Efficient preparation of highly pure chlorin e₆ and its photodynamic anti-cancer activity in a rat tumor model

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Abstract. Photodynamic therapy (PDT) is currently being used as an alternative therapeutic modality for a variety of malignant tumors. This study was performed to show an efficient preparation of second generation of photosensitizer chlorin e₆ (Ce₆) with high yield and purity, and to test antitumor activity of Ce₆-induced PDT (Ce₆-PDT) both in vitro and in vivo using a rat tumor model. Three-week-old male Sprague-Dawley (SD) rats were inoculated s.c. on the right flank with 5x10⁶ RK3E-ras cells. The animals were administered i.v. with Ce₆ (10 mg/kg) and 24 h later, PDT was performed using a laser diode at a light dose of 100 J/cm². Ce₆-PDT generated reactive oxygen species and led to significant growth inhibition in RK3E-ras cell. In addition, Ce₆-PDT induced apoptosis through the activation of caspase-3 and its downstream target, PARP cleavage. The protein level of anti-apoptotic bcl-2 was also reduced by Ce₆-PDT in RK3E-ras cells. In vivo experiments, application of Ce₆-PDT led to a significant reduction of tumor size. PCNA immunostaining and TUNEL assay revealed that Ce₆-PDT inhibited tumor cell proliferation and increased apoptosis. These findings suggest that the newly purified Ce₆-PDT can effectively arrest tumor growth by inhibiting cell proliferation and inducing apoptosis.

Introduction

Photodynamic therapy (PDT) is currently being used as a therapeutic alternative for a variety of malignant tumors. Compared with ionizing radiation therapy or chemotherapy, PDT is generally safer for the surrounding normal tissues because photosensitizers are preferentially accumulated in tumor cells (1). PDT involves the selective uptake and retention of a photosensitizer in the tumor followed by light irradiation of an appropriate wavelength to cause the destruction of tumor cells by the formation of cytotoxic reactive oxygen species (ROS) (1,2). The type I radicals including superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), hydroxyl radical (·OH), and type II ROS such as singlet oxygen have been implicated in the therapeutic or toxic responses of PDT (3). PDT produces cytotoxic effects through photodamage to subcellular organelles and molecules such as mitochondria, lysosomes, cell membranes, and nuclei of tumor cells, which are considered as potential targets. During light exposure, photosensitizers that are localized in mitochondria may induce apoptosis, while those localized in lysosomes and cell membranes may cause necrosis. Apoptosis is responsible for PDT-mediated tumor cell death in vitro and tumor ablation in vivo (2,4).

In clinical PDT, photosensitizers such as hematoporphyrin derivatives, Photofrin, Photosens, Levulan and Visudyne have been used exclusively. These compounds have low localization in tumors, resulting in low photodynamic effect and high skin photosensitivity (2,5). Therefore, development of compounds with higher selective affinity for tumors was required and consequently, chlorin e₆ (Ce₆) was developed as a second generation photosensitizer. It is a promising photosensitizer as it exhibits advantageous photophysical properties for PDT such as having long lifetimes in their photoexcited triplet states and high molar absorption in the red region of the visible spectrum. Moreover, a 664-nm laser light can penetrate tissue deeper than the 630-nm laser light used for Photofrin (6). However, a mixed form of Ce₆ derivatives has been prepared in the clinical application (7,8). Therefore, a standard process for the preparation of pure form of Ce₆ with high yield is required.
In this study, we developed an efficient preparation of Ce₆ with high yield and purity from chlorophyll a of a seawater live chlorella (Chlorella ellipsoidea) belonging to green algae. We investigated anticancer effect of PDT with the newly isolated Ce₆ through both in vitro and in vivo experiments.

