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Antimicrobial photodynamic therapy using chlorin e6 with halogen light for acne bacteria-induced inflammation

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ABSTRACT

Aims: The present study was designed to evaluate the therapeutic potential of antimicrobial photodynamic therapy (PDT) using chlorin e6 with halogen light against acne bacteria-induced inflammation.
Main methods: Highly purified chlorin e6 (Ce6), as a second generation photosensitizer, was synthesized from Spirulina chlorophyll. To evaluate the antimicrobial property of Ce6-mediated PDT with halogen light, the broth microdilution method and two-color fluorescence assay were used. The free radicals generated upon irradiating Ce6 with halogen light were measured using 2,7-dichlorofluorescin diacetate. *Propionibacterium acnes* was intradermally injected into the left ear of the ICR mice, and the anti-inflammatory effect of Ce6-mediated PDT with halogen light was measured by the histological examination. The expressions of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) as well as pro-inflammatory cytokines were also measured by Western blotting.
Key findings: Chlorin e6-mediated PDT with halogen light (30,000 lx) inactivated various skin bacteria, including *P. acnes* in a dose-dependent manner. The MIC_{99} value against *P. acnes* (KCTC3314) of Ce6 with light was >0.49 μg/ml, whereas the MIC_{99} for Ce6 alone was >31.25 μg/ml. Ce6-mediated PDT suppressed the expression of *P. acnes*-induced pro-inflammatory cytokines and iNOS, but not COX-2 in a mouse model.
Significance: This study showed a remarkable therapeutic effect of chlorin e6-mediated PDT with halogen light against *P. acnes*-induced inflammation. Our results suggest for the first time the potential of Ce6-mediated PDT with halogen light as a more effective and safer alternative treatment to antibiotic therapy against pathogenic infections of the skin.

Introduction

Photodynamic therapy (PDT), for the treatment of several diseases such as cancers, rheumatoid arthritis, age-related macular degeneration and skin disease, consists of three components; a photosensitizer (PS), a light source with a suitable wavelength and tissue oxygen [6,17,28,33].

The activation of the PS from its ground state into an excited state upon light illumination results in generation of reactive oxygen species, which subsequently leads to cell proliferation inhibition, cell cycle arrest, and cell death. Antimicrobial PDT, based on the overproduction of reactive oxygen species for the inactivation of pathogens and destruction of a cellular target, has attracted great attention as an effective approach to treat microbial infections [27,36].

The efficiency of a photosensitizer is one of the main factors for determining the feasibility of PDT. The use of several PSs has been clinically limited due to their poor cellular delivery, low biocompatibility, and low tumor targeting efficacy [27]. Chlorin e6 (Ce6), as a second-generation photosensitizer, has been reported to possess remarkable advantages; such as shorter photosensitizing period, selective accumulation in target tissue, relatively deep penetration within tissues via absorption of light of longer wavelength, simple synthesis and easy production as well as its minimal side effect [18,27].

Propionibacterium acnes is a Gram-positive anaerobe normally located in human sebaceous glands and has been associated with the inflammatory phase in acne vulgaris. Acne is the most common disorder of the human skin, with major pathophysiological features including androgen stimulated seborrhea, hyperkeratinization and obstruction of the follicular epithelium, proliferation of *Propionibacterium acnes*, and inflammation [24]. Recently, PDT using 5-aminolevulinic acid (ALA) as a photosensitizer has attracted great attention as a new acne

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treatment. However, limitations of ALA-mediated PDT, such as the relatively long photosensitizing period needed and common adverse effects, have been reported [5,29]. Indole-3-acetic acid (IAA) was also suggested as a potential photosensitizer in PDT for acne treatment [19,23]. Most PDT applications use laser light which, in itself, can induce thermal or photochemical damage to the retina and skin [26]. The availability of safer and more efficient light source is also imperative for clinically successful PDT applications.

We have demonstrated that antimicrobial PDT using chlorin e6 combined with halogen light can be an alternative therapy for acne vulgaris. The chlorin e6-mediated antimicrobial PDT using halogen light effectively inactivated various skin bacteria in this investigation. The therapeutic effect of chlorin e6-mediated PDT with halogen light on *P. acnes*-induced inflammation in a mouse model was also demonstrated.

Materials and methods

Synthesis of salt form chlorin e6 from Spirulina chlorophyll

100 g of Spirulina biomass was used for the production of chlorin e6. The ethanol extract of Spirulina was evaporated, and then equal volume of hexane was added. Distilled water equal to 50% of the volume of hexane extract was then added. The solution was stored at −20 °C overnight, then washed with 80% ethanol and distilled water. 1 N HCl was added to the extract until the pH was adjusted to pH 2 to make the Mg-free pheophytin. After stirring, 1 N NaOH was added to neutralize the solution and then stored at −20 °C with 80% ethanol overnight. This solution was evaporated to remove any remaining hexane and dissolved with acetone, and then filtered. The pH of the filtrate was adjusted to pH 12 with 1 N NaOH. After a 3 h reflux, 1 N HCl solution was used to neutralize the solution, and the solution was stored at −20 °C overnight [22]. Salt form of chlorin e6 with high purity was obtained following filtration and vacuum drying. HPLC (Agilent technologies, Inc. Santa Clara, CA) with reversed-phase 5 μ C18 column (4.6 × 150 mm) was used to check the purity of Ce6.

