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Molecular changes following topical photodynamic therapy using methyl aminolaevulinate in mouse skin

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ABSTRACT

Background: Photodynamic therapy (PDT) with aminolevulinic acid (ALA) or methyl aminolaevulinate (MAL) has been shown to enhance treatment of photoaged skin. However, there is little information about the molecular changes involved in dermal matrix remodeling following MAL-PDT for photorejuvenation.
Objective: We sought to analyze the molecular changes of the epidermal and dermal matrix after MAL-PDT in mouse skin.
Methods: Serial biopsy specimens were obtained at baseline and at various times after treatments with MAL-PDT, MAL alone and LED alone. To evaluate the molecular changes in the epidermal and dermal matrix, primary cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), transforming growth factor-β1 (TGF-β1), matrix metalloproteinases (MMPs), procollagen type I and III were evaluated by reverse transcriptase polymerase chain reaction, Western blot analysis, and immunohistochemistry assays.
Results: Elevation of primary cytokines and MMPs occurred at early points in time after one treatment with MAL-PDT based on the levels of mRNA and protein. On the other hand, procollagen type I protein increased later after MAL-PDT treatment.
Conclusions: MAL-PDT activates more quantifiable alterations in the molecules associated with epidermal and dermal remodeling compared to treatment with MAL or LED alone. MAL-PDT significantly induced the epidermal and dermal matrix molecules required for photorejuvenation.

1. Introduction

Photoaging is a multifactorial process and resulting in skin changes such as dyspigmentation, wrinkling, telangiectasia and cutaneous malignancies [1]. These changes result from intrinsic as well as environmental effects, especially ultraviolet (UV) radiation. The molecular changes of photoaging have been studied extensively in the field of photocarcinogenesis. The molecular process initiated by UV radiation is started by photochemical generation of the reactive oxygen species (ROS) [2]. UV-induced DNA mutations occur by ROS and are clinically related to wrinkling, as well as an increase in elastin and collagen damage [3]. During the early stage of UV exposure, receptors for cytokines such as IL-1 and TNF-α are activated via the generation of the ROS [4]. The activator protein 1 (AP-1) is expressed and regulates transcription of the matrix metalloproteinases (MMPs), responsible for degradation of the cellular matrix [5]. The most remarkable feature of photoaging is the reduced production of collagen in photoaged skin. After UV irradiation, the procollagen pool is markedly decreased *in vivo* [6]. AP-1 has been shown to decrease collagen synthesis by blocking the effects of transforming growth factor β (TGF-β) [7].

Non-ablative technologies are playing an important role in the management of skin photoaging for cutaneous rejuvenation. Photodynamic therapy with light-emitting diode (LED) and 5-aminolevulinic acid (ALA) or methyl aminolaevulinate (MAL) have added to this therapeutic armamentarium. In recent years, PDT used to enhance the effects of the light-based therapies involves application of a topical photosensitizer, such as ALA or MAL. ALA, the precursor of the endogenous photosensitizer, protoporphyrin IX (PpIX), is converted to PpIX by visible light, which then preferentially accumulates in rapidly proliferating skin cells. We previously reported that protoporphyrin IX (PpIX) production by ALA and its esters was induced in both normal and malignant skin cell lines [8]. The free radicals produced by activation of PpIX was found to cause apoptosis or necrosis in the cells exposed to ALA-PDT [9,10]. However, the mechanism underlying the efficacy of this

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treatment for improvement of the appearance of photoaged skin has not been fully elucidated.

In this study, we examined the molecular changes after MAL-PDT treatment in mice skin for understanding the mechanisms of photorejuvenation induced by ALA-PDT. The MMPs and cytokines, such as IL-1β and TGF-β1, were induced early after MAL-PDT treatment. Remarkably, protocollagen type I was increased around eight days after MAL-PDT treatment. These results provide quantitative information about the molecular changes after photo-based treatment with MAL on the skin that result in the understanding of the molecular mechanisms for underlying the clinical improvements with PDT.

2. Materials and methods

2.1. Photosensitizer and animals

We used 16% methylaminolevulinate cream (Metvix®, Galderma, UK) as a topical photosensitizer was used. Female ICR mice (BKW:BKW) were obtained from Dae Han Biolink Co., Ltd. (Cheongju, Korea) and maintained under individually ventilated cage (IVC) system. The normal mice were 9 weeks of age and weighed 25–30 g when the experiments were started.

2.2. Photodynamic therapy (PDT)

The ICR mice were divided into four groups: control group (group 1), MAL only group (group 2), light-emitting diode (LED) only group (group 3), and MAL-PDT group (group 4), with five mice in each group. As 3 days earlier, the hairs of the dorsal surface of all mice were removed with hair eraser. We used 0.25 g of the MAL cream applied to the dorsal skin in the corresponding group of mice on a spot of approximately 2 cm diameter of the dorsal surface of mice, and the application area was occluded by covering with a polyurethane dressing (Tegadem, 3M Healthcare, NL, USA). The mice kept in the dark during and after the MAL treatment (for 3 h). The area was cleansed with 0.9% sterile saline before LED irradiation. The skin of the mice was then exposed to a LED light with a peak at 635 nm in the range of 613–645 nm wavelength, (Philips Luxeon Lumileds, San Jose, CA, USA) at 35 mW cm^{-2} as measured with a Delta Ohm DO 9721 quantum photo-radiometer and thermometer data logger (Model DO9721, Padua, Italy). The total 25 J of light doses was exposed to all of the mice in each group.

