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ORIGINAL ARTICLE

Dihydroartemisinin inhibits angiogenesis in pancreatic cancer by targeting the NF-κB pathway

Shuang-Jia Wang · Bei Sun · Zhuo-Xin Cheng · Hao-Xin Zhou · Yue Gao · Rui Kong · Hua Chen · Hong-Chi Jiang · Shang-Ha Pan · Dong-Bo Xue · Xue-Wei Bai

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Abstract
Purpose Dihydroartemisinin (DHA) has recently shown antitumor activity in human pancreatic cancer cells. However, its effect on antiangiogenic activity in pancreatic cancer is unknown, and the mechanism is unclear. This study was aimed to investigate whether DHA would inhibit angiogenesis in human pancreatic cancer.
Methods Cell viability and proliferation, tube formation of human umbilical vein endothelial cells (HUVECs), nuclear factor (NF)-κB DNA-binding activity, expressions of vascular endothelial growth factor (VEGF), interleukin (IL)-8, cyclooxygenase (COX)-2, and matrix metalloproteinase (MMP)-9 were examined in vitro. The effect of DHA on antiangiogenic activity in pancreatic cancer was also assessed using BxPC-3 xenografts subcutaneously established in BALB/c nude mice.
Results DHA inhibited cell proliferation and tube formation of HUVECs in a time- and dose-dependent manner and also reduced cell viability in pancreatic cancer cells. DHA significantly inhibited NF-κB DNA-binding activity, so as to tremendously decrease the expression of NF-κB-targeted proangiogenic gene products: VEGF, IL-8, COX-2, and MMP-9 in vitro. In vivo studies, DHA remarkably reduced tumor volume, decreased microvessel density, and downregulated the expression of NF-κB-related proangiogenic gene products.
Conclusions Inhibition of NF-κB activation is one of the mechanisms that DHA inhibits angiogenesis in human pancreatic cancer. We also suggest that DHA could be developed as a novel agent against pancreatic cancer.

Keywords Pancreatic cancer · Dihydroartemisinin · Antiangiogenesis · Nuclear factor-κB

S.-J. Wang · B. Sun (✉) · Z.-X. Cheng · H.-X. Zhou · Y. Gao · R. Kong · H. Chen · H.-C. Jiang · S.-H. Pan · D.-B. Xue · X.-W. Bai
Department of Pancreatic and Biliary Surgery,
The First Affiliated Hospital of Harbin Medical University,
Harbin, 23 Youzheng Str, Nangang, Harbin,
People's Republic of China
e-mail: sunbei70@tom.com

S.-J. Wang
Department of Hepato-Biliary-Pancreatic and Vascular Surgery,
The First Affiliated Hospital of Xiamen University,
Xiamen, People's Republic of China

Y. Gao
Department of Surgery, University Hospitals,
Case Western Reserve University, Cleveland, OH 44106, USA

Introduction

Pancreatic cancer is currently the fourth most common cause for cancer death in western countries [1], and its incidence also trends up in China [2]. It is often detected at an advanced stage, which has a very poor prognosis, with a 5-year survival rate of less than 5% [3]. This could be due to the lack of early diagnosis, the highly aggressive behavior of the tumor and high resistance to chemotherapy and radiation. Therefore, effective therapeutic strategies are required to improve the prognosis of patients with pancreatic cancer.

Artemisinin, an effective antimalarial drug, is isolated from the traditional Chinese herb *Artemisia annua*. Dihydroartemisinin (DHA), the main active metabolite of artemisinin derivatives, has exhibited the strongest anticancer effects among the derivatives of artemisinin [4, 5]. Many studies as well as our recent studies have shown that DHA inhibits cell proliferation, induces cell cycle arrest, and promotes apoptosis in a wide variety of human cancer cell

lines [6–10]. Recently, a study has demonstrated that DHA has an antiangiogenic activity on in vitro and in vivo models of angiogenesis via down-regulating VEGF [11]. Moreover, DHA has also been shown to inhibit hypoxia inducible factor (HIF)-1α activation and down-regulate the expression of IL-8, MMP-9, angiogenin, fibroblast growth factor-2, and all of which are closely linked with angiogenesis [12–17].

NF-κB is constitutively activated in about 70% human pancreatic cancer cells and primary tumor specimens [18, 19]. NF-κB is closely involved in the progression of pancreatic cancer as it promotes the downstream expression of multiple genes involved in proliferation, angiogenesis, invasion, metastasis, and suppression of apoptosis [20, 21]. A critical role of NF-κB in carcinogenesis is in angiogenesis; however, the steps involved in angiogenesis (degradation of the vascular basement membrane, endothelial cell proliferation, and capillary tube formation) depend on the production of angiogenic factors by both tumor and stromal cells [22]. NF-κB seems to play a key role in regulating the expression of multiple angiogenic factors, such as VEGF, IL-8, COX-2, and MMP-9[21]. Therefore, targeting the NF-κB pathway seems to have the effect of antiangiogenic therapy on pancreatic and other cancers. Our recent reports demonstrated that DHA exerts inhibitory effect on NF-κB [7, 8]; however, little is known about its antiangiogenesis effects on pancreatic cancers. Thus, in this study, both in vitro and in vivo experiments were conducted to demonstrate whether, via inhibition of NF-κB and its downstream proangiogenic gene products, DHA would inhibit angiogenesis in pancreatic cancer.

Materials and methods

Materials

DHA (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in DMSO (Sigma) as a 100 mM stock solution and stored at 4°C. An electrophoretic mobility shift assay (EMSA) kit was purchased from Viagene Biotech Co. (Viagene, China). The antibodies against NF-κB/P65, VEGF, IL-8, COX-2, MMP-9, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). The anti-CD31 antibody was purchased from Lab Vision Co., (Thermo Scientific, CA, USA). 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), fibrinogen, and thrombin were purchased from Sigma–Aldrich, Inc.

