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Original Article

Dual wavelength 5-aminolevulinic acid photodynamic therapy using a novel flexible light-emitting diode unit

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ABSTRACT

Background: Photosensitizers used for photodynamic therapy (PDT) to treat dermatologic disease are metabolized into mainly protoporphyrin IX (PpIX), which has five absorption wavelength peaks: 410 nm, 510 nm, 545 nm, 580 nm, and 630 nm. Although only red light around 635 nm and blue light around 400 nm are used as light sources for PDT, the efficiency of PDT might be improved by using multiple wavelengths, including those that correspond to the other absorption peaks of PpIX. Furthermore, because the target disease often occurs on the face, a flexible-type light-source unit that can be fitted to the lesion without unnecessarily exposing the mucous membranes, e.g., the eyes, nostrils, and mouth, is preferred.

Objective: We investigated the efficacy of a flexible light-emitting diode (LED) unit that emits multiple wavelengths to improve PDT effects.

Methods: HaCaT cells were incubated with 5-ALA and subsequently irradiated with either a single wavelength or sequentially with two wavelengths. Cell viability and reactive oxygen species were analyzed. Nude mice were implanted with COLO679 cells by subcutaneous injection into the flank. 5-ALA was subcutaneously injected into the tumor. The tumor was irradiated with 50 J/cm^2 (day 0) and assessed daily until day 21.

Results: The synergistic PDT effects of dual-wavelength irradiation and reactive oxygen species production were highest with the 405-nm and 505-nm wavelength combination. This dual wavelength combination was also the most effective in vivo.

Conclusion: We could therefore conclude that dual-wavelength PDT is an efficient strategy for improving the therapeutic effects of PDT. Using a flexible LED unit is expected to achieve more uniform irradiation of uneven areas.

1. Introduction

Photodynamic therapy (PDT) is a promising, minimally invasive method of treating many dermatologic diseases. PDT was developed mainly to treat precancerous and cancerous lesions. The combination of a photosensitizer and visible light is used to destroy the target tissue [1]. In dermatology, PDT utilizing topically applied 5-aminolevulinic acid (5-ALA) was first reported by Kennedy et al. in 1990 [2]. 5-ALA [3,4] and methyl aminolaevulinate [5–7] are currently used as photosensitizers in PDT to treat skin diseases such as actinic keratosis [8,9], basal cell carcinoma [10,11], Bowen's disease [5–7], and acne vulgaris [12,13]. These drugs are metabolized within the target cells into photoactivatable porphyrins, especially protoporphyrin IX (PpIX). PpIX has five absorption peak wavelengths: 410 nm, 510 nm, 545 nm, 580 nm, and 630 nm [14]. Although the 410-nm wavelength has the highest absorption coefficient, it has a relatively low tissue penetration depth [15]. Generally, red light around 635 nm [4] or blue light around 400 nm [3] is used as the light source for PDT. In the case of red light, treatment duration must be extended due to its low absorption coefficient. Blue light is disadvantageous for deeply located diseases due to its low skin penetrability. Although only red light and blue light are available as PDT light sources, PDT efficiency might be improved by using multiple wavelengths, including those corresponding to the other absorption peaks of PpIX. Halogen lamps, xenon lamps, metal halide

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lamps, fluorescent lamps, and light-emitting diodes (LEDs) are used as PDT light sources for skin diseases [16]. The luminous efficiency of LEDs is now remarkably improved. Moreover, LEDs can be manufactured with highly selective emission wavelengths. Therefore, LEDs are a promising light source for supplying multiple wavelengths for PDT applications. Furthermore, because the target disease often occurs on the face and scalp, which have curved surfaces, a flexible-type light-source unit that can be fitted to the lesion for local treatment without undue exposure to the eyes, nose, and mouth is preferred. In this study, we investigated the efficacy of a flexible-type LED unit emitting multiple wavelengths to improve PDT effects.

