5′-GTC AGT GGT GGA CCT GAC CT-3′ (forward) and 5′-CCC TGT TGC TGT AGC CAA AT-3′ (reverse) [30].

Cell lysis and Western blot analysis

The whole-cell lysate was prepared as follows. Cells, cultured under hypoxia and pretreated with DHA for the indicated times for VEGF and ERK1/2 detection, were washed twice in cold phosphate-buffered saline (PBS) and lysed for 30 min with ice-cold lysis buffer containing 50 mmol/l Tris–HCl (pH 8), 150 mmol/l NaCl, 2 mmol/l ethylene diaminetetraacetic acid (pH 7.5), 2 mmol/l ethylene glycol-bis (*b*-aminoethyl ether), 25 mmol/l sodium fluoride, 25 mmol/l β-glycerolphosphate, 0.2% (v/v) Triton X-100 and freshly added protease inhibitors (0.1 mmol/l sodium vanadate, 5 μg/ml leupeptin, 0.1 mmol/l phenylmethyl sulfonyl fluoride, 0.3% NP-40). Cells were spun at 12 000 r.p.m. at 4°C for 30 min and supernatant containing proteins was collected. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, California, USA).

To detect the expression of VEGF and ERK1/2, p-ERK1/2, whole-cell lysate (40 μg/lane) was loaded and proteins were separated on a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and subsequently transferred onto nitrocellulose membranes. After blocking with 5% nonfat milk in T-PBS (0.01 mol/l PBS, pH 7.4, 0.1% Tween-20), membranes were probed with primary antibodies of VEGF (1:500), ERK2 (1:1000), p-ERK1/2 (1:500) and β-actin or α-tublin (1:1000) diluted in T-PBS with 5% nonfat milk for 2 h at room temperature or overnight at 4°C. After extensive washing, immunocomplexes were detected with horseradish peroxidase-conjugated species-specific secondary antiserum, followed by enhanced chemiluminescence (Biological Industries, Beit Haemek, Kibbutz Beit Haemek, Israel) reaction and then exposed to X-ray film (Eastman Kodak, Rochester, New York, USA). The intensity of immunoreactive bands was quantified with Quantity One software (Bio-Rad Laboratories).

MTT cell proliferation assay

The sensitivity to DHA of RPMI8226 cells was analyzed by MTT assay. Cells (1×10^4 cells/well) were plated in 96-well plates. Various concentrations (0–80 μmol/l) of DHA were added into each well in triplicates and cells were cultured under hypoxia. After exposure to DHA for 24 and 48 h, cell proliferation was determined by MTT assay. The optical density was read at 570 nm in the Universal Microplate Reader (EL_x800; Bio-Tek Instruments, Winooski, Vermont, USA). Experiments were repeated independently three to five times and the 50% inhibitory concentration values were calculated.

Flow cytometry assay

To evaluate cell cycle profile, the cells (about 1×10^6 cells), pretreated with DHA for 48 h, were harvested, washed twice with PBS and fixed in ice-cold 70% (v/v) ethanol overnight at 4°C. Prior to analysis, samples were washed again and incubated in PBS (pH 7.4) containing 0.1% (v/v) Triton X-100, 0.1 mmol/l ethylene diaminetetraacetic acid and 0.5 mg/ml RNase A (Sigma) for 5 min, and then incubated with 50 μg/ml propidium iodide at 37°C in the dark for 30 min. After filtration to remove cellular debris, the single-cell suspensions were analyzed on a flow cytometer (FACSCalibur; Becton Dickinson, San Jose, California, USA). Cell cycle parameters were analyzed by using Modfit software (Becton Dickinson).

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical significance of differences was determined using an unpaired Student's *t*-test. $P < 0.05$ denoted the presence of a statistically significant difference.