Cell culture

The human pancreatic cancer cell lines BxPC-3 and PANC-1 were obtained from the American Type Culture Collection (Rockville, Maryland, USA). Both cell lines were maintained in RPMI 1640 medium (Gibco, NY, USA), supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml). Human umbilical vein endothelial cells (HUVECs) were supplied by the Typical Animal Reserve Center of China. HUVECs were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 mg/ml), and 10 ng/ml VEGF (Sigma). All cells were incubated in a 37°C incubator with 5% CO_2.

Detection of cell proliferation

Cell proliferations were measured using the MTT assay and crystal violet assay. (a) MTT assay: Cells were seeded in 200 µl of RPMI1640 into 96-well plates and cultured overnight before certain concentrations of DHA (0–200 µM) were added and incubated. Cells treated with only DMSO were used as untreated control. After further incubation for designated time (24, 48, or 72 h), cells were washed before incubated with MTT at 37°C for 4 h. The medium was discarded, and DMSO was added into each well at room temperature for 20 min, before absorbance was measured at 490 nm on a plate reader. The cell proliferation index was calculated according to the formula: experimental OD value/control OD value × 100%. Experiment was repeated thrice. All cell lines underwent the same procedure. (b) Crystal violet assay: HUVECs (1×10^5/well) were seeded into 6-well plates and cultured overnight. Then the medium was replaced with complete culture medium containing DHA (25, 50 or 100 µM) for an additional 7 days. Cells were then washed twice with pre-warmed PBS, and then cells remaining were stained for 1 h with a crystal violet solution (0.5% crystal violet, 20% methanol). Plates were washed three times and left to dry at 37°, before the images were photographed.

In vitro angiogenesis (tubular formation) assay

Tube formation assay was performed as described previously with modification [23]. A 24-well plate was coated with 250 µl fibrinogen solution (1.0 mg/ml) and 10 µl thrombin solution (12.5 U/ml) per well, which was placed at 37°C for 60 min to form a layer of fibrin gel. HUVECs (5×10^4/well) were then seeded in 500 µl RPMI 1640 into 24-well plates. After 12 h, the culture medium was removed and then another layer of fibrin gel was added to each well. After polymerization, 500 µl complete culture medium containing DHA (12.5, 25, 50, or 100 µM) was added in the presence of 10 ng/ml VEGF. Or 6 h after transfection with pHK and psiVEGF, HUVECs were transferred on the fibrin gel on 24-well plates. Plates were then

incubated for 24 h. The images were photographed using an inverted phase contrast microscope (IX71, Olympus), and five random fields were counted for each well at a ×100 magnification. The experiment was repeated thrice.

Construction of siRNA expressing plasmid

A plasmid-based siRNA expression system containing VEGF siRNAs was constructed. The targeted sequence of human VEGF: 5′-ACC UCA CCA AGG CCA GCA C-3′ was designed. For target sequence, a pair of sense and anti-sense strands was designed; their respective complementary chains were then synthesized by annealing. The siRNA duplexes thus obtained were subcloned into the pEGFP vector, and the plasmids targeting VEGF were constructed, pEGFP-siVEGF. The control sequence which was named HK: 5′-TTC TCC GAA CGT GTC ACG T-3′ had no homology to any mammalian sequence. The plasmids were constructed by GenePharma Co., Ltd., Shanghai, China. HUVECs, BxPC-3, and PANC-1 cells were grown to 50% confluence in 6-well or 96-well plates; the plasmid DNA (pEGFP-siVEGF or pEGFP-siHK) and Lipofectamine™ 2000 (Invitrogen) diluted in serum-free medium without antibiotic were mixed, and the complex was added to the cell cultures. Growth medium was used as the control agent. The cells and the supernatants were harvested 48 h after transfection for Western blot analysis. Silencing of protein expression was confirmed by Western blot analysis. All the transfections were performed in triplicate.

Electrophoretic mobility shift assay (EMSA)

After treatment with DHA (12.5, 25 or 50 μM) for 72 h, BxPC-3 and PANC-1 cells were harvested. Nuclear extract, prepared according to a method described previously [8], was incubated with 1 μg of poly (dI-dC) in binding buffer for 30 min at 4°C. DNA-binding activity was confirmed with biotin-labeled oligonucleotide bio-NF-κB probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′) using an EMSA kit according to the manufacturer's instructions (Viagene, China). The resulting DNA–protein complex was separated from free oligonucleotide on a 4% polyacrylamide gel containing 0.25 × TBE (Tris/borate/EDTA) buffer and visualized with a Coollmger imaging system (IMGR002). The bands were quantified using Scion Image software.

Animal experimental design

All surgical procedures and care administered to the animals were in accordance with institutional guidelines. Male nude BALB/c mice, 6–8 weeks old, were obtained from the Animal Research Center, The First Clinical Medical School of Harbin Medical University, China. Tumors were established by subcutaneous injection of 5×10^6 BxPC-3 cells into the flanks of mice. When tumors reached around 120 mm^3, the mice were randomly assigned to 4 groups (each group had 7 mice): control (DMSO was dissolved in 200 μl PBS, once daily by i.p. injection) and three treatment groups (DHA at doses of 2, 10, or 50 mg/kg, respectively, once daily by i.p. injection). Tumor volumes were estimated according to the formula: $\pi/6 \times A^2 \times B$, where A is the short axis and B the long axis. The mice were closely monitored for 21 days, before *euthanized*, and the tumors removed.

Immunohistochemistry

The methodology has been described previously [24]. Briefly, 4-μm tumor sections were immunostained with primary Abs, and subsequently with appropriate secondary Abs using the Ultra Sensitive TMS-P kit (Zhongshan Co., Beijing, China), immunoreactivity developed with Sigma FAST DAB (3,3′-diam-inobenzidine tetrahydrochloride), and $CoCl_2$ enhancer tablets (Sigma). Sections were counterstained with hematoxylin, mounted and examined under microscopy.

