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ORIGINAL ARTICLE

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Antimalarial dihydroartemisinin also inhibits angiogenesis

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Abstract Dihydroartemisinin, a more water-soluble metabolite of artemisinin derivatives, is a safe and most effective antimalarial analog of artemisinin. In the present study, we investigated the antiangiogenic activity of dihydroartemisinin in vitro and in vivo, and investigated dihydroartemisinin-induced apoptosis in human umbilical vein endothelial cells (HUVEC). Dihydroartemisinin markedly reduced VEGF binding to its receptors on the surface of HUVEC. The expression levels of two major VEGF receptors, Flt-1 and KDR/flk-1, on HUVEC were lower following dihydroartemisinin treatment as shown by an immunocytochemical staining assay. The in vivo antiangiogenic activity was evaluated in the model of chicken chorioallantoic membrane (CAM) neovascularization. Dihydroartemisinin significantly inhibited CAM angiogenesis at low concentrations (5–30 nmol/100 μl per egg). We also investigated both qualitatively and quantitatively the induction of HUVEC apoptosis by dihydroartemisinin. A dose-related (5–80 μ*M*) and time-dependent (6–36 h) increase in dihydroartemisinin-induced HUVEC apoptosis was observed by flow cytometry. Our results suggest that the antiangiogenic effect induced by dihydroartemisinin might occur by induction of cellular apoptosis and inhibition of expression of VEGF receptors. These findings and the known low toxicity of dihydroartemisinin indicate that it might be a promising candidate angiogenesis inhibitor.

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Introduction

Angiogenesis is a process in which a network of new blood vessels is formed from pre-existing vessels. Highly regulated and transient angiogenesis plays an important role in embryogenesis, wound healing and corpus luteum formation. By contrast, uncontrolled and persistent angiogenesis is associated with diseases such as solid tumors, diabetes, rheumatoid arthritis, and atherosclerosis [1, 2]. In particular, the growth, intravasation and metastasis of tumors have been found to depend on tumor angiogenesis [3, 4]. Angiogenesis inhibitors would therefore be expected to be clinically useful for the treatment of those diseases related to angiogenesis, including tumors [5].

Angiogenesis can be divided into a series of temporally regulated responses, including protease induction, endothelial migration, proliferation and differentiation [6]. This is a highly complex process, in which a number of cytokines and growth factors released by endothelial cells (EC), tumor cells and matrix cells are involved. Vascular endothelial growth factor (VEGF) has been shown to be one of the most potent angiogenic factors. It binds to EC surface receptors and activates various functions of the cell including angiogenesis [7, 8, 9]. The major VEGF receptors expressed preferentially on EC are Flt-1 (fms-like tyrosine kinase) and KDR (kinase insert domain containing receptor)/Flk-1 [10, 11, 12]. Therefore, it has been suggested that inhibition of VEGF secretion or Flt-1 and KDR/Flk-1 expression may interrupt VEGF-induced angiogenesis. VEGF can be secreted by many human tumors and yet it is a growth factor specific for vascular EC. The secretion of VEGF in a variety of tumors suggests that the angiogenesis in tumors is VEGF-induced [13, 14]. This has

been demonstrated in several recent studies supporting the idea that inhibition of VEGF-induced angiogenesis may suppress tumor growth [5, 15].

Apoptosis is a highly ordered cell death process which involves the activation of a cell-intrinsic suicide program [16]. During apoptosis the nucleus and the cytoplasm condense to produce membrane-bound apoptotic bodies that are phagocytosed by macrophages or adjacent cells [17]. Apoptosis of EC is unique in several ways. First, the interaction between EC and extracellular matrix is exquisitely sensitive, whereby disruption of this interaction triggers apoptosis, and apoptosis also damages the interaction. Second, apoptotic EC are unlikely to be phagocytosed by adjacent cells; instead, because of the shear caused by blood flow, cells are more likely to detach from the vessel wall and embolize downstream where their debris can be processed [18, 19]. With these distinguishing features, apoptosis of EC is likely to represent a major physiological mechanism of EC death.

Artemisinin, the active principle of the Chinese herb *Artemisia annua*, and its derivatives form a promising class of antimalarial drugs, which are now commonly used in the treatment of chloroquine-resistant falciparum malaria [20, 21]. More effective analogs have been developed, among which the water-soluble dihydroartemisinin is the most effective [22, 23]. Recently, it has been reported that artemisinin analogs also show antitumor activity both in vitro and in vivo [24, 25, 26, 27, 28].

In a previous report, we have described the potent antiangiogenic effects of artemisinin derivatives as well as their antitumor effects in vitro [29]. In the study reported here, we confirmed and further investigated the effect of dihydroartemisinin on angiogenesis in vivo and in vitro. The in vitro antiangiogenic activity of dihydroartemisinin was assessed by immunocytochemical staining for VEGF receptors (Flt-1 and KDR/Flk-1) as well as VEGF binding to its receptors on the surface of human umbilical vein EC (HUVEC). The in vivo effects of dihydroartemisinin were investigated in an angiogenesis model of chorioallantoic membrane of the chick embryo (CAM). We also demonstrated that dihydroartemisinin significantly induced apoptosis in HUVEC, using TUNEL labeling, dual staining with acridine orange (AO) and ethidium bromide (EB), and activity quantification by flow-cytometric propidium iodide (PI) analysis.

