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The effects of Ce6-mediated sono-photodynamic therapy on cell migration, apoptosis and autophagy in mouse mammary 4T1 cell line

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ABSTRACT

Purpose: Sono-Photodynamic therapy (SPDT) is an alternative therapy which claims to enhance the anti-cancer effects by combining sonodynamic therapy (SDT) with photodynamic therapy (PDT). In the present study, we investigated the effects of chlorin e6 (Ce6) mediated SPDT on migration, apoptosis and autophagy in mouse mammary 4T1 cancer cells, and its underlying mechanisms.
Materials: Cell migration was determined by wound healing assay. Apoptosis was analyzed using annexin V-PE/7-ADD staining as well as Hoechst 33342 staining. Changes of mitochondria membrane potential (MMP) was evaluated by flow cytometry. Formation of acidic vesicular organelles (AVOs) during autophagy was observed with fluorescence microscope by MDC staining. Immunofluorescence assays were performed to detect the co-localization of LC3 and Lamp2. Western blotting was employed to analyze the activity of the apoptosis related proteins Caspase-3, PARP, Bax and Bcl-2, as well as the autophagy associated processing of LC3-I to LC3-II and Beclin-1 expression.
Results: Ce6 mediated SPDT further enhanced cell migration inhibition, significantly triggered cell apoptosis, nuclear condensation and MMP drop. Cleaved Caspase-3 and PARP increased dramatically after Ce6-SPDT, accompanied by decreased Bcl-2 expression, while the expression of Bax remained stable. Additionally, AVOs formation, co-localization of LC3 and Lamp2 occurred following Ce6-SPDT and simultaneously accompanied by LC3-II processing and increased Beclin-1 expression.
Conclusions: Ce6-SPDT could enhance cell migration inhibition, and induce mitochondria-dependent apoptosis as well as autophagy in 4T1 cells.

1. Introduction

Photodynamic therapy (PDT) involves the administration of photosensitizer followed by light irradiation in the target tissue, and has been used for the treatment of a wide range of cancers [1–3]. Sonodynamic therapy (SDT) is derived from PDT with distinct advantages of focusing ultrasound energy to target deep tissue sites and activating the sonosensitizing compound locally [4,5]. Recently, Kessel et al. reported that both apoptosis and autophagy were induced by PDT in L1210 cells [6]. Autophagic response to PDT with different photosensitizers has also been observed in many other cell lines [7]. Previous studies indicated that autophagy was involved in SDT induced cytotoxicity in L1210 cells [8,9].

Combining SDT with PDT, sono-Photodynamic therapy (SPDT) is a neoteric approach which provides therapeutic advantages through therapeutic synergism for anti-cancer therapy. Although few reports and information concerning the responses to SPDT has been reported, the combined therapy has shown more remarkable anti-cancer effects than either monotherapy [10–13].

As a second generation photosensitizer, Chlorin e6 (Ce6) has been reported to accumulate more effectively in tumors, absorb more strongly at longer wavelengths (650 and 670 nm) and clear faster from the organism [14]. Toxicological examinations have shown that Ce6 is basically nontoxic for experimental cells or animals, but it is characterized by a high sensitizing efficacy, being activated by both light and ultrasound to induce obvious inhibitory effect on cancers [15–17].

Breast cancer is one of the most fatal cancers threatening female health throughout the world for decades. Because of poor prognosis and serious side effects, none of the currently available, first line therapies (such as surgery, radiotherapy and chemotherapy) is the ideal treatment for it. Mouse mammary cancer 4T1 cell line, a highly metastatic tumor cell with its growth and metastasis in Bal

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b/c mice similar to breast cancer in human body, is a widely-used model in the study of tumor metastasis. In order to explore cell migration inhibition and the possible response of apoptosis and autophagy following Ce6-mediated SPDT, 4T1 cells were exposed to ultrasound with a frequency of 1.0 MHz for up to 1 min in combination with laser light with a total radiation dose of 1.2 J/cm^2 in the presence of 1 μg/ml Ce6. In this study, we found that the antitumor efficacy of Ce6-SPDT was remarkably enhanced accompanied by apoptotic and autophagic response. We proposed that mitochondria-dependent apoptosis pathway and autophagy might be associated with the cell damage induced by Ce6-SPDT. The findings may provide new insight in cellular responses to SPDT therapy.

2. Materials and methods

2.1. Chemicals

Ce6 was purchased from Sigma Chemical Company (St. Louis, MO, USA) and was dissolved in PBS at the concentration of 2.5 mg/ml, sterilized, and stored at −20 °C in the dark. Guava Nexin Reagent (4500–0450), Hoechst 33342 (HO), and rhodamine-123 (RHO123) were purchased from Millipore Company (Guava technologies Inc, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide etrazolium (MTT), Monodansylcadaverine (MDC), the primary antibodies of Poly ADP-ribose polymerse (PARP), Bcl-2, Bax, Microtubule associated protein light chain 3 (LC3) and Beclin-1 were purchased from Sigma Chemical Company (St. Louis, MO, USA); Lysosome-Associated Membrane Protein 2 (Lamp2) and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); IR Dye 680-labeled secondary antibodies were from Li-cor, Biosciences. Secondary antibodies of immunoglobulin FITC or TRITC conjugates were obtained from ZSGB-BIO, China. All other reagents were commercial products of analytical grade.