Assessment of tumor vascularity

The methodology has been described previously [24]. Briefly, 4-μm tumor sections were immunostained with anti-CD31 Ab. Stained vessels were counted in 10 blindly chosen random fields at 200× magnification, and the microvessel density was recorded.

Western blotting

The methodology has been described previously [8]. Briefly, 5×10^5 cells were sonicated in RIPS buffer and homogenized. Tumor tissues were excised, minced, and homogenized in protein lysate buffer. Debris was removed by centrifugation at 12,000×*g* for 10 min at 4°C. The samples containing 50 μg protein were electrophoresed on 12% polyacrylamide SDS gels and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 3% BSA, incubated with primary antibodies, and subsequently with alkaline phosphatase-conjugated secondary antibody, before developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Tiangen Biotech Co. Ltd, China). Blots were also stained with anti-β-actin antibody as internal control for the amounts of target proteins.

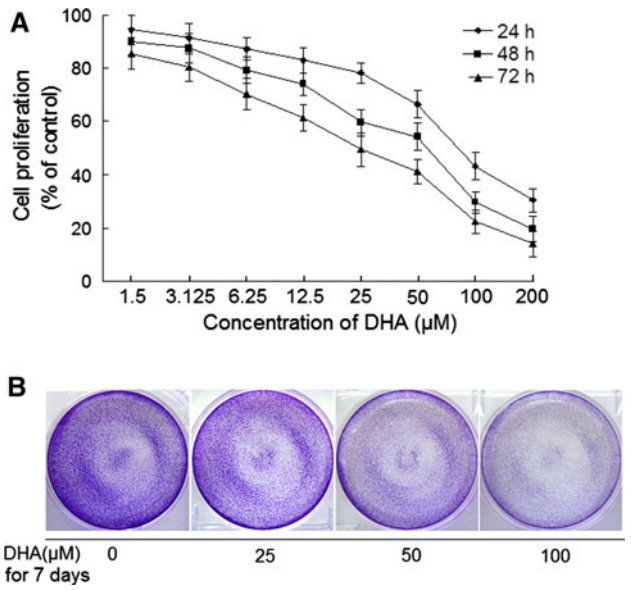

Fig. 1 Effects of DHA on the proliferation of HUVECs. HUVECs were incubated in the absence or presence of DHA at different concentrations for 24, 48, or 72 h before harvested. **a** The proliferation of cells was assessed by the MTT assay to calculate the proliferation rate (%). **b** The proliferation of cells was also measured by crystal violet assay. Representative images are shown

Statistical analysis

The growth patterns of tumors were compared using the analysis of variance (ANOVA) test. Other results were expressed as mean values ± standard deviation, and a Student's *t* test was used to evaluate statistical significance. $P < 0.05$ was defined as statistical significance.

Results

Effects of dihydroartemisinin on proliferation of HUVECs

HUVECs were treated with various concentrations of DHA (0–200 μM) for 24, 48, or 72 h, before the cell proliferation was assessed by MTT assay. As shown in Fig. 1a, DHA inhibited the proliferation of HUVECs in a time-dependent manner, compared with untreated cells. The proliferation of HUVECs could be effectively inhibited by 21.9% after being incubated with 25 μM of DHA for 24 h, when compared with control. This inhibition continued to increase to 40.3 and 50.6% after being incubated with 25 μM of DHA for 48 and 72 h, respectively. With a simple linear regression analysis, the IC50 value was calculated to be 89.4 ± 5.3, 39.4 ± 5.8, and 19.1 ± 4.6 μM when HUVESs cells were incubated with DHA for 24 h, 48 h, and 72 h, respectively. DHA also inhibited the proliferation of HUVECs in a dose-dependent manner for 24, 48, or 72 h, respectively, compared with control. These similar results were further confirmed by crystal violet assay (Fig. 1b). The data demonstrate that DHA may exhibit the antiangiogenic activity by inhibition of specific growth-related signals of vascular endothelial cell proliferation.

Effects of dihydroartemisinin on HUVECs tube formation

To investigate the effect of DHA on capillary tube formation of HUVECs, cells were determined with in vitro angiogenesis assay. In the presence of 10 ng/ml VEGF, HUVECs plated on the fibrin gel and formed capillary-like structures (Fig. 2a). Treatment of HUVECs with DHA resulted in

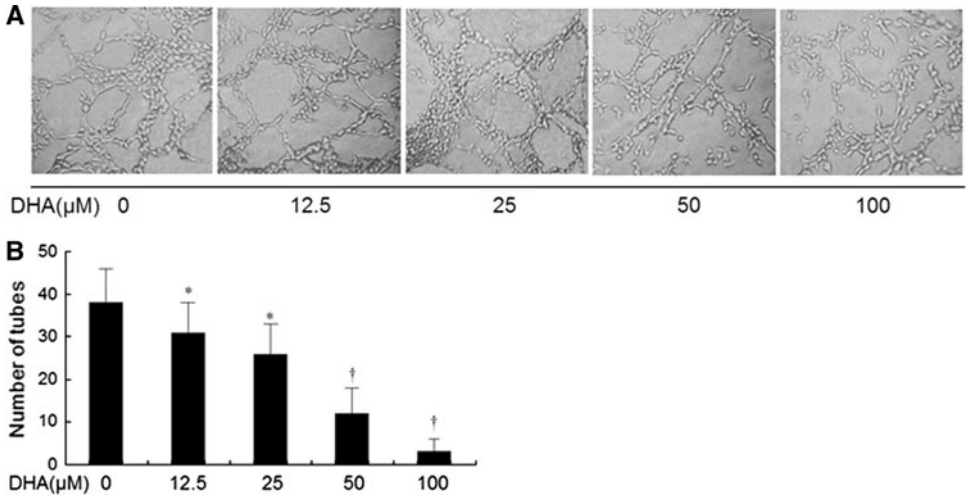

Fig. 2 Effects of DHA on tube formation of HUVECs. HUVECs were plated on the fibrin gel on 24-well plates and treated with various concentrations of DHA in the presence of 10 ng/ml VEGF for an additional 24 h. **a** The tube formation of HUVECs was determined with in vitro angiogenesis assay. The images were photographed by microscope at ×100 magnification. Representative images are shown. **b** The number of tubes was counted under microscope to calculate the tube formation of HUVECs. $^{*}P < 0.05$ and $^{\dagger}P < 0.01$ compared with untreated control

