ORIGINAL ARTICLE

Low-Level Light Therapy with 410 nm Light Emitting Diode Suppresses Collagen Synthesis in Human Keloid Fibroblasts: An *In Vitro* Study

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Background: Keloids are characterized by excessive collagen deposition in the dermis, in which transforming growth factor β (TGF-β)/Smad signaling plays an important role. Low-level light therapy (LLLT) is reported as effective in preventing keloids in clinical reports, recently. To date, studies investigating the effect of LLLT on keloid fibroblasts are extremely rare. **Objective:** We investigated the effect of LLLT with blue (410 nm), red (630 nm), and infrared (830 nm) light on the collagen synthesis in keloid fibroblasts. **Methods:** Keloid fibroblasts were isolated from keloid-revision surgery samples and irradiated using 410-, 630-, 830-nm light emitting diode twice, with a 24-hour interval at 10 J/cm^2. After irradiation, cells were incubated for 24 and 48 hours and real-time quantitative reverse transcription polymerase chain reaction was performed. Western blot analysis was also performed in 48 hours after last irradiation. The genes and proteins of collagen type I, TGF-β1, Smad3, and Smad7 were analyzed. **Results:** We observed no statistically significant change in the viability of keloid fibroblasts after irradiation. Collagen type I was the only gene whose expression significantly decreased after irradiation at 410 nm when compared to the non-irradiated control. Western blot analysis showed that LLLT at 410 nm lowered the protein levels of collagen type I compared to the control. **Conclusion:** LLLT at 410 nm decreased the expression of collagen type I in keloid fibroblasts and might be effective in preventing keloid formation in their initial stage. (Ann Dermatol 29(2) 149~155, 2017)

INTRODUCTION

Keloids are characterized by hyperproliferative growth of dermal fibroblasts and excessive collagen deposition in the dermis[1,2]. Previous studies suggested that the initial step in the development of the fibrotic reaction in keloids involves the expression of transforming growth factor β1 (TGF-β1) in neovascular endothelial cells, thus inducing the adjacent fibroblasts to express markedly high levels of TGF-β1/β2 and their receptors, as well as type I and VI collagen[3,4]. Recent studies emphasize a potential role for TGF-β1 intracellular signaling pathways, especially for the Smad pathway, in the pathogenesis of keloids[4]. Smad2 and Smad3 are overexpressed and the inhibitory Smad6 and Smad7 are reduced in keloid fibroblasts[1,3,5].

Treatments for keloids include surgical excision, intralesional corticosteroids, 5-fluorouracil, bleomycin and interferon, topical imiquimod, compression, cryotherapy, radiation, silicon sheeting and laser or light-based therapies, showing variable success. Recurrence is common, even with combination therapy[6]. Therefore, preventing keloid is an important issue.

Low-level light therapy (LLLT) uses low levels of visible to near infrared light. It has been widely used to reduce pain

and inflammation, to promote wound healing, and to prevent tissue necrosis. Many reports demonstrate that LLLT modulates normal dermal fibroblasts and the TGF-β family[1]. Recently, two clinical reports showed that LLLT was effective in preventing hypertrophic scars and keloids[7,8]. However, studies investigating the effect of LLLT on keloid fibroblasts are still rare, especially the ones investigating the effects of LLLT at molecular level. Herein, we aimed to evaluate the effect of LLLT using blue (410 nm), red (630 nm), and infrared (830 nm) light on the collagen synthesis in keloid fibroblasts, by observing the signaling molecules involved.

MATERIALS AND METHODS

Isolation and culture of keloid fibroblasts

Keloid fibroblasts were isolated from keloid-revision surgery samples after obtaining informed consents from the patients. Keloid fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% carbon dioxide (CO_2).