2. Materials and methods

2.1. Cells and cell culture

Human cancer keratinocyte cells (HaCaT) were a kind gift from Dr. Norbert Fusening (Institute of German Cancer Research Center, Heidelberg, Germany). Human melanoma cells (COLO679) were obtained from the Riken Bio Resource Center. The HaCaT cells were cultured with RPMI-1640 (Millipore Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), 1% HEPES solution (Millipore Sigma), 1% sodium pyruvate solution (Millipore Sigma), 1% antibiotic-antimycotic (Gibco Laboratories, Gaithersburg, MD), and 1% MEM Non-Essential Amino Acids Solution (Gibco) in 5% CO_2 at 37 °C in a humidified incubator. The COLO679 cells were cultured with RPMI-1640 (Millipore Sigma) supplemented with 10% fetal bovine serum (Gibco) and 1% L-glutamine (Gibco) in 5% CO_2 at 37 °C in a humidified incubator.

2.2. Light source

The plate-type light-source units comprised oval LEDs with peak wavelengths of 405 nm, 505 nm, 545 nm, 570 nm, 635 nm, or 405 nm + 505 nm (Imac, Shiga, Japan) (Fig. 1a). The flexible-type light-source units comprised LED chips, a flexible substrate, and a phosphor (Fig. 2) with peak wavelengths of 405 nm and 635 nm or 405 nm + 505 nm (SHARP, Osaka, Japan) (Fig. 1b). The relative spectral distribution and irradiances were measured using a spectral radiometer USR-45DA (USHIO, Tokyo, Japan).

2.3. PDT treatment and cell viability

HaCaT Cells were cultured in 96-well plates at a density of 5×10^4 cells/100 μL/well for 18 h and then coincubated with 5-ALA (1 mM) for 4 h in 5% CO_2 at 37 °C in a humidified incubator. After replacing 5-ALA with Dulbecco's phosphate-buffered saline (Millipore Sigma), the cells were irradiated with either a single wavelength or sequentially with two wavelengths of light using all combinations of two wavelengths (irradiated sequentially) with the plate-type LEDs at 405 nm (11 mW/cm^2), 505 nm (17 mW/cm^2), 545 nm (17 mW/cm^2), 570 nm (1 mW/cm^2), and/or 635 nm (19 mW/cm^2), and cultured further for 18 h in 5% CO_2 at 37 °C in a humidified incubator. Cell viability was evaluated using the Cell Proliferation Kit II (XTT Assay; Roche Diagnostics, Mannheim, Germany). The absorbance value at 450 nm was read with a microplate reader (SPECTRA MAX 340, Molecular Devices, San Jose, CA) to determine the viability ($OD_{treatment}/OD_{control}$), where $OD_{treatment}$ was the absorbance value at 450 nm of treated cells, and $OD_{control}$ was the absorbance value of sham-irradiated cells.

2.4. Detection of reactive oxygen species (ROS)

HaCaT cells at a density of 5×10^4 cells/mL were co-incubated with 5-ALA (1 mM) for 4 h in 5% CO_2 at 37 °C in a humidified incubator. Four hours later, the cells were evaluated using the CellROX™ Green Flow Cytometry Assay Kit (Thermo Fisher Scientific, Waltham, MA) and irradiated (total 0.4 J/cm^2) with one wavelength or sequentially with two wavelengths using the plate-type LEDs at 405 nm (12 mW/cm^2), 505 nm (18 mW/cm^2), 545 nm (14 mW/cm^2), 570 nm (1 mW/cm^2), and/or 635 nm (19 mW/cm^2). After irradiation, the cells were analyzed by fluorescence-activated cell sorting.