Materials and methods

Materials

Dihydroartemisinin was purchased from Guiling Pharmaceutical Company (Guangxi, China). Dulbecco's modified Eagle's medium (DMEM) was supplied by Gibco BRL (Merelbeke, Belgium). DMSO, penicillin, streptomycin, MTT, VEGF, AO, EB and PI were obtained from Sigma (St. Louis. Mo.). Rabbit anti-human Flt-1 antibody and KDR/Flk-1 antibody were bought from Santa Cruz Biotechnology (Santa Cruz, Calif.). A TUNEL assay kit was obtained from Boehringer Mannheim (Mannheim, Germany).

Cell culture

HUVEC were obtained from the American Type Culture Collection (ATCC, Rockville, Md.) and were grown in DMEM with 10% fetal bovine serum, antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) and 10 ng/ml VEGF at 37°C in an incubator under an atmosphere containing 5% CO_2. HUVEC were used within ten passages.

CAM assay

Effect on in vivo angiogenesis was evaluated using the CAM vessel development assay with slight modifications as described previously [30]. Briefly, fresh fertilized eggs were incubated at 37°C (humidity 55–60%) for 4 days before a window was opened in the eggshell, exposing the CAM. The window was covered with Cellophane tape and the eggs were returned to the incubator. On day 9, sterile filter paper disks saturated with dihydroartemisinin or 0.1% DMSO were placed on the CAMs. The windows were then covered, and the eggs were further incubated at 37°C for 48 h. CAMs were carefully isolated and fixed in 95% alcohol. (No microvessel branches were broken in the removal of the disks and non-viable embryos were discarded.) CAM vessels in 15 viable embryos of each treatment group were counted under a light microscope (Olympus, Tokyo, Japan) and the neovascular zones of the CAM under the disks were photographed.

Cell proliferation assay

EC were seeded at a density of 1×10^4 cells in 1.0 ml HUVEC medium and were stimulated with 10 ng/ml VEGF in 24-well plates. After 24 h incubation at 37°C in an incubator under an atmosphere containing 5% CO_2, cells were exposed to graded concentrations of dihydroartemisinin for 6, 12, 24 or 36 h. The cells were then incubated with 5 mg/ml MTT [3-(4.5-dimethylthiazol-2-yl-2.5-diphenyl)tetrazolium bromide] solution for 4 h. Then 100 μl 10% sodium dodecyl sulfate solution was added to the culture. Absorbance at 570 nm was determined using an ELISA reader (BioTek Instruments, Winooski, Vt.). By the MTT method, cell numbers were obtained in terms of absorbance values.

VEGF receptor binding assay

Levels of bound VEGF protein were measured in cell lysates of HUVEC using an ELISA kit (R&D Systems, Minneapolis, Minn.).This assay was sensitive to 9 pg/ml (0.2 p*M*) VEGF and did not crossreact with other homologous cytokines.

Briefly, HUVEC were seeded at a density of 1×10^5 cells/well into a 24-well culture plate in 10% FBS/DMEM with 10 ng/ml VEGF and incubated for 24 h allowing them to attach to the well bottom completely. After removal of culture medium, the HUVEC monolayers were rinsed twice with DMEM and incubated in 10% FBS/DMEM containing no VEGF with or without dihydroartemisinin for 24 h. After washing with DMEM, the HUVEC monolayers were treated with DMEM containing 20 ng/ml VEGF at 4°C for 1 h. Then unbound VEGF was removed by washing the cells four times with phosphate-buffered saline (PBS). Cell lysates were obtained by treatment with PBS containing 0.5% Triton X-100 and were centrifuged at 10,000 *g* for 10 min. The VEGF content of the supernatants was determined using a quantitative sandwich enzyme immunoassay technique, according to the manufacturer's instructions. The monoclonal antibody to human $VEGF_{165}$ was pre-added onto microtiter plates, and 200 μl of treated or control supernatant was added and incubated. Then polyclonal anti-VEGF antibody was added. VEGF absorbance values were determined using an ELISA reader (BioTek Instruments) and the

VEGF content of the samples was estimated from a standard curve determined from serially diluted VEGF standards. Two individual cell passages were analyzed in triplicate.

Immunocytochemical assay of VEGF receptors

The expression levels of the two VEGF receptors, Flt-1 and KDR/Flk-1, in HUVEC were determined using immunocytochemistry and image analysis [31]. Briefly, sterile glass slides were put into 75-mm dishes and then HUVEC suspended in 10% FBS/DMEM with 10 ng/ml VEGF were seeded at a density of 1×10^4/ml into the dishes containing the slides. The cells were allowed to grow into confluent monolayers on the slides by incubation at 37°C in an incubator under an atmosphere containing 5% CO_2. Subsequently, HUVEC were incubated for a further 24 h with or without dihydroartemisinin and the slides were taken out of the dishes, washed carefully with PBS, and fixed in 95% alcohol. Immunocytochemical staining for Flt-1 and KDR/Flk-1 was performed with anti-Flt-1 antibody and anti-KDR/Flk-1 antibody, respectively. Antibodies were used at a dilution of 1:100 with overnight incubation at 4°C. A three-step immunoperoxidase method using an LSAB kit (DAKO, Carpinteria, Calif.) was carried out according to the instructions provided by the manufacturer to detect immunostained cells. A negative control was performed by substituting PBS for the primary antibody. Then slides were lightly counterstained with hematoxylin (DAKO). An Olympus B×50 microscope was used to examine the slides, and the intensity and area of Flt-1 or KDR/Flk-1 staining were analyzed using an image analyzer (Q550 IW; Leica, Wetzler, Germany). The protein levels were quantified in terms of integral absorbance (IA) values.