Results

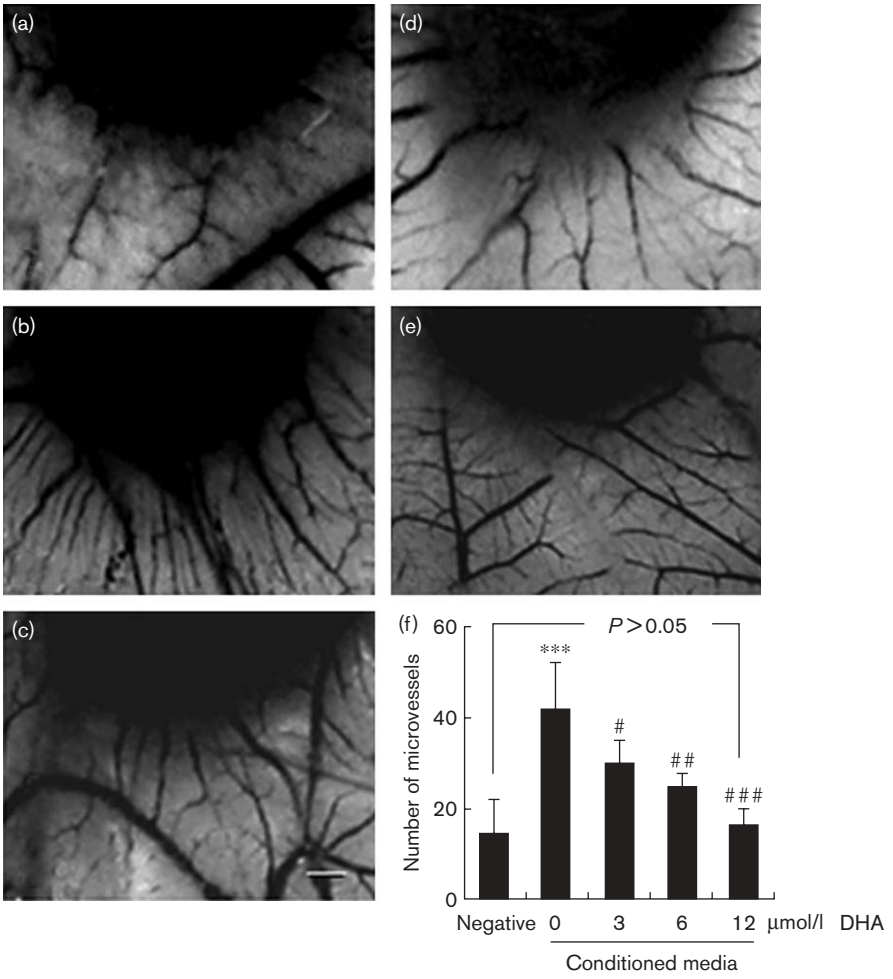
Inhibition of multiple myeloma-induced in-vivo angiogenesis

In this study, we first examined the angiogenic potential of myeloma RPMI8226 cells after pretreatment with DHA under hypoxic conditions using a modified CAM assay [26]. The gelatin sponges loaded with RPMI1640 medium alone or with CM from RPMI8226 cells pretreated with 0–12 μmol/l of DHA under hypoxic conditions were loaded onto the CAM on day 8 of development. CM-loaded sponges on day 12 displayed an intense capillary growth with numerous microvessels converging towards the sponges in a 'spoked wheel' pattern (Fig. 1b–d). The angiogenic activity was decreased in response to the CM from RPMI8226 cells pretreated with DHA in a dose-dependent manner (Fig. 1f). Compared with CM from normal control RPMI8226 cells, numbers of microvessels stimulated by CM from RPMI8226 cells pretreated with 3, 6 and 12 μmol/l of DHA was reduced by approximately 28.6 ($P < 0.05$), 41.3 ($P < 0.01$) and 61.4% ($P < 0.001$), respectively. In addition, there was no significant difference in numbers of microvessels between the negative control group (RPMI1640 medium alone, Fig. 1a) and the CM group from 12 μmol/l DHA-pretreated RPMI8226 cells (Fig. 1e) ($P > 0.05$). These results demonstrated the efficacy of DHA to strongly inhibit the angiogenic potential of RPMI8226 cells.

Suppression of vascular endothelial growth factor secretion of RPMI8226 cells

To evaluate the effect of DHA on VEGF secretion by RPMI8226 cells under 3% O_2 hypoxic conditions, we

Fig. 1



Inhibition of multiple myeloma-induced in-vivo angiogenesis by dihydroartemisinin (DHA) on chicken chorioallantoic membrane (CAM). The CAM of a 8-day-old chick embryo incubated for 4 days with gelatin sponge loaded with RPMI1640 medium alone was used as the negative control (a) or with conditioned medium (CM) from RPMI8226 cells pretreated with 0, 3, 6 and 12 μmol/l DHA in 3% O_2 for 48 h, respectively (b–e) (200 ng total protein/10 μl per egg). (f) The microvessels of CAMs around the gelatin sponges were quantified using the image analysis program Image-Pro Plus 5.0. Results are expressed as the mean ± standard deviation ($n = 12$). $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ versus the normal CM group (b). $^{***}P < 0.001$ versus the negative control group (a). Bar = 7 μm.