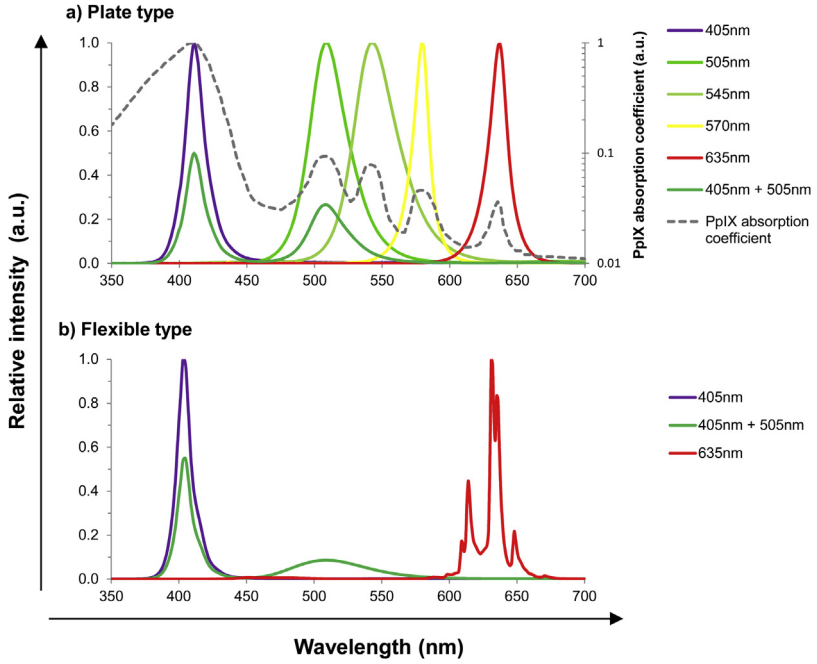

Fig. 1. Relative spectral distribution of a) the plate-type LEDs and the absorption coefficient of PpIX, b) the flexible-type LEDs.