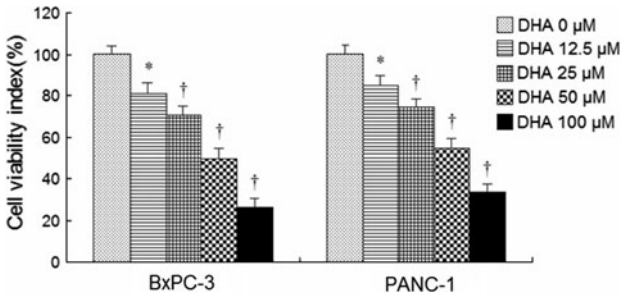

Fig. 3 DHA inhibits the viability of pancreatic cancer cell lines in vitro. BxPC-3 and PANC-1 cells were incubated in the absence or presence of DHA at different concentrations for 72 h before harvested. The viability of cells was assessed by the MTT method to calculate the viability index (%). $^{*}P < 0.05$ and $^{\dagger}P < 0.01$ compared with untreated control

dose-dependent inhibition of tube formation induced by VEGF. At the concentration of 50 μM, the capillary-like network of the fibrin gel was completely disrupted and inhibited the tube formation of HUVECs by 70% (Fig. 2b) compared with control cultures ($P < 0.01$). These results suggest that DHA efficiently inhibiting angiogenesis in vitro may be due to the effect on the angiogenic differentiation of vascular endothelial cells.

Dihydroartemisinin reduces viability of pancreatic cancer cells

BxPC-3 and PANC-1 cells were treated with various concentrations of DHA (12.5, 25, 50, or 100 μM) for 72 h, and cell viability was determined by MTT assay. As shown in Fig. 3, DHA reduced the viability of two types of cells incubated with DHA for 72 h in a dose-dependent manner when compared with untreated cells. At the concentration of 12.5, 25, 50, or 100 μM, DHA inhibited the growth of BxPC-3 and PANC-1 cells by 19.1, 28.9, 50.5, 74.6, and 15.3, 24.9, 46.1, 66.2%, respectively. With a simple linear regression analysis, the IC50 value was calculated to be 40.6 ± 6.8 μmol/l when BxPC-3 cells were incubated with DHA for 72 h, and the IC50 value for PANC-1 cells was 48.9 ± 6.1 μmol/l.

Dihydroartemisinin inhibits NF-κB activation and down-regulates its downstream proangiogenic gene products in pancreatic cancer cells

After the above treatments, nuclear extract was obtained for determining NF-κB DNA-binding activity by EMSA, while total protein extract was obtained for its downstream gene expression by Western blot. As shown in Fig. 4a, BxPC-3 and PANC-1 cells constitutively expressed NF-κB activity. However, DHA significantly reduced its DNA-binding activity in a dose-dependent manner. At the concentration of 12.5, 25, or 50 μM, DHA significantly inhibited NF-κB DNA-binding activity compared with untreated cells ($P < 0.05$), in both BxPC-3 and PANC-1 cells. As shown in Fig. 4c, incubation with DHA inhibited NF-κB activation and down-regulated the expression of VEGF, IL-8, COX-2, and MMP-9 in a dose-dependent manner, in BxPC-3 and PANC-1 cells. These results suggested that DHA reduces NF-κB DNA-binding activity and down-regulates its downstream proangiogenic gene products, which are believed to be responsible for exerting its antiangiogenic activity in pancreatic cancer cells.

Dihydroartemisinin suppresses the growth of pancreatic tumors in mice

As shown in Fig. 5, tumors in the control group grew remarkably faster, reaching 730.6 ± 90 mm^3 in volume 21 days after treatment. In contrast, the tumors of mice treated with 2, 10, or 50 mg/kg DHA were significantly ($P < 0.05$) smaller than control tumors, reaching only 569 ± 69, 389 ± 44, and 244 ± 36 mm^3 in volume, respectively, 21 days after treatment.

Dihydroartemisinin inhibits angiogenesis in vivo

Tumor sections prepared from the above tumors were stained with an anti-VEGF and IL-8 antibody which detects the expression of angiogenic factors, or an anti-CD31 Ab to view tumor microvessels. Immunohistochemical analysis revealed reductions in the expression of VEGF and IL-8 in tumors from the three DHA treatment groups compared with the control group (Fig. 6a). Additionally, the inhibitory effects on the expression of VEGF and IL-8 of DHA therapy displayed a dose-dependent manner. As shown in Fig. 6b, there were fewer microvessels in tumors treated with 2, 10, or 50 mg/kg DHA therapies compared with the control tumors. The CD31-stained microvessels were counted to calculate the microvessel density. DHA therapy at the dose of 2 mg/kg resulted in a significant decrease in the microvessel density by 21.7% ($P < 0.05$), compared with control, and at the dose of 10 or 50 mg/kg significantly decreased the microvessel density by two- to threefold ($P < 0.01$) (Fig. 6b). We further detected tumoral expression of angiogenesis-related proteins by Western blot and found that DHA therapy inhibited NF-κB activation and down-regulated the expression of VEGF, COX-2, and MMP-9 in a dose-dependent manner, in accordance with the findings in vitro (Fig. 6c).

