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Potent proapoptotic actions of dihydroartemisinin in gemcitabine-resistant A549 cells

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ABSTRACT

Our recent studies have demonstrated the key roles of reactive oxygen species (ROS)-mediated caspase-8- and Bax-dependent apoptotic pathways in dihydroartemisinin (DHA)-induced apoptosis of A549 cells. This report is designed to investigate the proapoptotic mechanisms of DHA in gemcitabine (Gem)-resistant A549 (A549GR) cells. A549GR cells exhibited lower basal antioxidant capacity, higher level of basal ROS and intracellular Fe^{2+} than Gem-sensitive A549 (A549) cells. In contrast to the sluggish ROS generation induced by Gem, DHA induced a rapid ROS generation within 30 min. Moreover, Gem induced similar ROS generation in both cell lines, while DHA induced more ROS generation in A549GR cells than in A549 cells. More importantly, after treatment with DHA, A549GR cells showed more potent induction in Bax activation, loss of mitochondrial membrane potential ($\Delta\Psi m$), caspase activation and apoptosis than A549 cells. Furthermore, NAC pretreatment potently prevented DHA-induced ROS generation and loss of $\Delta\Psi m$ as well as apoptosis, and silencing Bax by shRNA or inhibition of one of caspase-3, -8 and -9 also significantly prevented DHA-induced apoptosis in both cell lines, indicating the key roles of ROS and Bax as well as the caspases. Collectively, DHA presents more potent proapoptotic actions in A549GR cells preferentially over normal A549 cells via ROS-dependent apoptotic pathway, in which Bax and caspases are involved.

1. Introduction

Gemcitabine (Gem) is one of the most important chemotherapeutic drugs for therapy of advanced human lung cancer [1,2]. Its major action mechanisms include DNA chain termination, cell cycle arrest and apoptosis [3,4]. Although Gem does provide an improvement in the quality of life, side effects and acquired resistance seriously limit its clinical efficacy [4–7]. Mechanisms of resistance to Gem involve resistance-related proteins like P-glycoprotein, multidrug resistance related protein, glutathione-dependent enzymes, topoisomerases and thymidylate synthase [8]. However, some unclear mechanisms have been reported to contribute to Gem resistance [7–10]. In fact, resistance to chemotherapy drugs such as cisplatin, Gem and gefitinib is a crucial aspect in anticancer therapy [9,11–15]. Currently, exploring or developing novel drugs with slight effects that can be used alone or in combination with chemotherapeutic drugs to intensely induce apoptosis for drug-resistant cancer cells remains a major clinical challenge [16,17].

Artemisinin (ART) and its derivatives (ARTs) such as dihydroartemisinin (DHA) and artesunate (ARTS), the most effective antimalaria drugs, have been demonstrated to have potent anticancer activity without significant side effects [18–27]. Notably, ARTs were also reported to be effective in many refractory or drug-resistant cancer cell lines [22,23,28,29], which is similar with the intense action of ARTs against multidrug-resistant *Plasmodium falciparum* and *Plasmodium vivax* strains [30] and against ganciclovir-resistant human cytomegaloviruses [31]. Modulation of multidrug resistance by ARTS and DHA has been studied in a number of drug-resistant cancer cell lines [21–23,32]. Drug-resistant cell lines with high expression of MDR1, MRP1 and ABC transporters or p53 mutation do not show potent resistance to ARTs [20,28,29,32]. DHA can increase the efficacy of Gem against human hepatoma cells [20] and pancreatic cancer [17] with minimal effects on normal cells, and ARTS does not induce cross-resistance in multidrug-resistant cells [23,29,33].