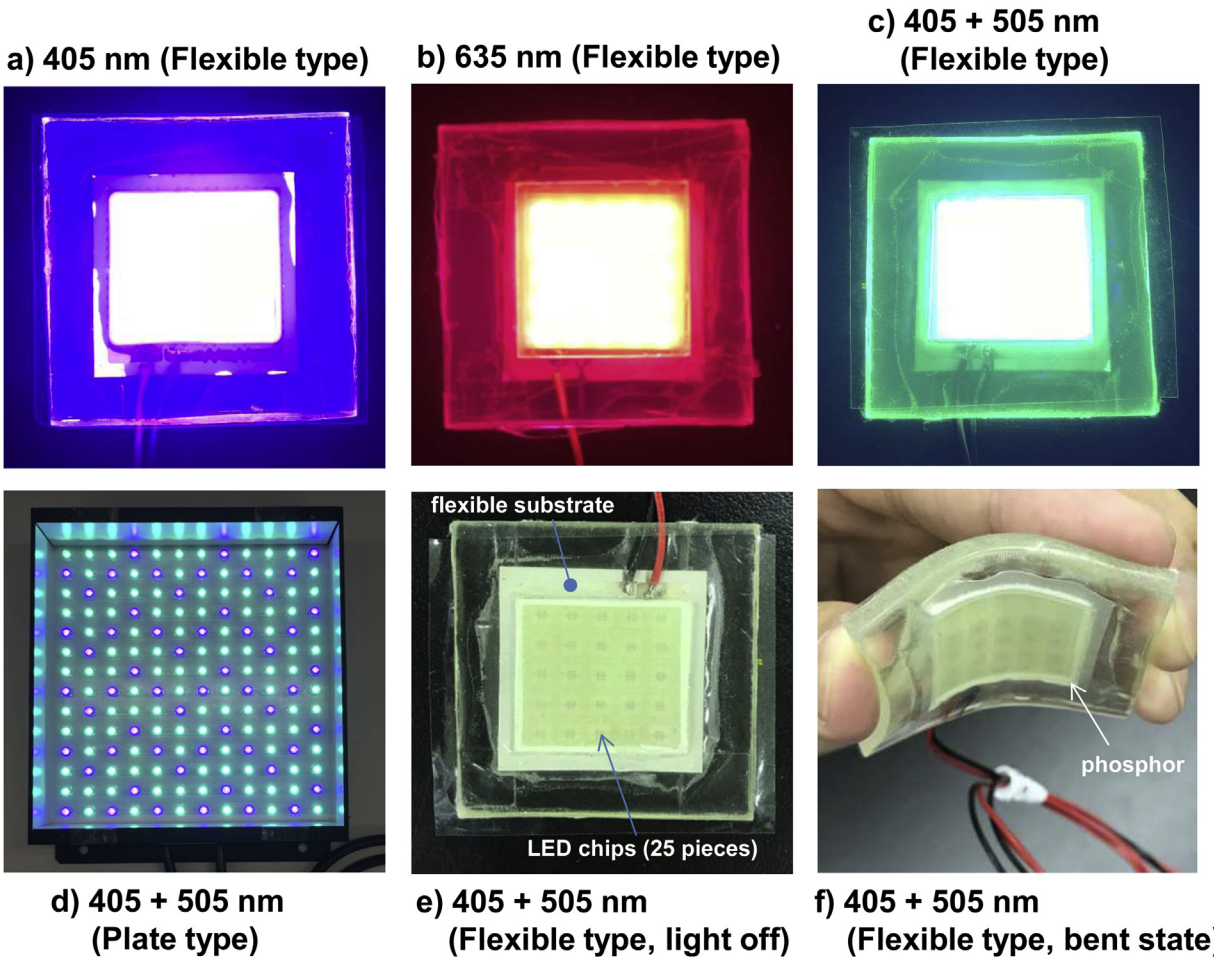

Fig. 2. Irradiation devices. a) 405 nm (flexible type), b) 635 nm (flexible type), c) 405 + 505 nm (flexible type), d) 405 + 505 nm (plate type), e) 405 + 505 nm (flexible type, light off), f) 405 + 505 nm (flexible type, bent state).

2.5. PDT in vivo

Nude mice (CAnN.Cg-Foxn1nu/CrlCrlj; Charles River, Wilmington, MA) were implanted with COLO679 cells (2×10^6 cells) via subcutaneous injection into the flank. At 13 days after COLO679 transplantation, the tumor size reached 5–7 mm in diameter. 5-ALA (250 mg/kg) was subcutaneously injected into the tumor. Four hours later, the tumor site was irradiated with 50 J/cm^2 using one of two types of LEDs, the plate type as a conventional type or the flexible type as a novel type at 405 nm (plate type: 68 mW/cm^2, flexible type: 63 mW/cm^2), 635 nm (plate type: 63 mW/cm^2, flexible type: 69 mW/cm^2), or 405 nm + 505 nm (plate type: 26 mW/cm^2, flexible type: 60 mW/cm^2), to determine the optimal irradiation method (day 0). While the mice were irradiated, a light-shading mask made using an aluminum sheet with a 20-mm square hole was placed between the light source and the mouse to protect the healthy regions and face from the light. Tumor size was measured daily until day 21 using calipers. Relative tumor volume was calculated using the following equation: relative tumor volume = tumor volume on day measured/tumor volume on day 0. The formula for computing tumor volume applies to the standard volume of an ellipsoid, where volume = $4\pi/3$ (length/2$\times$ width/2$\times$ depth/2). Assuming that depth equals the width and $\pi = 3$, volume = 1/2$\times$ (major axis)$\times$ (minor axis)^2.

2.6. Statistics

To determine the relative tumor volume in mice and the total ROS, the statistical significance of differences was calculated with the Tukey test, and the values are expressed as the mean ± standard error (SE) or mean ± standard deviation (SD), respectively. A *p*-value less than 0.05 compared with the control was considered statistically significant.

3. Results

3.1. The 405 + 505 nm combination produced the highest synergistic effect in HaCaT cells

To evaluate the synergistic effects of dual-wavelength irradiation, PDT was conducted using the plate-type LEDs with either one wavelength or sequentially with two wavelengths in HaCaT cells. Dose-survival curves of the single-wavelength irradiation roughly corresponded to the absorption coefficients of PpIX. We defined a standard dose-survival line to compare single-wavelength irradiation and dual-wavelength irradiation. If there were no synergistic effect, the results of irradiation with two wavelengths would match the standard dose-survival line. The standard dose-survival line was created by graphing a standard point ($S_{\lambda n+\lambda n,\ D}$), which was defined by the following formula: $S_{(\lambda 1\ +\ \lambda 2,\ D)} = 1 - ((1 - \text{Cell Survival}_{(\lambda 1,\ D/2)}) + (1 - \text{Cell Survival}_{(\lambda 2,\ D/2)}))$, where D was the dose, $S_{(\lambda 1+\lambda 2,\ D)}$ was the standard point of dual-wavelength irradiation with λ_1 and λ_2 at D, $\text{Cell Survival}_{(\lambda 1,\ D/2)}$ was the cell viability of single-wavelength irradiation with λ_1 at D/2, and $\text{Cell Survival}_{(\lambda 2,\ D/2)}$ was the cell viability of single-wavelength irradiation with λ_2 at D/2 (Supplemental Fig. 1).

