Dihydroartemisinin inhibits growth of pancreatic cancer cells *in vitro* and *in vivo*

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Dihydroartemisinin (DHA), a semisynthetic derivative of artemisinin, has recently shown antitumor activity in various cancer cells. Its effect on pancreatic cancer is, however, unknown and the mechanism is unclear. The study aims to investigate its antitumor activity and underlying mechanisms in human pancreatic cancer BxPC-3 and AsPC-1 cells *in vitro* and subcutaneous BxPC-3 xenograft tumors in mice. The MTT assay was used to evaluate cell viability, and flow cytometry and laser scanning confocal microscopy were used to detect apoptosis, for cultured cells. Pancreatic tumors were established by subcutaneous injection of BxPC-3 cells in nude BALB/c mice, and DHA was administered intraperitoneally to the mice. The size of tumors was monitored and they were harvested after the mice had been killed. Tumor sections were immunostained with an anti-Ki-67 Ab to assess the proliferation index, or stained with TUNEL to evaluate in-situ cell apoptosis. The gene expression in cells and tumors was evaluated by western blot analysis. In the cultured cells, DHA inhibited cell viability, downregulated the expression of proliferating cell nuclear antigen and cyclin D1, and upregulated $p21^{WAF1/CIP1}$; and induced apoptosis by reducing the ratio of Bcl-2/Bax and increasing the activation of caspase-9, in a dose-dependent manner. Similarly, in mice bearing BxPC-3 xenograft tumors, administration of DHA inhibited tumor growth in a dose-dependent manner, and modulated tumoral gene expression consistent with the in-vitro observations. This study indicates that DHA may be a potent and promising agent to combat pancreatic cancer. *Anti-Cancer Drugs* 20:131–140 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Pancreatic cancer patients have a very poor prognosis with a 5-year survival rate of less than 1% and a median survival of 4–6 months. Even after surgical resection, the 5-year survival rate is at best 15% without adjuvant therapy or 25% with adjuvant therapy [1]. Pancreatic cancer is highly resistant to chemotherapy and other forms of treatments [2]. Therefore, novel treatment modalities are worth investigation.

In seeking novel anticancer drugs, an effective antimalarial drug, artemisinin, which is isolated from the traditional Chinese herb *Artemisia annua* and its derivatives, have recently drawn attention. Dihydroartemisinin (DHA), a semisynthetic derivative of artemisinin, has exhibited the strongest anticancer activity among the derivatives of artemisinin. A number of studies have investigated the use of DHA in inhibiting growth and/or inducing apoptosis of cells of breast cancer [3], cervical cancer [4], uterus chorion cancer [4], embryo transversal cancer [4], ovarian cancer [4–6], glioma [7], lung cancer [8,9], leukemia [10,11], fibrosarcoma [12], osteosarcoma [13] and oral cancer [14]; or enhancing radiosensitivity for glioma cells [15], cytotoxicity of pirarubicin and doxorubicin for leukemic and lung cancer cells [16], cytotoxicity of sodium butyrate for leukemic cells [10], and cytotoxicity of temozolomide for glioma cells [17]. More recently, DHA has displayed significant anticancer effects against human hepatoma cells with minimal effects on normal cells [18]. Mechanisms that might explain the antitumor activity of DHA include its ability to induce apoptosis of lymphatic endothelial cells by regulating apoptosis-related proteins and downregulating vascular endothelial growth factor-3, thus inhibiting lymphangiogenesis [19], lung cancer cells by activating P38 MAPK and increasing intracellular Ca^{2+} [9] or downregulating survivin expression [8], and ovarian cancer cells by regulating apoptosis-related proteins of Bcl-2 family [6]; or lead to DNA fragmentation in U2OS osteosarcoma cells by interfering with fortilin [13]; or inhibit growth of C6 glioma cells by increasing the reactive oxygen species and inhibiting activation of hypoxia inducible factor-1α [7]; or inhibit angiogenesis by reducing extracellular signal-regulated kinase1/2 activation [20], downregulating expression of vascular endothelial growth

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factor [11] or inhibiting proliferation, migration, and tube formation of vascular endothelial cells [4]. More importantly, DHA has shown selective toxicity on breast cancer cells but not on normal human breast cells [3], and exerted potent cytotoxicity on ovarian carcinoma cells but had minimal effects on nontumorigenic human ovarian surface epithelial cells [5], suggesting that DHA is well tolerated and represents a potent promising therapeutic agent to treat cancers. However, little is known about its effects on pancreatic cancers. Therefore, we designed this study to evaluate the activity of DHA in pancreatic cancer cells *in vitro* and *in vivo*.

Materials and methods

Mice, cell lines, and reagents

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. The human pancreatic cancer cell lines BxPC-3 and AsPC-1 were obtained from the American Type Culture Collection (Rockville, Maryland, USA). The cells were routinely cultured at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a CO_2 incubator. DHA was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The antibodies against Bcl-2, Bax, caspase-9, $p21^{WAF1/CIP1}$, proliferating cell nuclear antigen (PCNA), cyclin D1 and β-actin were purchased from Santa Cruz Biotechnology, California, USA. The anti-Ki-67 Ab was purchased from Abcam (Cambridge, Massachusetts, USA).

