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Remodeling of dermal collagen in photoaged skin using low-dose 5-aminolevulinic acid photodynamic therapy occurs via the transforming growth factor- β pathway

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Abstract

5-Aminolevulinic acid photodynamic therapy (ALA-PDT) is known to be effective in the treatment of photoaged skin. However, the molecular mechanisms still remain elusive. Protoporphyrin IX (PpIX) fluorescence is primarily located in the epidermis while ALA-PDT affects the dermal collagen, presumably by an indirect mechanism. This study aimed to investigate the molecular communication in low-dose ALA-PDT occurring between epidermal keratinocytes and dermal fibroblasts. Western blotting and enzyme-linked immunosorbent assays were performed to evaluate collagen expression and transforming growth factor- β (TGF- β) signaling in human keratinocytes and dermal fibroblasts. The impact on fibroblast proliferation was assessed by morphology and proliferating cell nuclear antigen immunofluorescence. Skin biopsies from mice were used to analyze the histological changes in dermal collagen and PpIX distribution. When fibroblasts were cocultured with keratinocytes treated with low-dose ALA-PDT, collagen synthesis and fibroblast proliferation were enhanced. Low-dose ALA-PDT stimulated TGF- β 1 expression in keratinocytes. Fibroblasts cocultured with low-dose ALA-PDT-treated keratinocytes also showed activation of the TGF- β pathway. In vivo, PpIX fluorescence was densely distributed in photoaged mouse epidermis while collagen in the mouse dermis underwent remodeling. This

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Conflicts of interest

The authors have no conflicts of interest to declare.

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study suggests that low-dose ALA-PDT can stimulate keratinocytes to release TGF- β 1, activating the TGF- β pathway in dermal fibroblasts to remodel collagen in the dermis.

Graphical abstract

Keywords

5-aminolevulinic acid; collagen; photoaging; photodynamic therapy; TGF- β

1 | INTRODUCTION

Several clinical studies have shown that 5-aminolevulinic acid photodynamic therapy (ALA-PDT) can improve skin photoaging in humans [1–5]. ALA-PDT improves fine wrinkles and tightens slackness in photoaged skin possibly through the upregulation of collagen production [6]. However, the underlying mechanisms are still unclear.

PDT is a 2-step therapy, which combines application of a nontoxic photosensitizer (PS) together with irradiation with harmless visible light. Singlet oxygen and other reactive oxygen species (ROS) are produced that can damage biomolecules. ALA is commonly used as a precursor for PS synthesis in PDT, especially in dermatology. When ALA is topically applied to the skin, it is converted into intracellular protoporphyrin IX (PpIX). Our previous study demonstrated that the intense PpIX fluorescence was primarily located in the epidermis, rather than in the dermis [7], which suggests that the action of ALA-PDT should primarily affect the epidermis, and not the dermis. However, the fibroblasts producing collagen in the skin are embedded in the dermis and not the epidermis. Therefore, ALA-PDT probably affects the dermis indirectly. Increasing recent evidence suggests that there are “cross-talk” pathways occurring between keratinocytes and fibroblasts which are mediated by secreted cytokines [8, 9]. Some cytokines and soluble mediators may play a role in the remodeling dermal collagen occurring after low-dose ALA-PDT.

The transforming growth factor- β (TGF- β) pathway regulates aspects of cell growth and extracellular matrix synthesis, including collagen synthesis by dermal fibroblasts [10, 11]. TGF- β 1, one of the TGF- β family members, is a key factor in collagen synthesis. TGF- β 1 is synthesized as a latent precursor, which is subsequently activated by destruction of the

