In Vitro Percutaneous Absorption and *In Vivo* Protoporphyrin IX Accumulation in Skin and Tumors after Topical 5-Aminolevulinic Acid Application with Enhancement Using an Erbium:YAG Laser

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ABSTRACT: 5-Aminolevulinic acid (ALA) is used as a precursor of protoporphyrin IX (PpIX) for photodynamic therapy (PDT) of superficial skin cancers and subcutaneous metastases of internal malignancies. The permeability of ALA across intact skin is always low, making it difficult to achieve the desired therapeutic benefits. Hence new methods for enhancing ALA permeation are urgently needed. The aim of this study was to determine the *in vivo* kinetics of PpIX generation in mouse tissues after topical ALA application enhanced by an erbium (Er):yttrium-aluminum-garnet (YAG) laser. The *in vitro* permeation of ALA was also used to screen the optimal method for the *in vivo* study. The efficacy of the improved drug delivery was determined as a function of various laser fluences and cancer models. ALA applied to laser-treated skin produced a higher accumulations of PpIX within superficial skin and subcutaneous tumors as compared to those of the non-treated group (t-test, $p < 0.05$). The enhancement ratios (ER) of laser-treated skin ranged from 1.7 to 4.9 times as compared to the control depending to the fluences used. The enhanced PpIX level of laser-treated skin was generally more pronounced in normal and lesional skin than in subcutaneous nodular tumors. Confocal laser scanning microscopy (CLSM) of laser-treated skin revealed intense red fluorescence within the epidermis and upper dermis, and a much-weaker fluorescence within the bottom layers of the skin. On the other hand, the fluorescence intensity of the control group was much lower than that of laser-treated group. The barrier properties of the skin irradiated by the laser had completely recovered within 3 days. Pretreatment of skin using an Er:YAG laser was useful in increasing the amount of Pp IX within skin tumors.
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INTRODUCTION

Skin cancer is the most common of all pathologies related to cancerous disease. Nearly two million

new cases are diagnosed annually in the entire world, and this number continues to rise.[1] Administration of 5-aminolevulinic acid (ALA) is an increasingly popular method of photosensitization for photodynamic therapy (PDT) of skin tumors. ALA is a prodrug to form the photosensitizer—protoporphyrin IX (PpIX) after the exogenous application of ALA. Systemic ALA administration may cause liver damage as well as neuropsychiatric disorders similar to symptoms of acute intermittent porphyria.[2] Topical application of ALA is still the most-promising technique in PDT of skin tumors due to a lack of severe side effects.[3] Complete destruction of a tumor by PDT critically depends on a sufficiently high concentration and homogenous distribution of PpIX in the malignant tissue. ALA is a hydrophilic molecule with a stratum corneum (SC)/water partition coefficient of 0.1.[4] As expected from skin permeability theory, ALA permeates poorly across intact skin.[5]

Lasers are physical devices that have been used for medical diagnosis and therapeutic purposes. The erbium:yttrium-aluminum-garnet (Er:YAG) laser is very popular nowadays, because it can ablate the SC with minimal residual thermal damage to the skin. It is currently used for the resurfacing of rhyrides, scars, and photodamage.[6] We recently suggested that the Er:YAG laser could effectively enhance and control *in vitro* drug delivery via the skin, including: nalbuphine, indomethacin,[7] 5-fluorouracil,[8] vitamin C,[9] and ALA.[10] Although laser-mediated enhancement of drug delivery has been demonstrated for a number of *in vitro* studies, the effect of the Er:YAG laser has not been characterized on an *in vivo* model of PDT. The aim of this present study was to assess whether *in vivo* topical ALA delivery was enhanced using a single pulse of the Er:YAG laser.

The present study investigated the tumor selectivity, tissue distribution, and permeation of PpIX content in skin and tumors after induction by *in vivo* topical ALA application. Franz cells for evaluating the *in vitro* topical delivery by laser treatment were also utilized to correlate the *in vitro* and *in vivo* results. Confocal laser scanning microscopy (CLSM) was employed to visually characterize ALA transport via the skin irradiated by the laser. The safety of the laser and recovery of the skin barrier function were examined by transepidermal water loss (TEWL).

MATERIALS AND METHODS

Materials

ALA, PpIX, phorbol 12-myristate 13-acetate (TPA), and 9,10-dimethyl-1,2-benzathracene (DMBA) were all purchased from Sigma Chemical (St. Louis, MO). The basal cell carcinoma (BCC) cell line was kindly provided by Professor Hsin-Su Yu (Department of Dermatology, National Taiwan University, Taipei, Taiwan).