Our recent studies have demonstrated that the reactive oxygen species (ROS)-mediated caspase-8- and Bax-dependent apoptotic pathways play an important role in DHA-induced apoptosis of both ASTC-a-1 and A549 cell lines, two non-small-cell lung cancer (NSCLC) cell lines [34–37]. Our results also demonstrate potent synergistic efficacy of the

Abbreviations: A549GR cells, gemcitabine-resistant A549 cells; ART, artemisinin; ARTs, artemisinin derivatives; ARTS, artesunate; CCK-8, cell counting kit-8; DHA, dihydroartemisinin; FCM, flow cytometry; FITC, fluorescein isothiocyanate; Gem, gemcitabine; NAC, N-acetylcysteine; NSCLC, non-small-cell lung cancer; PS, phosphatidylserine; Rho 123, rhodamine 123; ROS, reactive oxygen species; DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; shRNA, short hairpin RNA; STS, staurosporine; T-AOC, total antioxidant capacity; $\Delta\Psi m$, mitochondrial membrane potential.

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combined treatment with DHA and low-dose ionizing radiation (IR) for A549 cells in which IR potentiates DHA-induced apoptosis largely by enhancing the caspase-8-mediated extrinsic pathway [35]. In addition, although the Bax-mediated intrinsic pathway and the Fas-caspase-8-mediated extrinsic pathway are involved in the DHA-induced apoptosis of A549 cells, the Bak-mediated intrinsic pathway plays key role in the proapoptotic synergy of the combined treatment with Gem and DHA in A549 cells [34].

This report aims to investigate the proapoptotic mechanisms of DHA in Gem-resistant A549 (A549GR) cells. Our study indicates that A549GR cells have lower basal antioxidant capacity and higher intracellular Fe^{2+} than Gem-sensitive A549 (A549) cells, which may be associated with the higher level of both endogenous basal ROS and exogenous ROS decomposed from DHA in A549GR cells. Moreover, our data demonstrate that the ROS-mediated Bax- and caspase-dependent apoptosis pathways are involved in the more potent proapoptotic actions of DHA in A549GR cells.

2. Materials and methods

2.1. Agents

DHA was obtained from Holley-Cotec (Chongqing, China). Working solutions were prepared by dissolving the compound in dimethyl sulphoxide (DMSO, Sigma) before experiments. The final concentration of DMSO was less than 1% in all experiments. Gem was purchased from Eli Lilly Pharmaceuticals (Indianapolis, IN). Working solutions were prepared by dissolving the compound in PBS before experiments. Staurosporine (STS) was purchased from Alexis Biochemicals (Switzerland). N-acetylcysteine (NAC), propidium iodide (PI), RNase A and Rhodamine 123 (Rho 123) were obtained from Sigma (St. Louis, USA). Caspase-3, -8 and -9 inhibitors zDQMD-fmk, zIETD-fmk and zLEHD-fmk were purchased from Merck-Calbiochem (USA). Cell lysis buffer (Cat. No. P0013) was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Primary antibodies of Bak, Bax, and β-actin were from Cell Signaling Technology (Beverly, MA, USA). Mouse monoclonal anti-Bak (Ab-2) and anti-Bax (Ab-6) were purchased from Calbiochem (San Diego, CA). Secondary goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) antibody was purchased from Invitrogen (eBioscience).

2.2. Cell culture

A549 cell line was purchased from Cell Bank of Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (CAS, Shanghai, China). A549GR and ASTC-a-1 cell line were gifts by the Department of Medicine, Jinan University (Guangzhou, China). 95D and H446 cell lines were obtained from the Experimental Animal Center, Sun Yat-sen University (Guangzhou, China). A549, A549GR, ASTC-a-1 and H446 cell lines were cultured in DMEM and 95D cells were propagated in RPMI 1640. All cells were supplemented with 10% fetal calf serum at 37 °C with a humidified 5% CO_2 incubator. DMEM and RPMI 1640 medium were purchased from Gibco (Grand Island, USA).