Irradiation

Keloid fibroblasts were seeded in a 6-well plate at 3.0×10^5 cells/well. APL® (Medro Medical Division, Seoul, Korea) was used for this study. This device produces blue (410 nm), red (630 nm), and infrared (830 nm) light emitting diode (LED) light. The power of each wavelength was 205 mW/cm^2 for blue, 172 mW/cm^2 for red, 50 mW/cm^2 for infrared. Keloid fibroblasts were irradiated twice, with the second irradiation 24 hours after the first one. Preliminary experiment was performed to determine the lowest fluence to inhibit the proliferation of keloid fibroblasts. Cells were maintained in 400 μl Dulbecco's phosphate-buffered saline during irradiation and transferred in DMEM after irradiation for 24 or 48 hours.

Cell viability assay

To determine the direct effect of LLLT on the proliferation of viable cells, we counted the number of viable cells using the EZ-CyTox cell viability assay kit (Daeillab, Seoul, Korea). Twenty-four hours after the last irradiation, the cells were treated with 40 μl of Ez-CyTox solution and incubated for additional 3 hours at 37°C. Absorbance was measured at 450 nm using an ELISA reader (Molecular Device, Menlo Park, CA, USA). Viability was expressed as percentage of viable cells compared to the non-irradiated control. The experiment was repeated twice with two set of fibroblasts.

Real-time quantitative reverse transcription polymerase chain reaction

The mRNA of collagen type I, TGF-β1, Smad3, and Smad7 were measured in 24 and 48 hours after last irradiation. Total RNA was isolated using RNAasy® mini kit (Quiagen, Hilden, Germany). cDNA transcription was performed using the Superscript® III First-Strand (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was analyzed by real-time quantitative reverse transcription polymerase chain reaction using ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster, CA, USA). Fluorescent signals were collected during extension phase, Ct values of the sample were calculated, and the transcript levels were analyzed by $2^{-\Delta\Delta Ct}$ method. Primers and internal probes for collagen type I, TGF-β1, Smad3, and Smad7 were purchased as assays on demand primer-probe sets (Applied Biosystems). The experiment was repeated five times.

Western blot analysis

The protein of collagen type I, TGF-β1, Smad3, and Smad7 were measured in 48 hours after last irradiation. Cells were lysed in lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.1% NP 40, 1% sodium dodecyl sulfate [SDS], 0.5% deoxycholic acid, 1 mM EDTA, protease and phosphatase inhibitors). Samples were separated on 10% SDS-polyacrylamide gels and transferred onto polyvinyldenefluoride membranes. The protein blots were incubated with antibodies against collagen type I (Abcam, Cambridge, MA, USA), TGF-β1 (GeneTex, Irvine, CA, USA), Smad3 (Epitomics, Burlingame, CA, USA), Smad7 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and actin (Santa Cruz Biotechnology). Detection was performed via enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK). The intensity of each band was quantified using Image J and the data were reported as relative intensity according to actin.

Statistical analysis

IBM SPSS Statistics Desktop ver. 20.0.0 (IBM Co., Armonk, NY, USA) was used for statistical analysis. Results are expressed as mean and standard deviation. Mann-Whitney U test was used for statistical evaluation between control and experimental groups in the study. A p-value lower than 0.05 was considered statistically significant.

RESULTS

Determination of the fluence of LLLT

Keloid fibroblasts were irradiated once with 3, 10, 30

J/cm^2 fluences of the three wavelengths of 410-, 630-, and 830-nm as preliminary experiment. Tryptan blue assay exhibited proliferations rates of keloid fibroblasts tend to decrease only at 10 and 30 J/cm^2, showing rather increased proliferation rate at 3 J/cm^2, in all wavelengths, although it was not significant (Appendix). In the condition of similar proliferation rate, using lower dose is more suitable and convenient for conducting experiment. Therefore, we determined to irradiate 10 J/cm^2 for keloid fibroblasts.