Er:YAG Laser Assembly

The Er:YAG laser (Continuum Biomedical, Dublin, CA) has a wavelength of 2,940 nm and a pulse duration of 250 μs. An articulated arm was used to deliver the laser beam onto the skin. Output energies of $0.35 \sim 1.20$ Joule (J)/pulse with a beam spot size of 7 mm in diameter achieved fluences of $0.9 \sim 3.1$ J/cm^2. The energy of the laser pulse was monitored with an energy meter (Nova Display, Ophir, Israel) before and after treatment.

In Vitro Topical Delivery of ALA

The diffusion cell used in this *in vitro* experiment was a Franz diffusion assembly. A piece of excised female BALB/C mouse skin ($7 \sim 8$ weeks old) was mounted with the SC side facing towards the donor compartment. After pretreatment with the Er:YAG laser, the skin surface was wiped with a cotton wool swab several times. The receptor compartment (10 mL) was filled with pH 5 citrate-phosphate buffer because of stability considerations.[11] The donor compartment was filled with 8 mL of 3% (w/v) ALA in pH 5 buffer. The available area of the cell was 1.76 cm^2. The compartments were maintained at 37°C, and contents were stirred by a magnetic bar at 600 rpm. At appropriate intervals, 300-μL aliquots of receptor medium were withdrawn and immediately replaced by an equal volume of fresh receptor solution.

The amount of ALA retained in the skin was determined at the end of the *in vitro* experiment (8 h). The application site on the skin was washed 10 times using a cotton cloth immersed in water. A sample of skin was weighed, cut with scissors, positioned in a glass homogenizer containing 1 mL of 0.1 M HCl, and ground for 5 min with an electric stirrer. The resulting solution was centrifuged for 10 min at 10,000 rpm. The supernatant was

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filtered across a 0.45-μm PVDF membrane and then analyzed by HPLC.

HPLC Analysis for ALA

The fluorescence derivation of ALA samples was based on a modification of the Hantzsch reaction.[12] The ALA content of samples was analyzed by HPLC as cited previously.[10] To a test tube, 3.5 mL acetylacetone and formaldehyde solution were added and mixed with a vortex mixer for 3 s. This mixture was heated for 10 min at 100°C with an aluminum-block heater. The test tube was then cooled in an ice bath. A 25-cm-long, 4-mm inner diameter stainless steel RP-18 column (Merck, Darmstadt, Germany) was used. The mobile phase, consisting of methanol:water:acetic acid (50:50:1) was used at a flow rate of 0.7 mL/min. The wavelength of the fluorescence detector was set to excitation at 378 nm and emission at 467 nm. The column oven was set to 40°C. The injection volume was 20 μL. Under these conditions, the ALA retention of approximately 8.5 min was obtained. Standard solution of ALA were prepared in the range $1 \sim 20$ μg/mL and analyzed to obtain a calibration curve ($r = 0.99$).

Animals and Tumor Models

Female BALB/C mice $7 \sim 8$ weeks old were used in this study. A BCC cell line was maintained at 37°C in RPMI 1640 medium containing 10% fetal calf serum and was subcultured twice a week. The back of each mouse was shaved with electric clippers. Tumors were obtained by a subcutaneous injection of 6×10^5 cells into the back of a mouse. Experiments were performed on day 8 after implantation. In the tumor model induced by chemicals, mice were topically treated with DMBA at a dose of 0.1 mM in 50 μL acetone as an initiation treatment twice a week. One week after initiation with DMBA, mice were promoted by the application with 5 nm of TPA in DMSO three times a week. This process continued for 6 weeks. The designed protocol for using animals in this study followed internationally recognized ethical guidelines and was approved by the Institutional Animal Care and Use Committee of Chang Gung University.

In Vivo Topical Delivery of ALA

For pharmacokinetics of ALA-induced PpIX, a glass cylinder with an available area of 0.785 cm^2 was placed on the shaved back skin of a female BALB/C mouse with glue. ALA/pH 5 buffer (0.2 mL) at a concentration of 3% was added to each cylinder. The vehicles were applied either on the skin overlying the tumor or on an area of normal skin. The application times of the vehicle were 2, 4, or 6 h. After excising the skin or the solid tumor under the skin on which the vehicle was applied, the skin was washed 10 times with a cotton cloth immersed in methanol. All procedures were carried out in the dark to prevent the influence of ambient light.