The dose-survival curves for the dual irradiation were below the standard dose-survival line for 405 nm + 505 nm, 405 nm + 545 nm, 505 nm + 545 nm, 505 nm + 570 nm, 505 nm + 635 nm, 545 nm + 570 nm, 545 nm + 635 nm, and 570 nm + 635 nm (Fig. 3a,b,e,f,g,h, i,j, respectively).

In Fig. 4, we calculated the synergistic effects using the following formula to compare wavelength combinations: $E_{(\lambda1+\lambda2,\ D)} = S_{(\lambda1+\lambda2,\ D)}$ - Cell $Survival_{(\lambda1+\lambda2,\ D)}$, where $E_{(\lambda1+\lambda2,\ D)}$ was the synergistic effect of dual-wavelength irradiation with λ_1 and λ_2 at dose D, $S_{(\lambda1+\lambda2,\ D)}$ was the standard survival point of dual-wavelength irradiation with λ_1 and λ_2 at dose D, Cell $Survival_{(\lambda1+\lambda2,\ D)}$ was the cell viability of dual-wavelength irradiation with λ_1 and λ_2 at dose D. The dose was set to the value of the transition phase for each combination of wavelengths. A large synergistic effect was observed for 405 nm + 505 nm, 405 nm + 545 nm, and 505 nm + 545 nm (Fig. 4). The other combinations did not produce synergistic effects. Evaluation of the effect of using all five wavelengths revealed no synergistic effect (Supplemental Fig. 2).

3.2. Total ROS production was highest using dual-wavelength irradiation

To clarify the mechanisms by which the synergistic effects occurred, we focused on the total ROS. The mean fluorescence intensity of the total ROS roughly corresponded to the absorption coefficients of PpIX (Fig. 5). The level of total ROS at 405 nm + 505 nm was significantly higher than that of the control and was comparable with 405 nm alone, but the difference between 405 nm + 505 nm and 405 nm alone was statistically significant.

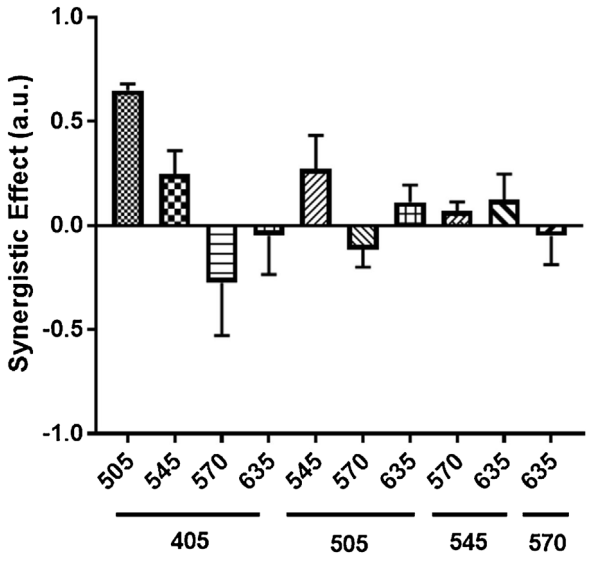

Fig. 4. Comparison of the synergistic PDT effects of dual-wavelength irradiation calculated from the standard-dose line obtained with single irradiation.

3.3. Dual-wavelength irradiation was the most effective in vivo

To test our hypothesis that dual-wavelength irradiation improves the effects of PDT, we conducted PDT using mouse xenograft models with human melanoma cells. 5-ALA was subcutaneously injected into the tumor. Four hours later, the tumor site was irradiated with 50 J/cm^2 using one of two types of