latency-associated peptide (LAP) by a variety of mediators, including ROS. There is a report in the literature suggesting that keratinocytes can express more TGF- β 1 after ALA-PDT treatment [12]. We hypothesized that ALA-PDT may target the keratinocytes and activate the dermal fibroblasts indirectly via the TGF- β pathway. In the present study, we treated human keratinocyte cells with ALA-PDT and then cocultured them with human dermal fibroblasts (HDF) in a transwell coculture system. We found that low-dose ALA-PDT stimulated keratinocytes to release TGF- β 1 and dermal collagen was remodeled by activation of the TGF- β pathway in fibroblasts.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The immortalized human epidermal keratinocyte cell line (HaCaT, ZQ0044, Shanghai Zhong Qiao Xin Zhou Biotechnology Co. Ltd., Shanghai, China) was cultured in DMEM medium (Gibco, Carlsbad, CA USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA USA), 100 U/mL penicillin, and 100 U/mL of penicillin/streptomycin (Gibco, Carlsbad, CA USA) at 37°C and 5% CO₂. The human dermal fibroblasts-adult (HDF-a, 2320, Sciencell, San Diego, CA USA) cells were cultured in fibroblast medium (Sciencell, San Diego, CA USA) at 37°C and 5% CO₂.

2.2 | Cell viability assay

Cell viability was determined using the MTT tetrazolium dye colorimetric assay for mitochondrial activity. Keratinocytes were seeded in 96-well plates at a density of 2×10^3 cell/well and incubated overnight. Twenty-four hours after ALA-PDT treatments, MTT solution (Sigma, St. Louis, MO, USA) was added to each well. Then cells were incubated for an additional 4 hours. Subsequently, the medium was removed and replaced with 150 microliters DMSO solvent, and plates were incubated for 30 minutes with shaking to dissolve the precipitate. Absorbance was measured at 570 nanometers using a spectrophotometer.

2.3 | Intracellular ROS fluorescence imaging

Keratinocytes were seeded into 35-millimeter diameter dishes. After the ALA-PDT treatment, the medium was removed and the cells were washed 3 times with PBS. Then the cells were treated with 10-millimolar 2,7-dichlorofluorescein diacetate (DCFH-DA; Beyotime Biotechnology Co. Ltd., Shanghai, China) and incubated in dark for 30 minutes at 37°C. ROS production was visualized using a Fluorescence microscopy (Olympus, excitation 494 nanometers, emission 518 nanometers).

2.4 | Keratinocyte-fibroblast coculture system and ALA-PDT treatment

Six well transwell plates (Corning, NY USA) with 0.4-micrometer pore polyester (PET) membrane inserts were used in our study. The porous membrane separating the upper and lower chambers acts in a similar manner to the basement membrane separating epidermal keratinocytes from dermal fibroblasts in skin. While the cells cannot pass through the membrane, soluble factors produced by keratinocytes are able to permeate this membrane and influence the fibroblasts. The upper compartments on their own were seeded with

keratinocytes 16 hours before. After removing the growth medium, serum-free medium containing 1 millimolar ALA was added to the upper compartments. Keratinocytes were allowed to take up ALA for 6 hours. Following removal of the ALA medium, the cells were rinsed and phosphate-buffered saline was added. Irradiation with various energy densities of light ($0.5\text{--}4\text{ J/cm}^2$) was performed for one time immediately afterward. Irradiation of the cells was performed using a 630 ± 15 nanometers LED light (Philips, Netherlands). Power density is 0.01 mW/cm^2 . After the irradiation, the upper compartments were inserted into the lower compartments that had been seeded with fibroblasts 16 hours before and cocultured for specified periods of time.

2.5 | Western blot analysis

Total protein was extracted from treated cells using RIPA buffer. Equal amounts of cell extracts were fractionated by SDS-PAGE and analyzed by Western blotting and visualized by enhanced chemiluminescence. The following primary antibodies were employed: anti-pro-COL1A1 (SC-8782, Santa Cruz, Dallas, TX, USA), anti-TGF- β 1 (ab92486, Abcam, Cambridge, MA, USA), anti-TGF- β receptor I antibody (ab31013, Abcam, Cambridge, MA, USA), anti-TGF- β receptor II (ab186838, Abcam, Cambridge, MA, USA), antiphospho-Smad2/Smad3 (8828, Cell Signaling, Danvers, MA, USA), anti-Smad2/3 (3102, Cell Signaling, Danvers, MA, USA), anti-CTGF (SC-14939, Santa Cruz, California) and anti-GAPDH antibody (sc-47724, Santa Cruz, Dallas, TX, USA). The quantification of protein bands was carried out using GelPro Analysis Software (Media Cybernetics).