determined the levels of VEGF in CM using a human VEGF ELISA assay kit. CM was prepared as described in Materials and methods. Basal VEGF secretion by RPMI8226 cells under hypoxia was 587 ± 22 pg/ml per 10^6 cells. A significant dose-dependent decrease in VEGF secretion by RPMI8226 cells was induced by DHA treatment [mean ± SD versus control: 495 ± 27 (3 μmol/l), 218 ± 42 (6 μmol/l) and 123 ± 14 (12 μmol/l) versus 587 ± 22 pg/ml per 10^6 cells] (Fig. 2a). Compared with control, RPMI8226 cells pretreated with 3, 6 and 12 μmol/l DHA showed significant lower secretions of VEGF protein by 15.7 ± 4.7 ($P < 0.05$), 62.9 ± 2.0 ($P < 0.001$) and $79.0 \pm 2.3\%$ ($P < 0.001$), respectively. The results of this experiment demonstrated that inhibition of the angiogenic potential of RPMI8226 cells by DHA was correlated with the decrease of VEGF secretion.

Decrease of vascular endothelial growth factor mRNA expression in RPMI8226 cells

Next, we analyzed the effect of DHA on VEGF mRNA expression of RPMI8226 cells under hypoxia by reverse transcription-PCR. RPMI8226 cells were pretreated with 0.1% DMSO or DHA for 48 h. PCR productions revealed three bands corresponding to the splice variants: 516 bp of $VEGF_{121}$, 588 bp of $VEGF_{145}$ and 648 bp of $VEGF_{165}$. Compared with the control group, DHA significantly reduced VEGF mRNA expression in a dose-dependent manner (Fig. 3a). Even at the lower concentration of 3 μmol/l DHA, the levels of $VEGF_{165}$, $VEGF_{145}$ and $VEGF_{121}$ mRNA under pretreated with DHA in hypoxia were decreased by 12.7, 37.9 and 15.4% ($P < 0.05$, 3 μmol/l) (Fig. 3b). The results implied that DHA could downregulate VEGF expression at gene level.

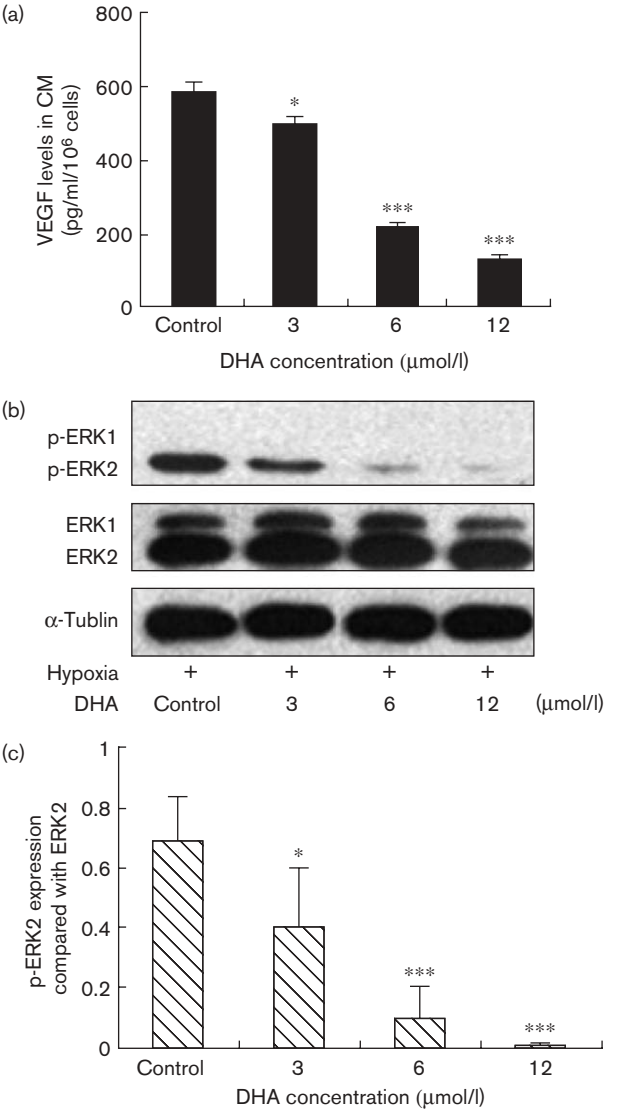
Downregulation of vascular endothelial growth factor protein expression in RPMI8226 cells