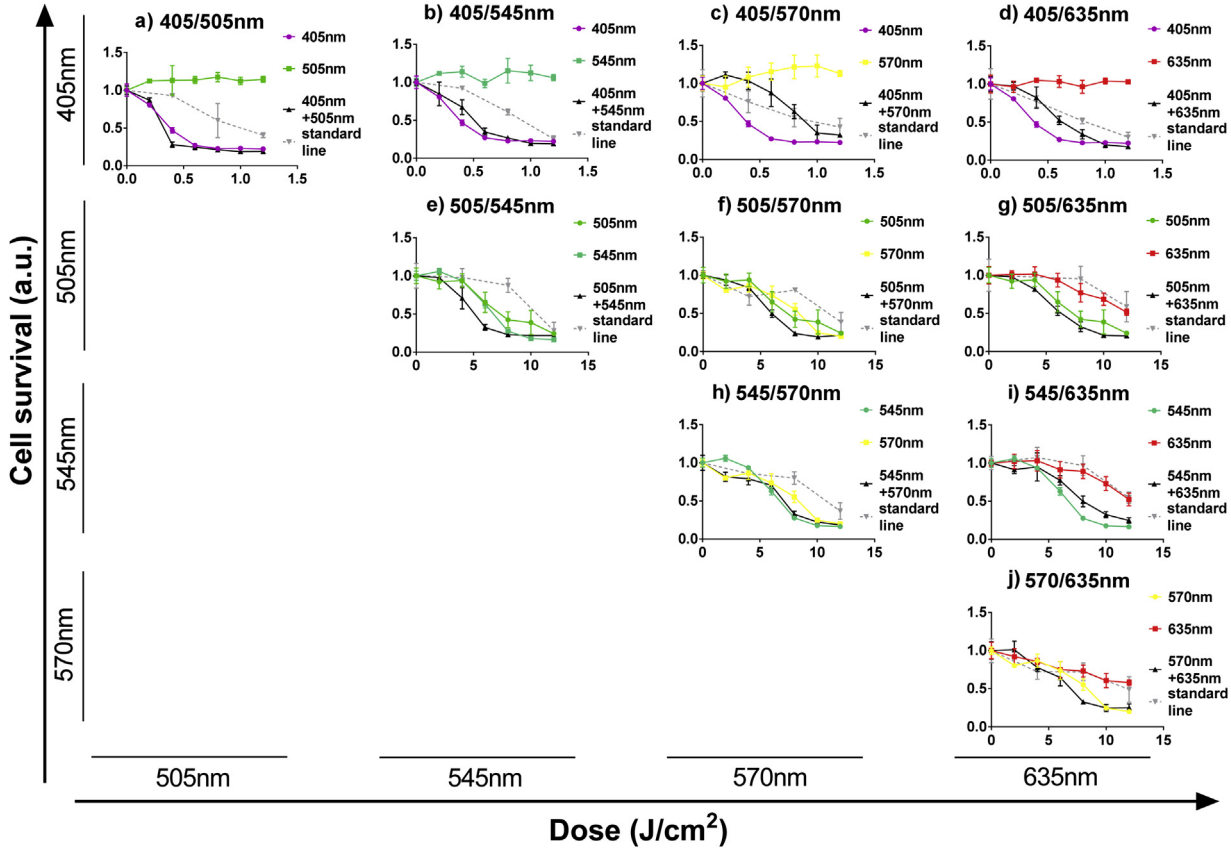

Fig. 3. Dose survival curves of in vitro-irradiated HaCaT cells with a) 405 nm and/or 505 nm, b) 405 nm and/or 545 nm, c) 405 nm and/or 570 nm, d) 405 nm and/or 635 nm, e) 505 nm and/or 545 nm, f) 505 nm and/or 570 nm, g) 505 nm and/or 635 nm, h) 545 nm and/or 570 nm, i) 545 nm and/or 635 nm, j) 570 nm and/or 635 nm.

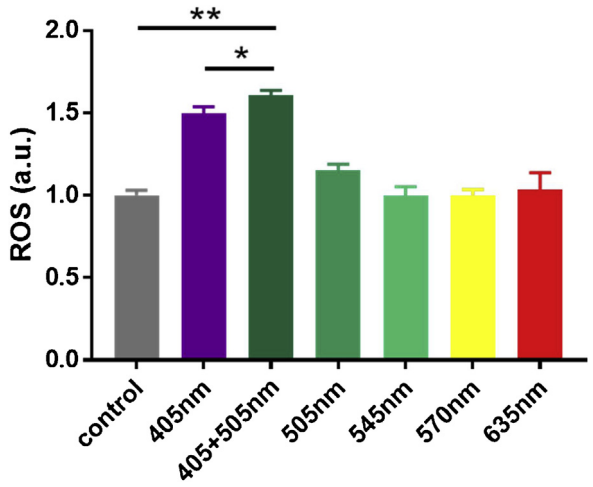

Fig. 5. Comparison of the total ROS production in vitro. The statistical significance of differences was calculated using the Tukey test. Values are expressed as mean ± SD. *P < 0.05, ** P < 0.01 (n = 6/group).

