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Cisplatin and photodynamic therapy exert synergistic inhibitory effects on small-cell lung cancer cell viability and xenograft tumor growth

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ABSTRACT

Lung cancer is the leading cause of cancer death worldwide. Small-cell lung cancer (SCLC) is an aggressive type of lung cancer that shows an overall 5-year survival rate below 10%. Although chemotherapy using cisplatin has been proven effective in SCLC treatment, conventional dose of cisplatin causes adverse side effects. Photodynamic therapy, a form of non-ionizing radiation therapy, is increasingly used alone or in combination with other therapeutics in cancer treatment. Herein, we aimed to address whether low dose cisplatin combination with PDT can effectively induce SCLC cell death by using in vitro cultured human SCLC NCI-H446 cells and *in vivo* tumor xenograft model. We found that both cisplatin and PDT showed dose-dependent cytotoxic effects in NCI-H446 cells. Importantly, co-treatment with low dose cisplatin (1 μM) and PDT (1.25 J/cm^2) synergistically inhibited cell viability and cell migration. We further showed that the combined therapy induced a higher level of intracellular ROS in cultured NCI-H446 cells. Moreover, the synergistic effect by cisplatin and PDT was recapitulated in tumor xenograft as revealed by a more robust increase in the staining of TUNEL (a marker of cell death) and decrease in tumor volume. Taken together, our findings suggest that low dose cisplatin combination with PDT can be an effective therapeutic modality in the treatment of SCLC patients.

1. Introduction

Lung cancer remains the most common malignancy in the world with an estimated 1.6 million new cases per annum, and is the leading cause of cancer-related death [1]. Small-cell lung cancer (SCLC) accounts for 10–15% of all lung cancer cases [2], but compared to non-small lung cancer (NSCLC) SCLC is poorly differentiated and show a rapid growth rate, strong aggressiveness, early metastases, and poor prognosis with an overall 5-year survival rate below 10% [3]. SCLC patients are optimally treated with combination chemotherapy with thoracic radiation. Cyclophophamide was the first cytotoxic chemotherapy agent used in the treatment of SCLC patients [4]. Cisplatin is an evolving type of cytotoxic agent that has been commonly used in the treatment of limited-stage SCLC patients as well as in the first-line chemotherapy for extensive-stage SCLC patients [5,6]. The cytotoxic effect of cisplatin is multifold. It was initially thought that cisplatin induces the generation of nuclear DNA adducts, which causes cell death as a result of DNA replication and transcription blockage [7]. It was later found that cisplatin induces endoplasmic reticulum (ER) stress [8,9]. Recent studies further demonstrate that cisplatin causes mitochondrial dysfunction and subsequent production of reactive oxygen species (ROS) [10,11]. Conversely, despite of the effectiveness of cisplatin in chemotherapy treatment, clinical evidence has shown severe adverse effects [12]. Therefore, the use of an alternative treatment modality in combination with low-dose cisplatin, which renders less toxicity, may potentially be effective in the treatment of SCLC patients.

Photodynamic therapy (PDT) is a form of non-ionizing radiation therapy that exerts anti-cancer activity through the use of a

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photosensitizer and illumination [13]. Following induction by light, photosensitizer in tumor cells produces reactive singlet oxygen and ROS, which causes cellular cytotoxicity that eventually leads to tumor cell apoptosis and/or necrosis [13,14]. PDT is an emerging treatment modality that is increasingly used to treat thoracic malignancies, including lung cancer [14,15]. So far, PDT is primarily utilized in patients with early stage NSCLC. PDT can also be employed to palliate obstructing endobronchial lesions or to reduce the extent of operation in patients with advanced-stage NSCLC. In contrast, SCLC has a high propensity for distant metastatic spread, causing the use of PDT as curative modality problematic. Nevertheless, PDT can be considered for palliation of symptomatic patients with SCLC who show progressive diseases following standard treatments, such as chemotherapy [16,17]. In the present study, we evaluated the effects of cisplatin-based chemotherapy in combination with PDT on SCLC cell viability and xenograft tumor growth in nude mouse model. We demonstrated that PDT and cisplatin play synergistic roles in promoting SCLC cell death probably dependent on ROS production.

2. Materials and methods

2.1. Cell culture

Human small cell lung cancer NCI-H446 cell line was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences. Cells were cultured in RPMI 1640 medium (HyClone, South Logan, UT) supplemented with 10% fetal bovine serum (Gibco, BRL) and 1% streptomycin/penicillin (Gibco, BRL) in a 5% CO_2 humidified incubator at 37 °C.