Extraction of PpIX

PpIX in tissue samples was extracted and quantified using procedures modified from Fritsch et al. and Tsai et al.[3,4] One milliliter of a 1.5 M perchloric acid:methanol (1:1 v/v) mixture was added to the excised tissues in a glass homogenizer and ground for 5 min. The resulting solution was centrifuged for 10 min at 10,000 rpm. The fluorescence of the supernatant was determined spectrofluorometrically (Hitachi F-2500, Tokyo, Japan) at an excitation wavelength of 403 nm and emission at 602 nm. The extracted amount could exhibit a good linearity to the original PpIX amount in an *in vitro* test ($r = 0.97$). The recovery in the *in vitro* status was $93.2 \pm 6.8\%$.

Confocal Laser Scanning Microscopy (CLSM)

Skin samples obtained following *in vivo* treatment were examined for PpIX fluorescence images by CLSM. The full skin thickness was optically scanned at $10 \sim 16$-μm increments through the Z-axis of a Leica TCS SP2 confocal microscope (Wetzlar, Germany). Optical excitation was carried out with a 488-nm argon laser beam, and the fluorescence emission was detected at 590 nm.

Skin Barrier Recovery

The recovery of laser-treated skin was evaluated for 3 days using live mice. The treated skin was examined for its TEWL. TEWL was measured quantitatively with an evaporimeter (Tewameter 300, Courage and Khazaka, Köln, Germany). Measurements were taken 30 min, and 1, 2, and 3 days after laser treatment. TEWL values were automatically calculated and are expressed in $g/m^2/h$. An adjacent untreated site was used as a baseline standard for each determination.

Statistical Analysis

The statistical analysis of differences between different treatments was performed using the unpaired Student's *t*-test. A 0.05 level of probability was taken as the level of significance. The ANOVA test was also utilized in this study.

RESULTS

In Vitro Topical Application of ALA

The *in vitro* permeated amount of ALA ($\mu g/cm^2$) in the receptor compartment was plotted as a function of time. The slopes of the resulting plots were computed, and the fluxes ($\mu g/cm^2/h$) were calculated from the slopes (Tab. 1). The results in Table 1 demonstrate that the Er:YAG laser was effective in enhancing skin permeability of ALA at all intensities studied. Partial ablation of the SC by the laser, which reduced the inherent barrier of the skin is a possible reason for the increase in flux after exposure to the laser. An increase in laser intensity led to a further enhancement of ALA flux of from 0.9 to 2.6 J/cm^2. However, the use of the highest fluence (3.1 J/cm^2) did not further increase the *in vitro* ALA permeation. Deposition of ALA within the skin was also enhanced by laser irradiation; however, the ratio of enhancement was lower than the ratio of flux for ALA permeation (Tab. 1).

PpIX Kinetics in Normal Skin after *In Vivo* Topical ALA Application

The time course (2 h, 4 h, and 6 h) of PpIX levels in the skin after the *in vivo* topical application of 3% ALA is shown in Figure 1a. The maximal accumulation of PpIX was found 6 h after administration in the non-treated control group in this present study. There was no significant difference (*t*-test, $p > 0.05$) in PpIX contents at 2 and 4 h between the control group and the group treated with 1.4 J/cm^2. On the other hand, PpIX accumulation in the skin at 6 h was lower for 1.4 J/cm^2-treated mice compared to the controls. Both 1.7 J/cm^2- and 2.0 J/cm^2-treated groups showed higher PpIX deposition than the control group. Skin treated with a fluence of 2.0 J/cm^2 showed a different manner of PpIX deposition compared to that treated with 1.7 J/cm^2. There was no significant difference (ANOVA test, $p > 0.05$) among PpIX levels of the 2.0 J/cm^2-treated group with various application times.

PpIX Kinetics in the BCC after *In Vivo* Topical ALA Application

As shown in Figure 1b, ALA-induced PpIX in BCCs peaked at between 4 and 6 h post-application in the control group. As compared to Figure 1a, the PpIX concentration after administration of ALA was more pronounced in the skin than in the tumor. All treatments of the Er:YAG laser at $1.4 \sim 2.0$ J/cm^2 enhanced the PpIX concentration within the BCC at 2 h post-application (Fig. 1b). On the other hand, there was no significant difference (*t*-test, $p > 0.05$) in PpIX accumulation at 4 and 6 h between laser and non-laser treatment. There was a tendency for the PpIX concentration at 4 h to be higher for the 1.4 J/cm^2-treated group than for the non-treated group, but this was not statistically significant (*t*-test, $p > 0.05$).