Apoptosis assessment

Morphological assessment of apoptosis

For dihydroartemisinin-induced apoptosis, HUVEC at 5×10^4 cells/well were incubated with dihydroartemisinin at various concentrations (0–80 μM). All cell suspensions were plated in triplicate in 24-well plates. After 24 and 36 h of incubation, a mixture of EB (100 μg/ml) and AO (100 μg/ml) was added according to a modification of the protocol described by Cotter and Martin [32]. Since the emission spectra of these two dyes are different, they could be used simultaneously in the same cells. Briefly, 60 μl of the EB/AO cocktail mixed with 1 ml PBS was added to every well, and the plates were incubated at room temperature in the dark for 3–5 min. Fields of stained cells were selected and focused under a fluorescence microscope (Nikon Eclipse E800). The apoptotic cells were graded qualitatively by their staining characteristics as previously described [32] and outlined as follows:

1. Viable: bright green nucleus with intact structure
2. Early apoptosis: bright green nucleus showing condensation of chromatin as dense green areas in the nucleus
3. Late apoptosis: orange nucleus showing condensation of chromatin as dense orange areas, and reduced cell size

Detection of apoptosis by TUNEL

The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP biotin nick end-labeling (TUNEL) assay characterizes an important biochemical feature of apoptosis—DNA fragmentation [33]. To confirm that dihydroartemisinin-treated HUVEC were undergoing apoptosis, HUVEC adherent to the coverslip were stained using a TUNEL assay kit according to the manufacturer's instructions. A negative control, without the addition of TdT enzyme, was included in each experiment. Finally, the cells were slightly counterstained with hematoxylin. Analysis was performed by light microscopy at magnifications of ×1000 and ×2000.

Quantification of apoptosis by flow cytometry

Apoptotic cells can be distinguished from necrotic cells and quantified using flow cytometric analysis of the cellular DNA [34]. The apoptotic cell population was identified by the DNA-specific fluorochrome PI. Briefly, Detached cells were combined with adherent cells after lifting with trypsin/EDTA, centrifuged at 1000 *g* and washed with ice-cold PBS. The resultant cell pellet was fixed with cold 70% ethanol, centrifuged and then 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 FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, N.J.).

Data analysis

The results are expressed as means ± SD and the significance of differences between two groups were assessed by Student's *t*-test. *P* values less than 0.05 were considered to be statistically significant.

Results

Dihydroartemisinin suppresses CAM angiogenesis

The CAM assay in the chick embryo is perhaps the most widely used vessel development assay in vivo. It is particularly suitable for the initial screening of potential inhibitors of angiogenesis in a living organism because it is simple and quick, and vessels grow spontaneously, with no need for the addition of external growth factors. New vessel growth was significantly suppressed by treatment with dihydroartemisinin (5–30 nmol/100 μl per egg) in a dose-dependent manner (Fig. 1), while the control CAM showed a well-developed neovascular network. Dihydroartemisinin at 1 nmol/100 μl per egg exerted no inhibitory effect on angiogenesis of the CAM.

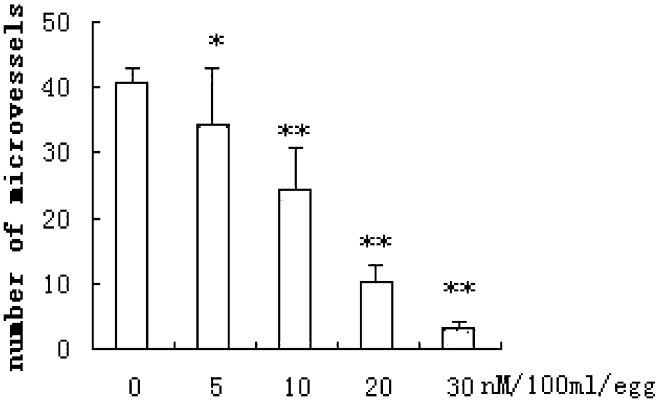


Fig. 1 Inhibition of angiogenesis by dihydroartemisinin in the CAM. Disks containing dihydroartemisinin were placed on selected areas of the CAM on day 9 and the eggs were incubated at 37°C for 48 h. One control and one drug-containing disk were placed on every CAM. The microvessel numbers under the disks were counted under a light microscope (*$P < 0.05$, **$P < 0.01$)

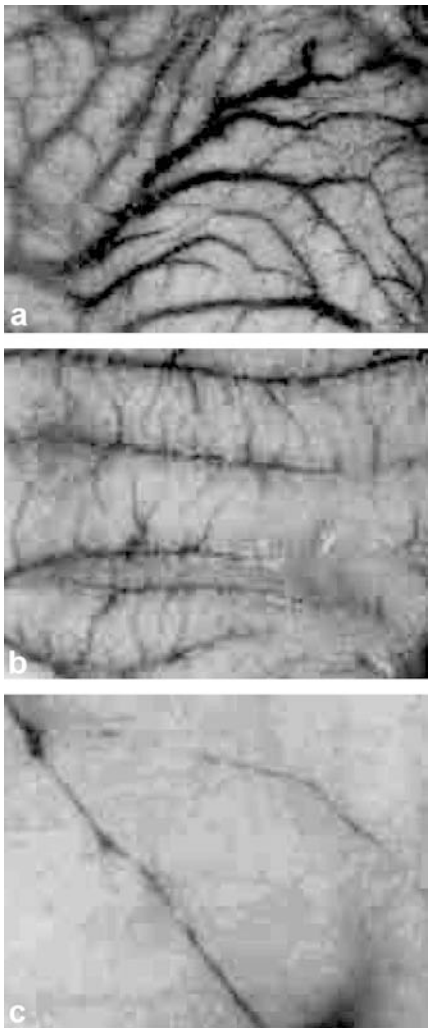


Fig. 2a–c Effect of dihydroartemisinin on CAM angiogenesis. Membranes were treated with (**a**) 0.1% DMSO (control), (**b**) 15 nmol/100 μl per egg dihydroartemisinin, and (**c**) 30 nmol/100 μl per egg dihydroartemisinin

It should be noted that the evaluation method used did not distinguish between newly formed microvessels (after addition of dihydroartemisinin to the CAM) and those already present on day 9. Therefore, 100% reduction of vascular density was not attainable. In fact, no newly formed microvessels could be detected on the CAM under the filter saturated with dihydroartemisinin at 30 nmol/100 μl per egg and the reductions with 30 nmol/100 μl per egg can be considered complete inhibition of neovascularization (Fig. 2).