LEDs, the plate type as a conventional type or the flexible type as a novel type at 405 nm, 635 nm, or 405 nm + 505 nm to determine the optimal irradiation method. The relative tumor volume was smaller in all irradiation conditions compared with the control (Fig. 6). In the 405-nm + 505-nm wavelength combination with either the flexible- or plate-type, the tumor was completely disappeared up to day 15 (Fig. 6a). The relative tumor volume on day 21 was 13.0 (405 nm, plate type), 13.0 (405 nm flexible type), 3.8 (405 nm + 505 nm, plate type), 0.3 (405 nm + 505 nm, flexible type), 18.5 (635 nm, plate type), and 10.9 (635 nm, flexible type) (Fig. 6b). The effect of PDT was significantly greater when using the 405-nm + 505-nm wavelength combination with either the flexible- or plate-type LED units, and when using the 635-nm wavelength with the flexible-type LED unit compared with the control at days 7, 14, and 21 (Fig. 6b).

4. Discussion

In the present study, we examined the synergistic effects of dual-wavelength irradiation for PDT. Dual-wavelength irradiation combining 405-nm and 505-nm light produced the greatest synergistic effects in HaCaT cells. The 405 nm wavelength is the most toxic given the absorbance of PpIX, but has low penetration into skin. For that reason, in an attempt to reduce the amount of the shorter wavelength as much as possible, long wavelength light was added, and we searched for a combination that achieved the same effect as 405 nm alone. The results confirmed that the combination of 405 nm and 505 nm was more effective, based on comparison with the standard-dose line, and as effective as 405 nm alone. The level of total ROS at 405 nm + 505 nm was also significantly higher than that of the control and was comparable with 405 nm alone, but the difference between 405 nm + 505 nm and 405 nm alone was statistically significant. These findings suggest that the combination of 405-nm and 505-nm light produced the greatest synergistic effects in HaCaT cells due to high ROS production. Moreover, the 405 nm + 505 nm combination for PDT was also the most effective in vivo. In both the in vitro and in vivo studies, we aimed to confirm the difference in effect due to the differences in the photobiologic characteristics of the light in each study. Therefore, even if the cells are different, it is an appropriate approach for this study. Although mice can be inoculated with HaCaT, the cells do not become attached, which is why we chose to use melanoma cells for the in vivo studies. In a previous study on PDT, we also used HaCaT cells in vitro and COLO679 in vivo [17]. Other groups routinely use similar experimental systems [18]. Mouse skin is approximately 200-μm thick [19]. Based on the small slope of the light penetration curve crossing skin, the skin had relatively little effect on light penetration [20]. Therefore, the difference in transmittance due to the difference in wavelength is larger than the variation in transmittance due to the variation in the depth of the target cells. Thus, this experimental system is suitable for this study. Skin cancer occurs frequently in the face and neck due to sun exposure,

