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DHA regulates angiogenesis and improves the efficiency of CDDP for the treatment of lung carcinoma

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ABSTRACT

Dihydroartemisinin (DHA), a semisynthetic derivative of artemisinin, has been shown to exhibit anti-angiogenic and anti-tumor effects apart from its antimalarial activity. In this study, we demonstrate that the combined treatment of cisplatin (CDDP) and DHA exerts a strong, synergistic anti-proliferative effect in human lung carcinoma cells, including A549 and A549/DDP cells, with an average combination index below 0.7. Moreover, the *in vivo* anti-tumor efficacy of CDDP treatment was increased by DHA. The enhanced anti-cancer activities were also accompanied by reduced tumor microvessel density, increased CDDP concentration within A549 and A549/DDP xenograft BALB/c athymic mice models and suppressed expression of the vascularization-related proteins HIF-1α and VEGF both *in vivo* and *in vitro*. Furthermore, the level of apoptosis in the tumor cells increased with the combined treatment of DHA and CDDP. Taken together, our results indicate that a combination of DHA and CDDP treatments synergistically affects tumor angiogenesis, and these results provide a clear rationale for the investigation of these drugs in future clinical trials.

Introduction

The tumor microenvironment has been recognized as a major factor that not only influences the response of a tumor to conventional anti-cancer therapies but also helps define the potential of a tumor for malignant progression and metastasis (Guise, 2010; Solyanik, 2010). In particular, hypoxia is now considered to be a fundamentally important characteristic of the tumor microenvironment. Hypoxia is common in tumors (Adrian, 2002), and hypoxia-inducible factor-1 alpha (HIF-1α), which mediates the adaptive response by controlling the expression of numerous hypoxia-inducible genes, is frequently overexpressed in tumors (Maynard and ohh, 2007). Several approaches have shown that the expression of vascular endothelial growth factor (VEGF) is induced by HIF-1α in response to hypoxia (Kallergi et al., 2009). VEGF is a potent endothelial cell mitogen that is very important in tumor angiogenesis (Chen et al., 2004; Folkman, 1995).

Anti-angiogenic drugs are considered to be generally capable of inhibiting vasculature formation as well as inducing hypoxia and malnutrition. Jain first proposed that anti-angiogenic therapies could normalize the tumor vasculature before causing its destruction (Jain, 2005). The transient normalization of the tumor vasculature produces a temporary increase in oxygen and nutrient delivery to the cancer cells that surround the "normalized" vessels. Therefore, when antiangiogenic drugs are given in combination with chemotherapeutic agents, a great increase in the therapeutic efficacy resulted. However, currently, the ideal chemotherapeutic agent or the best combination of agents that result in the strongest cytotoxicity in cancer cells with minimal effects on normal cells has not yet been developed.

Lung cancer is a leading cause of cancer-related deaths worldwide (Parkin et al., 2005). Non-small cell lung cancer (NSCLC) accounts for up to 80% of total pulmonary malignancies (Zhang et al., 2008). Approximately 16% of NSCLC patients are diagnosed with early stage or localized disease and are treated with surgical resection. Systemic chemotherapy is indicated in adjuvant treatment (Pignon et al., 2008) as well as in advanced stages of NSCLC. The most active chemotherapeutic agent for the treatment of NSCLC is cisplatin (CDDP) which is used in a doublet with other agents such as paclitaxel, gemcitabine and docetaxel (Schiller et al., 2002). But its use is limited by drug resistance and severe toxic side effects. In this study, we investigate how dihydroartemisinin (DHA) regulates angiogenesis and improves the efficiency of CDDP for the treatment of lung carcinoma.

Artemisinin is a sesquiterpene lactone endoperoxide found in the traditional Chinese medicinal plant *Artemisia annua*. The derivatives of artemisinin are used worldwide as anti-malarial drugs. DHA, a

Abbreviations: DHA, dihydroartemisinin; CDDP, cisplatin; HIF-1α, hypoxia-inducible factor-1 alpha; VEGF, vascular endothelial growth factor; NSCLC, non-small cell lung cancer; DMSO, dimethyl sulfoxide; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; ICP-MS, inductively coupled plasma mass spectrometry; CI, combination index.

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semi-synthetic derivative of artemisinin, is a more effective, water-soluble anti-malarial drug than artemisinin. Studies have shown that DHA inhibits cell proliferation, and induces apoptosis in various human cancer cell lines via downregulating cyclin (D1, E), Bcl-2, Bcl-xL, caspase (3, 9), and VEGF, while upregulating P21 and P27 (Chen et al., 2003, 2009; Fujita et al., 2008; Hou et al., 2008; Mu et al., 2008). Our recent studies also indicate that DHA inhibits cell growth, promotes apoptosis and improves the efficiency of chemotherapeutics in lung carcinomas *in vivo* (Li and Zhou, 2006; Zhou et al., 2010).

In this study, we examine the possibility of utilizing DHA alone or in combination with CDDP treatment as a lung cancer therapy and evaluate the underlying mechanisms of DHA that facilitate its efficacy against cancer.

Materials and methods

Materials

The dihydroartemisinin used in this study was a kind gift from Jiaxing Pharmaceutical Co. (Zhejiang, China). Cisplatin was obtained from Qilu Pharmaceutical General Factory, China. Penicillin, streptomycin, dimethyl sulfoxide (DMSO), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), ethidium bromide and propidium iodide were purchased from Sigma (St Louis, MO, USA). The polyvinylidene difluoride membranes were obtained from Millipore Co. (Billerica, MA, USA). The EliVision™ plus IHC Kit was obtained from Maixin Biological (Fuzhou, China). The rabbit anti-VEGF, mouse anti-HIF-1α, goat anti-actin (I-19) and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA).