Dihydroartemisinin inhibits cell proliferation

The presence of dihydroartemisinin at concentrations more than 5 μ*M* for 6, 12, 24 and 36 h inhibited the growth of HUVEC in a concentration- and time-dependent manner as shown in the MTT assay (Fig. 3). The lowest concentration used in this study (5 μ*M*) had

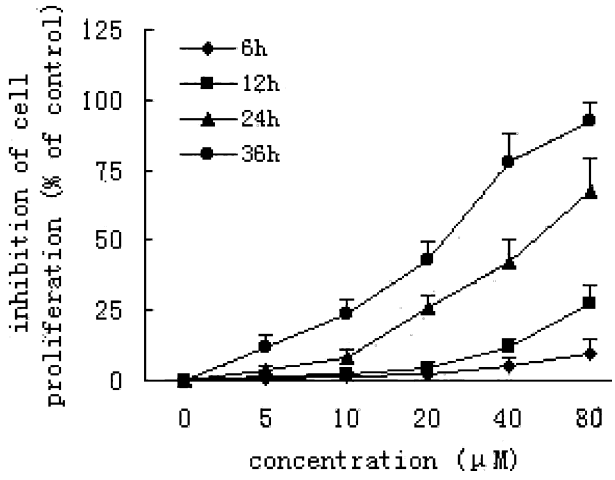


Fig. 3 Inhibition of VEGF-induced HUVEC growth by dihydroartemisinin. Cells were seeded at a density of 1×10^4 cells/well in 24-well plates and were stimulated with VEGF. After 24 h, cells were treated with dihydroartemisinin at various concentrations for 6, 12, 24 or 36 h. Then the numbers of cells were determined by the MTT assay. The results are expressed as a percent of control. Error bars represent SD ($P<0.01$ ANOVA)

no effect on cell growth after 6 and 12 h treatment ($P>0.05$). Dihydroartemisinin at 10, 20, 40 and 80 μ*M* inhibited the growth of cells at 24 h by 8.4%, 25.6%, 41.8% and 67.5%, respectively, and at 36 h by 23.3%, 42.6%, 77.4% and 92.1%, respectively. In the time-course study, significant inhibition of the growth of HUVEC treated with dihydroartemisinin was observed after 6 h (at 80 μ*M*),12 h (at 40 μ*M*) and 24 h (at 10 μ*M*) treatment. Moreover, growth inhibition of the HUVEC treated with these five concentrations of dihydroartemisinin increased further with increasing incubation time.

Dihydroartemisinin inhibits VEGF binding to its receptors

Whether dihydroartemisinin could inhibit VEGF binding to its cell surface receptors was determined. The binding assay was performed with various concentrations of dihydroartemisinin up to 5 μ*M*. Dihydroartemisinin at concentrations as low as 0.1 μ*M* significantly reduced VEGF binding ($P<0.05$). Treatment with 0.5 μ*M* dihydroartemisinin reduced VEGF binding by 40% (Table 1).

Dihydroartemisinin suppresses VEGF receptor expression

By immunostaining, Flt-1-positive or KDR/Flk-1-positive HUVEC could be distinguished by the brown-stained cytoplasm. Most control HUVEC showed strong expression of Flt-1 and KDR/Flk-1, but the expression in experimentally treated HUVEC was

Table 1 Inhibitory effect of dihydroartemisinin on VEGF receptor binding on HUVEC. After incubation in 10% FBS/DMEM containing no VEGF with dihydroartemisinin for 24 h, HUVEC were treated with DMEM containing 20 ng/ml VEGF for 1 h. Then unbound VEGF was removed by washing with PBS and bound VEGF was obtained by cell lysis. Concentrations of bound VEGF were determined using an enzyme-linked immunosorbent assay. The data are expressed as the means ± SD ($n = 3$) of bound VEGF concentrations

	Dihydroartemisinin (μM)			
	0	0.1	0.5	5
Bound VEGF (pg/ml per 10^5 cells)	45 ± 7	39 ± 11*	27 ± 9**	22 ± 8**

*$P < 0.05$, **$P < 0.01$

weaker (Fig. 5). There were significant differences between the control HUVEC and HUVEC treated with both concentrations of dihydroartemisinin ($P < 0.05$). The Flt-1 protein levels in the cells treated with dihydroartemisinin at 0.05 and 0.1 μM were reduced by 16% and 39%, respectively, compared with the control cells. The KDR/Flk-1 levels in the cells treated with dihydroartemisinin at 0.05 and 0.1 μM were reduced by 32% and 71%, respectively (Fig. 4). The results suggest that dihydroartemisinin suppressed the expression of both Flt-1 and KDR/Flk-1.