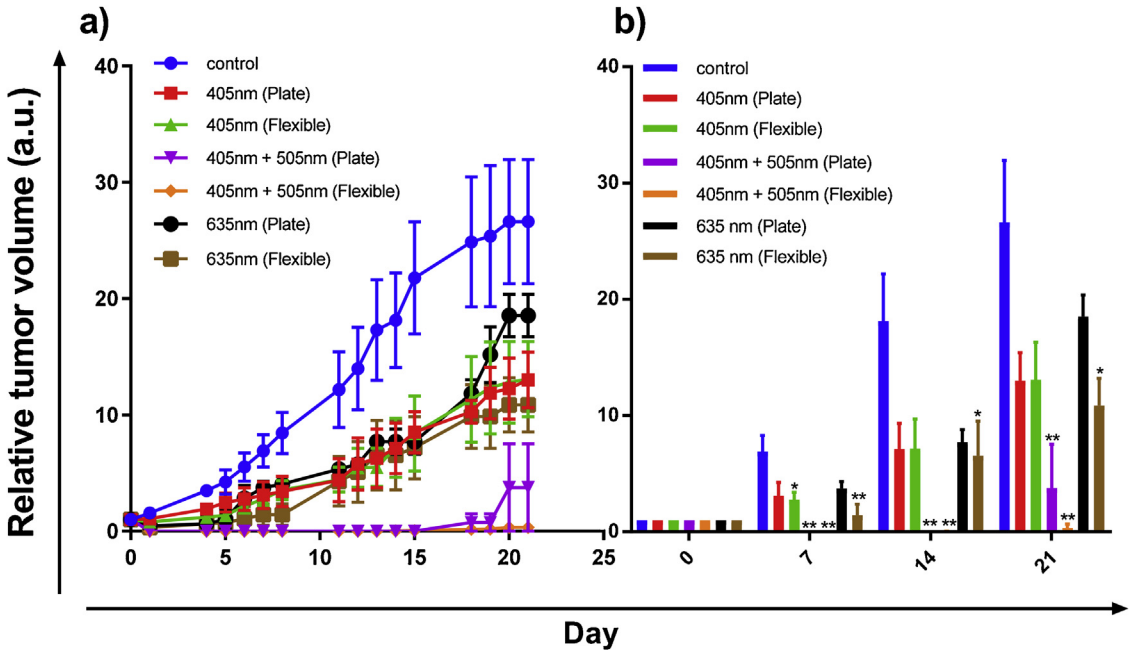

Fig. 6. Effect of PDT in vivo. a) Daily relative tumor volume compared with that before light irradiation, b) Comparison of relative tumor volume on days 0, 7, 14, and 21. The statistical significance of the differences was calculated using the Tukey test. Values are expressed as mean ± SE. A p-value less than 0.05 compared with the control was considered statistically significant. *P < 0.05, ** P < 0.01 (n = 3/group).

and the face is not a flat surface, but rather has an uneven shape. To improve the irradiance uniformity using a light source composed of many LED chips, a large divergence angle LED (e.g., a full divergence angle >135°) is used. On the other hand, with LEDs having a large divergence angle, irradiance on the surface of the concave portion is lower than that on the surface of the convex portion, so it is difficult to uniformly treat the lesion. Further, when LEDs with a small divergence angle (e.g., an entire divergence angle of ˜30°) are used, the dependency of the irradiance on the irradiation distance becomes small, but because the uniformity of the irradiance is low, it is difficult to treat the lesion uniformly. In this study, we used a flexible-type light source unit comprising 405-nm LED chips, a flexible substrate, and a phosphor to convert the light to 505 nm, which was designed to produce uniform irradiance and a spectral distribution at the contact surface. This flexible-type light source unit may solve the problem of uniformly irradiating an uneven surface.

Pain is a side effect of PDT. Pain may be experienced after irradiation and/or post-treatment, and is intense for some patients. Studies of the mechanisms underlying the pain produced during PDT irradiation suggest that the receptor channels expressed at the nerve terminals of the pain fibers are involved in detecting the oxidative stress induced by the treatment [21]. Patients tend to experience more pain with red-light than with blue-light PDT, and a lower pain score is reported with shorter wavelength light, such as green light compared with red light [22]. Whether methyl aminolaevulinate is less painful than 5-ALA has also been discussed [23,24]. For pain relief, various methods, such as cooling [25], topical anesthetics (EMLA [26], lidocaine [27], tetracaine [28], or morphine [29]), lower irradiance/daylight-mediated PDT [30], and two-step irradiance [31,32], have been used in clinical trials. A flexible-type light source unit can be affixed to lesions, so treatment at home as well as at a hospital is possible. In the case of home use, irradiation duration may be increased because treatment can be conducted while performing other activities. Therefore, irradiation with low irradiance becomes possible, which might reduce pain.

In this study, we examined whether dual wavelengths produced a synergistic effect, and whether the same effect as the conventional plate-type LEDs can be achieved using flexible-type LEDs. When considering clinical applications, it is desirable that the light source be flexible to achieve more uniform irradiation of uneven areas. We could therefore conclude that dual-wavelength PDT is an efficient strategy for improving the therapeutic effects of PDT. Using a flexible LED unit is expected to achieve more uniform irradiation of uneven areas.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2018.12.006.

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