Effects of psiVEGF on proliferation and angiogenesis in vitro

As shown in Fig. 7a, the cells exhibited green fluorescence indicating that the plasmids were successfully transfected into HUVECs, BxPC-3, and PANC-1 cells, and three stably

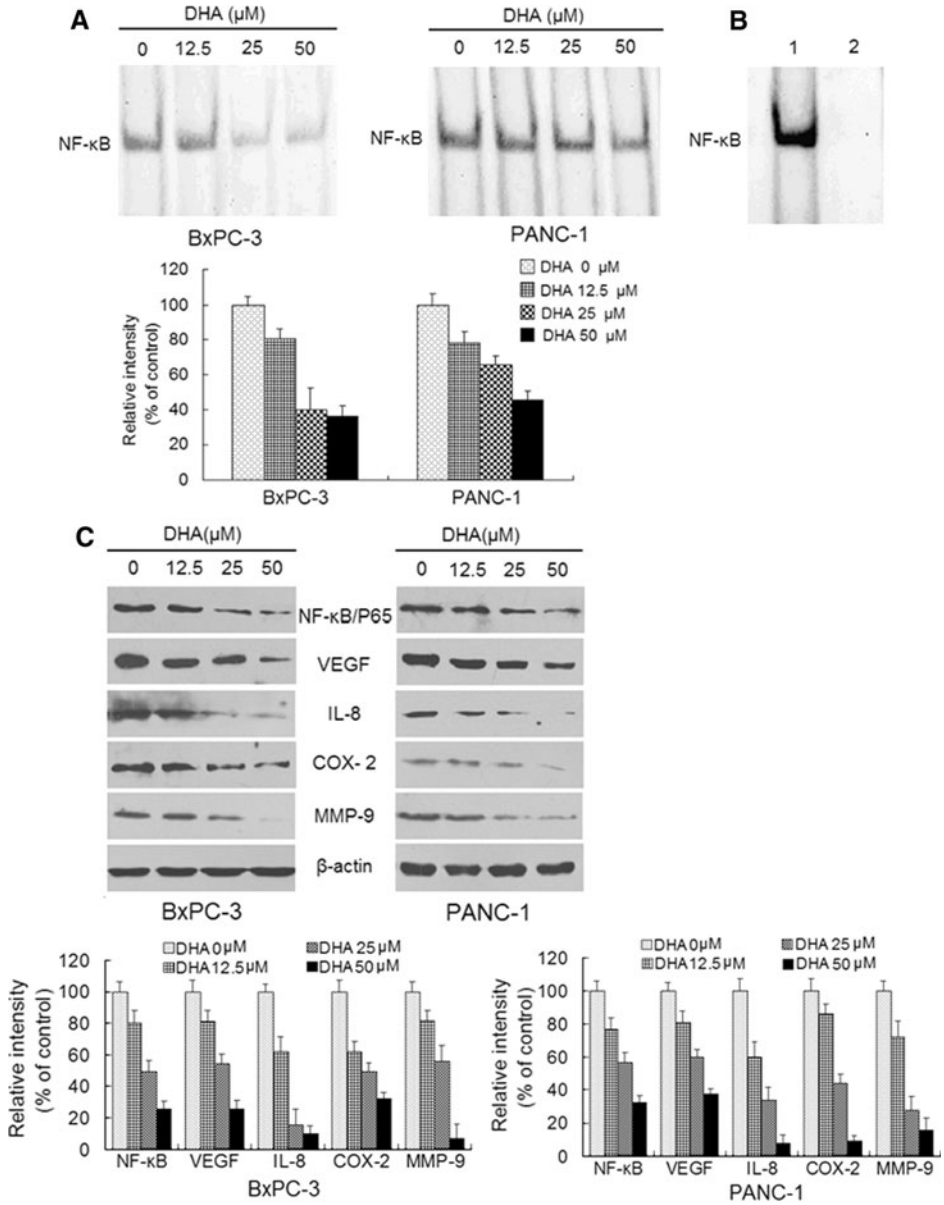

Fig. 4 NF-κB DNA-binding activity and NF-κB-targeted gene expression. Nuclear extracts and total protein extracts were prepared from BxPC-3 and PANC-1 cells treated with DHA for 72 h. **a** EMSA for NF-κB DNA-binding activity on respective nuclear extracts. *Bottom panel*: densitometric quantification of data presented in the *top panel*. $^{*}P < 0.05$ and $^{\dagger}P < 0.01$ compared with untreated control. **b** Competitive assay confirmed the specificity of NF-κB binding to the DNA. *Lane 1*: control; *lane 2*: excess unlabeled ("cold") NF-κB oligonucleotide (50-fold). **c** Western blotting analysis on the expression of p65, VEGF, IL-8, COX-2, and MMP-9 from respective cell homogenate, with β-actin as protein loading control. The histogram in each *panel* indicates the relative band intensity generated from densitometric scans of 3 independent experiments on arbitrary densitometric units