MTT assay

BxPC-3 (2×10^3) and AsPC-1 (8×10^3) cells were seeded in 200 μl of RPMI 1640 medium into 96-well plates, and cultured overnight. Then the medium was replaced with fresh RPMI 1640 or the same media containing different concentrations of DHA (0–160 μmol/l). After a further incubation for 72 h, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (20 μl) was added to each well followed by 4-h incubation. The medium was discarded and 150 μl of dimethyl sulfoxide was added into each well, and incubated for 20 min. The OD490 nm was measured. The cell viability index was calculated according to the formula: experimental OD value/control OD value × 100%. The experiments were repeated thrice.

Cell apoptosis assay

The cells were incubated with DHA at different concentrations as above for 72 h, harvested, washed, the numbers of cells were counted, and 1×10^5 cells were suspended in 100 μl binding buffer, 5 μl of annexin V, and 5 μl of propidium iodide (PI) were added, and incubated for 15 min at room temperature in dark, according to the manufacturer's instruction (BD Biosciences, San Jose, California, USA). Then the cells were subjected to flow cytometry to measure the apoptosis rate (%) with a Beckman Coulter Epics Altra II cytometer (Beckman Coulter, California, USA), or viewed under a laser scanning confocal microscope (LSM-510 Meta; Carl Zeiss Jena GmbH, Jena, Germany). The experiments were repeated thrice.

Animal experimental design

All surgical procedures and care administered to the animals were in accordance with institutional guidelines. Tumors were established by subcutaneous injection of 5×10^6 BxPC-3 cells into the flanks of mice. 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. When tumors reached around 120 mm^3 volume at about 2 weeks, the mice were randomly assigned to four groups (each group had seven mice): control and three treatment groups. The mice in the control group received daily intraperitoneal injection of 100 μl of PBS, and the mice in the three treatment groups received intraperitoneal injection of DHA at doses of 2, 10, or 50 mg/kg, respectively. The mice were closely monitored. Eighteen days later, all the mice were euthanized, and the tumors removed. Each tumor was split into two parts: one part was fixed with 10% buffered formalin, and the other part kept at −80°C freezer for further analysis.

Quantitation of Ki-67 proliferation index

Formalin-fixed tumor specimens were transferred to 70% ethanol, and subsequently paraffin embedded and sectioned. The tumor sections were rinsed with PBS, blocked with 3% bovine serum albumin for 2 h, and incubated overnight with an anti-Ki-67 Ab. They were subsequently incubated for 30 min with the appropriate secondary Ab using the Ultra Sensitive TMS-P kit (Zhongshan Co., Beijing, China), and immunoreactivity developed with Sigma FAST DAB (3,3′-diaminobenzidine tetrahydrochloride) and $CoCl_2$ enhancer tablets (Sigma-Aldrich, Shanghai, China). Sections were counterstained with hematoxylin, mounted, and examined by microscopy. The Ki-67 positive cells were counted in 10 randomly selected × 400 high-power fields under microscopy. The Ki-67 proliferation index was calculated according to the following formula: the number of Ki-67-positive cells/the total cell count ×100%.

In-situ detection of apoptotic cells

Tumor sections above were stained with the TUNEL agent (Roche, Shanghai, China), and the TUNEL-positive cells were counted in 10 randomly selected × 400 high-power fields under microscopy. The apoptosis index was calculated according to the following formula: the number of apoptotic cells /total number of nucleated cells ×100%.

Western blotting

A total of 5×10^5 cells were sonicated in RIPS buffer (1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride,

45 μg/ml aprotinin, 100 mmol/l sodium orthovanadate) and homogenized. The tumor tissues were excised, minced, and homogenized in protein lysate buffer as described earlier [21]. Debris was removed by centrifugation at $10\,000 \times g$ for 10 min at 4°C. The content of protein in the homogenates from cells or tumor tissues was determined, samples containing 50 μg total protein were resolved on 12% polyacrylamide SDS gels, and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with 3% bovine serum albumin, incubated with primary antibodies, and subsequently with alkaline phosphatase-conjugated secondary antibody. They were developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Tiangen Biotech Co. Ltd., Beijing, China). Blots were stained with anti-β-actin Ab to confirm that each lane contained similar amounts of tumor homogenate.

Statistical analysis

The half maximal inhibitory concentration (IC_{50}) was calculated with a simple linear regression analysis. The growth patterns of tumors were compared using the analysis of variance test. Other results were expressed as mean values ± standard deviation, and the Student's *t*-test was used to evaluate statistical significance. A value of less than 0.05 ($P < 0.05$) was used for statistical significance.