We further examined the effect of DHA on expression of VEGF protein of RPMI8226 cells under hypoxia. Whole-cell lysate was analyzed by Western blot. As shown in Fig. 4, we found that DHA led to a reduction of VEGF expression in a dose (3–12 μmol/l)- and time (6–48 h)-dependent fashion. Noticeably, when incubated under hypoxic conditions for 48 h, VEGF protein expression was decreased by 24.4 ± 5.5 (3, $P < 0.05$) and $90.7 \pm 2.2\%$ (12 μmol/l $P < 0.001$) versus the control group. Furthermore, with the exposure to hypoxic conditions for 6, 12, 24 and 48 h, the expression of VEGF protein in RPMI8226 cells treated with 12 μmol/l DHA was significantly downregulated by 41.0 ± 11.1 (12 h, $P < 0.05$), 84.1 ± 10.8 (24 h, $P < 0.001$) and $91.5 \pm 11.8\%$ (48 h $P < 0.001$) as compared with the control group. These results confirmed that DHA could significantly downregulate VEGF expression.

Blockage of extracellular signal-regulated kinase 2 activation in RPMI8226 cells

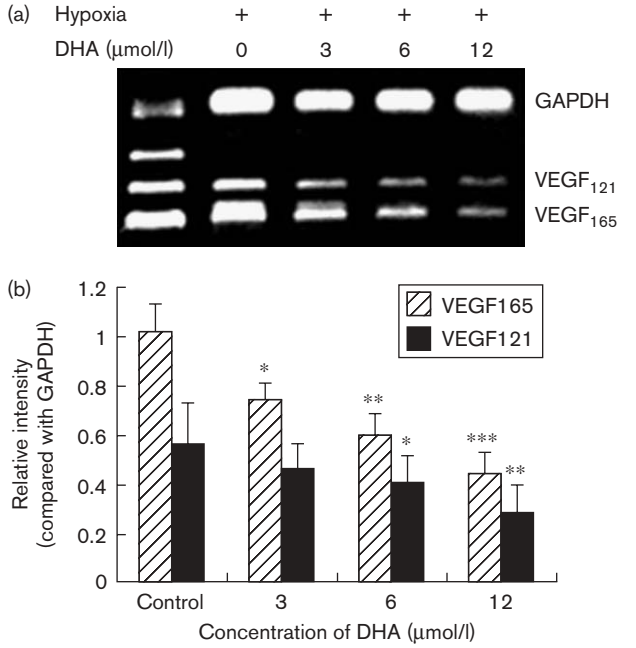
We also investigated whether ERK1/2 and p-ERK1/2 proteins were altered upon DHA treatment. Normal RPMI8226 cells (1×10^5/ml) were incubated with various concentrations of DHA under hypoxic conditions for 6 h, and collected for protein extraction and analyzed by Western blot as described in Materials and methods. As shown in Fig. 2(b and c), when normal RPMI8226 cells were incubated with DHA, ERK1/2 expression did not change significantly. A lower level of phospho-ERK2,

Fig. 2



Suppression of vascular endothelial growth factor (VEGF) secretion and blockage of extracellular signal-regulated kinase (ERK) 2 activation by dihydroartemisinin (DHA) in RPMI8226 cells under hypoxia. (a) Conditioned media (CM) were collected as described in Materials and methods. Levels of VEGF in CM were quantified by enzyme-linked immunosorbent assay. (b) RPMI8226 cells were pretreated with DHA under hypoxia for 6 h. Forty micrograms of total protein were used for Western blot analysis, probed with mouse dual-anti-p-ERK1/2 mAb or rabbit anti-ERK2 polyclonal antibody. α-tublin was analyzed as control. (c) Activation of ERK1/2 was assessed by relative intensity of p-ERK2 compared with ERK2. The data are shown as mean ± standard deviation from three independent experiments. *$P < 0.05$; ***$P < 0.001$ versus the control.

