Ultrasonics Sonochemistry 20 (2013) 667–673

Contents lists available at SciVerse ScienceDirect

Ultrasonics Sonochemistry

journal homepage: www.elsevier.com/locate/ultson

The tumor affinity of chlorin e6 and its sonodynamic effects on non-small cell lung cancer

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ARTICLE INFO

Article history:
Received 1 July 2012
Received in revised form 22 September 2012
Accepted 23 September 2012
Available online 2 October 2012

Keywords:
Sonodynamic therapy
Chlorin e6
Tumor affinity
Human lung adenocarcinoma cell
SPCA-1
Non-small cell lung cancer

ABSTRACT

Objective: Sonodynamic therapy (SDT) is a promising new approach for cancer therapy. The aim of this study was to investigate the tumor affinity of chlorin e6, a photosensitizer, and its sonodynamic effects on NSCLC.

Methods: Human lung adenocarcinoma cells SPCA-1 and mice bearing SPCA-1 tumor xenograft were exposed to ultrasound in the presence or absence of chlorin e6. Chlorin e6 distribution was detected by laser scan confocal microscope. Cell apoptosis and necrosis were studied by flow cytometry analysis. Tumor size and weight were measured after different treatments.

Results: The concentration of chlorin e6 in tumor tissue was remarkably higher than that in normal muscle near tumor, and the difference was greatest at 18 h (the fluorescence intensity was 5.38-fold higher in tumor than in muscle, $P < 0.05$). *In vivo*, ultrasound (0.4–1.6 W/cm^2) or chlorin e6 (10–40 mg/kg) alone had no remarkable anti-tumor effects, but the combination of ultrasound (1.6 W/cm^2) with chlorin e6 (SDT) hampered tumor growth significantly ($P < 0.05$). Intraperitoneal injection of 40 mg/kg chlorin e6 exerted no notable side effect on blood, liver and kidney function. Flow cytometry analysis showed that chlorin e6-mediated sonodynamic effect was mainly through the induction of cell necrosis.

Conclusion: Chlorin e6 is a promising sonosensitizer and chlorin e6-mediated SDT may provide a new approach for NSCLC therapy.

1. Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer death in the world [1]. 70% of patients with NSCLC present locally advanced or distant metastatic diseases when they are first diagnosed. Platinum-based combination chemotherapy is the first-line therapy for advanced NSCLC patients with wide-type epidermal growth factor receptor (EGFR) or those patients with unknown EGFR state. However, these combination chemotherapies have reached a therapeutic plateau, and the side effects were somewhat inevitable and remarkable. Although targeted therapies inhibiting EGFR have been introduced in the treatment of NSCLC, the therapeutic efficacy greatly depends on gene mutation and aberrant expression of EGFR, with a median resistance time of 8–10 months [2]. Therefore, seeking new therapeutic approaches with higher efficiency and lower side effect is becoming a great challenge in front of us.

Sonodynamic therapy (SDT), which is developed from photodynamic therapy (PDT), is a promising new approach for cancer treatment. It functions by activating tumor-localizing sonosensitizer such as porphyrins through acoustic cavitation by ultrasound [3,4]. The activation of sonosensitizers leads to conversion of molecular oxygen to various highly reactive oxygen species (ROS), which causes irreversible damages of tumor cells directly or damages the tumor associated vasculature [5,6]. Comparing with the laser light or microwave used in PDT, ultrasound has an appropriate tissue attenuation coefficient that allows it to reach internal targets without losing the ability to focus energy into small volumes [7–9]. This unique advantage makes SDT a more promising approach in non-invasive treatment of non-superficial tumors. Even though lots of pre-clinical studies showed that SDT could exert selective anti-tumor effects [10–13], the clinical application is limited based on the reason that sonosensitizers adopted before such as haematoporphyrins (Hp) and haematoporphyrin derivatives (HpD) have relatively low tumor affinity and long clearance time, which may cause prolonged photosensitivity and toxicity in normal tissues [14,15]. To solve these problems, quite a few investigations have been carried out with respect to the identification of new sonosensitizers with more preferential uptake in tumor

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http://dx.doi.org/10.1016/j.ultsonch.2012.09.008

and rapid clearance in normal tissues. However, up to now, no such ideal sensitizer has been developed.

It has been well established that Mono-1-aspartyl chlorin e6, a chlorophyll derivative, is a photosensitizer with high tumor affinity and clear quickly from normal tissues, which make it have lower phototoxic side effects [16–19]. The photodynamic therapy of chlorin e6 has been used in clinical (phaseIclinical trial) for the treatment of melanoma [20]. As most photosensitizers exhibited sonochemical activity [20,21], chlorin e6 is also found recently to be sonosensitive in leukemia cells. It can be activated by ultrasound to induce apoptosis as well as necrosis in human promyelocytic leukemia HL-60 cells [21–23]. Previously in our lab, chlorin e6 was used for the first time as a sonosensitizer for SDT of NSCLC *in vitro*. Our results showed that chlorin e6 combined with ultrasound significantly inhibited the proliferation of human lung adenocarcinoma cells SPCA-1 in a dose-dependent manner, while had little toxicity on normal peripheral mononuclear cells (PMNC) [24]. However, the *in vivo* sonodynamic effect of chlorin e6 on animal model is still unknown. So, the first aim of this study is to investigate the tumor affinity of chlorin e6. The second aim is to study the sonodynamic effect of chlorin e6 *in vivo* and its possible cellular mechanism.