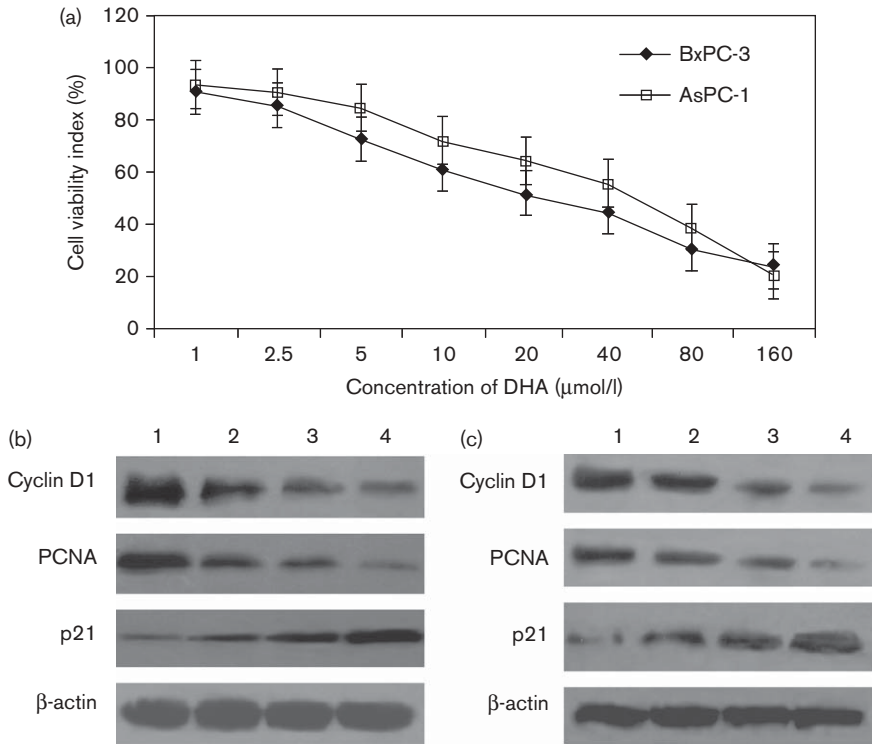
Results

Dihydroartemisinin reduces viability of pancreatic cancer cells

The effect of DHA on the viability of BxPC-3 and AsPC-1 cells was determined with MTT assay. As shown in Fig. 1, DHA reduced the viability of two types of cells incubated with DHA for 72 h in a dose-dependent manner, compared with untreated cells. With a simple linear regression analysis, the IC_{50} was calculated to be 33.6 ± 6.8 μmol/l when BxPC-3 cells were incubated with DHA for 72 h; and the IC_{50} for AsPC-1 cells was 49.9 ± 7.0 μmol/l.

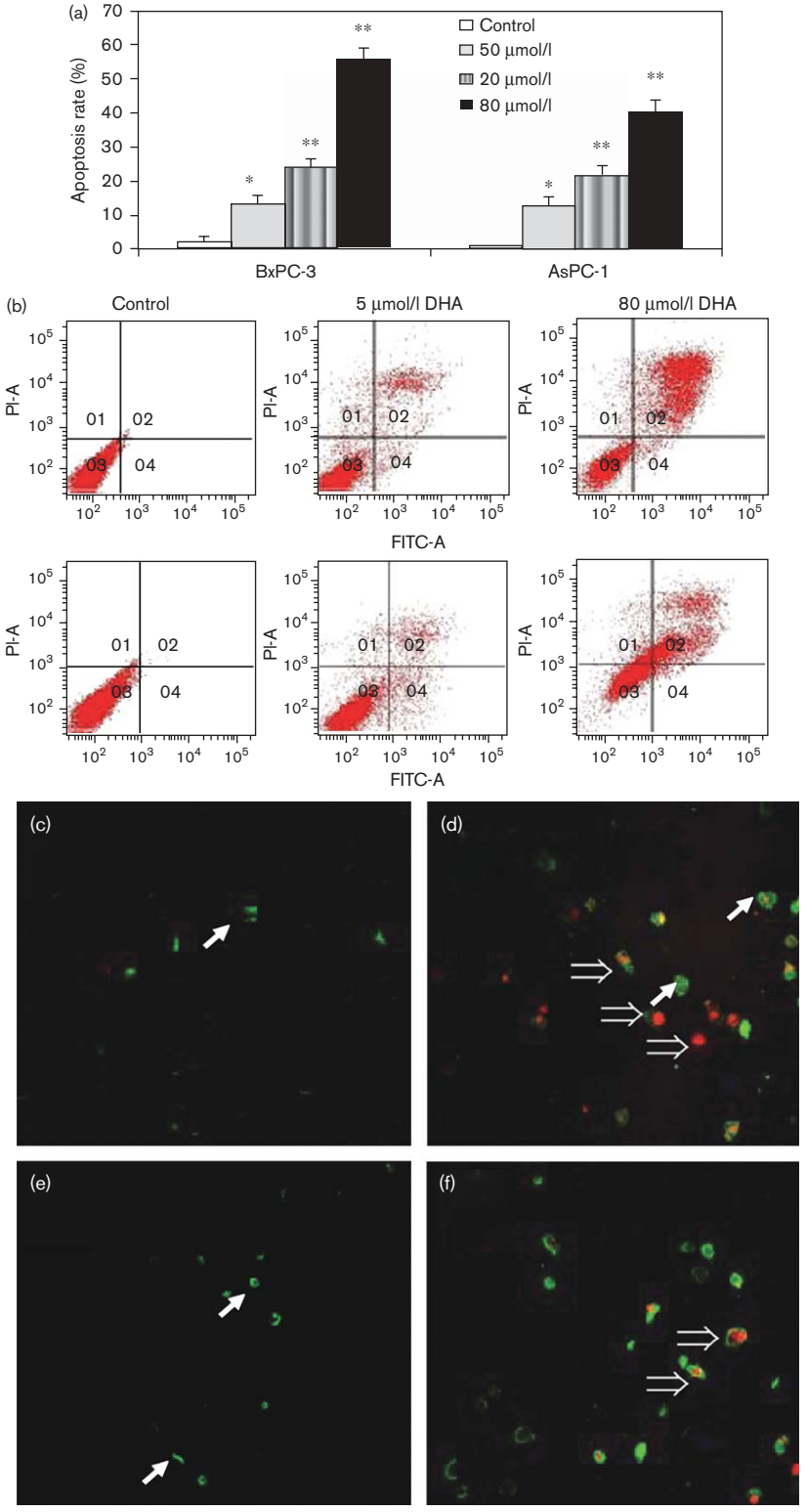
To investigate the mechanisms accounting for its proliferative inhibitory effects, the expression of cyclin D1, $p21^{WAF1/CIP1}$, and PCNA was detected with western blot analysis. As shown in Fig. 1b and c, incubation with DHA downregulated the expression of cyclin D1 and PCNA, and upregulated $p21^{WAF1/CIP1}$, in a dose-dependent manner, in BxPC-3 (Fig. 1b) and AsPC-1 (Fig. 1c) cells.