Fig. 3



Decrease of vascular endothelial growth factor (VEGF) mRNA expression induced by dihydroartemisinin (DHA) under hypoxia. RPMI8226 cells were pretreated with or without DHA in hypoxia for 48 h. (a) The polymerase chain reaction products were electrophoresed on 1.5% agarose gel and visualized with ethidium bromide staining. (b) Relative gene expression levels of $VEGF_{121}$ and $VEGF_{165}$ mRNA were assessed as the relative intensity compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are representative of three independent experiments. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ versus the control.

however, was observed in RPMI8226 cells pretreated with various concentrations of DHA under hypoxic conditions. The expression intensity of phospho-ERK2 protein, in comparison with control, was significantly lowered by 40.6 (3), 85.4 (6) and 98.2% (12 μmol/l), respectively. The results showed that the activation of ERK2 protein in RPMI8226 cells was rapidly inhibited by DHA treatment. Take together, the above data implied that the ERK1/2 signal transduction pathway may play an important role in VEGF-mediated MM cells growth and MM cell-induced angiogenesis.

Inhibition of RPMI8226 cell proliferation under hypoxia

As in the context of the BM microenvironment, binding of VEGF to MM cells through VEGF receptors, in particular Flt-1, can trigger its downstream ERK signaling pathway mediating cell proliferation. Next, we evaluated effects of DHA on proliferation of human myeloma RPMI8226 cells cultured under hypoxia by MTT assay. The 50% inhibitory concentration of DHA on RPMI8226 cells in hypoxia was 30.24 ± 6.71 and 16.76 ± 5.82 μmol/l for 24 and 48 h, respectively. DHA inhibited proliferation of myeloma RPMI8226 cells in a dose (0–80 μmol/l)- and time (24–48 h)-dependent manner (Fig. 5). The data suggested that, to some extent, the inhibition of VEGF expression and VEGF secretion resulted in the inhibition of proliferation of RPMI8226 cells under hypoxia through an autocrine mechanism.

Growth of multiple myeloma RPMI8226 cells under hypoxia arrested by dihydroartemisinin

Finally, we investigated effects of DHA on the cell growth cycle of RPMI8226 cells under hypoxia. RPMI8226 cells (1×10^5/ml) were pretreated with 3, 6 and 12 μmol/l of DHA or DMSO (vehicle control) under hypoxia for 48 h, and cell cycle was analyzed by FACS. As shown in Fig. 6(A) [(a) control, (b) 3 μmol/l DHA, (c) 6 μmol/l DHA and (d) 12 μmol/l DHA] and Fig. 6(B), treatment with DHA significantly decreased the S phase (42.86 versus 14.58%), with corresponding increases of cells in the G_2/M phase (8.06 versus 30.37%) and in the sub-G_1 phase (4.59 versus 23.28%). DHA induced growth arrest in a dose-dependent fashion and it was directly correlated with the inhibition of cell proliferation (Fig. 5). These results demonstrated that DHA-induced growth arrest of RPMI8226 cells was partially due to cell cycle block, which caused cells to accumulate in the G_2/M phase and partially to the apoptotic sub-G_1 phase.

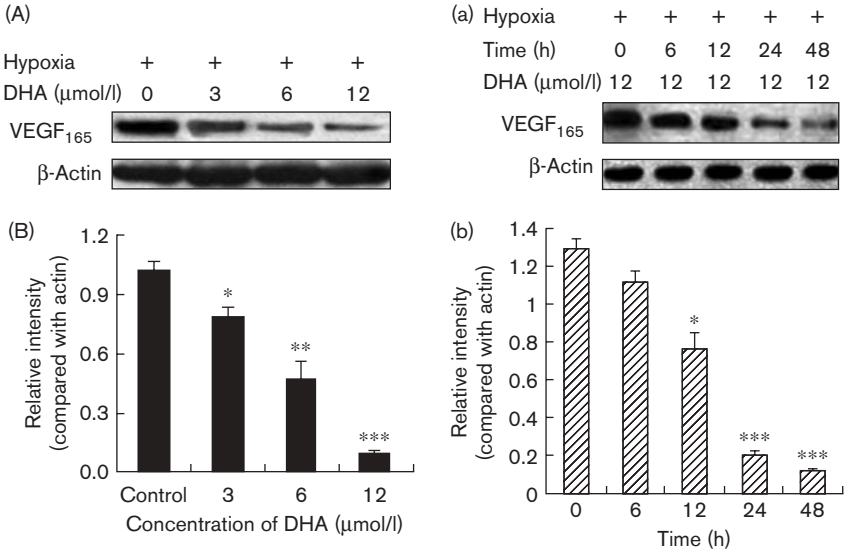
Discussion

The present study has demonstrated that DHA inhibits human myeloma RPMI8226 cell-induced angiogenesis under hypoxia. The proposed mode of inhibition action by DHA was mediated through downregulating VEGF expression, inhibiting VEGF protein secretion under hypoxia conditions.