Materials and methods

Preparation of chlorin e₆. Live chlorella (Chlorella ellipsoidea) 100 g (dried weight) was sequentially washed with 500 ml of water and 300 ml of 50% ethanol in water to remove polar materials and the residue was extracted twice with 500 ml of 100% ethanol to obtain chlorophyll a rich fraction (extraction yield 4.3%). Stirring the combined ethanol solution of chlorophyll a in 1 N HCl (pH 2.5) for 3 h at room temperature afforded phaeophytin in the form of precipitates. The precipitate was dissolved in dichloromethane washed with distilled water, dried with anhydrous sodium sulfate, and rotary-evaporated to dryness. The residue was purified by a chromatography using neutral alumina (Aldrich, Brockmann, ~150 mesh) with a gradient elution from 30% dichloromethane in n-hexane to 100% dichloromethane. The main green band was collected and evaporated to dryness. The crystalline powder was dissolved in acetone, adjusted pH 12.0 with 1 N NaOH, and stirred for 12 h. The precipitated Ce₆ was filtered, washed with acetone and dissolved in 100 ml of water, and filtered to remove insoluble impurity. After lyophilization of the filtered water solution, a fine black powder of Ce₆ was obtained. The purity of Ce₆ is 93-98% (yield of Ce₆: 1% from dried weight of chlorella).

Cell culture. RK3E-ras cells, rat kidney epithelial cells transformed with K-ras gene, were maintained in DMEM medium containing 5% fetal bovine serum, 100 U/ml penicillin-streptomycin (Invitrogen, CA, USA) and subsequently incubated at 37˚C in an atmosphere containing 5% CO₂. RK3E-ras-Fluc cells were kindly provided by Dr Eric Fearon (University of Michigan Medical School, Ann Arbor, MI) and were described previously (9).

In vitro photodynamic treatment. RK3E-ras cells (2x10⁴ cells/well) were cultured overnight in 12-well plates and treated with different doses of Ce₆ for 24 h. Subsequently, PDT was performed using a laser diode (Geumgwang Co., Ltd., Daejeon, Korea) at a wavelength of 664 nm. The total laser energy was determined with a laser diode at a dose of 10 mg/kg. After 24 h, PDT was performed using a laser diode at a light dose of 100 J/cm² and wavelength of 664 nm. The animals were administered i.v. with Ce₆ at a dosage of 10 mg/kg. After 24 h, PDT was performed using a laser diode at a light dose of 100 J/cm² and wavelength of 664 nm. For bioluminescence imaging, the animals were anesthetized with the combination of ketamine (50 mg/kg) and xylazine (5 mg/kg) and then given i.p. with luciferin.
Molecular Probes, Palo Alto, CA) at a dose of 80 mg/kg body. The animals were imaged with the Xenogen IVIS imaging system (Xenogen Co., Alameda, CA) to record the bioluminescent signal emitted from the tumor. The IVIS-100 equipped with CCD camera system was used for emitted light acquisition, and Living Image software (Xenogen) was used for data analysis.

Histopathology, immunohistochemistry, and TUNEL assay. The animals were sacrificed on day 15 and the tumors were removed carefully and fixed in 10% formalin over 24 h. The tissues were then dehydrated in an alcohol-xylene series and embedded in paraffin wax. From each block, sections 2 μm thick were prepared and stained with haematoxylin and eosin for histological examination. For immunohistochemistry, the sections were incubated in 3% H2O2 in methanol for 10 min to remove endogenous peroxidase and blocked with 1% BSA in PBS for 1 h. The sections were then incubated with anti-PCNA antibody (Dako) overnight at 4˚C. After washed 3 times with PBS-T, the sections were subjected to avidin-biotin peroxidase complex (ABC) method (Vector) and peroxidase activity was evaluated with 3,3'-diaminobenzidine (Vector). Finally, the sections were counterstained with hematoxylin. The PCNA-positive cells were counted from 5 randomly selected areas under x200 magnifications and represented as mean ± SD.

The terminal deoxynucleotidyl transerase-mediated dUTP nick end-labeling (TUNEL) assay was done using an Apoptosis detection kit (Intergen, Purchase, NY) according to the manufacturer’s protocol.

Statistical analysis. The differences in mean values among groups were tested, and the values were expressed as mean ± SD. All of the statistical calculations were carried out using Microsoft Excel.