PpIX Kinetics in Superficial Lesions after *In Vivo* Topical ALA Application

As shown in Figure 1c, the superficial intralesional PpIX levels were relatively lower than the

Table 1. Fluxes, Skin Deposition, and Enhancement Ratios of ALA across Skin Treated by Er:YAG Laser

Fluence (J/cm^2)	Flux ($\mu g/cm^2/h$)	ER_{flux}[a]	Skin Deposition (ng/mg)	$ER_{deposition}$[b]
0	6 ± 2	—	142 ± 27	—
0.9	184 ± 34	30	682 ± 178	5
1.2	351 ± 56	57	582 ± 67	4
1.4	418 ± 41	68	612 ± 232	4
1.7	493 ± 107	81	587 ± 186	4
2.0	512 ± 98	84	796 ± 9	6
2.6	815 ± 108	133	677 ± 200	5
3.1	659 ± 109	108	672 ± 117	5

[a]The enhancement ratio (ER) was the ALA flux of laser treatment group/ALA flux of control group.
[b]The ER was the ALA deposition in skin of laser treatment group/ALA deposition in skin of control group.
Each value represents the mean $\pm$ SD ($n = 3 \sim 6$).

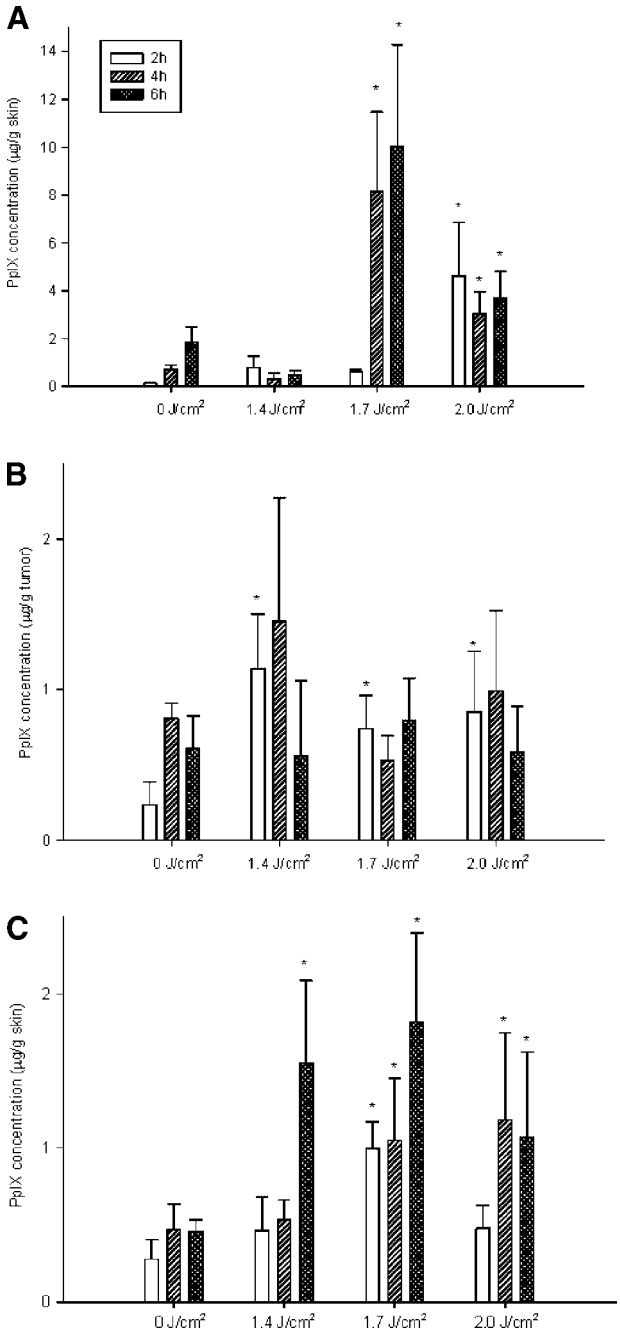

Figure 1. PpIX concentrations in normal skin (A), subcutaneous BCCs (B), and superficial cancerous skin (C) at 2, 4, and 6 h after *in vivo* topical application of 3% ALA into Er:YAG laser-treated skin at various fluences. *A significantly higher value ($p < 0.05$) as compared to the control group. Each value represents the mean ± SD ($n = 3 \sim 6$).