MM is an incurable disease with the accumulation of malignant plasma cells into the BM. Recent evidence indicated that in the malignant BM microenvironment, the angiogenetic process is critically involved in the physiopathology of MM by promoting plasma cell growth and survival [2,31,32]. Angiogenesis in MM patients is mainly promoted by VEGF. VEGF is either produced by myeloma cells or BM stroma cells as a result of myeloma-cell-induced upregulation [9,12]. It has also been demonstrated that BM of MM is in a low oxygen environment [14–17]. As a key regulator of VEGF expression, hypoxia mediates angiogenesis in the BM microenvironment. Several research groups have shown the antitumor growth efficacy through downregulating VEGF expression in MM cells. Therefore, targeting VEGF production in the hypoxic BM microenvironment of MM is a promising therapeutic approach.

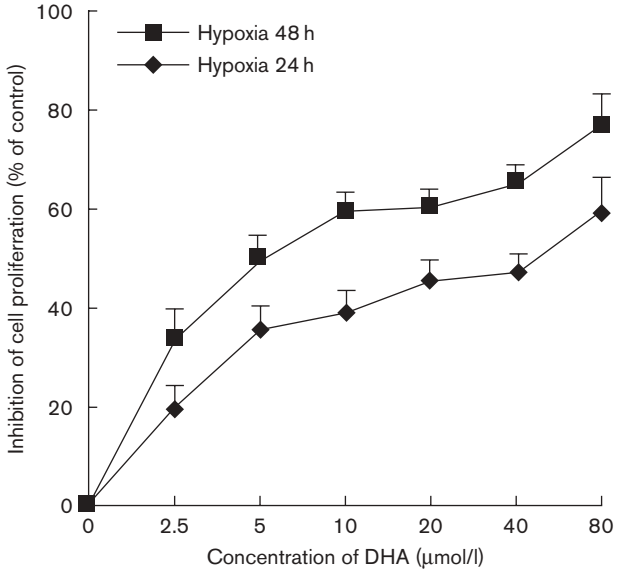
In order to investigate whether the antimalarial agent DHA was able to inhibit MM-induced angiogenesis, the present study examined such effects using a well-established in-vivo CAM assay [26,33]. In this model,

Fig. 4



Downregulation of vascular endothelial growth factor (VEGF) protein expression induced by dihydroartemisinin (DHA) in hypoxia. (A) RPMI8226 cells were pretreated with various concentrations of DHA for 48 h under hypoxia. $VEGF_{165}$ (46 kDa) was analyzed by Western blot. β-Actin (43 kDa) was analyzed as internal controls. (a) RPMI8226 cells were pretreated with 12 μmol/l DHA under hypoxia for 0, 6, 12, 24 and 48 h. $VEGF_{165}$ was analyzed by Western blot. (B, b) Relative expression levels of $VEGF_{165}$ were expressed as the relative intensity compared with β-actin. Values are the mean ± standard deviation of data from three independent experiments. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ versus the control.