Fig. 1



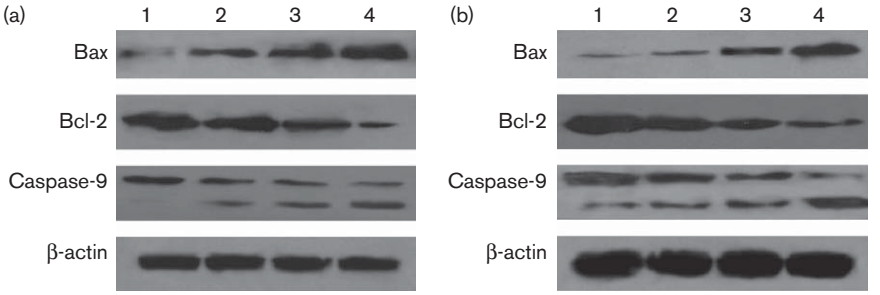
Dihydroartemisinin (DHA) inhibits the viability of pancreatic cells and regulates proliferation-related proteins *in vitro*. (a) BxPC-3 and AsPC-1 cells were incubated in the absence or presence of DHA at different concentrations, and harvested 72 h later. The viability of cells was assessed by the MTT method to calculate the viability index (%). (b,c) The BxPC-3 (b) or AsPC-1 (c) cells incubated in the absence of DHA (lane 1) or presence of DHA at concentrations of 5 (lane 2), 20 (lane 3), or 80 (lane 4) μmol/l, were harvested, homogenized, and subjected to western blot analysis to detect the expression of cyclin D1, proliferating cell nuclear antigen (PNCA), and $p21^{WAF1/CIP1}$. β-actin served as an internal control.

Fig. 2



Dihydroartemisinin (DHA) induces cell apoptosis. BxPC-3 and AsPC-1 cells were incubated with different concentrations of DHA for 72 h. Untreated cells served as control. (a) Flow cytometry was performed to measure apoptosis rates. *Indicates a significant difference at $P<0.05$, and **a highly significant difference at $P<0.001$, compared with control. (b) Representative histograms were from cytometrically analyzed BxPC-3 (upper panel) or AsPC-1 (lower panel) cells incubated with control medium (control) or media containing 5 or 80 μmol/l of DHA. (c–f) Representative photographs ($\times$400 magnification) for BxPC-3 (c, d) or AsPC-1 (e, f) cells incubated with control medium (c, e) or media containing 80 μmol/l DHA (d, f) stained with annexin V/propidium iodide and examined under laser scanning confocal microscopy to detect apoptotic cells. The cells at the early phase of apoptosis or programmed cell death are indicated by '→', and at the late phase by '⇒'.

Fig. 3



Dihydroartemisinin (DHA) regulates apoptosis-related proteins *in vitro*. The BxPC-3 (a) or AsPC-1 (b) cells were incubated in the absence of DHA (lane 1) or presence of DHA at concentrations of 5 (lane 2), 20 (lane 3), or 80 (lane 4) μmol/l, were harvested, homogenized, and subjected to western blot analysis to detect expression of Bax, Bcl-2, and caspase-9. β-actin served as an internal control.

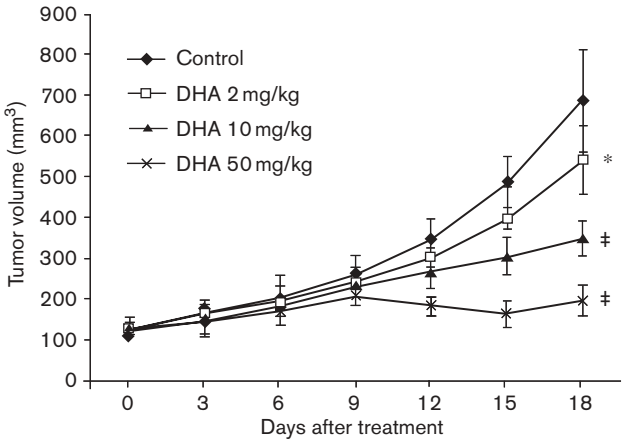
Dihydroartemisinin induces apoptosis of pancreatic cancer cells

BxPC-3 and AsPC-1 cells were incubated with DHA at different concentrations for 72 h, and flow cytometric analysis was used to measure the apoptosis rates. As shown in Fig. 2a, DHA induced apoptosis of BxPC-3 cells in a dose-dependent manner. When the concentration of DHA reached 5 μmol/l, the apoptosis rate of BxPC-3 cells was significantly higher than that of untreated cells ($P < 0.05$). Furthermore, 20 or 80 μmol/l of DHA resulted in a highly significant difference in apoptosis rate between DHA-treated and untreated cells ($P < 0.001$). Similarly, DHA also induced apoptosis of AsPC-1 cells in a dose-dependent manner (Fig. 2a). The representative histograms of flow cytometry shows that the apoptosis rates of BxPC-3 cells were 1.2, 13.1, or 55.4% when they were treated with DHA at concentrations at 0, 5, 80 μmol/l, respectively (Fig. 2b upper panel), and that of AsPC-1 cells were 1.5, 12.3, and 39.8% (Fig. 2b lower panel).