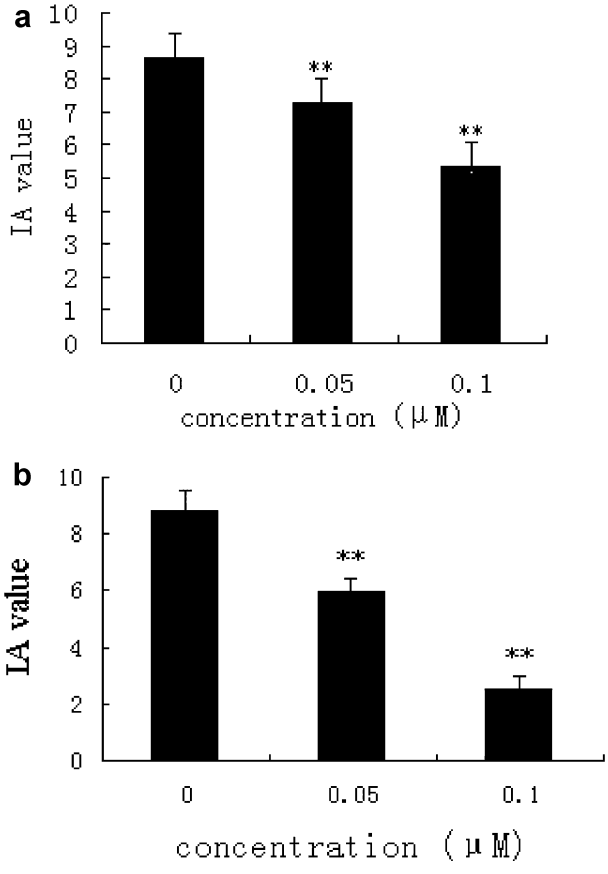


Fig. 4A, B Inhibitory effects of dihydroartemisinin on the expression of Flt-1 (**A**) and KDR/Flk-1 (**B**). The expression levels of Flt-1 and KDR/Flk-1 in HUVEC were determined by immunocytochemistry and image analysis. The protein levels are expressed as integral absorbance (IA) values. *$P < 0.05$, **$P < 0.01$, compared to control

Dihydroartemisinin induces HUVEC apoptosis

Based on the results of the growth inhibition and cytotoxicity studies, further studies were done to explore and quantitate dihydroartemisinin-induced apoptosis in HUVEC.

Fluorescence microscopy

Figure 6 shows comparative morphological alterations of apoptosis in HUVEC treated with dihydroartemisinin for 24 h. Dual staining of HUVEC with AO/EB dyes discriminated between early (AO_{low}/EB^-, green) and late apoptotic cells (AO_{low}/EB^+, orange) according to their membrane integrity. Cells with normal morphology are shown in Fig. 6A. When treated with 20–80 μM dihydroartemisinin for 24 h, cell shrinkage, membrane blebbing, chromatin condensation, and formation of apoptotic bodies were seen (Fig. 6B, C), all of which are morphological changes characteristic of apoptosis.

TUNEL assay

To confirm the reliability of the fluorescence dye analysis, the experiment was repeated using the TUNEL assay. The fluorescence dye staining data correlated significantly with those of the TUNEL assay, which detects fragmented DNA. There were very few apoptotic cells among the control cells detected by the TUNEL method. Dihydroartemisinin treatment induced a significant increase in apoptotic cells as shown in Fig. 6. HUVEC started to show positive TUNEL staining after treatment with dihydroartemisinin at 40 μM for 12 h. These cells showed more condensed nuclei than adjacent TUNEL-negative cells. Almost all of the cells treated with 80 μM dihydroartemisinin for 36 h were TUNEL-positive, and some had become detached from the glass coverslip.

Quantification by flow cytometry

The dose-related (5–80 μM) and time-dependent (6–36 h) increase in dihydroartemisinin-induced HUVEC apoptosis was initially analyzed using flow cytometry (Fig. 7). A significant, time-dependent increase in HUVEC apoptosis was noted in the first 12 h after treatment with dihydroartemisinin at 80 μM ($P < 0.01$; Fig. 7D), and the proportion of apoptotic HUVEC after treatment with dihydroartemisinin at 80 μM for 36 h was up to 85%. Further, dose-related increases in HUVEC apoptosis were demonstrated after dihydroartemisinin treatment for 24 h and 36 h.