2.2. Cell viability assay

Cell viability was determined by using the Cell Counting Kit-8 (CCK-8, JOJINDO, Japan) according to the manufacture's instruction. NCI-H446 cells were seeded in 96-well plate at the density of 10^4 cells/well for 24 h, followed by the indicated treatments for 24 h. Ten μl CCK-8 solution was added to the culture medium of each well for 2 h, and the 450-nm absorbance was measured with Infinite® M200 PRO (Tecan, Switzerland).

2.3. PDT treatment of cultured cells

PDT treatment of cultured cells in the presence of photosensitizer molecule EtNBS-COOH (gift of Dr. Xiujun Fu, Shanghai Jiaotong University) has been described in a previous study [18]. In brief, NCI-H446 cells, cultured for 24 h in 96-well plate, were treated with 2 μM EtNBS-COOH for 5 min prior to illumination with 640 ± 10 LED light at the density of 25 mW/cm^2 for 0, 50, 100, 200, 300 or 400 s. Cytotoxicity was determined by using CCK-8 method as the above described.

2.4. Cell migration assay

NCI-H446 cells grown to 80% confluence were treated with or without 1 μM cisplatin (Qilu Pharmaceutical, China) for 24 h. Cells were then incubated with 2 μM EtNBS-COOH for 5 min, followed by PDT treatment for 50 s to evaluate the combined effect of cisplatin and PDT. To determine the rate of cell migration, a lesion was created on the monolayer cells and changes in the distance between the lesion edges area were monitored at 12 h and 24 h. The migration rate (%) is presented as the distance that cells migrate *vs.* the original distance of the lesion.

2.5. Propidium iodide (PI) staining

PI staining was performed to assess cell death. Briefly, cultured NCI-H446 cells at 80% confluence, rinsed with phosphate-buffered saline (PBS), were incubated with 5 μg/ml Hoechst 33258 and 15 μg/ml PI for 10 min at 37 °C. Cells were washed with PBS for three times, and the staining result was examined under Eclipse Ti-S (Nikon, Japan) fluorescence microscope.

2.6. Reactive oxygen species (ROS) detection

Intracellular ROS level was determined by using 2′,7'-dichlorodihydrofluorescin diacetate (H_2DCF-DA, ThermoFisher, USA). Cells were treated with cisplatin and/or PDT, rinsed with RPMI 1640 medium, and then incubated with 10 μM H_2DCF-DA for 20 min at 37 °C. After three washes with RPMI 1640 medium, fluorescence signal was examined at excitation wavelength of 488 nm.

2.7. Animal treatment

NCI-H446 cells (10^7 cells in 100 μl PBS) were injected subcutaneously into the back of a nude mouse (SLRC Laboratory Animal Company, Shanghai, China). Cisplatin and PDT were given when tumor size reached approximately 1-cm diameter. Cisplapin was injected intravenously at the dose of 5 mg/kg body weight (i.v.). For the combined treatment group, 24 h later, EtNBS-COOH (2 μM in 200 μl) was injected into the tumor and mice were subjected to PDT for 10 min at the rate of 25 mW/cm^2 (15 J/cm^2 in total). The illumination power density inside the tumor was monitored by FieldMaster power meter (Coherent Inc., CA). The size of each tumor was measured on Day 1, 3, 7, 10 and 14, and the volume of the tumor was calculated by the following formula: Volume = π x ½ long diameter x short diameter x thickness [19]. A subgroup of the mice were sacrificed on Day 7, and tumors were isolated for histological examination.

2.8. Hematoxylin and eosin (H&E) and TUNEL staining

Isolated tumors were fixed in 4% paraformaldehyde, paraffin embedded, and processed as 5-μm thick sections. Paraffin sections were deparaffinized and hydrated prior to H&E staining. In situ cell death assay was performed by using a TUNEL kit (Beyotime, China) according to the manufacture's instruction. Briefly, sections were incubated with fluorescein-labelled dUTP solution, followed by incubation with an HRP-labeled anti-fluorescein antibody. Tissues were subjected to DAB substrate reaction, mounted, and death signal was examined under microscope.

2.9. Statistical analysis

Graphics were created by GraphPad Prism (GraphPad Software, USA). Counting of PI staining signal was done by Image J (National Institute of Health, USA). All results are presented as mean ± SD. Statistical significance was determined by unpaired two-tailed t-tests or two-way analysis of variance. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Dose-dependent effects of cisplatin and PDT on cell viability

We first assessed the dose-dependent cytotoxic effects of cisplatin and PDT on NCI-H446 cells. Cells were treated with cisplatin for 24 h, and cell viability was determined by using CCK-8. As shown in Fig. 1A, a slight but significant decrease (80.77 ± 5.49%

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