2.2. Cell culture

Mouse mammary cancer 4T1 cell line was obtained from the department of basic medicine, Union Medical College, Beijing, China. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies, Inc., USA) supplemented with 10% fetal bovine serum (Hyclone, USA), 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mM L-glutamine, in a incubator with 5% CO_2 and 100% humidity at 37 °C. Cells in the exponential phase of growth were used in each experiment.

2.3. Laser light and ultrasound treatment protocols

4T1 cells cultured in 35 mm culture dishes (Corning Company) at 70% of confluence were incubated with Ce6 in serum-free DMEM in dark for 4 h, allowing sufficient time for cell uptake of Ce6 to a maximum level, then subjected to laser light and ultrasound treatment protocol.

The laser light (excitation wavelength: 650 nm; manufacturer: Institute of Photonics & Photon-technology, Department of Physics, Northwest University, Shaanxi, China) was used as a source for evocation of the photodynamic effect. Irradiance was measured by the radiometer system (Institute of Photonics & Photon-technology, Department of Physics, Northwest University). For the laser light, the power output: 1.3 mW; power intensity: 10.4 mW/cm^2; irradiation time: 120 s; emitting aperture area: 0.125 cm^2; area of the beam: 9.62 cm^2; The power intensity (10.4 mW/cm^2) = the output power (1.3 mW)/the detector area (0.125 cm^2) and the energy intensity was calculated using the following equation: energy intensity (1.2 J/cm^2) = power intensity (10.4 W/cm^2) × irradiation time (120 s), so the final light dose was 1.2 J/cm^2 in this experiment.

A 35 mm diameter planar transducer (Institution of Applied Acoustics, Shaanxi Normal University) was submerged in an acrylic container filled with cold degassed water (4 °C). No significant variation of temperature was detected (⩽2 °C) to avoid thermal effect. Continuous-wave ultrasound was generated by the generator (T&C Power Conversion, Inc., Rochester, NY). Cells in 35 mm culture dish were placed in the water bath and 10 mm above the top of the transducer in a horizontal position for sonication. The frequency of 1.0 MHz and duration of 1 min was used for ultrasound treatment.

For MTT assay, Ce6 mediated PDT groups were incubated with different Ce6 dose (0.25, 0.5, 1, 2, 5 and 10 μg/ml); Ce6 mediated SDT groups were incubated with 1 μg/ml Ce6, and then exposed to different ultrasound intensity (0.36, 0.504, 0.54 and 0.72 W/cm^2).

For other analysis, cells were divided randomly into four groups: (1) control (no treatment), (2) SDT (Ce6 plus ultrasound), (3) PDT (Ce6 plus laser light), and (4) SPDT (Ce6 plus ultrasound plus laser light). Cells were incubated with 1 μg/ml Ce6 in serum-free DMEM for 4 h and then subjected to PDT or/and SDT protocol, cells were incubated for an equivalent time without Ce6 and exposed to neither ultrasound nor light for control. SDT group were exposed to ultrasound with intensity of 0.36 W/cm^2 for up to 1 min. PDT group were irradiated with 1.2 J/cm^2 laser light. Cells were exposed to ultrasound then immediately irradiated with laser light under the same condition for SPDT. Cells were then cultured for an additional time as specified and subjected to analysis. All procedures were carried out with low-level light to minimize any influence of photo-activation.

2.4. Cytotoxicity

The cytotoxic effect was determined by MTT assay 4 h after different treatments. Cells were washed by PBS then harvested by trypsinization and re-suspended in DMEM with 10% FBS, and added to 96-well culture plates (100 μl/well), followed by adding 10 μl MTT solution (5 mg/ml, dissolved in PBS), and the mixture was incubated at 37 °C in a CO_2 incubator for 4 h. The MTT solution was carefully removed after centrifugation and 150 μl dimethyl sulfoxide (DMSO) was added in order to solubilize the violet formazan crystals. The absorbance of the resulting solution was measured in a 96-well microplate reader (BIO-TEK ELX800, USA) at 570 nm against the reference value at 630 nm. The results were determined as percentage of control.

2.5. Wound healing assay for cell migration analysis

Cell migration ability was assessed by wound healing assay. Cells were cultured in 35 mm dishes when reached 90–100% confluence, and were wounded by scratching with a pipette tip or by a 1 ml micro-pipette tip linking vacuum equipment. A rectangular or circular blank area without cells then formed with the aid of mechanical damage or the help of negative pressure. The cells were subjected to SDT or/and PDT, then incubated with individual medium and allowed to migrate for 24–48 h. The wounds of both initial and 24, 48 h post different treatments were recorded with phase-contrast microscopy (Nikon, TE2000-S) at a magnification of 10× or 4×, each wound area was measured by automatic analysis software Gene Tools. The migration ability was expressed as percentage of migration and was calculated from the following formula: % of migration = (1-average of the wound area/average of the initial wound area) × 100%.

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