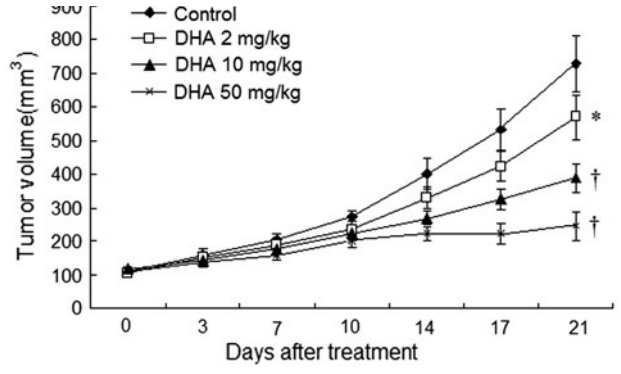

Fig. 5 Administration of DHA inhibits tumor growth. Tumor volume comparison measured using Vernier calipers and calculated by formula: $V = /6 \times A^2 \times B$ at indicated time points. $^{*}P < 0.05$ and $^{\dagger}P < 0.01$ compared with untreated control

transfected cell lines could be constructed. To evaluate specificity and potency of the targeting sequence in BxPC-3 and PANC-1 cells, we examined its effects on VEGF expression in vitro. As shown in Fig. 7b, Western blotting analysis indicated that significant suppression of VEGF expression in BxPC-3 and PANC-1 cells was distinct 48 h after transfection with siVEGF, whereas VEGF expression was not affected by siHK. The biological effects of the VEGF siRNA were determined using cell proliferation assays and tubular formation assay. As shown in Fig. 7c, the proliferation of HUVECs, BxPC-3, and PANC-1 cells could be effectively inhibited by 29.9, 27.7, and 35.5%, respectively, 48 h after transfection with siVEGF, when compared with untransfected cells ($P < 0.01$). Whereas there was little difference between the negative control cells

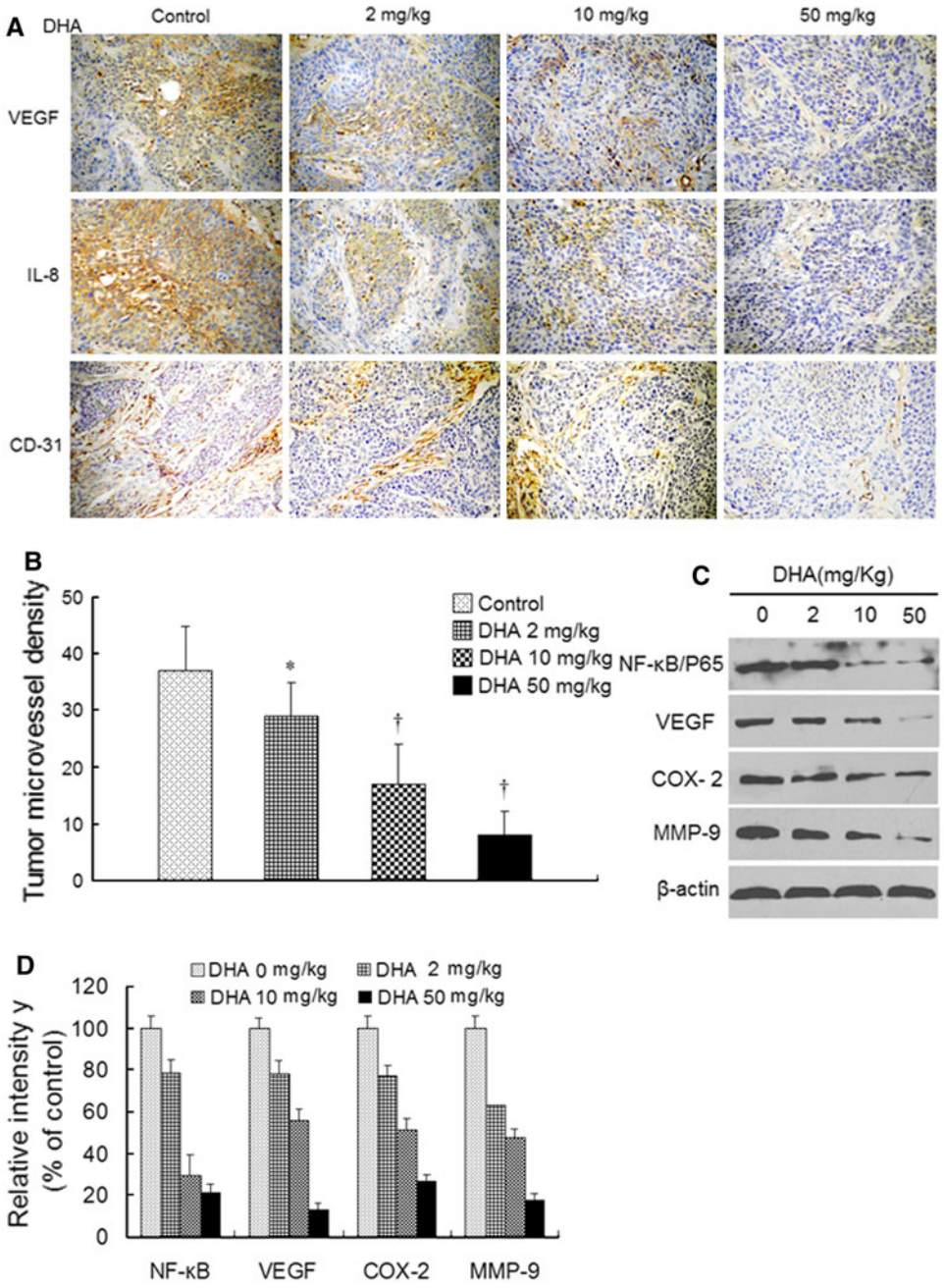

Fig. 6 DHA inhibits angiogenesis and regulates pro-angiogenic proteins in vivo. **a** Tumor sections were immunostained for expression of VEGF, IL-8, and CD31 (to view microvessels). The images were photographed by microscope at ×200 magnification. **b** CD31-stained microvessels were counted under microscope to record microvessel density. Seven tumors from each group of mice were assessed. $^{*}P < 0.05$ and $^{\dagger}P < 0.01$ compared with untreated control. **c** Tumors from mice were homogenized and subjected to western blot analysis on the expression of p65, VEGF, COX-2, and MMP-9, using β-actin as protein loading control. **d** The histogram in each panel indicates the relative band intensity generated from densitometric scans of 3 independent experiments on arbitrary densitometric units

(siHK) and untransfected cells over the entire experimental period ($P > 0.05$). As shown in Fig. 7d, the number of tubule-like structures of the fibrin gel in transfected-siVEGF HUVECs was significantly reduced by 85.1% compared with the negative control cells (siHK).