2.6 | Immunofluorescence staining

Fibroblast cells were grown on slides in the lower chambers of transwell plates. Forty-eight hours after coculturing with keratinocytes, cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature. After washing, the fixed cells were permeabilized with 1% Triton-X 100 for 20 minutes at room temperature, then blocked with 5% bovine serum albumin at 37°C for 30 minutes, and incubated with primary antibodies against proliferating cell nuclear antigen (PCNA; 13110 s, Cell Signaling, Danvers, MA, USA) at 4°C overnight. The following day, the slides were incubated with FITC-labeled secondary antibody (WH1116, Jackson ImmunoResearch Lab., West Grove, PA, USA) in the dark at 37°C for 30 minutes and 4',6-diamidino-2-phenylindole (DAPI) for 20 minutes. Slides were mounted with antifade mounting medium (Beyotime Biotechnology Co. Ltd., Shanghai, China). Representative images were taken by fluorescence microscope (BX51TF, Olympus). The objective lens was $40\times$. The slides were imaged and analyzed using Image-Pro Plus software (Media Cybernetics).

2.7 | ELISA for determination of TGF- β 1

The concentrations of TGF- β 1 in the medium were determined using commercially available ELISA kit (eBioscience, San Diego, CA, USA) according to the manufacturer's protocol.

2.8 | Hairless mouse model of photoaged skin and ALA-PDT treatment

Adult male SKH-1 hairless mice (6-8 weeks old, 20-30 grams, Jackson Laboratories) were used. A SS-03AB ultraviolet (UV) phototherapy light source (Shanghai Sigma High Technology Co. Ltd, Shanghai, China) was used for UVA irradiation (wavelength 340-400 nanometers, power density 0.6 mW/cm²) and UVB irradiation (wavelength 311-313 nanometers, power density 0.26 mW/cm²). Five daily sessions of irradiation were applied each week for 8 weeks. The UVA irradiation dose was 36 mJ/cm² in the 1st and 2nd week, the dose was increased by 6 mJ/cm² every 2 weeks. The UVB irradiation dose was 15.6 mJ/cm² in the first and second week, the dose was increased by 2.6 mJ/cm² every 2 weeks, UV irradiation was discontinued after 8 weeks, and PDT was delivered. The PDT group of mice were anesthetized by intraperitoneal injection of chloral hydrate (280 mg/kg). ALA 3% cream (Shanghai Fudan-Zhangjiang BioPharmaceutical Co. Ltd., Shanghai, China) was uniformly applied on the back of each mouse and was followed by application of an occlusive dressing. After 3 hours of incubation in the dark, excess ALA cream was removed and the ALA-applied area of skin was irradiated by a red LED light (Wuhan Yage Optic and Electronic Technique Co. Ltd., Wuhan, China) at 633 ± 15 nanometers at 100 mW/cm², the total light dose for each mouse was 6 J. The irradiation parameters of the control (red light alone) group were identical to that of the PDT group. The PDT group of mice received 3 sessions of PDT (once a week), The red light group of mice received 3 sessions of red light irradiation (once a week).

2.9 | Histopathological examination

Mice from each group were sacrificed 12 hours after PDT or red light treatment and the skin tissues were collected and subjected to routine H&E (Haematoxylin and Eosin) and Masson's trichrome staining for histological analysis. The IOD (integrated optical density) values were measured using Image-Pro Plus software (Media Cybernetics).

2.10 | PpIX fluorescence imaging

Cryosections were visualized under a fluorescence microscope (BX51TF, Olympus) equipped with a mercury lamp for excitation. The fluorescence excitation wavelength was set at 410 nanometers and the emission wavelength at 630 nanometers. The objective lens was 40×. Fluorescence images were captured using Image-Pro Plus software (Media Cybernetics).

2.11 | Statistical analysis

The nonpaired *t* test was used to assess statistical differences for all quantitative comparisons between the control and PDT groups.