Results and Discussion

The yield of chlorophyll \( \alpha \) extracted from undisrupted chlorella cells with ethanol was 10 times more than that from a commercially available chlorella powder (data not shown). The retention time of chlorophyll \( \alpha \) in HPLC system (Xenogen Co., Alameda, CA) to record the bioluminescent signal emitted from the tumor. The IVIS-100 equipped with CCD camera system was used for emitted light acquisition, and Living Image software (Xenogen) was used for data analysis.

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Results and Discussion

The yield of chlorophyll \( \alpha \) extracted from undisrupted chlorella cells with ethanol was 10 times more than that from a commercially available chlorella powder (data not shown). The retention time of chlorophyll \( \alpha \) in HPLC system was 7.8 min (Fig. 1A). Treatment of the ethanol to chlorophyll \( \alpha \) in an acidic condition (1 N HCl, pH 2.5) enable to remove the Mg\( ^{2+} \) ion easily to afford a crude pheophytin in the form of precipitates, which could be further purified with alumina column chromatography (Fig. 1B). As the final step, pheophytin was hydrolized by reacting with 1 N NaOH (pH 12.0) to give Ce6 as a fine powder which is a water soluble sodium salt form with 95-98% purity (Fig. 1C). The molecular weight of Ce6 (596.2) was confirmed by an observation of 597.2 in LC-MS analysis as the (M+H) + form (Fig. 1D). The total yield of Ce6 is 1%, based on the calculation as a percentage of obtained Ce6 weights to the dried weight of chlorella. In this study, we established an efficient isolated procedure of highly pure Ce6 utilizing undisrupted live
Figure 2. Ce6-PDT induces growth inhibition and increases ROS content on RK3E-ras cells. (A) RK3E-ras cells were incubated with the indicated concentrations of Ce6 followed by exposure to 10 J/cm² of laser light for 24 h. (B and C) RK3E-ras cells were incubated with 5 μM of Ce6 followed by exposure to 10 J/cm² of laser light for the indicated time periods. (A and B) Cell viability was evaluated by MTT assay. (C) RK3E-ras cells were incubated with 5 μM of Ce6 followed by exposure to 10 J/cm² of laser light. Quantitative amount of ROS production was measured using the scanning multimode reader and software. The data are expressed as the means ± SD. *p<0.05, **p<0.01.

Figure 3. Ce6-PDT induces the apoptosis through caspase-3 activation in RK3E-ras cells. RK3E-ras cells were incubated with 5 μM of Ce6 for 24 h followed by exposure to 10 J/cm² of laser light. (A) Apoptosis was evaluated by the flow cytometric method by staining the cells with Annexin-V-FLUOS and propidium iodide. (B) Cells were lysed and caspase-3 activity was measured as described in Materials and methods. (C) The protein fraction was prepared and resolved by 12% SDS-PAGE. The expression levels of bcl-2, cleaved PARP, and actin were detected by Western blot analysis. *p<0.01.
Chlorella, a key rich source of the starting material chlorophyll a.

The cytotoxic effect of PDT on the survival of Ce6-treated cells was examined in a time- and dose-dependent manner using MTT assays. The viability of RK3E-ras cells was decreased by Ce6-PDT depending on Ce6 concentration (Fig. 2A). When Ce6 was treated for 12 and 24 h, PDT most effectively reduced cell viability (Fig. 2B). The excited photosensitizer interacts with molecular oxygen and results in the production of ROS, which can lead to damage of the cellular constituents and subsequent cell destruction (12-14). It is believed that ROS plays a direct role in damaging cells subjected to PDT (2,4). Therefore, we examined intracellular ROS production by Ce6-PDT in the RK3E-ras cells. ROS generation was induced immediately after Ce6-PDT and increased in a time-dependent manner (Fig. 2C).