To further confirm cell apoptosis induced by DHA, we used the annexin V/PI method to stain the cells, and examined them under laser confocol microscopy. As shown in Fig. 2c (BxPC-3 cells) and Fig. 2e (AsPC-1 cells), only a small number of apoptotic cells were detected among the untreated cells, and most were at the early phase of apoptosis. The early-staged apoptotic cells were stained green by annexin V, as these cells display phosphatidylserine on their outer surface membranes, which is readily detectable by annexin V. However, among the DHA-treated cells, we detected more apoptotic cells, including late-staged apoptotic cells, which had their nuclei stained red by PI as plasma membrane becomes increasingly permeable during the later stage of apoptosis, allowing PI to move across the cell membrane and bind to DNA (Fig. 2d, BxPC-3 cells; Fig. 2f, AsPC-1 cells).

Next we used western blot analysis to examine the changes of apoptosis-related proteins in the two types of cells. DHA treatment upregulated the expression of Bax and downregulated the expression of Bcl-2, thus reduced the ratio of Bcl-2/Bax, and also increased the activation of caspase-9, in the two types of cells in a dose-dependent manner (Fig. 3a, BxPC-3 cells; Fig. 3a, AsPC-1 cells).

Fig. 4



Administration of dihydroartemisinin (DHA) inhibits tumor growth. BxPC-3 tumors were established subcutaneously in the flank of mice. When the tumors reached approximately 120 mm^3 in volume, the mice received daily injection of 100 μl PBS (control), or the equal volume of DHA at the doses of 2, 10, or 50 mg/kg, as indicated. The sizes (mm^3) of tumors were monitored and recorded. A significant difference in tumor volumes from control is denoted by '*', and a highly significant difference by '‡'.

Administration of dihydroartemisinin suppresses the growth of pancreatic tumors

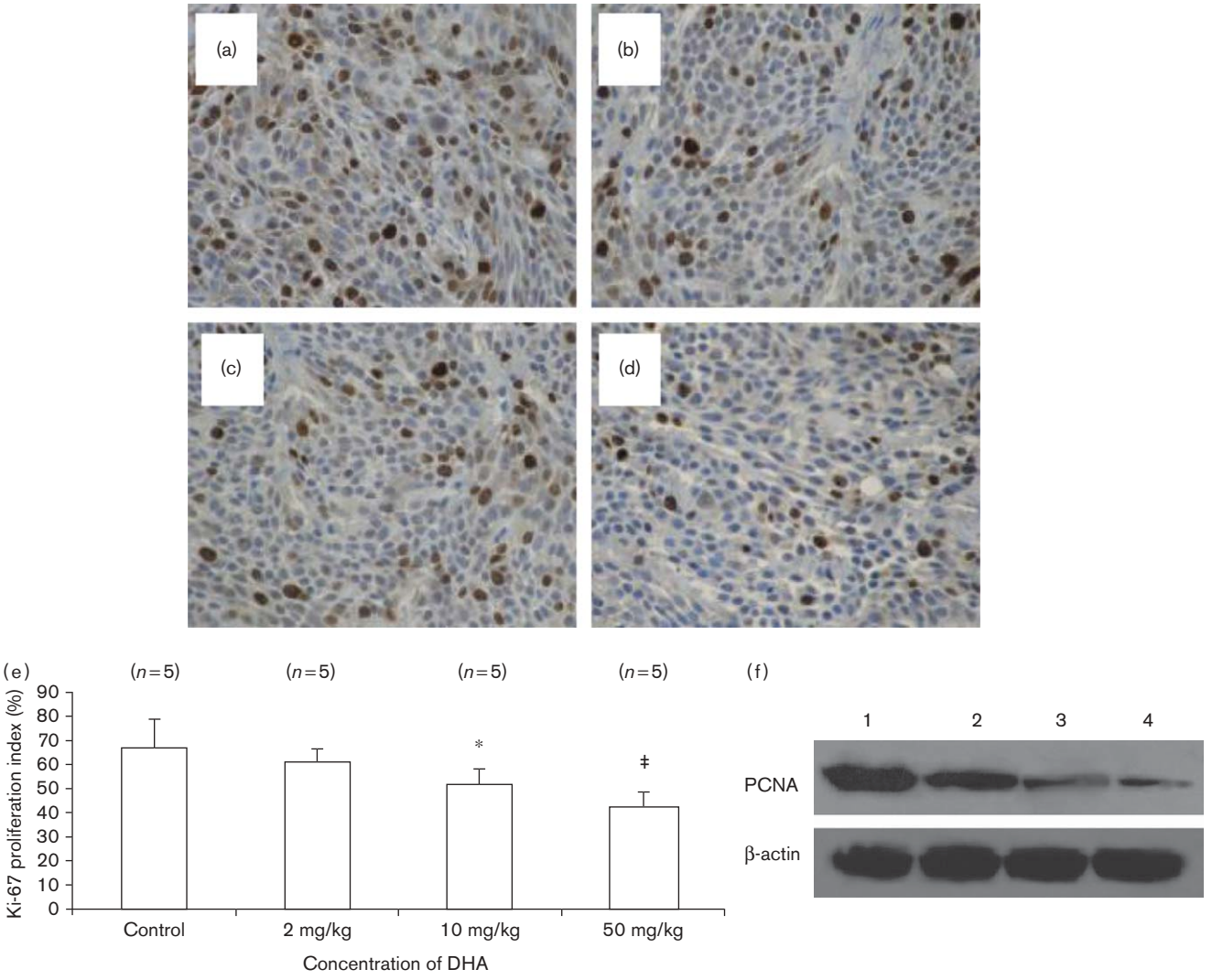
BxPC-3 tumors were established in mice. When the tumors reached a volume of around 120 mm^3, the mice were randomly assigned to four groups to receive injection of PBS (control), 2, 10, or 50 mg/kg DHA, respectively. As shown in Fig. 4, tumors in the control group grew remarkably quickly, reaching 685 ± 123 mm^3 in volume 18 days after injection. In contrast, the tumors