PpIX levels in normal skin. The laser with a fluence of 1.7 J/cm^2 showed the highest energy efficiency for enhancing the PpIX accumulation in superficial lesions. A significant difference (t-test, $p < 0.05$) was observed for all application durations (2, 4, and 6 h). In contrast to that of normal skin, 1.4 J/cm^2 of fluence was sufficient to produce enhancements in the amounts of PpIX at 6 h (Fig. 1c).

Confocal Images of Skin after Topical Application of ALA

Figure 2 shows confocal images obtained from control and laser-treated (1.7 J/cm^2) samples of BALB/C mice skin after ALA application for 1 h. The full skin thickness was optically scanned at $10 \sim 16$-μm increments for 16 fragments from the surface of skin (left to right, top to bottom). Besides PpIX signaling, the fluorescence intensity may also depend on the laser intensity, thickness of fragments, and scattering within the skin. Hence a blank skin only applied by pH 5 buffer was monitored by CLSM. No or negligible signal could be detected in the sample. Hence the influence of such factors may be excluded. Compared to the control (Fig. 2a), increased red fluorescence in skin treated with the Er:YAG laser can clearly be seen with intense signals (Fig. 2b). Considering that PpIX has excitation wavelengths around 400 nm and an emission peak at 630 nm, this red fluorescence appears to arise from PpIX. This result of CLSM may relate to the result of PpIX extraction from normal skin in Figure 1a: laser treatment can increase ALA deposition within skin. For the site exposed to laser irradiation, there was a broad band of red fluorescence that extended from the epidermis into the upper dermis. Moderate fluorescence was also observed in the upper dermis. There was almost no fluorescent signal in the bottoms layers of either the laser-treated or non-treated skin.

Skin Barrier Recovery

Baseline values of non-treated sites were subtracted from the achieved measurements to give actual changes in TEWL (ΔTEWL). Figure 3 shows the recovery of ΔTEWL after laser ablation. The value of ΔTEWL at day 0 increased following an increase in the laser fluence. There was an excellent relationship ($r = 0.99$) between fluences and ΔTEWL on day 0. The skin had recovered to a normal status 3 days after laser treatment regardless of the fluences used (ANOVA test, $p > 0.05$).