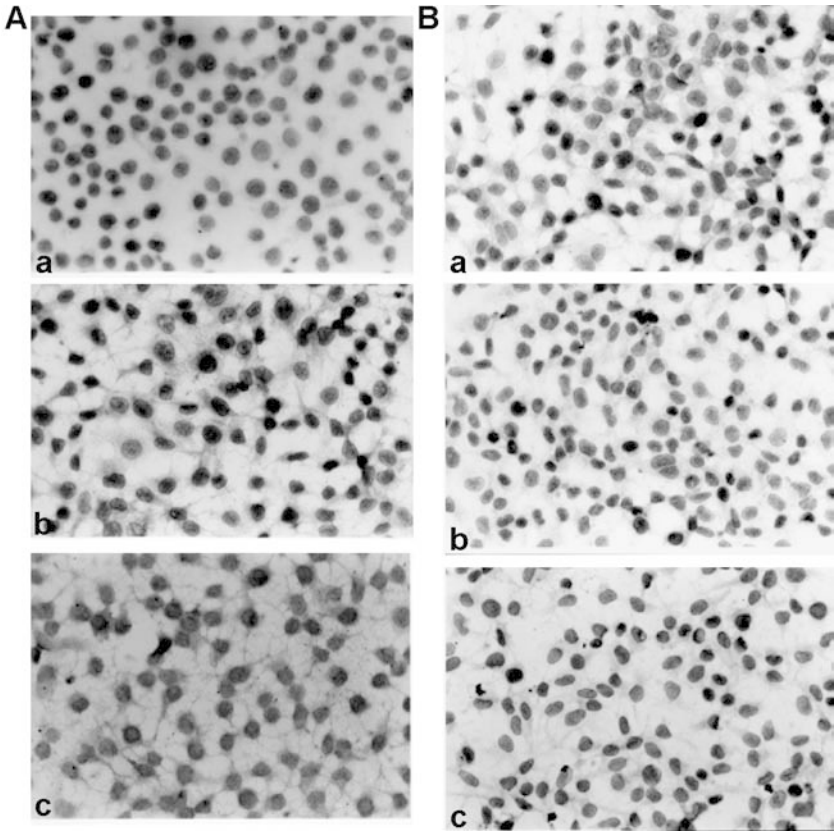


Fig. 5A, B Immunostaining for Flt-1 and KDR/flk-1. Microscopic morphology (×400) of HUVEC treated as for the results described in Fig. 4. **A** Effect of dihydroartemisinin on Flt-1 expression: *a* negative control, *b* control, *c* 0.1 μ*M* dihydroartemisinin. **B** Effect of dihydroartemisinin on KDR/Flk-1 expression: *a* control, *b* 0.05 μ*M* dihydroartemisinin, *c* 0.1 μ*M* dihydroartemisinin

Following control treatment, the proportions of PI-positive HUVEC were 4.3 ± 0.6% and 4.7 ± 1.3% at 24 and 36 h, respectively, and following treatment with dihydroartemisinin at 10, 20, 40 and 80 μ*M* the proportions were 11.5 ± 2.1%, 24.3 ± 5.0%, 38.4 ± 7.2% and 64.8 ± 13.6%, respectively, at 24 h, and 22.0 ± 4.6%, 35.3 ± 8.7%, 60.9 ± 10.8% and 84.8 ± 12.6%, respectively, at 36 h ($P < 0.01$; Fig. 7D).

Moreover, we also found that the dihydroartemisinin-induced apoptosis of HUVEC occurred in the S phase of the cell cycle (the cell-cycle profile was determined using program M software on the flow cytometer) which is partly shown in Fig. 7E, F. After dihydroartemisinin treatment, the percentage of cells in S phase was decreased while the percentage of cells in the other phases did not change significantly. The results indicate that almost all apoptotic cells were in S phase.

Discussion

Artemisinin is a sesquiterpene lactone peroxide containing an endoperoxide moiety which forms free radicals when induced by iron. The antimalarial action of artemisinin is due to its reaction with intraparasitic heme iron, generating free radicals which lead to cell death [35, 36]. Iron plays a vital role in cell growth (i.e. energy metabolism and DNA synthesis). Due to their high rates of division, rapidly growing cells such as most cancer cells and perhaps activated EC have a high iron intake, which contributes to the selective toxicity of artemisinin in rapidly growing cells [37, 38]. Many studies have utilized this property of artemisinin analogs and targeted them towards cancer cells. It has previously been shown by us and others that the antimalarial artemisinin derivatives such as artesunate and dihydroartemisinin also possess antineoplastic activity [27, 29, 39, 40]. We also initially reported the potent antiangiogenic activity of artemisinin derivatives in vitro, by demonstrating it in in vitro models of angiogenesis: proliferation, migration and tube formation in cultured HUVEC [29].

In the present investigation, we tried to confirm the antiangiogenic activity of dihydroartemisinin in vivo as well as its effect on the expression of the major VEGF receptors Flt-1 and KDR/Flk-1. We evaluated the in vivo antiangiogenic effect of dihydroartemisinin in the CAM model of neovascularization. Dihydroartemisinin started to inhibit angiogenesis at doses as low as 5 nmol/100 μl per egg and completely inhibited it at a dose of 30 nmol/100 μl per egg, which indicates that dihydroartemisinin also has antiangiogenic potential in vivo.

VEGF is considered to be a specific and fundamental regulator of EC growth and differentiation [9, 41]. VEGF-induced angiogenesis is mediated mainly by two VEGF receptors, Flt-1 and KDR/Flk-1. Therefore, we examined whether dihydroartemisinin could inhibit VEGF receptor binding and VEGF receptor expression in HUVEC. The results of the VEGF binding assay and