Fig. 5



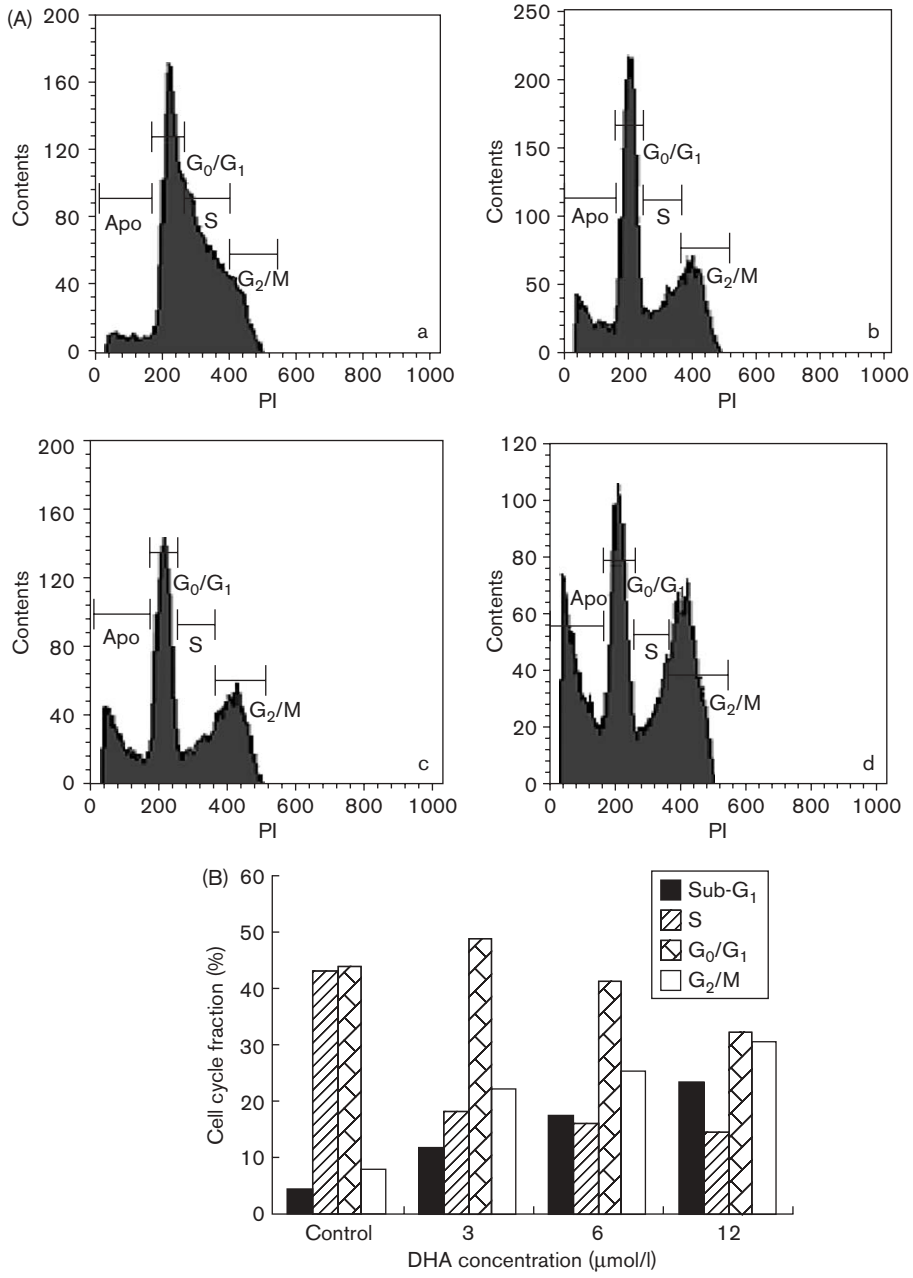
Inhibition of proliferation of myeloma RPMI8226 cells under hypoxic conditions. RPMI8226 cells were treated with 0–80 μmol/l dihydroartemisinin (DHA) for 24 and 48 h. Cell proliferation was detected by the MTT assay as described in Materials and methods. Graphs are representative of three experiments in triplicate. Results are expressed as mean ± standard deviation.

the complete process of angiogenesis was studied, including endothelial cell detachment, dissolution of the extracellular matrix, proliferation, migration, alignment, reattachment and maturation [34]. As RPMI8226 cells express high levels of the VEGF, secrete soluble form of VEGF protein and express the VEGF receptor Flt-1 [7,35], this cell line was used as a model system. We first investigated the angiogenic potential of CM of RPMI8226 cells pretreated with DHA under 3% O_2 hypoxic conditions on the CAM model. At a lower concentration of DHA (3 μmol/l), we observed a significant reduction in microvessel growth by approximately 28.6% ($P < 0.05$). These results indicated that DHA effectively suppressed angiogenesis induced by RPMI8226 CM under hypoxia.

Effects of DHA on VEGF secretion were also determined in the present study. We examined levels of soluble VEGF secreted into CM by ELISA under a low oxygen condition, which mimics the BM microenvironment. A significant inhibition of VEGF secreted by RPMI8226 cells was observed after DHA treatment in a dose-dependent manner. These results correlated well with reduction in microvessel growth induced by RPMI8226 CM on CAM.