Discussion

The complex process of angiogenesis is mediated in part by the transcription factor NF-κB and several proangiogenic factors including VEGF, IL-8, COX-2, and MMP-9 [24, 25]. The key finding from the present study is that DHA has antiangiogenic effect both on in vitro angiogenesis models and on in vivo pancreatic carcinoma-derived tumor models. Furthermore, we show that these effects are likely mediated through inhibition of the NF-κB pathway as well as its downstream proangiogenic growth factors.

Antiangiogenic therapy, as an evolving anticancer strategy, is directed against tumor endothelial cells to block revascularization. Since the successful efforts to inhibit vascular endothelial cell growth were reported, many new agents have been developed for the inhibition of tumor angiogenesis. DHA, an effective antimalarial drug with little

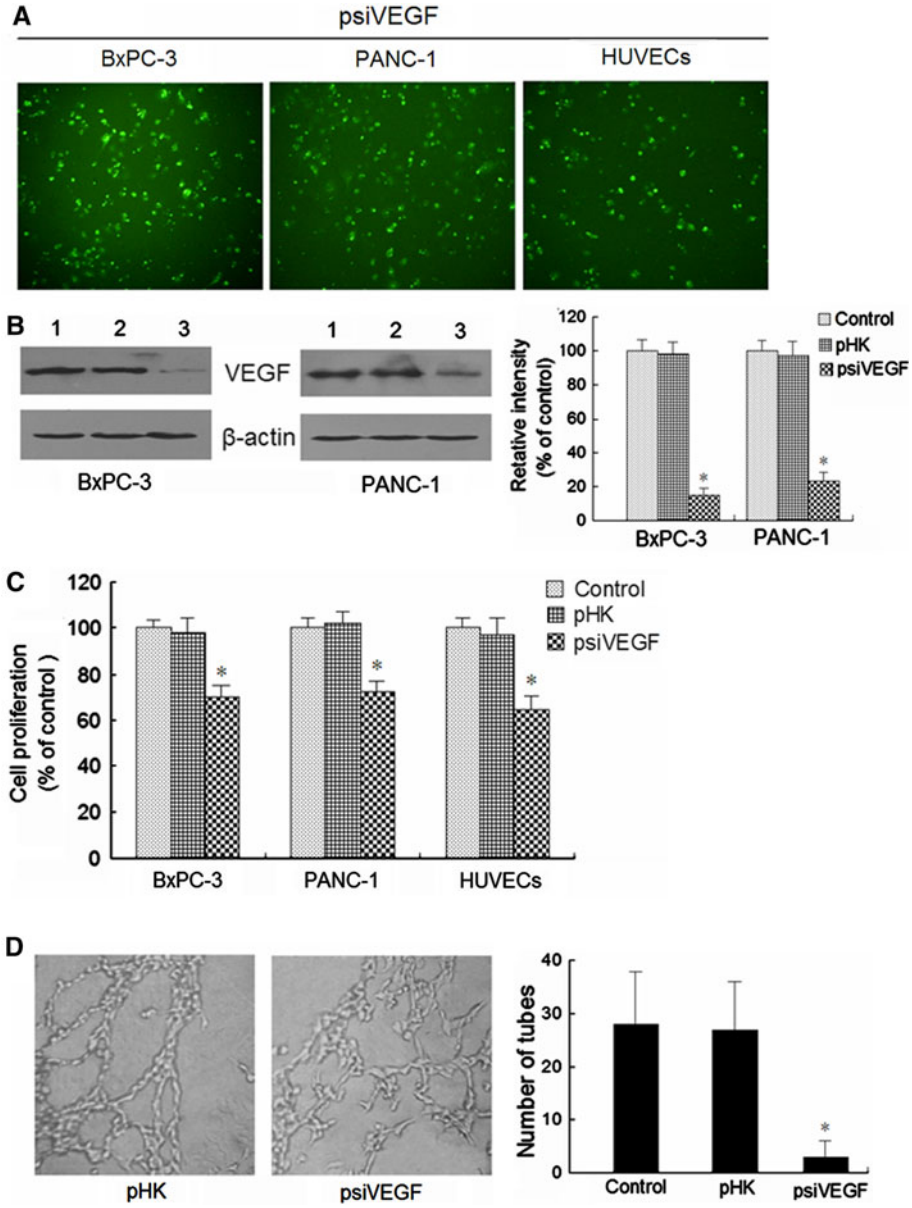

Fig. 7 Effects of psiVEGF on proliferation and angiogenesis in vitro. **a** HUVECs, BxPC-3 and PANC-1 cells exhibited green fluorescence after 48 h of transfection, indicated that the plasmids were successfully transfected into cells. **b** *Left panel* shows the results of western blotting analysis on the expressions of VEGF in BxPC-3 and PANC-1 cells 48 h after transfection with siVEGF, with *β*-actin as protein loading control. *Lane 1*: control; *lane 2*: pHK; *Lane 3*: psiVEGF. The histogram of right panel indicates the relative band intensity generated from densitometric scans of 3 independent experiments on arbitrary densitometric units. $^*P < 0.01$ compared with control and pHK. **c** pHK and psiVEGF were transiently transfected into HUVECs, BxPC-3 and PANC-1 cells. Forty-eight hours after transfection, cell proliferation rate was determined by the MTT assay. $^*P < 0.05$, compared with control and pHK. **d** Effects of psiVEGF on HUVECs angiogenesis in vitro. Six hours after transfection with pHK and psiVEGF, HUVECs (5×10^4/well) were transferred on the fibrin gel on 24-well plates and incubated for another 24 h. The images were photographed by microscope at $\times 100$ magnification. Representative images are shown. The number of tubes was counted under microscope to calculate the tube formation of HUVECs. $^*P < 0.01$ compared with control and pHK