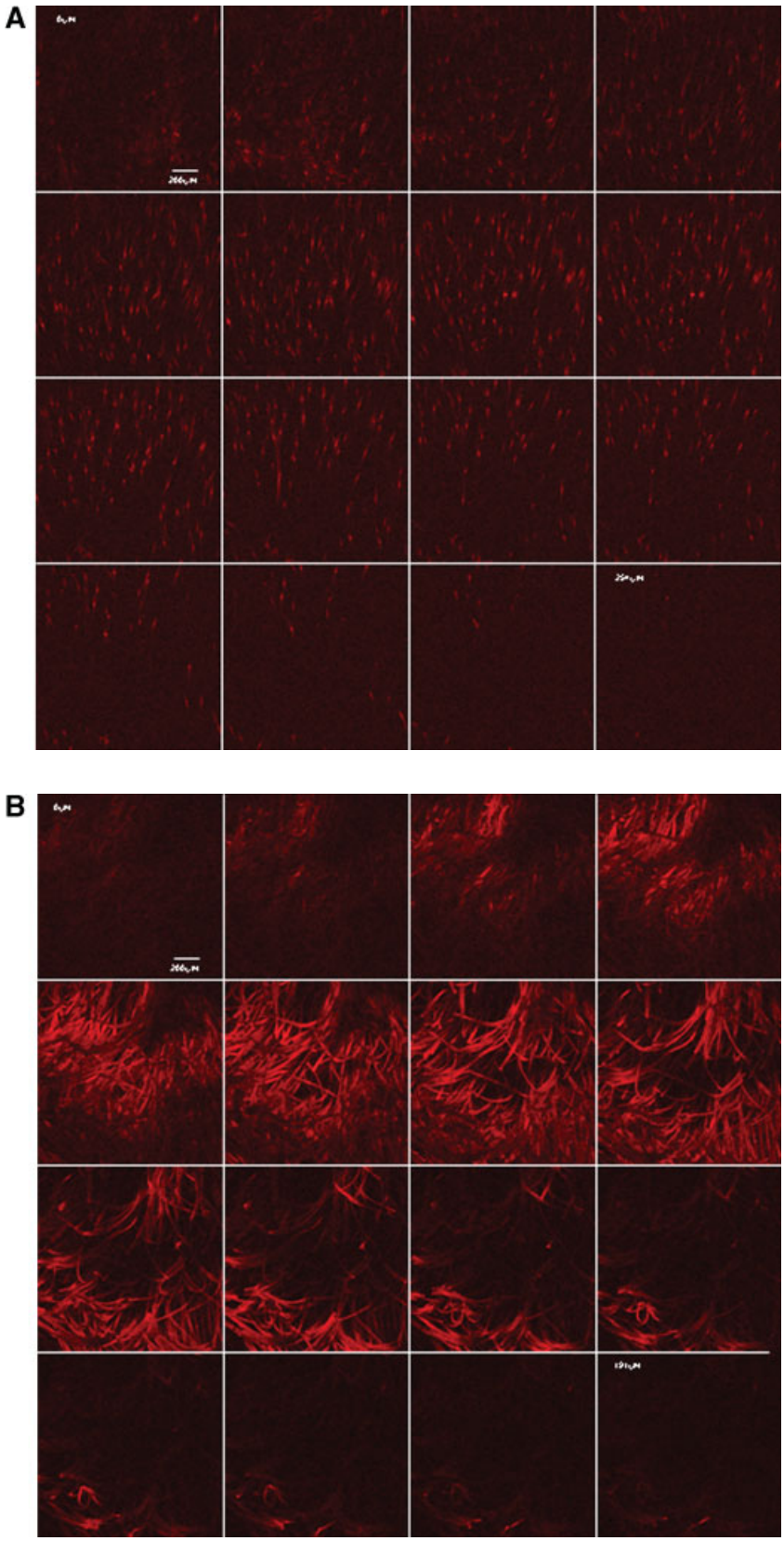

Figure 2. CLSM micrographs of BALB/C mouse skin after application of 3% ALA without (A) and with (B) treatment of Er:YAG laser at 1.7 J/cm^2.

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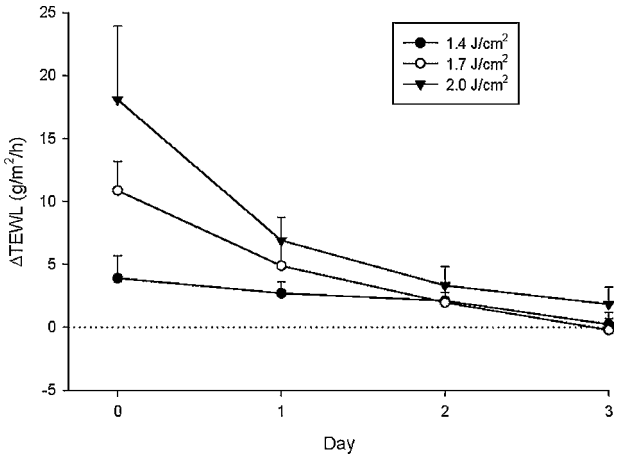

Figure 3. Kinetics of ΔTEWL of BALB/C mouse skin during 3 days after Er:YAG laser treatment at various fluences. Each value represents the mean ± SD ($n = 6$).

DISCUSSION

The fluences of the laser used varied between 0.9 and 3.1 J/cm^2 in the *in vitro* study, which are lower than those utilized in clinical therapy.[13,14] There was a linear relationship between the ALA flux and the laser fluences tested from 0.9 to 2.6 J/cm^2 (correlation coefficient, $r = 0.98$). This linear relationship suggests that ALA permeation can be precisely controlled using an Er:YAG laser with fluences of $0.9 \sim 2.6$ J/cm^2. However, this correlation was reduced ($r = 0.90$) when including the profile of the highest fluence (3.1 J/cm^2). ALA is first partitioned into the SC, after which it progresses through the skin to deeper layers. Although removal of a part of the SC can reduce the inherent barrier properties of the skin and thus increase permeation, the partitioning of drugs into the SC should be deceased after removal by the laser, which results in an offset effect.[8,9] Two mechanisms, including optical breakdown (photomechanical stress) and direct ablation, can be generated by Er:YAG laser irradiation.[7,9] The photomechanical stress induces expansion of the lacunar spaces within the highly tortuous intercellular domains leading to the formation of transient channels.[8,15] The Er:YAG laser acts on the lipid-rich pathways by photomechanical waves, but also on the intracellular regions by disrupting the corneocytes.[8] Both pathways may contribute to the enhancement of permeation by the laser.