2.3. Western blot analysis

Tissue extracts were prepared by sonication in RIPA lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA, pH 8.0, and protease inhibitor cocktail). After centrifugation (12,000 × *g*, 15 min at 4 °C), the protein concentration of the supernatant was measured by using a BCA protein assay kit (Pierce, Rockford, IL). The extract was separated in 6–10% SDS-PAGE and transferred onto a PVDF membrane (0.45 mm, Millipore, MA, USA). After incubation with blocking solution (5% skim milk in Tris-buffered saline with 0.1% Tween-20, TBS-T) for 1 h at room temperature (RT), the membranes were incubated with relevant primary antibody for overnight at 4 °C. The primary antibodies used were anti-Procollagen Type I (Y-18, A-17), anti-MMP-1/8 (H-300) (Santacruz, Biotechnology, Inc., CA, USA), anti-MMP-9 (AF911, R&D systems, MN, USA), anti-MMP-2 (MAB3308, Millipore, MA, USA), anti-MMP-3 (ab53015, Abcam, Cambridgeshire, UK) and mouse monoclonal β-actin (ab6276, Abcam). After incubation with HRP-conjugated goat anti-rabbit IgG (Jackson Imunoresearch, West Grove, PA, USA), or anti-mouse IgG (Jackson Imunoresearch), immune complexes were visualized using the ECL kit (Millipore, MA, USA).

Table 1
Sequences of primers used for PCR amplification.

Gene	Forward primer/reverse primer (5′ → 3′)	Amplicon size (bp)
Procollagen I	CTTTGCTTCCCA GATGTCCTAT CGGTGTCCCTTCATTCCAG	327
Procollagen III	CCACCCCGAACTCAAGAGTGG CCATCCTCTAGAACTGTGTAAGTG	443
IL-1β	GTGGCCATGGACAAGCTGAG TTTGGTCCCTCCCAGGAAGA	453
TNF-α	GGAGGTCTACTTTGGAGTCATTGC TCCCTTTGCAGAACTCAGGAATGG	320
TGF-β1	TGACGTCACTGGAGTTGTACGG GGTTCATGTCATGGATGGTGC	169
MMP1	GACTTCTACCCATTTGATGG TTAGGGTTGGGGTCTTCATC	307
MMP2	AGAAAAGATTGACGCTGTGT CTTCACGCTCTTGAGACTTT	396
MMP3	TGTACCCAGTCTACAAGTCCTCCA CTGCGAAGATCCACTGAAGAA	658
MMP9	GCATACTTGTACCGCTATGG TATGATGTTATGATGGTCCC	224
P-Actin	TCATGAAGTGAGACGTTGACATCCGT CCTAGAAGCACTTGCGGTGCACGATG	284

2.4. Semi-quantitative RT-PCR

Total RNA was isolated by using the RNeasy mini kit (Qiagen, Fremont, CA, USA) according to the manufacturer's instructions. cDNA was obtained from 500 ng of total RNA using the Omniscript RT kit (Qiagen). For the semi-quantitative PCR, PCR-premixture kits (ELPIS, Taejeon, Korea) were used. The sequences of the specific primers for collagen, MMPs and cytokines are listed in Table 1. The PCR products were analyzed using a 1.5% agarose gel electrophoresis, stained with cyber safe DNA gel stain buffer (Invitrogen), and visualized under luminescent image analyzer (LAS 3000, Fujifilm). Band intensities were relatively quantified using densitometry analysis and normalized to the β-actin values.

2.5. Hematoxylin & eosin (H&E) and immunohistochemical staining

The skin tissue samples were embedded in paraffin, cut into 4 μm slices, and stained with hematoxylin and eosin (H&E) or used for immunohistochemical staining with the LSAB2® System-HRP (DakoCytomation, Carpenteria, CA, USA) The sections were deparaffinized in xylene and rehydrated in graded ethanol. To block endogenous peroxidase activity, we fixed the sections for 5 min in methanol containing 3% hydrogen peroxide. The sections were washed with Tris-buffered saline (TBS) and incubated with rabbit antibody against MMP-3 (abcam, 1:100) for 10 min at RT. After washing three times with TBS, the sections were incubated with biotinylated secondary antibody for 15 min at RT, and washed three times. The slides were incubated with streptavidin horseradish peroxidase for 20 min, then washed with TBS for 5 min. Sections were developed using 3–3′ diaminobenzidine substrate-chromogen solution for 7 min, and washed three times with distilled water. The slides were mounted with a fluorescent mounting medium (DakoCytomation®).

2.6. Gelatin zymography for MMP-2 and -9 activities

Tissue extracts (10 μg) were subjected to 8% SDS-PAGE containing 0.25% gelatin. The gel was washed with renaturation buffer (2.5% Triton X-100) for 1 h at RT, and then incubated overnight in the development buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij-35) at 37°C. Subse-