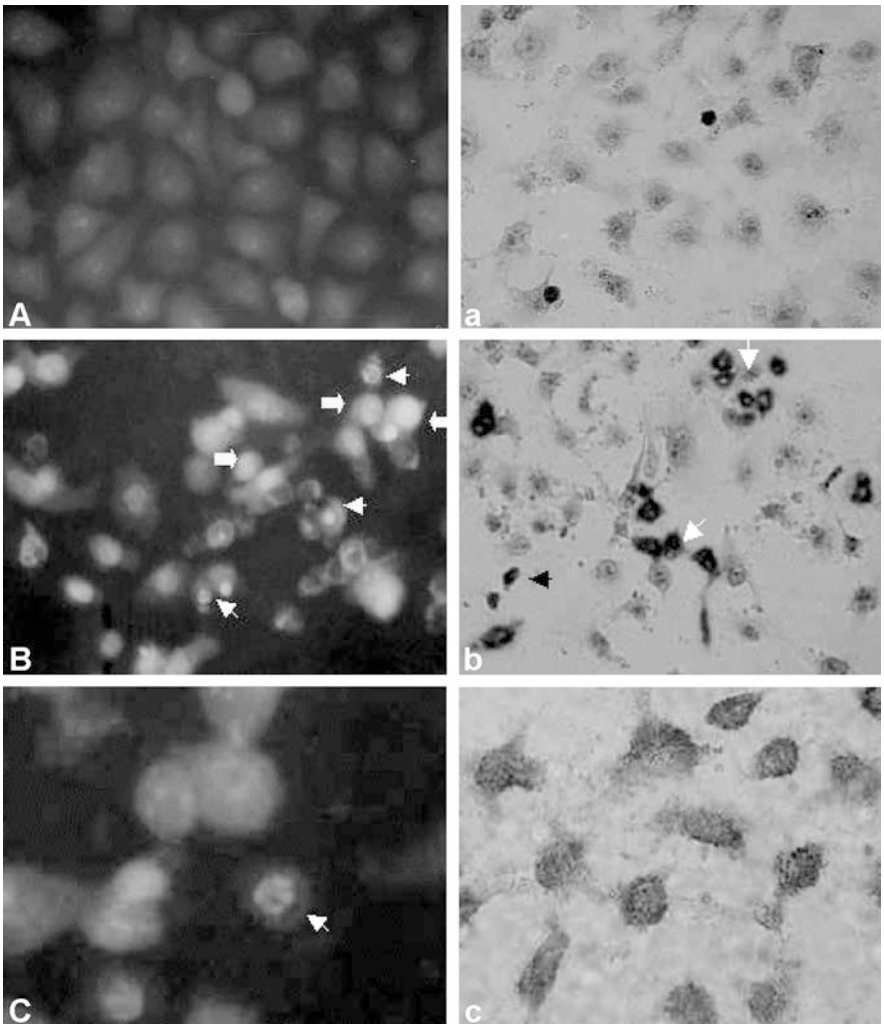
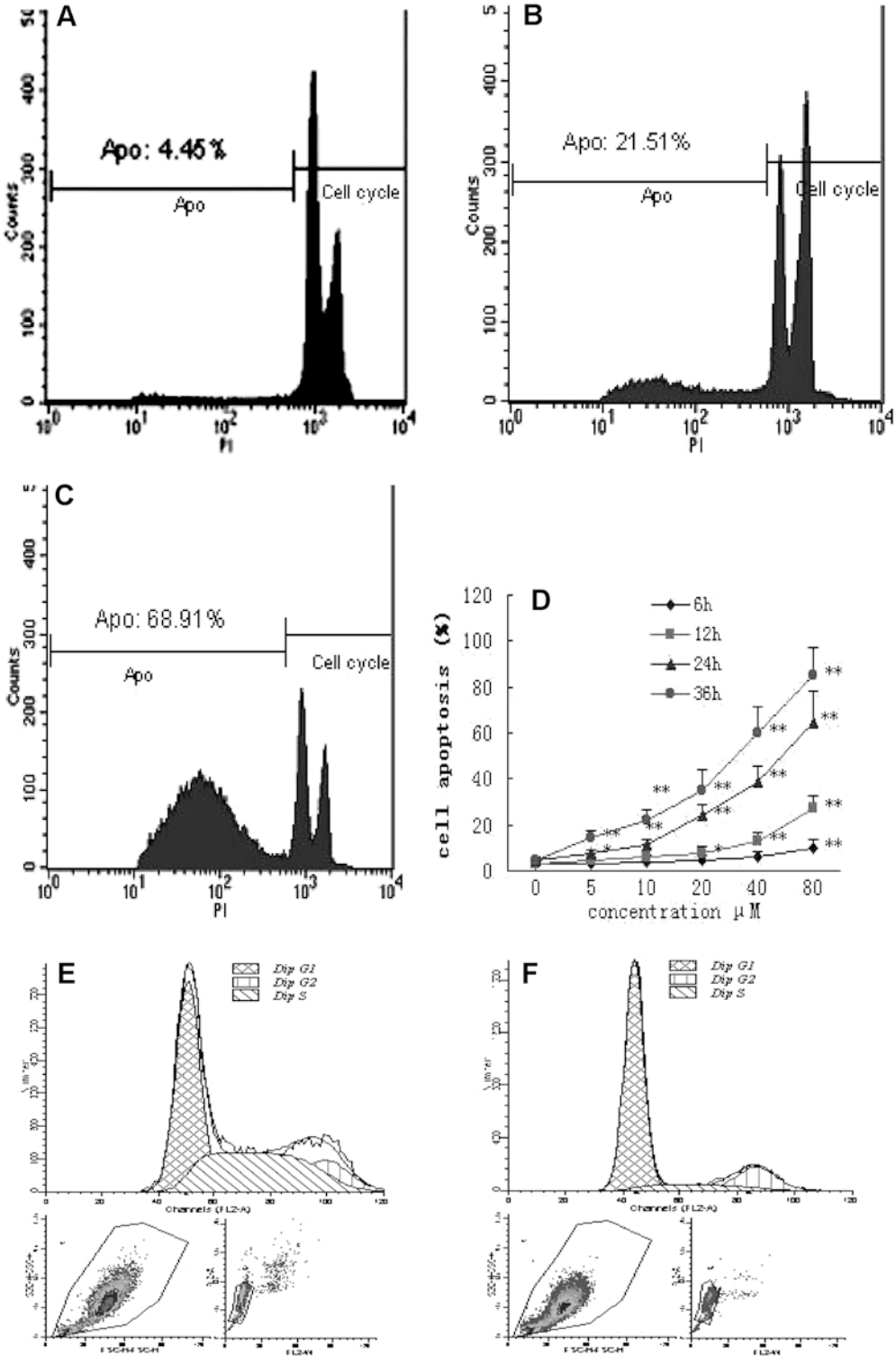