Cell culture

The human non-small cell lung cancer (NSCLC) A549 cells were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), and the cisplatin-insensitive NSCLC A549/DDP cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The A549 and A549/DDP cells were both maintained in RPMI1640 standard medium supplemented with 10% fetal calf serum and antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin) in a humidified atmosphere with 5% CO_2/95% air (normoxia) at 37 °C. For the hypoxic conditions, the cells were incubated in a hypoxic incubator with a humidified atmosphere of 5% CO_2/1% O_2 balanced with N_2 (hypoxia) at 37 °C. Exponentially growing cells were used throughout the study.

Testing the antitumor effects on non-small cell lung cancer A549 and A549/DDP xenograft BALB/c athymic mice models

For the xenograft mouse models, 5- to 6-week-old BALB/c female mice were purchased from the National Rodent Laboratory Animal Resource (Shanghai, China). All of the animals were raised under specific pathogen-free conditions in the Animal Center of Zhejiang University according to the procedures outlined in the Guide for the Care and Use of Laboratory Animals. The A549 or A549/DDP cells were injected subcutaneouly (4×10^6 cells per mouse) into the right axillary region of 4 BALB/c nude mice. When the subcutaneous tumors reached approximately 1.5 cm in diameter, the mice were sacrificed, and small pieces of the tumor tissues (~1 mm^3) were implanted into the right axillary region of new recipient mice. The BALB/c nude mice were monitored regularly after the tumor-tissue implantation. When the solid tumor volumes reached 30 to 50 mm^3, the mice were randomly placed into 8 experimental groups of 8 mice each, and the administration of the two drugs was initiated. The eight experimental groups included mice that were administered with saline (control group), CDDP (2 mg · kg^{-1} · 3 $days^{-1}$, intraperitoneal injection), DHA (50, 100, or 200 mg · kg^{-1} · $days^{-1}$, intragastric administration), and CDDP + DHA (drug combination group). The CDDP was administered i.p. once every 3 days for 12 days (A549) or 28 days (A549/DDP). The tumor volumes were measured with calipers and calculated according to the following equation as previously described (Lee et al., 2006): 0.5 × (width) ^{2} × (length). The tumor volume at day n was expressed as the RTV according to the following formula: $RTV = TV_n/TV_0$, where TV_n is the tumor volume at day n and TV_0 is the tumor volume at day 0. The tumor growth inhibition rate (IR) was calculated using the following formula: $IR\ (\%) = (1 - TW_t/TW_c) \times 100$, where TW_t and TW_c are the mean tumor weights of the treated and control groups, respectively. The weight of the mice and the tumor volumes were recorded every 2 days until the animals were sacrificed at 12 days (A549) or 28 days (A549/DDP).

Determining the cisplatin concentration in the A549/DDP xenograft tumor and blood plasma of BALB/c athymic nude mice

The concentration of cisplatin in the tumors and blood was assayed using inductively coupled plasma mass spectrometry (ICP-MS). All of the reagents used were of analytical grade or higher. High purity deionized water was obtained from a Milli-Q system (Millipore; Molsheim, France) and used throughout this work. To determine the cisplatin concentration, the tumor tissue samples were dissolved in HNO_3 (Sinopharm Chemical Reagent Co. Ltd.; Shanghai, China), which was purified in a sub-boiling system before use. The method used for the determination of the cisplatin concentration was based on the method described by Brouwers et al. (2008).

Immunohistochemical analysis for the expression of angiogenic factors

The expression levels of HIF-1α and VEGF were determined using an immunohistochemical analysis of paraffin-embedded specimens fixed in 4% buffered formalin. Histological slides, 4 μm in thickness, were deparaffinized in xylol and then incubated with anti-HIF-1α or anti-VEGF monoclonal antibodies (1:50) overnight at 4 °C. The slides were then washed three times with PBS, incubated with an HRP-labeled polymer-conjugated secondary antibody, treated with DAB as a substrate, rinsed in distilled water and counterstained with hematoxylin.

The quantitation of the vessel density and pericyte coverage

The tumor vessel density was measured as described previously (Sun et al., 2001). Briefly, the tumor sections were immunostained with an anti-CD31 antibody. The stained blood vessels were counted in five blindly chosen, random fields at ×40 magnification, and the mean of the highest three counts was calculated.

NG2, one of the proteoglycans in vessel pericytes, was detected by immunofluorescence. Cryosections (10 μm) from tumors were equilibrated in PBS three times for 5 min. Then, the nonspecific-binding sites were blocked using a serum-free protein block for 10 min at room temperature. Next, the sections were incubated in PBS

Fig. 1. The synergistic effect of DHA and CDDP treatment on A549 and A549/DDP xenograft models. The mice transplanted with A549 or A549/DDP xenografts were randomly divided into 8 groups. (I) In A549 xenograft model, after 7 days, DHA was administered i.g. daily at 50 (DHALD), 100 (DHAMD), or 200 (DHAHD) mg/kg, and CDDP was administered i.p. every third day for 5 consecutive times at 2 mg/kg. (II) In A549/DDP xenograft model, after 10 days, DHA was administered i.g. daily at 50 (DHALD), 100 (DHAMD), or 200 (DHAHD) mg/kg, and CDDP was administered i.p. every third day for 10 consecutive times at 2 mg/kg. The data are expressed as the mean ± SD. * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$ compared to the control group. # = $p < 0.05$ and ## = $p < 0.01$ compared to the CDDP-treated group. ▲ = $p < 0.05$ and ▲▲ = $p < 0.01$ compared to the DHA treated group.

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