of mice treated with 2, 10, or 50 mg/kg DHA were significantly ($P < 0.01$) smaller than control tumors, reaching only 525 ± 86, 349 ± 43, and $188 \pm 37\,mm^3$ in volume, respectively, 18 days after treatment.

Dihydroartemisinin inhibits cell proliferation *in situ*

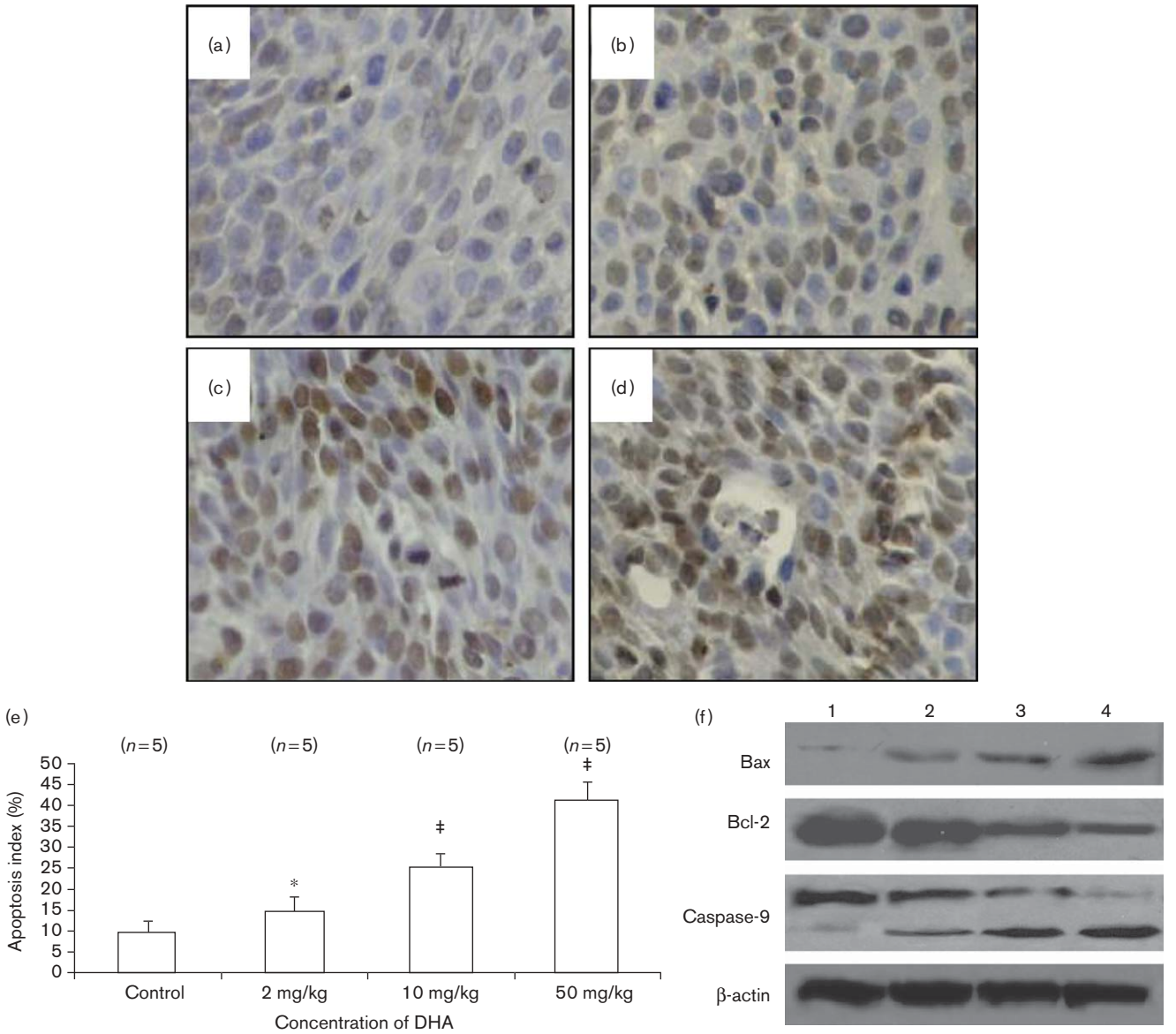
We further showed that DHA inhibited the proliferation of BxPC-3 cells *in situ*. Tumor sections from above were stained with an Ab, which detects the cell proliferation marker Ki-67. Fewer Ki-67 positive cells were observed in tumors in the three DHA treatment groups, compared with the control group (Fig. 5a). Additionally, the inhibitory effects on cell proliferation of DHA therapy displayed a dose-dependent manner, as there were fewer Ki-67 positive cells in the 10 mg/kg DHA group (Fig. 5c) than in the 2 mg/kg DHA group (Fig. 5b), and even fewer Ki-67 positive cells in the 50 mg/kg group (Fig. 5d) than in the 10 mg/kg group. Ki-67 positive cells in sections were counted to record the proliferation index. DHA therapy at the dose of 2 mg/kg resulted in a slight reduction in the proliferation index compared with control, but the difference did not reach significance. DHA therapy at the dose of 10 mg/kg reduced the proliferation index by 26% ($P < 0.05$), and at dose of 50 mg/kg, by 42% ($P < 0.001$), compared with control (Fig. 5e). We further detected tumoral expression of