Cell viability assay

Viable keloid fibroblasts decreased with irradiation with all wavelengths compared to non-irradiated control. However, it was not statistically significant (Fig. 1).

mRNA expression of TGF-β/Smad signaling components

Collagen type I expression was significantly decreased with irradiation at 410 nm in 48 hours ($p < 0.005$). We also observed lower Smad3 expression after irradiation at 410 nm in 48 hours, but it was not statistically significant ($p = 0.065$). There were no differences between irradiated
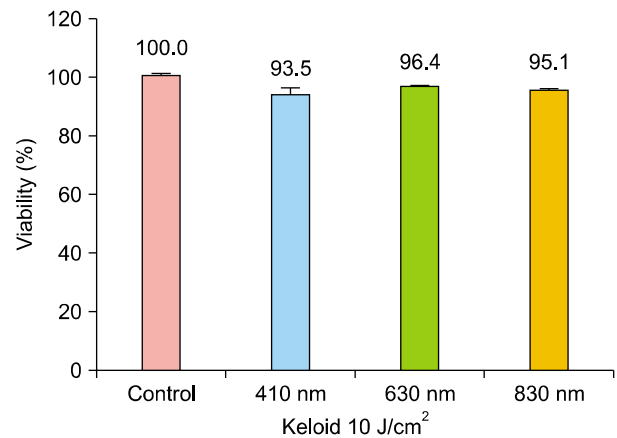
Fig. 1. Viable keloid fibroblasts had tendency to decrease after irradiation with all wavelengths compared to non-irradiated keloid control. However, there was no statistically significant decrease compared to the control. The results are represented mean ± standard error from two independent experiments with two set of fibroblasts.
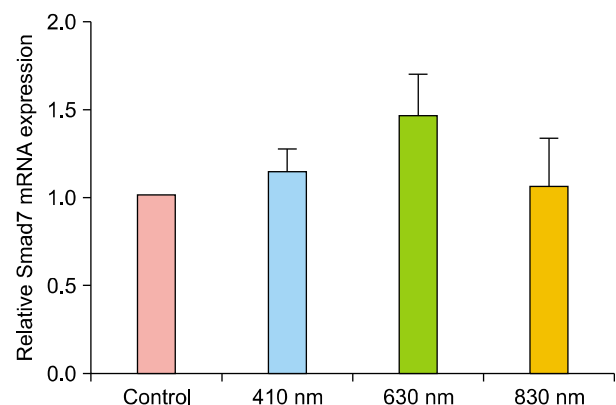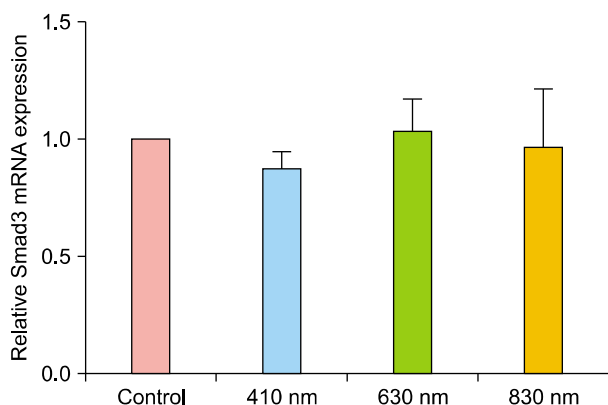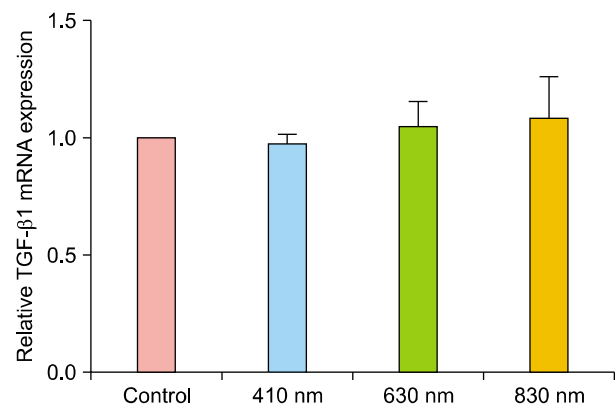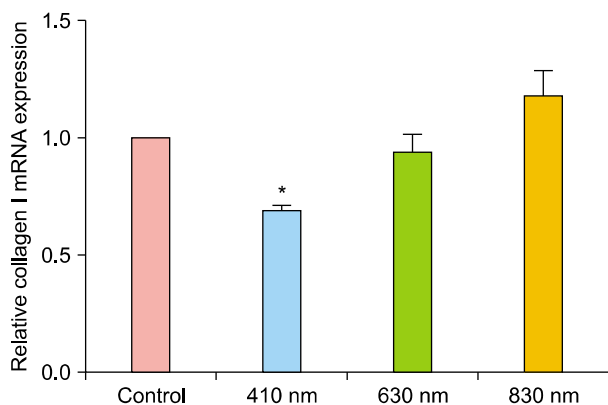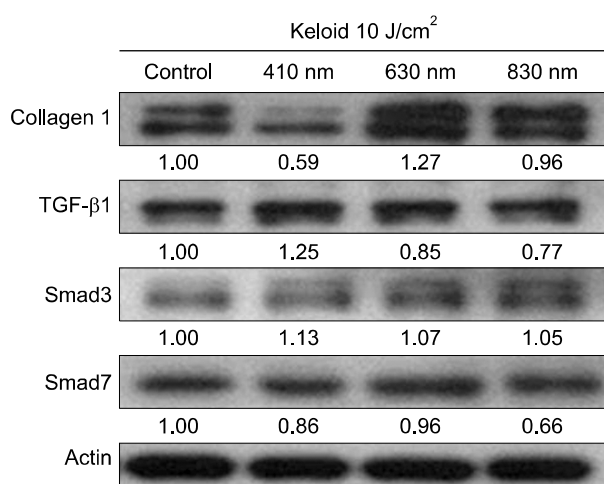