2.3. Cell viability and apoptosis assay

Cell viability was assessed by Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. Cell apoptosis detection was performed by flow cytometry (FCM) analysis using Annexin V-FITC/PI apoptosis detection kit (Bender MedSystems, Vienna, Austria) as previously described [36] and for FCM analysis, 10,000 events were recorded in each sample. Apoptotic cells are those stained with Annexin V-$FITC^+/PI^-$ (early apoptotic cells) and Annexin V-$FITC^+/PI^+$ (late apoptotic cells).

2.4. Transfection and RNA interference

For silencing experiments, cells were seeded at 1×10^5 cells per well in 6-well plates and cultured overnight to reach ~50% confluence on the day of transfection. Then the cells were transiently transfected with constructs containing short hairpin RNA (shRNA) for Bak, Bax, or negative control shNC, respectively, using HilyMax (Dojindo, Kumamoto, Japan) according to the manufacturer's recommendations. Twenty-four hours after transfection, the cells were split into 96-well plates for cell viability assay. The constructs containing shRNA were purchased from GenePharma (Shanghai, China) and the efficiency of shRNA was measured by Western blotting analysis as described previously [34,38]. The target sequences of shRNAs were synthesized as follows: shBax: 5′-GGGACGAACTGGAC AGTAACATTCAAGAGATGTTACTGTCCA GTTCGTCCCTT-3′; shBak: 5′-GCCTGTTTGAGAGTG GCATCATTCAAGAG ATGATGCCACTCTCAAACAG-GCTT-3′; shNC: 5′-GTTCTCCGAACGTGT CACGTCAAGAGATTACGTGACACGTTCGGAG AATT-3′.

2.5. Measurement of mitochondrial membrane potential (ΔΨm)

Rho 123, a potential-sensitive dye, was used to evaluate changes in ΔΨm by FCM assay as previously described [38]. Results were expressed as the proportion of cells with lost or low ΔΨm which was estimated by reduced fluorescence intensity from Rho 123.

2.6. Cell cycle analysis

The proportions of cells in Sub-G_1 (apoptosis), G_0/G_1, G_2/M and S phases were determined by FCM analysis of DNA content as described previously [35]. To evaluate cell cycle profile, the cells (about 1×10^6 cells) were harvested, washed twice with PBS and fixed in ice-cold 70% (v/v) ethanol for 4 h at 4 °C. Prior to analysis, the samples were washed again and incubated in PBS containing 10 μg/ml RNase A for 30 min, and then incubated with 5 μg/ml PI at 37 °C in the dark for 30 min. Data were analyzed by ModFit LT software. For each analysis, 10,000 events were recorded.

2.7. FCM analysis of the activation of Bak and Bax

The cells were grown on 6-well dishes and treated under different conditions. Briefly, the cells were harvested and fixed with 4% formaldehyde in PBS for 10 min at 37 °C. Afterwards, the cells were treated with ice-cold 100% methanol for 10 min to permeabilize the cells. Fixed cells were blocked in PBS solution containing 1% bovine serum for 10 min at room temperature and then were incubated with either anti-Bax (6A7) or anti-Bak (Ab-2) (1:50) at room temperature for 1 h and then incubated with FITC-conjugated goat-anti-mouse IgG (1:200) for 30 min in the dark. After washing, the samples were analyzed by FCM. The results for each condition were calibrated by values for cells stained with mouse IgG as the primary antibody. Values for untreated controls were normalized to 100%. In parallel, the cells for each condition were stained with antibodies to total Bax or Bak for comparison.

2.8. Fluorometric determination of caspase enzymatic activation

Activities of caspase-3, -8 and -9 were measured using Ac-DEVD-AFC, Ac-IETD-AFC and Ac-LEHD-AFC (Alexis, Switzerland), respectively, according to the manufacturer's instructions as described previously [18]. The cells were harvested in caspase lysis buffer. Caspase activity was assessed by monitoring the release of fluorogenic AFC using an auto microplate reader (Infinite M200, Tecan, Austria). Caspase-like activity is reported as the ratio of the fluorescence output in treated samples relative to controls.