Fig. 5



Administration of dihydroartemisinin (DHA) inhibits tumor cell proliferation *in situ*. Illustrated are representative tumor sections prepared from mice receiving daily injection of 100 μl PBS (control) (a), or the equal volume of DHA at the dose of 2 (b), 10(c), or 50 (d) mg/kg, as indicated in Fig. 3. Tumor sections were stained with an anti-Ki-67 Ab to detect proliferating cells. (e) Cells expressing Ki-67 were counted to calculate the proliferation index. A significant difference in the proliferation index from control is denoted by '*', and a highly significant difference by '‡'. (f) Tumors from mice receiving daily injection of 100 μl PBS (lane 1), or an equal volume of DHA at the doses of 2 (lane 2), 10 (lane 3), or 50 mg/kg (lane 4), were homogenized and subjected to western blot analysis to detect expression of proliferating cell nuclear antigen (PCNA). β-actin served as an internal control. *n*, number of tumors assessed.

Fig. 6



Administration of dihydroartemisinin (DHA) induces cell apoptosis. Illustrated are representative tumor sections prepared from mice receiving daily injection of 100 μl PBS (control) (a), or the equal volume of DHA at the dose of 2 (b), 10 (c), or 50 (d) mg/kg, as indicated in Fig. 3. Tumor sections were stained with the TUNEL agent to view apoptotic cells. (e) TUNEL-positive cells were counted to calculate the apoptosis index. A significant difference in the apoptosis index from control is denoted by '*', and a highly significant difference by '‡'. (f) Tumors from mice receiving daily injection of 100 μl PBS (lane 1), or an equal volume of DHA at the doses of 2 (lane 2), 10 (lane 3), or 50 (lane 4) mg/kg, were homogenized and subjected to western blot analysis to detect the expression of Bax, Bcl-2, and caspase-9. β-actin served as an internal control. *n*, number of tumors assessed.

PCNA, which was downregulated after DHA treatment in a dose-dependent manner, in accordance with the findings *in vitro* (Fig. 5f).

Dihydroartemisinin induces cell apoptosis *in situ*

Tumor sections from the above experiments were stained with the TUNEL agent and examined by microscopy. A small number of apoptotic cells were detected in tumors in the control group (Fig. 6a), whereas a greater number of apoptotic cells were detected in tumors in the DHA groups at doses of 2 (Fig. 6b), 10 (Fig. 6c), or 50 mg/kg (Fig. 6d). The apoptotic cells in sections were counted to record the apoptosis index. DHA therapy at the dose of 2 mg/kg resulted in a significant increase in the apoptosis index by 31% ($P < 0.05$), compared with control, and at the dose of 10 or 50 mg/kg significantly increased the apoptosis index by two- to three-fold ($P < 0.001$) (Fig. 6e). We further detected tumoral expression of apoptosis-related proteins

and found that DHA therapy upregulated the expression of Bax and downregulated the expression of Bcl-2, thus reduced the ratio of Bcl-2/Bax, and also increased the activation of caspase-9, in a dose-dependent manner, in accordance with the findings *in vitro* (Fig. 6f).

Discussion

After the discovery of artemisinin and its derivatives, especially DHA, as a novel and promising treatment for cancer, a large number of studies have investigated the use of DHA in the treatment of cancers [3–14,18], but the effect of DHA on pancreatic cancer is unknown, and most of those studies examined the antitumor activity in cultured cancer cells. This study has for the first time demonstrated that DHA exhibits antitumor activity in treating pancreatic cancer *in vitro* and *in vivo*. Our data suggest that DHA shows antiproliferative and proapoptotic activities by downregulating PCNA and cyclin D1, upregulating $p21^{WAF1/CIP1}$, reducing the ratio of Bcl-2/Bax, and increasing the activation of caspase-9.