toxicity, has been reported to be highly effective against a wide variety of tumors [6, 9, 26]. In the present study, we showed that treatment of HUVECs with DHA resulted in a dose-dependent inhibition of the cell proliferation and capillary tube formation. Furthermore, after incubation with 12.5 and 25 μM DHA for 24 h, the viability of HUVECs was effectively reduced by 16.5 and 21.9%, while the tube formation was suppressed and the tube formation index reduced by 21.1 and 31.5%, respectively. This discrepancy indicates that the antiangiogenic effect cannot be completely attributed to the inhibitory effect of DHA upon HUVESs cell viability; instead, here is a specific antiangiogenic effect on the angiogenic differentiation of endothelial cells working along. Therefore, these results suggest that DHA efficiently inhibits

angiogenesis in vitro, and this antiangiogenic activity may be attributed more to its specific inhibition on the angiogenic differentiation of vascular endothelial cells, rather than antiproliferative activity. To evaluate the antiangiogenic potential of DHA in pancreatic cancer, the antiangiogenic effect in vivo was evaluated in BxPC-3 tumor-bearing mice. We demonstrated that DHA strongly inhibited local tumor growth, decreased the expression of VEGF and IL-8, and also reduced the microvessel density. All of which revealed a significant reduction in the neoangiogenic phenotype.

Studies showed that NF-κB could enhance the angiogenic potential of pancreatic cancer cells through inducing the production of proangiogenic factors [27]. Several in vitro and in vivo studies illustrate that blocking NF-κB in pancreatic cancer affects angiogenesis. For example, blockade of NF-κB activation by using NF-κB inhibitors MG-132 or transfecting mutant IκB-α have shown efficacy in inhibition of neovascularization and tumor growth in pancreatic cancer [20, 27]. In this study, our EMSA results demonstrated that NF-κB activity was constitutively expressed in pancreatic cancer cell lines, which directly correlated with the angiogenic potential of pancreatic cancer. On the other hand, our study, for the first time, demonstrated DHA suppresses pancreatic cancer-associated angiogenesis through inhibition of NF-κB activity. In both cells, DHA significantly reduced its DNA-binding activity so that abrogated its inducing effect on angiogenesis, which was exactly consistent with both the inhibition of DHA on HUVECs proliferation and tube formation in vitro, and the reduction in microvascular density in vivo. Together, these evidences indicate that NF-κB blockade by DHA could be an effective method to prevent angiogenesis in pancreatic cancer.

Of the numerous proangiogenic molecules discovered so far, VEGF and IL-8 have been identified as key mediators of pancreatic cancer angiogenesis [28, 29]. VEGF via binding to its high-affinity receptors promotes endothelial cells proliferation and increases vascular permeability, thereby playing a crucial role in tumor neovascularization [30]. IL-8 triggers angiogenesis by stimulation of endothelial cell growth as well as by leukocyte-dependent effects [31]. Recent study demonstrated that the pleiotropic transcription factor NF-κB regulates the expression of multiple genes, including VEGF and IL-8 [32]. Our present study showed that constitutive NF-κB activity drives constitutive overexpression of VEGF and IL-8, which contributes to the angiogenic phenotype of human pancreatic cancer. Under DHA, the decreased expression of VEGF and IL-8 in vitro and in vivo correlated with decreased proliferation and neovascularization. These similar results were further confirmed by siRNA interference assay. After transfection with siVEGF, significantly decreased expression of VEGF correlated with suppressed proliferation of HUVECs and pancreatic cancer cells, and inhibited angiogenesis of HUVECs in vitro. Furthermore, our study is the first to indicate that DHA inhibited constitutive NF-κB activity and suppressed NF-κB-targeted VEGF and IL-8 gene transcription, which is at least partially responsible for DHA-mediated inhibition of proliferation and angiogenesis in human pancreatic cancer.

MMPs were reported to enhance proteolytic degradation of the basement membrane, which is deeply involved in tumor growth, metastasis, and angiogenesis [31]. Studies have demonstrated that MMP-9 contributes to angiogenic switch and is essential to angiogenesis and progressive growth of orthotopic human pancreatic cancer cells implanted into nude mice [25, 33]. COX-2 is overexpressed in malignancies including pancreatic cancer [34], and its expression is regulated by NF-κB [35]. Previous study has suggested that expression of COX-2 contributes to tumor growth by inducing angiogenesis [36]. Several pieces of evidence demonstrated that COX-2 inhibitors decrease tumor-induced angiogenic responses of endothelial cells in vitro as well as in vivo [36–38]. Our present study showed that DHA down-regulated the expression of MMP-9 and COX-2 in vitro and in vivo in pancreatic cancer, which correlated with decreased neovascularization. Furthermore, our current study exploited the ability of DHA to block NF-κB activation and suppress NF-κB-regulated MMP-9 and COX-2 gene transcription, which is at least partially responsible for DHA-mediated inhibitory effects on growth and angiogenesis in human pancreatic cancer.

In conclusion, our present study demonstrated DHA suppressed angiogenesis and tumor growth of human pancreatic cancer, which is at least partially through the blockade of NF-κB activity and down-regulation of NF-κB-regulated proangiogenic gene products. The effect of DHA on antiangiogenic activity and its toxicity profile open up the possibility that DHA might be a candidate inhibitor to angiogenesis. Furthermore, our results, together with those of several previous studies, further strengthened the wide chemotherapeutic potential of DHA and its subsequent development as a potential therapeutic agent against human pancreatic cancer.

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Conflict of interest No conflict of interest.

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