The skin deposition of ALA was enhanced by laser irradiation; however, the ratio of enhancement was lower than the data for flux (Tab. 1). This may have been due to the lack of an SC drug reservoir within the skin, resulting in insufficient space for the deposition of ALA. Discrepancies in skin deposition among various fluences of the Er:YAG laser were not large (ANOVA test, $p > 0.05$). A very low correlation ($r = 0.32$) was calculated between the fluence and its enhancement ratio (ER), $ER_{\text{deposition}}$ value. The offset effect by the reduced skin volume in which ALA can be deposited at higher fluences may be responsible for this low correlation.

PpIX levels following *in vivo* topical delivery of ALA were determined in BALB/C mice over a period of 6 h. In the control group without laser treatment, maximal accumulation was found in skin 6 h after administration. The PpIX accumulation in skin at 6 h was lower for 1.4 J/cm^2-treated mice as compared to the control. This suggests that the effect of the devoid SC volume by laser ablation was superior to the effect of inherent barrier reduction at this lower fluence. An opposite trend was observed at higher fluences since PpIX deposition was significantly increased although ablation of the SC layer was also greater (Fig. 1a). The 1.7 J/cm^2 fluence was generally the most-efficient energy level for enhancing *in vivo* ALA delivery. By determining PpIX deposition within skin, the 1.7 J/cm^2-treated group showed ER values of 4.39, 11.31, and 5.42 at 2, 4, and 6 h, respectively. The PpIX within the skin was higher at 2 h and lower at 4 and 6 h for the 2.0 J/cm^2-treated group than for the 1.7 J/cm^2-treated one. This suggests that reduction of the skin barrier by photomechanical stress and direct ablation is important for topical ALA absorption in the beginning of application since the higher fluences produced greater disruption of the skin. After that, the ALA molecules should partition into the limited spaces of the skin reservoir.

The literature has reported a complete initial response rate of >85% for superficial BCCs by ALA. However, for subcutaneous nodular BCCs, a much-lower complete response rate of 50% has been obtained.[1,16] PpIX production in subcutaneous BCCs, after topical delivery of ALA with or without laser treatment, was thus investigated. The concentration of PpIX after administration of ALA was more pronounced in the skin than in the tumor for the control group. The increased permeability of some cutaneous neoplasms due to a damaged skin barrier further enhances the activity of ALA when it is applied topically.[1] However, this phenomenon was not observed in this present study. Topical ALA PDT is restricted when used against thick lesions.[17] The efficacy of topical ALA

with respect to nodular and nodulo-ulcerative lesions by invasion of overlying tumor skin with a thickness of more than $2 \sim 3$ mm dramatically decreased.[1,18] This may result in limited PpIX distribution to deeper-lying or nodular BCCs.

Another possible reason for the low accumulation of PpIX in the tumor may be the predominant location of topical ALA in tissues. It has been shown that after topical ALA application to mice, red fluorescence was observed mainly to be localized in the epidermis of the skin and in the upper part of subcutaneously located carcinomas, but no fluorescence was seen at the bottom of tumors.[18,19] The increased efflux of ALA and PpIX into the circulation from tumor tissues may also contribute to the lower efficiency of topical ALA movement into the tumor.[19]

All treatments with the Er:YAG laser at $1.4 \sim 2.0$ J/cm^2 enhanced the PpIX concentration within BCCs at 2 h post-application (Fig. 1b). This may suggest that the Er:YAG laser can accelerate ALA permeation into tumors at the initial stage of application, and thus, this can reduce the onset time of topical ALA delivery. It was evident that the transport and enhancement of ALA in the *in vitro* experiments were higher than those in the *in vivo* studies in BALB/C mice. It was found that for hydrophilic molecules, correlations between *in vitro* and *in vivo* studies are generally poor; *in vitro* studies give higher rates than *in vivo* results.[20,21] This may be due to the intact clearance processes (vascular and enzymatic) in the *in vivo* status.

The experimental group which received treatment with DMBA and TPA showed 100% incidence of superficial papillomas and carcinomas within 6 weeks. The superficial intralesional PpIX levels were relatively lower than the PpIX levels in normal skin (Fig. 1c). The hyperkeratotic and thicker skin surrounding the lesions may be the reasons for the lower accumulation of PpIX. The average skin weight in the same area (0.785 cm^2) for normal skin and lesional skin was 12.81 ± 6.22 and 37.14 ± 12.00 mg, respectively, indicating the massive tissues of the skin associated with the superficial carcinomas. In contrast to normal skin, the 1.4 J/cm^2 fluence was sufficient to produce enhancement in the amounts of PpIX at 6 h (Fig. 1c). Both normal skin and lesional skin showed an enhancing trend of 1.7 J/cm^2 $>$ 2.0 J/cm^2 $>$ 1.4 J/cm^2, indicating a similar mechanism of the Er:YAG laser on both skin tissues.