2. Material and methods

2.1. Chemicals

RPMI-1640 culture medium and bovine serum were from Gibco (NY, USA). The ultrasonic exposure machine was from Angel Ltd. (Beijing, China). Chlorin e6 was from Scientific Co. Ltd. (Salt Lake, UT, USA). The absorbance spectra of chlorin e6 were detected and the prominent absorption bands located around 400 and 653 nm were observed, which was consistent with other literatures [25].

2.2. Cell cultures

Human lung adenocarcinoma cell SPCA-1 was kindly given by Institute of Biochemistry and Cell Biology (Shanghai, China). The study was approved by the ethics committee and review board of our hospital. Cells were cultured in RPMI-1640 medium supplemented with 10% bovine serum (Gibco, Grand Island, NY, USA) in a humidified atmosphere (Forma Scientific, Marietta, OH, USA) containing 5% CO_2 at 37 °C as previously described [26].

2.3. Sonodynamic treatment in SPCA-1 cells

The apparatus for *in vitro* ultrasonic exposure is shown schematically in Fig. 1A. An ultrasound transducer with a focal length of 2 cm was submerged in the bottom of a glass container filled with cold degassed water. The polystyrene sample test tube containing 0.5 ml SPCA-1 cell suspension (2.5×10^5 cells/ml) was placed into the focal area of the transducer for insonation. Cells were divided into 4 groups and treated as follows (Fig. 1B): ① Ultrasound group (U): cells were treated with 1.0 MHz × 1.0 W/cm^2 ultrasound for 60 s. ② Chlorin e6 group (E6): cells were incubated with 0.2 mg/ml chlorin e6 for 45 min. ③ SDT group (U + E6): cells were incubated with 0.2 mg/ml chlorin e6 for 45 min and then subjected to 1.0 MHz × 1.0 W/cm^2 ultrasound for 60 s. ④ Control group (C): cells were kept in the same condition with test groups without chlorin e6 or ultrasound insonation.

2.4. Flow cytometry analysis

Freshly prepared SPCA-1 cells were transferred to cryopreservation tubes. Each group consisted of 3 tubes. After treatments, cells were washed twice with PBS and then fixed with 70% alcohol at 4 °C overnight. After centrifugation at 1000 rpm for 5 min, cells were incubated with propidium iodide (Sigma–Aldrich, St. Louis, MO, USA) at 4 °C for 30 min in the dark before being subjected to a flow cytometer (FACScan, Becton Dickinson, Mountain View, CA). Cell cycle was analyzed using Multicycle-DNA Cell Cycle Analyzed software.

Apoptosis was assessed by detecting membrane redistribution of phosphatidylserine with fluorescent Annexin V. Cells were treated as previously described and then washed twice with PBS and resuspended in 500 μl of staining solution contatining fluorescein isothiocyanate (FITC)-conjugated annexin V antibody (5 μl, BD PharMingen, San Diego, CA) and 5 μl propidium iodide. After 5 min of incubation at room temperature in dark, cells were immediately analyzed in a flow cytometer (FACScan, Becton Dickinson, Mountain View, CA) using CellQuest software. The percentages of apoptotic cells and necrotic cells were determined by three independent experiments. Cells in quadrant 4 were identified as apoptotic cells, and cells in quadrant 2 were necrotic cells.

2.5. Animal model

Four weeks old Kunming (KM) female mice weighing 18–20 g were obtained from Nanfang Medical University and were maintained in the Experimental Animal Center of our hospital. A total of 0.2 ml SPCA-1 cell suspension (10^7 cells/ml) was injected subcutaneously in the right upper flank of each mouse. All of the studies were performed according to the international guidelines for animal care.

2.6. Chlorin e6 distribution analysis

When tumors reached approximately 6 mm in diameter, 35 mice were intraperitoneally injected with chlorin e6 (10 mg/kg) and then randomly divided into 7 groups (5 in each group). Mice of different groups were raised away from light and were killed 3 h, 6 h, 12 h, 18 h, 24 h, 48 h and 72 h respectively after the injection. Tumor and muscle tissues were taken and frozen sections were prepared. The fluorescence intensity of chlorin e6 in different tissues at different time points was determined by fluorescence confocal microscopy (LSM 710, ZEISS, Germany) at an excitation wavelength of 400 nm and an emission wavelength of 653 nm.

2.7. Safety analysis

When tumors reached approximately 6 mm in diameter, 42 mice were randomly divided into 2 groups (21 in each group) and were intraperitoneally injected with 40 mg/kg chlorin e6 or PBS. Mice were raised away from light and blood samples were taken from the eyeball 3 h, 6 h, 12 h, 18 h, 24 h, 48 h and 72 h after the injection, respectively. Levels of RBC, WBC, hematoglobin (Hb), platelet (PLT), direct bilirubin (DBIL), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (Cr) and blood urea nitrogen (BUN) were determined.

2.8. In vivo sonodynamic treatment

When tumors reached approximately 6 mm in diameter, 60 mice were randomly divided into 12 groups (5 in each group) (Fig. 1B): Control group (C): mice were intraperitoneally injected with PBS; Ultrasound groups: mice were treated with ultrasound alone of 0.4 W/cm^2 (U1), 0.8 W/cm^2 (U2) and 1.6 W/cm^2 (U3) respectively; Chlorin e6 groups: mice were intraperitoneally injected with chlorin e6 of 10 mg/kg (e1), 20 mg/kg (e2) and 40 mg/kg (e3) respectively; Ultrasound + Chlorin e6 groups: mice were treated with ultrasound of 1.6 W/cm^2 combined with chlorin