Fluorescence measurement for chlorin e6 following halogen light irradiation

Chlorin e6 (Ce6), dissolved in phosphate buffered saline at a concentration of 100 μg/ml, was used for the fluorescence measurements. A halogen lamp (12 V/50 W) was used as a source of irradiation of 30,000 lx, a perceived form of illumination produced by the strength of sunlight in the spring in Asan City, Korea. A Perkin-Elmer LS 50B Luminescence Spectrometer (Perkin Elmer, Inc. Waltham, MA) was used to examine the fluorescence emission spectra of Ce6 after irradiation with the halogen light and the fluorescence excitation spectra at the wavelength corresponding to the peak of fluorescence emission were recorded [6]. Light at the wavelength of the fluorescence excitation maximum was used for excitation. Diode laser (10 J/cm^2) was also used to compare the fluorescence properties of Ce6.

DCF assay for ROS generation by chlorin e6 with halogen light irradiation

To examine whether Ce6 might produce free radicals upon irradiation with halogen light (30,000 lx), any generated free radicals were measured using 2,7-dichlorofluorescin diacetate (DCFH-DA), which is oxidized by free radicals to the fluorescent dichlorofluorescein (DCF). Reaction mixtures contained activated DCFH-DA solution and Ce6 (100 μg/ml) which were irradiated with halogen light for 5 min interval. The negative controls were light irradiated DCFH-DA solution without Ce6 and light shielded DCFH-DA solution with Ce6 [29,35].

Determination of MIC using chlorin e6 and halogen light irradiation

Gram-positive *P. acnes* (KCTC 3314, KCTC3320, and KCTC5527), and *Staphylococcus aureus* subsp. *aureus* (KCTC 1927) were obtained from the Korean Culture Center of Microorganisms, Seoul, Korea. The minimum inhibitory concentration (MIC) testing was performed according to the recommendations of the clinical laboratory standard institute [2]. *P. acnes* strains and *S. aureus* were cultured and treated with Ce6 with or without light irradiation. The broth microdilution method using 96-well microtiter plate was used to measure the antimicrobial effects of Ce6-mediated PDT with halogen light [8,14]. The bacterial suspensions were diluted to 1×10^6 CFU/ml [24], and irradiated with halogen light (30,000 lx) for 30 min after adding Ce6. Incubation proceeded at 37 °C under their corresponding anaerobic conditions for 72 h or aerobic conditions for 12 h. The bacterial suspensions' absorbance at 620 nm was measured to estimate bacterial growth. MIC_{99} is the concentration of Ce6 at which microbial growth was inhibited by more than 99%. The antimicrobial activity of 5-ALA was also measured at the same experimental condition as Ce6.

Bacterial viability assay

Bacterial viability was evaluated using the LIVE/DEADBacLigh™ Bacterial Viability Kit (Thermo Fisher Scientific, Inc. Waltham, MA). This is a two-color fluorescence assay that is based on the mixture of a green fluorescent nucleic acid stain SYTO 9 and the red-fluorescent nucleic acid stain propidium iodide (PI) [11,28]. Bacterial suspensions (1×10^6 CFU/ml) were irradiated with halogen light (30,000 lx) for 30 min after adding Ce6 (0.1 μg/ml). Incubation proceeded at 37 °C under their corresponding anaerobic conditions for 72 h. The number of bacteria stained with the fluorescent dyes was counted and represented as the average of the five highest areas within a single 200× field [28].

Mouse model for P. acnes-induced inflammation

7-week-old male ICR mice were purchased from the Orient Bio Co. Ltd. (Seoul, Korea). Room temperature was maintained at 20 ± 2 °C and the relative humidity at 60 ± 10%. The animals were maintained on a 12:12 h light/dark cycle. All animal procedures were conducted with the approval of the Animal Research Ethics Committee at Soonchunhyang University (approval number: SCH13-04-01).

P. acnes (1×10^7 CFU per 20 μl in PBS, KCTC 3314) was intradermally injected into the left ear of the ICR mice. The right ear received an equal amount (20 μl) of PBS. For epicutaneous application, 200 μl of Ce6 (1, 25 and 50 μg/ml in saline) was applied on the surface of the ear skin after intradermal injection with the *P. acnes*. After the Ce6 treatment, the test area was irradiated with halogen light for 30 min (30,000 lx). Moreover, N-(3-(aminomethyl)benzyl)acetamidine, dihydrochloride (1400 W, Sigma-Aldrich Corp. St. Louis, MO), a specific and highly selective iNOS inhibitor, was administered as previously described [1,30]. Briefly, 1400 W was used for pre-treatment that consisted of a daily intraperitoneal injection (i.p.) of 5 mg/kg/day for 2 days. On the 3rd day, 1400 W was injected into mice 30 min before intradermal injection of *P. acnes*. 24 h after the bacterial injection, the mice were sacrificed with zoletil and rompun mixture and the ears were cut off and punched with an 8 mm biopsy punch. The ears were stored at −70 °C until the next experiments [24,34].

Western blot analysis

The protein from ear was separated by 10% SDS-PAGE, and then transferred onto polyvinylidene fluoride (Bio-Rad Laboratories, Inc. Hercules, CA) membrane. The membranes were incubated overnight with COX-2 (Santa Cruz Biotechnology, Inc. Dallas, Texas) and iNOS (Abcam Ltd, Cambridge, UK), TNF-α (Abcam Ltd, Cambridge, UK),