3 | RESULTS

3.1 | Viability analysis and intracellular ROS detection of keratinocytes treated with ALA-PDT

To determine the suitable light dose for low-dose ALA-PDT, we performed an MTT assay to measure the viability of keratinocytes: control group (without treatment) and PDT groups (1,

2, 4, 8 and 16 J/cm²). The PDT group of cells were incubated for 6 hours with 1-millimolar ALA. As shown in Figure 1A, ALA-PDT suppressed the viability of keratinocytes in a dose-dependent manner compared with the control group. The viability of the 2 J/cm² and 4 J/cm² groups were 92.3% and 68.3%, respectively. To ensure more than 90% of keratinocytes were alive, the experimental assays were performed at 2 J/cm² or below. To confirm that the cultured keratinocytes indeed internalized ALA and generated intracellular ROS, we examined the intracellular ROS production using a fluorescent probe DCFH-DA. The intracellular ROS green fluorescence was shown in all ALA-PDT groups and positive control group (Rosup group). This result suggested that ALA-PDT occurs in the cytoplasm of keratinocytes (Figure 1B).

3.2 | Collagen synthesis by fibroblasts was enhanced after coculture with keratinocytes treated with low-dose ALA-PDT

Collagen regeneration is a key indicator of skin rejuvenation. To investigate the indirect effect of ALA-PDT on collagen synthesis in fibroblasts, we treated keratinocytes with low-dose ALA-PDT and then cocultured them with fibroblasts. We measured the production of type I procollagen in fibroblasts by Western blot analysis as shown in Figure 2. Compared with the control group, there were clear increases in type I procollagen expression in fibroblasts in the 0.5 J/cm² and 1 J/cm² groups, but higher doses to the keratinocytes (2 J/cm² and 4 J/cm²) did not result in any increase in collagen production, and the levels were comparable to those in control fibroblasts. Our data suggest that coculturing fibroblasts with low-dose ALA-PDT-treated keratinocytes can increase fibroblasts produce of procollagen I.

3.3 | Proliferation of fibroblasts was enhanced after coculture with keratinocytes treated with low-dose ALA-PDT

The proliferation of fibroblasts is closely associated with collagen synthesis [13]. So we further examined the proliferation of fibroblasts which had been cultured with keratinocytes that had been treated with ALA-PDT, using PCNA immunofluorescence. PCNA, a cell cycle-related protein, which is known as a specific molecular marker of proliferation. It is a processivity factor for DNA polymerase, synthesized shortly prior to the S-phase of the cell cycle [14]. As shown in Figure 3, low-dose ALA-PDT (0.5 and 1 J/cm²) to keratinocytes increased the PCNA expression in the fibroblasts compared with the control group, with no significant effects seen at 2 and 4 J/cm² on the expression of PCNA. Our results suggest that coculture of fibroblasts with keratinocytes treated with low-dose ALA-PDT may enhance their proliferation as well as their collagen synthesis.

3.4 | Low-dose ALA-PDT stimulates TGF-β1 expression in keratinocytes

We first investigated the expression of TGF-β1 in low-dose ALA-PDT-treated keratinocytes by Western blot analysis. As shown in Figure 4A, TGF-β1 expression in keratinocytes was increased significantly when compared with control group, 48 hours after ALA-PDT at low doses (0.5, 1, 2 J/cm² groups), whereas there was no increase in the high dose group (4 J/cm²). Similar results were produced by the ELISA assays (Figure 4B). Results from both the Western blot analysis and ELISA assays agreed showing that relatively low-dose ALA-PDT stimulated the expression of TGF-β1 in keratinocytes.

3.5 | The TGF- β pathway in fibroblasts was activated after coculture with keratinocytes treated with low-dose ALA-PDT

To further explore the underlying mechanism of increased collagen expression in fibroblasts, we examined the expression of intermediates in the TGF- β signaling pathway. The TGF- β signaling cascade is activated by binding of TGF- β 1 to TGF- β type I and type II receptors, following which, the signal is transduced inside the cell, activating the downstream effector Smad proteins, and connective tissue growth factor (CTGF). Therefore, we performed Western blot analysis to evaluate the expression of TGF- β pathway related proteins. The protein expression of TGF- β type I receptor (T β R-I) and TGF- β type II receptor (T β R-II) in fibroblasts was significantly higher when they were cultured with keratinocytes in the 0.5 and 1 J/cm² groups, while there was no increase in the 2 and 4 J/cm² groups (Figure 5A–C). The Smad2/3 protein levels in the 0.5 and 1 J/cm² groups were lower than the control group, however, the phosphorylated p-Smad2/3 in the 0.5 J/cm² group was significantly higher than the control group (Figure 5A,D,E). The 0.5 and 1 J/cm² groups also showed significantly higher protein expression of CTGF than the control group, however, no significant difference was seen for the 2 and 4 J/cm² groups (Figure 5A,F). These results clearly indicate that coculture of fibroblasts with low-dose ALA-PDT-treated keratinocytes can activate the TGF- β signaling pathway and its downstream mediators, consistent with the upregulation of TGF- β 1 in keratinocytes.