Uncontrolled cell cycle progression and evasion of apoptosis are hallmarks of cancers [22], thus therapeutic drugs such as DHA, which can impede cell cycle progression and promote apoptosis simultaneously in cancer cells, are highly desired. DHA inhibited proliferation of BxPC-3 and AsPC-1 pancreatic cancer cells *in vitro* and *in vivo* by regulating cyclin D1, $p21^{WAF1/CIP1}$, and PCNA, which are involved in the uncontrollable proliferation of cancer cells [23]. PCNA is a 36 kDa, highly conserved nuclear protein required for DNA synthesis by DNA polymerases δ and ε. PCNA is synthesized in the early G_1 and S phases of the cell cycle. In the early S phase, PCNA has a very granular distribution and is absent from the nucleoli. In the late S phase, PCNA is prominent in the nucleoli. PCNA is also required for postreplicative DNA repair through interactions with MSH2 and MLH1 [24,25]. By downregulating PCNA expression, DHA could inhibit DNA synthesis and repair, thus inhibiting cell proliferation. Overexpression of cyclins provides a selective growth advantage to tumor cells [23,26]. Therefore, targeting cyclins that promote tumor progression is therapeutically relevant for treatment of cancer. D-type cyclins are required for cell cycle entry and early G_1 phase cell cycle progression [27], and inhibition of cyclin D1 specifically triggers G_1 phase cell cycle arrest [28]. Cyclin D1 is overexpressed in many human cancers, including pancreatic cancer [29]. Cyclin D1 forms a complex with either of its catalytic partners, cdk4 or cdk6, to promote G_1 cell cycle progression [26]. By inhibiting the expression of cyclin D1, DHA induce cells arrested in G_1 phase [28].

$p21^{WAF1/CIP1}$, an important mediator of cell proliferation, impedes cell cycle progression by inhibiting cyclin E-cdk2 complexes that promote G_1–S phase cell cycle progression [30]. p21 also inhibits DNA synthesis through inhibition of PCNA [31]. Dergham *et al.* [32] showed that pancreatic tumors expressing the $p21^{WAF1/CIP1}$ protein tend to have a longer survival than patients whose tumors are $p21^{WAF1/CIP1}$ negative, especially when treated with chemotherapy or radiation therapy. Transfection of $p21^{WAF1/CIP1}$ into pancreatic cancer cells induced G_0/G_1 cell cycle arrest [33]. In this study, DHA upregulated the expression of $p21^{WAF1/CIP1}$ in both BxPC-3 and AsPC-1 cells, indicating that $p21^{WAF1/CIP1}$ is involved in the inhibition of proliferation by DHA in pancreatic cancer. In contrast, p21 has recently been shown to be a proapoptotic protein in human bronchial epithelial cells [34], suggesting that p21 might be involved in DHA-induced cell apoptosis as well.

Cellular apoptosis can be triggered by the death-receptor-induced extrinsic pathway or the mitochondrial–apoptosome-mediated intrinsic pathway [35,36]. The released cytochrome c from mitochondria binds to Apaf-1, resulting in proteolytic processing and activation of procaspase-9. Active caspase-9 then directly activates procaspase-3, initiating a cascade of additional caspase activation that culminates in apoptosis [37]. Disbrow [38] has reported that DHA induced apoptosis of cervical cancer Hela cells by increasing activity of caspase-9, but had no effect on the activity of caspases-8, -10 and, -12, suggesting that DHA induces cell apoptosis through mitochondrial pathway. Chen [5], however, reported that DHA increased the activity of both caspase-8 and -9 in ovarian cancer cells in a dose-dependent manner, indicating that DHA induces cell apoptosis both through the death-receptor extrinsic and mitochondrial intrinsic pathways. This study has shown that DHA induced apoptosis of BxPC-3 and AsPC-1 cells by increasing the activation of caspase-9 in a dose-dependent manner, indicating that mitochondrial pathway may be involved in the apoptosis of pancreatic cancer cells induced by DHA. However, whether the death receptor pathway is also involved in DHA-induced apoptosis in pancreatic cancer cells is unknown and further investigation is required in the future.

The members of the Bcl-2 family are the most prominent regulators of apoptosis in a variety of cell types, including cancer cells [39–41]. A large number of anticancer drugs induce apoptosis through regulation of the Bcl-2 family [42]. Bcl-2, located on the membrane of mitochondria, is a proapoptotic protein, whereas Bax directly binds to Bcl-2 and inhibits its function [43,44]. Wang reported that exposure to DHA led to downregulation of Bcl-2 mRNA and upregulation of Bax mRNA in cultured murine lymphatic endothelial cells [19]. Similarly, Jiao [6] showed that DHA downregulated Bcl-2 and upregulated Bax at both the mRNA and protein levels in ovarian cancer cells, and knock-down of Bcl-2 increased the sensitivity of cells to DHA-induced apoptosis whereas knock-down of Bax decreased this sensitivity. Our data have shown that DHA-

induced apoptosis was accompanied by a decrease in Bcl-2 and a concomitant increase in Bax in pancreatic cancer cells. We propose that DHA induces apoptosis by regulating the expression of Bcl-2 and Bax, leading to the release of cytochrome *c* from the mitochondria to trigger the activation of downstream initiator caspase-9, resulting in the activation of the effector caspases, thereby inducing apoptosis. This study suggests that the DHA-induced apoptotic signal is passed from Bcl-2 family members to the mitochondria to induce caspase-9 activation.

In conclusion, this study, to our knowledge, is the first systemic one to investigate anticancer effects of DHA and the underlying mechanisms in pancreatic cancers *in vitro* and *in vivo*. Compared with conventional chemotherapeutic agents, DHA, a natural resource, is inexpensive and safe, and without serious adverse cytotoxicity [45]. Moreover, DHA has exhibited the potential to combat multidrug resistance in some tumors [46]. All these features have made DHA a promising and attractive anticancer agent in combating pancreatic cancer, although more preclinical trials are required before clinical application.

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