Apoptosis is responsible for PDT-mediated tumor inhibition in certain cell lines (15,16). To examine whether the growth-inhibitory effect of Ce6-PDT in RK3E-ras cells is caused by apoptosis, flow cytometry was performed 24 h after Ce6-PDT. Among Ce6-PDT-treated cells, 51.5% cells were shown to be apoptotic and 39.71% is necrotic or late apoptotic (Fig. 3A).

Although cell death can be induced by PDT with different photosensitizers, the molecular characteristics involved in each death event might be different (14,17). To examine the molecular changes associated with cell death in RK3E-ras cells by Ce6-PDT, we investigated caspase-3 activity, which is a hallmark of apoptosis. Ce6-PDT enhanced caspase-3 activity in a time-dependent manner (Fig. 3B), implicating that Ce6-PDT triggers caspase-3 activation in RK3E-ras cells. Particularly, 12 h after PDT, caspase-3 activity showed an 88.6% increase compared with those in control group.

Caspase-3 plays an important role in the execution of apoptosis and is primarily responsible for the cleavage of PARP during cell death (18-22). The activation of PARP by DNA strand breaks contributes to the consumption of NAD and ATP that occurs in cells undergoing apoptosis (23,24). In this study, the cleavage of PARP was detected in RK3E-ras cells 12 and 24 h after Ce6-PDT (Fig. 3C). Moreover, the protein level of anti-apoptotic bcl-2 was reduced 24 h after Ce6-PDT (Fig. 3C). These findings suggest that Ce6-PDT might induce growth inhibition in RK3E-ras cells through apoptosis.

We previously demonstrated an animal model for rapid induction of malignant tumor (25). This animal model has the advantage of allowing short-term screening of antitumor agents. We examined the effect of Ce6-PDT on in vivo tumor growth using this rat model with subcutaneous injection of RK3E-ras cells. Tumor volume was measured every three days after Ce6-PDT and the results showed that Ce6-PDT treatment arrested tumor progression (Fig. 4A). On gross, Softex X-ray, and bioluminescence images, the tumor almost disappeared in the rats treated with Ce6-PDT (Fig. 4B). These results suggest that only a single application of Ce6-PDT can inhibit effectively the progression of a solid tumor in vivo.

Histology of control tumors demonstrated solid growth of anaplastic undifferentiated carcinoma showing many mitotic figures with hemorrhage. However, Ce6-PDT treatment led to

Figure 4. Ce6-PDT inhibits tumor growth in vivo. RK3E-ras cells (5x106/rat) were injected s.c. into the right flank of SD rats. (A) Tumor volume was measured every 3 days and calculated using the formula V=(ab^2)/2, in which a is the largest diameter and b is the shortest diameter of the tumor. Results are expressed as mean ± SD. (B) Gross (left), soft X-ray imaging (middle), and in vivo bioluminescence images (right) obtained on day 15 after Ce6-PDT.
loss of cohesiveness and extensive cell death showing nuclear pyknosis and cytoplasmic eosinophilia (Fig. 5A, top). Most of tumor cells in the control and treated with Ce6 or laser alone were PCNA-positive, suggesting a high proliferative rate. However, the number of PCNA-positive cells significantly decreased in tumor treated with Ce6-PDT (Fig. 5A and B). We also performed TUNEL staining to assess the amount of the apoptotic cells in vivo. TUNEL-positive cells significantly increased in tumor treated with Ce6-PDT compared with the control untreated (Fig. 5A and C). These results confirmed in vitro data that Ce6-PDT effectively arrested the tumor growth by inhibiting cell proliferation and inducing apoptosis.

In summary, we showed herein that PDT using newly purified second-generation photosensitizer Ce6 exerted cytotoxic effect via the intracellular ROS production on the RK3E-ras cell line that induces rapid malignant tumor growth in SD rats. Ce6-PDT also induced apoptosis through the activation of caspase-3, and its downstream target, PARP cleavage. In addition, Ce6-PDT effectively arrested the tumor growth by inhibiting cell proliferation and inducing apoptosis. Therefore, the present study suggests that the newly purified Ce6-PDT may be useful in the clinical application to control solid tumors.

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References