3.6 | Low-dose ALA-PDT can remodel dermal collagen in a hairless mouse model of photoaged skin

To confirm the remodeling effect of low-dose ALA-PDT on dermal collagen, we then moved on to an in vivo investigation using a hairless mouse model of photoaged skin. A total of 40 SKH-1 mice were randomly divided into 4 groups: the control group ($n = 10$) were SKH-1 mice that received no treatment; the photoaged group ($n = 10$) were photoaged hairless mice which only received UVB radiation; the PDT group ($n = 10$) were photoaged hairless mice that received 3 sessions of low-dose ALA-PDT treatment; the red light group ($n = 10$) were photoaged hairless mice that received 3 sessions of red light treatments without ALA. The skin of the control group presented a normal appearance, while the UVB-treated photoaged group showed typical signs of photoaging consisting of coarse wrinkles and tactile roughness (Figure 6A). For the PDT group, the skin on the treated area was obviously smoother and softer with fewer wrinkles than the untreated photoaged group, the red light alone group also showed some improvement but this was less marked (Figure 6A). H&E staining (Figure 6C) and Masson-trichrome staining (Figure 6D) showed that the content of collagen fibers decreased and broken collagen fibers increased in the photoaged group. The red light group showed modest improvement in these histological changes characteristic of photoaging. There were greater improvements visible in the ALA-PDT group, with collagen fibers markedly increased and arranged more regularly and tightly. The IOD measured for the collagen is a quantitative measurement of the total amount of collagen in the delineated region. The IOD value of the photoaged group were obviously lower than the control group, the IOD value of the PDT group were significantly higher than the photoaged group (Figure 6E). These results indicated that low-dose ALA-PDT increased dermal collagen amount in hairless mouse model of photoaged skin.

As shown in the PpIX fluorescence microscopic images and H&E stained images (Figure 6B), the red PpIX fluorescence was primarily located in the epidermal layer and the hair follicles in photoaged hairless mouse skin. In contrast, the dermal layer showed little fluorescence. Taken together these results indicate that low-dose ALA-PDT can improve the photoaged appearance through remodeling dermal collagen, while since ALA-PDT mainly occurs in the epidermis the effect is likely to operate via an indirect mechanism.

4 | DISCUSSION

In this study, we found that low-dose ALA-PDT remodeled dermal collagen in photoaged skin, while since the PpIX fluorescence was confined to the epidermis; the mechanism was likely to be indirect. We tested the hypothesis that low-dose ALA-PDT could stimulate keratinocytes to release TGF- β 1 and activate the TGF- β signaling pathway in fibroblasts in a coculture system. The skin rejuvenation effect of ALA-PDT has been demonstrated by several clinical studies [1–5]. In clinical applications for treating photoaged skin, modulation of collagen synthesis and improvement of collagen fiber structure is the major therapeutic objective. An increase of collagen I fibers after topical ALA-PDT was already shown by another study [15]. Our histological results proved that the collagen fibers markedly increased in PDT-treated photoaged mice. In addition, the proliferation of fibroblasts may also reflect the capacity of dermal fibroblasts to produce collagen. So we measured proliferation of human dermal fibroblasts and the expression of type I pro-collagen in fibroblasts. Both of these endpoints were enhanced after coculture of fibroblasts with low-dose ALA-PDT-treated keratinocytes.