In addition, VEGF mRNA and protein levels were evaluated by semiquantitative reverse transcription-PCR and Western blot under hypoxia. The results demonstrated that the expression of VEGF was significantly downregulated in treated PRMI8226 cells in a time- and dose-dependent manner. When RPMI8226 cells were incubated with DHA under hypoxic conditions for 48 h,

Fig. 6



Growth of multiple myeloma RPMI8226 cells in hypoxia arrested by dihydroartemisinin (DHA). (A) RPMI8226 cells were cultured for 48 h in the presence of 3 (b), 6 (c) and 12 μmol/l (d) DHA or absence (a) of DHA under hypoxic conditions. Cells were then stained with propidium iodide (PI), and cell cycle profiles were determined by flow cytometry. (B) Cells in sub-G_1, S, G_0/G_1 and G_2/M phases of the cell cycle fractions are expressed as percentages of the total cell population. The graph is a representative of three separate experiments.

VEGF mRNA expression levels were decreased significantly by DHA treatment even at a low concentration of 3 μmol/l ($P < 0.05$). Importantly, under the same conditions the expression of VEGF protein was reduced by 24.4 ± 5.5 (3, $P < 0.05$) and $90.7 \pm 2.2\%$ (12 μmol/l $P < 0.001$) versus the control group. Furthermore, when treated with 12 μmol/l DHA for 12 h, the expression of VEGF protein in RPMI8226 cells was significantly downregulated by $41.0 \pm 11.1\%$ (12 h, $P < 0.05$). These results indicated that DHA effectively downregulated VEGF expression in RPMI8226 cells. In addition, it suggested that under hypoxia conditions MM-induced angiogenesis was inhibited by DHA through downregulation of VEGF production.

Moreover, the present study investigated the expression and activation of ERK1/2 upon DHA treatment by Western blot. The results showed that DHA (3–12 μmol/l) rapidly suppressed the phosphorylation of ERK2 at 6 h and the suppression correlated with the inhibition of the secretion of VEGF protein by RPMI8226 cells under hypoxia. Therefore, the inhibitory effect of DHA on VEGF secretion by RPMI8226 cells under hypoxic conditions was likely to be correlated with suppression of ERK2 activation.

The present study therefore examined effects of DHA on cell growth under hypoxia by MTT and flow cytometry assays. DHA (2.5–80 μmol/l) inhibited proliferation of RPMI8226 cells both in a dose- and time-dependent manner under hypoxia. In addition, cell cycle analysis revealed that DHA induced growth arrest in RPMI8226 cells under hypoxia by decreasing S phase, and increasing G_2/M and sub-G_1 phases. This effect also correlated with a lower expression of VEGF protein. This result is consistent with the published data by Podar and Anderson [13] and Shweiki *et al.* [14], suggesting that inhibition of VEGF secretion reduces myeloma-induced angiogenesis by downregulating phospho-ERK1/2 expression. Taken together, the suppressive effect of DHA on RPMI8226 cell proliferation was likely due to a decrease of VEGF expression and ERK2 activation mediated by an autocrine mechanism of the VEGF/VEGFR pathway [13,36]. Further experiments, however, remain to be carried out to elucidate the mechanism of action of growth/cycle inhibition. In summary, we have demonstrated that DHA inhibited MM-induced angiogenesis under hypoxic conditions via downregulating VEGF expression and inhibiting VEGF secretion in MM cells. Moreover, our studies suggested that VEGF in human myeloma RPMI8226 cells under hypoxia is a potential target for DHA.

Artemisinin is a sesquiterpene lactone peroxide containing an endoperoxide moiety. It was reported that in addition to its antimalaria effect, artemisinin and derivatives also showed antitumor activity [36,37]. In our previous studies, we showed the potent anti-angiogenesis effect of DHA in solid tumors and its downregulatory effect on VEGF expression in chronic myeloid leukemia K562 cells. The present study further demonstrated the ability of DHA to inhibit MM-induced angiogenesis, downregulate VEGF expression and secretion, and induce growth arrest in human MM under hypoxic conditions. To our knowledge, this is the first report on the effect of antimalarial DHA on MM-induced angiogenesis and on VEGF expression and secretion. As a result of its antiangiogenic effect in the BM microenvironment and that it is well tolerated [38,39], DHA may possess great potential as an anti-MM drug and thereby improve patient outcome.

In summary, we have demonstrated the ability of DHA to inhibit MM-induced angiogenesis and induce myeloma cell growth arrest under the hypoxic BM microenvironment through downregulation of VEGF production. More importantly, our studies have provided a framework for future clinical evaluation of the potential of DHA as an antitumor agent or as an adjunct treatment in MM to improve patient outcome.

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