Fig. 6A–C Detection of dihydroartemisinin-induced apoptosis in HUVEC. **A, B, C** Cells at 5×10^4 cells/well were incubated with dihydroartemisinin at various concentrations in 24-well plates for 24 and 36 h. The cells were then stained with AO/EB and their morphology immediately assessed using florescence microscopy. **A** Control: viable cells with green nuclei and intact structure. **B** Dihydroartemisinin at 40 μM for 36 h: *thick arrows* early apoptotic cells with bright green nuclei showing condensation of chromatin as dense green areas in the nucleus, *thin arrows* late apoptotic cells with orange nucleus showing condensation of chromatin as dense orange areas and reduced cell size. **C** Typical apoptotic changes such as cell membrane blebbing, chromatin condensation, and formation of apoptotic bodies were observed in dihydroartemisinin-treated cells (*thin arrows*). **a, b, c** Dihydroartemisinin-treated HUVEC adherent to the coverslips were stained by TUNEL according to the manufacturer's instructions. Fragmental DNA, developed in a peroxidase substrate system, is labeled brown, and all nuclei were counterstained with hematoxylin. Analysis was performed by light microscopy at a magnification of ×1000 and ×2000. **a** Control: TUNEL-positive cells not detected; no evidence of DNA fragmentation. **b** Dihydroartemisinin at 40 μM for 24 h: relative to the control group, the number of TUNEL-positive cells (*thin arrows*) is significantly increased. **c** Dihydroartemisinin at 80 μM for 36 h: almost all the treated cells are TUNEL-positive

immunohistochemical staining of its two receptors suggested that dihydroartemisinin suppressed VEGF binding to its receptors and Flt-1 and KDR/Flk-1 expression on HUVEC at a concentration of 0.1 μM. However, HUVEC growth was not inhibited at this concentration. So inhibiting Flt-1 and KDR/Flk-1 levels might be one mechanism by which dihydroartemisinin exerts its antiangiogenic activity.

In conclusion, dihydroartemisinin might inhibit angiogenesis in vitro and in vivo by inhibiting EC proliferation and differentiation [29] or by suppression of VEGF receptor expression and VEGF binding to its receptors. It is known that dihydroartemisinin is the major metabolite of artemisinin in humans. This together with our results indicates that other artemisinin derivatives might be active, and indeed more active, against angiogenesis after metabolization.

In the present study, we also demonstrated that dihydroartemisinin induces apoptosis in HUVEC based on the following observations: (1) distinct cell morphological alterations including cell shrinkage, cell membrane blebbing, chromatin condensation and formation of apoptotic bodies; (2) DNA fragmentation as determined by the TUNEL assay; and (3) dose- and time-dependent increases in PI-positive cells as determined by flow cytometry. Moreover, comparing the results of the quantification of HUVEC apoptosis with those of



dihydroartemisinin-induced inhibition of HUVEC proliferation, the percentage of dead cells was not significantly larger than that of apoptotic cells indicating that the cytotoxic effect of dihydroartemisinin in HUVEC is a consequence of its ability to induce apoptosis, not necrosis.

Angiogenesis is dependent on the stimulation of EC proliferation. Cell proliferation is a fundamental process common to both angiogenesis and tumor development. Both CAM neovascularization and HUVEC angiogenesis require rapid cell division controlled by growth factors/cytokines such as VEGF and bFGF. VEGF was

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Fig. 7A–F Flow cytometric analysis of DNA content in control and dihydroartemisinin-treated HUVEC. Cells were incubated with or without dihydroartemisinin for 6, 12, 24 or 36 h and analyzed after the addition of PI solution for staining (**A** control cells, **B** cells treated with 20 μM dihydroartemisinin for 24 h, **C** cells treated with 80 μM dihydroartemisinin for 24 h). **D** Percentages of apoptotic cells among HUVEC treated with dihydroartemisinin at various concentrations ($^{*}P < 0.05$, $^{**}P < 0.01$, ANOVA, $n = 3$). Cell cycle profiles were calculated from the number of nuclei in each cell cycle phase using the multicycle software and are expressed as a percentage in relation to the total number of cells. Compared with control cells (**E**), cells treated with dihydroartemisinin (**F**) show a significant decrease in the percentage of nuclei in S phase

first shown to be a survival factor for retinal EC [42]. A fundamental cellular mechanism by which VEGF promotes the formation of new blood vessels and maintains their integrity is the activation of EC survival or antiapoptotic signaling, and the inhibitory effects of VEGF on EC apoptosis are probably mediated through upregulation of components of the antiapoptotic cellular machinery [43, 44]. In this study we showed that dihydroartemisinin inhibits angiogenesis via an apoptotic mechanism, and induces apoptosis of HUVEC partly by inhibiting VEGF binding and lowering the expression of Flt-1 and Flk-1. These apoptotic effects of dihydroartemisinin on proliferating cells indicate that it may possess great potential as an anticancer drug as a result of both its cytotoxic action on tumor cells and its antiangiogenic effects.

It has been reported [26] that the cytotoxic activity of artemisinin derivatives is associated with the production of reactive oxygen species and artesunate carbon-centered free radicals both of which affect cellular proteins and lipids of the parasites, and with the alteration of basal mRNA expression of different gene classes such as cell cycle-regulating genes, growth factors and their receptors, oncogenes, and tumor suppressor genes. However, the mechanism of artemisinin derivative-induced apoptosis is not understood completely and needs further study.

Its antiangiogenic activity together with its known low toxicity [20, 22, 45] open the possibility that dihydroartemisinin might be a candidate angiogenesis inhibitor.

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