Previous studies have investigated the direct effects of ALA-PDT on proliferation and collagen synthesis of fibroblasts [16–18]. Recently, researchers have focused on the cross talk between keratinocytes and fibroblasts within the skin. Several studies have shown the indirect effect of PDT through induction and release of cytokines and growth factors [19, 20]. The transwell plate coculture system used in our study was a useful model to detect the indirect effects of ALA-PDT. The microporous membrane may act in a similar manner to the basement membrane of the skin. The cytokines and growth factors could pass through the membrane while the cells cannot. Importantly, our previous study reported that the PpIX fluorescence was primarily located in the epidermal layer with very little PpIX penetrating down to the dermis [4]. Our present PpIX fluorescence microscopic results also confirmed this observation in photoaged hairless mouse skin. This finding suggests that effects of ALA-PDT should theoretically be confined to the epidermis, which is clearly not the case considering its application to reversal of photoaging in the dermis. *in vitro* we used the transwell coculture system to ensure that ALA-PDT could only work on the epidermal keratinocytes.

TGF- β s are small secreted signaling proteins that are acknowledged to be important regulators of cellular growth and differentiation. TGF- β 1, one of the TGF- β isoforms, is closely related to collagen metabolism [21]. A previous study reported that ALA-PDT could stimulate keratinocytes to express more TGF- β 1 [9]. In accordance with these findings, our results also showed that the expression of TGF- β 1 in keratinocytes was stimulated by relatively low-dose ALA-PDT. Furthermore, we examined the molecular mechanisms that

could occur in dermal fibroblasts after coculture with PDT-treated keratinocytes. TGF- β 1 initiates TGF- β signaling by binding to T β R-I and T β R-II, then goes on to activate the Smad pathway [22]. CTGF also acts as a downstream mediator of the TGF β -Smad signaling pathway [23]. Our study assessed the activation of the TGF- β pathway in human dermal fibroblasts cocultured with PDT-treated keratinocytes. We showed that the expression of T β R-I, T β R-II, phospho-Smad2/3 and CTGF were markedly increased in the low-dose ALA-PDT group, which suggests that TGF β -Smad and its downstream pathway were activated after low-dose (but not high dose) ALA-PDT. Previous studies have suggested that UV radiation causes reduction of TGF- β 2 and T β R-II receptor and diminishes TGF- β signaling, which probably explains the decrease of procollagen in photoaged skin [24]. In our study, we showed that low-dose ALA-PDT indirectly activated TGF- β pathway in fibroblasts thus contributing to restoration of the dermal collagen.

ALA-PDT generates intracellular ROS, which often cause cytotoxic effects such those seen in treatment of skin cancers [25] and condyloma acuminata [26]. However, several studies have shown that low concentrations of ROS can also act as intracellular second messengers and as signaling molecules in various intracellular processes [27, 28]. Previous studies have shown that relatively low concentrations of ROS can stimulate cellular proliferation or regulate signaling pathways [29–31]. Furthermore, a recent study has shown that PDT with low MAL concentrations can promote a moderate production of ROS, which efficiently stimulated cell growth in HaCaT cells [32]. The biphasic nature of ROS effects on cells (stimulation at low doses and inhibition at high doses) [33] probably explains why only low doses of ALA-PDT had beneficial effects on TGF- β signaling and collagen production in fibroblasts, and high doses to the keratinocytes had no effect. We speculate that low-dose ALA-PDT produces low concentrations of ROS, which may regulate signaling pathways in keratinocytes leading to TGF- β production. Although our study did not specifically distinguish between latent TGF- β and active TGF- β , it should be remembered that one of the multiple mechanisms for activation of latent TGF- β centers on the action of ROS [34] in oxidizing methionine at position 253 on the LAP thus releasing active TGF- β [35]. It is at present not possible to say how much of the increase in TGF- β activity caused by ALA-PDT induced ROS is due to the stimulation of new TGF- β protein synthesis in keratinocytes, and how much is due to ROS activation of already synthesized latent TGF- β . Nevertheless, the mechanisms of ROS activity in ALA-PDT skin rejuvenation should be further studied.