Fig. 2. Only expression of collagen type I showed a significant decrease in cell irradiate with 410 nm light compared to non-irradiated control after 48 hours. The results are represented as mean ± standard error from five independent experiments. TGF-β1: transforming growth factor β1. *$p < 0.05$ vs. non-irradiated control.

Table 1. Relative mRNA level of collagen type I, TGF-β1, Smad3, and Smad7 after LLLT at 10 J/cm^2 in keloid fibroblasts

	Collagen I		TGF-β1		Smad3		Smad7	
	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
Control	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
410 nm	1.18±0.41	0.68±0.03*	1.35±0.62	0.97±0.10	1.70±1.24	0.87±0.17	1.35±1.24	1.14±0.13
630 nm	1.16±0.42	0.93±0.18	1.38±0.69	1.04±0.24	1.57±0.79	1.03±0.31	1.04±0.79	1.46±0.24
830 nm	1.22±0.46	1.18±0.24	1.15±0.47	1.07±0.18	1.64±1.36	0.96±0.25	1.30±1.36	1.06±0.12

Values are presented as mean only or mean±standard error. TGF-β1: transforming growth factor ß1, LLLT: low-level light therapy. *$p<0.05$ vs. non-irradiated control.

Fig. 3. Low-level light therapy suppressed the expression of collagen type I in keloid fibroblasts irradiated with 410 nm light. TGF-β1: transforming growth factor β1.

samples in 24 hours and non-irradiated controls (Fig. 2, Table 1).

Protein levels of TGF-β/Smad signaling components

Irradiation at 410 nm reduced the protein levels of collagen type I in 48 hours. However, we observed no differences between irradiated samples and the control for TGF-β1, Smad3, and Smad7 (Fig. 3).

DISCUSSION

In the present study, we investigated whether LLLT could affect the collagen synthesis in keloid fibroblasts. Irradiation with 410 nm decreased collagen type I expression in keloid fibroblasts.

In normal fibroblasts, the effect of LLLT can be explained by photobiomodulation. Wavelengths above 500 nm are widely used to stimulate wound healing[9]. Their energy is absorbed by the cytochrome C oxidase in the mitochondrial membrane and activates various cellular signaling pathways through inducing the production of nitrogen ox-

ide, adenosine triphosphate and reactive oxygen species (ROS)[10]. It can induce the transcription of various growth factors including platelet-derived growth factor, TGF-β, and fibroblast growth factor, and cytokines such as interleukins and tumor necrosis factor-alpha. On the other hand, blue light induces a higher production of intracellular ROS in a dose-dependent manner, which might act as a mediator of cellular effects of blue light[11]. Because of their relative close wavelengths, it is possible that blue and ultraviolet A light has similar biological effect such as ROS generation and immunomodulation[11]. Flavins are photo-acceptors and involved in the generation of ROS. Flavins are small, water-soluble molecules that absorb wavelengths below 500 nm and initiate free radical reactions when excited by light. Flavin-mediated photosensitization generates cytotoxic free radicals and is responsible for significant biological effects such as mutagenesis[12,13].