CLSM was utilized to examine the fluorescence resulting from photosensitizer action in skin specimens.[22] For the site exposed to laser irradiation, there was a broad band of red fluorescence that extended from the epidermis into the upper dermis. The fluorescence gradient in laser-treated skin decreased with tissue thickness from the epidermis to the dermis. Epidermal cells synthesize a much-higher quantity of porphyrins than fibroblasts. Also, ALA-induced porphyrins are preferentially localized in the epithelial lining of the skin. The moderate fluorescence shown in the upper dermis may be an artifact of the vestigial appendages left behind in the dermis when the skin is split.[4,23] There was almost no fluorescence signal at the bottom of the laser-treated skin. It seems reasonable to suggest that limited ALA permeation into the deeper skin layers where the nodular tumors are located contributes, at least in part, to the limited enhancement of PpIX accumulation in BCCs.

One of the characteristics of an ideal permeation-enhancing method is that the skin should recover its normal barrier properties following removal of the enhancing method. The safety of an enhancer is crucial for its applicability. The TEWL values of skin after laser treatment with various fluences were thus measured. TEWL refers to the rate at which water migrates from viable tissues through the layer of the SC to the external environment. It has demonstrated a good correlation between destruction of the skin barrier and increases in TEWL.[8] A higher ΔTEWL was determined by a more-persistent skin structural alteration resulting from a deeper level of ablation by higher laser fluences (Fig. 3). There was an excellent relationship ($r = 0.99$) between the fluences and ΔTEWL on day 0, indicating that the Er:YAG laser with various energies could precisely control the skin barrier properties. The rapid healing after Er:YAG laser treatment within 3 days is presumably related to the superficial nature of Er:YAG laser ablation and the minimally induced thermal damage.

Er:YAG laser could increase ALA permeation by a $30 \sim 130$-fold depends on the fluences applied. The potential of other enhancing method for increasing ALA permeation are also examined by investigators, such as chemical enhancers, iontophoresis, and patch forms.[22,24–27] It is reported that dimethyl sulfoxide (20%) can double ALA flux across hairless mouse skin.[22] The combination of anionic lipophilic counter-ions with 6-ketocholestanol, a permeation enhancer, can totally increase ALA delivery by an approximately sevenfold.[27] A $5 \sim 10$-fold increase of ALA permeation can be

achieved by iontophoresis or self-adhesive thin film formulations.[24,26] By increasing the skin temperature from 31°C to 36°C, about a 50% increase of the PpIX fluorescence is observed.[25] It should be noted that the permeation procedures and evaluation methods are different in these investigations. Hence a comparison among various enhancing methods should be cautious. Nevertheless, Er:YAG laser may have been proven as an efficient method for improving ALA permeation. Although SC-stripping technique may also produce high ALA permeation in clinical situation, laser may offer a more-precise and quicker method to control the enhancement.

CONCLUSIONS

This study shows that Er:YAG laser irradiation of the skin increased the *in vitro* and *in vivo* efficacy of topical ALA delivery. The transport of ALA in the *in vitro* study was higher than the transport in the *in vivo* study in mice. The lower enhancement of PpIX kinetics in superficially normal and lesional skin was much higher than that in subcutaneous BCCs. The threshold of laser fluences for enhancing PpIX deposition was lower in lesional skin and the subcutaneous tumor than in normal skin. SC barrier ablation, ultrastructural alteration of the remaining SC layer, and drug partitioning efficiency into the skin may contribute to the mechanisms influencing the permeation of ALA across laser-treated skin. The recovery results by TEWL determination showed that the complete reversibility of disruption to laser-treated skin at various fluences was achieved within 3 days. SC barrier removal and ALA permeation with this laser were controlled with a single pulse. Utilization of the Er:YAG laser for enhancing PpIX accumulation should be recommended for the treatment of more-superficial lesions such as superficial BCCs, keratoacanthomas, superficial SCCs, and Bowen's disease, because the laser can induce higher PpIX deposition in the epidermis and upper dermis. Er:YAG laser is a quick and controllable method to ablate SC, followed by application of PDT to induce photochemical reaction.

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