Our results are the first to show that collagen synthesis and proliferation of fibroblasts were enhanced after coculture with keratinocytes treated with low-dose ALA-PDT. Furthermore, we found that low-dose ALA-PDT induced the expression of TGF- β 1 in keratinocytes. In addition, we further confirmed that the TGF- β pathway in fibroblasts was activated by this indirect effect of low-dose ALA-PDT. Remodeling of the dermal collagen of photoaged skin while the PpIX was confined to the epidermis was also demonstrated in an animal model. Our findings represent mechanistic evidence for the indirect effects of low-dose ALA-PDT in skin rejuvenation.

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FIGURE 1.

Viability evaluation and intracellular ROS detection of keratinocytes after ALA-PDT. (A) Cultured keratinocytes were treated with different doses of ALA-PDT (1, 2, 4, 8 and 16 J/cm²). Viability (optical density, OD, value) in keratinocytes was detected by MTT assay at 24 hours after ALA-PDT treatment. (B) Cultured keratinocytes were treated with different doses of ALA-PDT (0.5, 1, 2 and 4 J/cm²), only red light and only ALA. Intracellular ROS generation was measured by fluorescence imaging using the DCFH-DA. The second row was the morphological images. Rosup group was used as a positive control. Magnification of microscopic image: 400×

FIGURE 2.

Coculture of fibroblasts with keratinocytes treated with low-dose ALA-PDT enhanced protein expression of collagen in fibroblasts. Cultured keratinocytes were treated with different doses of ALA-PDT (0.5, 1, 2, 4 J/cm²). After coculture with keratinocytes for 60 hours, the fibroblast cell lysates were analyzed by western blotting to measure the expression of type I procollagen protein. Data are mean $\pm$ SD from triplicate determinations of at least 3 independent experiments for each case. * $P < .05$ and ** $P < .01$ compared with the untreated control

FIGURE 3.

Coculture of fibroblasts with keratinocytes treated with low-dose ALA-PDT enhanced fibroblast PCNA expression. Cultured keratinocytes were treated with different dose of ALA-PDT (0.5, 1, 2, 4 J/cm²). After coculture with keratinocytes for 60 hours, fibroblast cell proliferation analyzed by PCNA immunofluorescence staining. DAPI (blue) was used as a nuclear stain. Magnification of microscopic image: 400×. Bar = 20 mm

FIGURE 4.

Treatment of low-dose ALA-PDT stimulated the expression of TGF- β 1 in keratinocytes. Cultured keratinocytes were treated with different doses of ALA-PDT (0.5, 1, 2, 4 J/cm²). (A) After 48 hours, cell lysates were analyzed by western blotting to measure the expression of TGF- β 1. (B) TGF- β 1 protein concentrations in supernatants after 48 hours evaluated by ELISA. Data are mean $\pm$ SD from triplicate determinations of at least 3 independent experiments in each case. * P < .05 and ** P < .01 compared with the untreated control

FIGURE 5.

Coculture of fibroblasts with keratinocytes treated with low-dose ALA-PDT induced the activation of TGF-β pathway in fibroblasts. Cultured keratinocytes were treated with different doses of ALA-PDT (0.5, 1, 2, 4 J/cm²). After coculture with keratinocytes for 60 hours, the fibroblast cell lysates were analyzed by western blotting to measure the expression of TβR-I and TβR-II protein (A-C), p-Smad2/3 and Smad2/3 protein (A,D,E), CTGF protein (A,F). Data are mean ± SD from triplicate determinations of at least 3 independent experiments for each case. **P* < .05 and ***P* < .01 compared with the untreated control

FIGURE 6.

Treatment of low-dose ALA-PDT remodeled dermal collagen in photoaged hairless mouse skin. (A) The appearance of SKH-1 mice in control group, photoaged group, PDT group and red light group is shown by representative gross photographs. (B) The PpIX fluorescence is shown by a fluorescence microscopic image and H&E staining from a SKH-1 mouse after applying 3% ALA for 3 hours. E = epidermis, D = dermis, F = hair follicle. The epidermal-dermal junction was represented by white dashed lines. The histological changes of collagen fibers were evaluated by (C) H&E staining and (D) Masson-trichrome staining. Magnification of microscopic image: 400 $\times$.

(E) The IOD values of the collagen in the dermis. Data are mean $\pm$ SD from triplicate determinations of at least 3 different mice in each case. * $P < .05$ and ** $P < .01$ compared with the untreated control or the photoaged group