Previous reports indicate that 410 nm LED irradiation is cytotoxic for normal fibroblasts. Opländer et al.[14] demonstrated that single irradiation with 410 or 420 nm LED at 5~10 J/cm^2 inhibits the proliferation of dermal fibroblasts. Bonatti et al.[15] also reported a significant reduction in the number of normal fibroblasts irradiated with 470 nm light at 18 J/cm^2. According to Seo et al.[16], red (630 nm, 9.5 J/cm^2) and green (530 nm, 9.8 J/cm^2) LED-irradiated cells were significantly more proliferated than cells irradiated with blue light (460 nm, 27 J/cm^2). Also, blue light did not increase the expression of collagen type I and TGF-β1 in the dermis compared to the control, whereas red and green light enhance the expression of both genes.

In contrast to studies on normal fibroblasts, reports on the effect of LLLT on keloid fibroblasts are extremely rare and disappointing. Bonatti et al.[15] showed that a single irradiation with 470 nm LED at 6, 12, and 18 J/cm^2 does not induce significant differences in the number of keloid fibroblasts. To our knowledge, no study reports that LLLT affects the molecules investigated here in keloid fibroblasts. Our results showed that collagen mRNA levels decreased

after irradiation in keloid fibroblasts, which means that LLLT inhibited the transcription of this gene. Unfortunately, it is uncertain whether the TGF-β/Smad signaling pathway is inhibited in keloid fibroblasts, because other downstream signaling components, except collagen type I, did not show any significant changes in their expression after LLLT. It should be considered that LLLT also affects other signaling pathways in keloid, such as the mitogen-activated protein kinase (MAPK), which is suggested to mediate TGF-β/Smad signal transduction[17,18]. The influence of MAPK on keloid fibroblasts was evaluated after pulsed dye laser (PDL) treatment in a few reports. Activation of extracellular signal-regulated kinase and the p38 signal transduction pathway is involved in the suppression of keloid fibroblast proliferation and the induction of apoptosis after PDL treatment[19,20]. Recently, possible role of wingless type (Wnt)/β-catenin signaling pathway in keloid pathogenesis has been reported, which was known to play a key role in cellular proliferation. Wnt5a and β-catenin, its effector, were highly expressed in keloid fibroblasts and mediate the effect of TGF-β in wound healing process[21]. In addition, unlike normal fibroblasts, abnormal TGF-β signaling and autocrine loop are important in keloid fibroblasts[1,4]. For example, keloid fibroblasts show marked sensitivity to TGF-β, causing an accelerated increase in collagen and fibronectin synthesis compared to normal fibroblast[22,23]. Therefore, the peak time of the TGF-β/Smad signaling components in keloid fibroblasts might be different from that of normal fibroblasts and it might be difficult to predict their expression pattern[1,4].

This study has significance, in that it demonstrated that LLLT with 410 nm light inhibited collagen synthesis in keloid fibroblasts *in vitro*. Considering the initial pathophysiology of keloid, in which the expression of the TGF-β1 activates the adjacent fibroblasts to produce collagen, LLLT might be effective in preventing keloid formation at the initial stage. Corresponding clinical studies with blue LED and further investigative studies are needed to demonstrate the signaling mechanisms in keloid fibroblasts after LLLT. We hope this study can be a starting point for clinical application of LLLT to patients with keloid scars.

ACKNOWLEDGMENT

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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Appendix. Proliferation rate of keloid fibroblasts irradiated at 3 J/cm^2 was higher than control, while proliferation rate at 10 and 30 J/cm^2 is lower than control.