Green light emitting diodes accelerate wound healing: Characterization of the effect and its molecular basis in vitro and in vivo

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ABSTRACT

Because light-emitting diodes (LEDs) are low-coherent, quasimonochromatic, and nonthermal, they are an alternative for low level laser therapy, and have photobiostimulative effects on tissue repair. However, the molecular mechanism(s) are unclear, and potential effects of blue and/or green LEDs on wound healing are still unknown. Here, we investigated the effects of red (638 nm), blue (456 nm), and green (518 nm) LEDs on wound healing. In an in vivo study, wound sizes in the skin of ob/ob mice were significantly decreased on day 7 following exposure to green LEDs, and complete reepithelialization was accelerated by red and green LEDs compared with the control mice. To better understand the molecular mechanism(s) involved, we investigated the effects of LEDs on human fibroblasts in vitro by measuring mRNA and protein levels of cytokines secreted by fibroblasts during the process of wound healing and on the migration of HaCat keratinocytes. The results suggest that some cytokines are significantly increased by exposure to LEDs, especially leptin, IL-8, and VEGF, but only by green LEDs. The migration of HaCat keratinocytes was significantly promoted by red or green LEDs. In conclusion, we demonstrate that green LEDs promote wound healing by inducing migratory and proliferative mediators, which suggests that not only red LEDs but also green LEDs can be a new powerful therapeutic strategy for wound healing.

INTRODUCTION

Light therapy is often utilized in dermatology as one of the most classic therapeutic modalities. Although solar and ultraviolet (UV) light have been popular for many decades, they can damage tissues by long time exposure. Other artificial light sources, including halogen lights, can cause thermal damage to the irradiated tissues because they also include a broad wavelength spectrum light. Recently, as a nonthermal and narrow wavelength spectrum light source, nonablative laser therapy and low-level laser therapy have been clinically and experimentally reported to be useful for various medical conditions, such as wound healing.[1–4] However, laser systems are expensive, relatively immobile, and require frequent repair. On the other hand, light-emitting diodes (LEDs), which are low-coherent, quasimonochromatic, and nonthermal, have many advantages related to safety and superior portability, compared with UV and laser therapies. Although the National Aeronautics and Space Administration (NASA) first studied the effects of LEDs on plant growth and later several studies indicated their efficacy for wound treatment,[5,6] the mechanism(s) of their effects remains unclear. In addition, newly developed innovations continue to provide more powerful and sophisticated light sources of LED, and a new narrow-band LED with a half-band width around 10 nm is

DMEM	Dulbecco's modified Eagle's medium
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FGF	Fibroblast growth factor
GaAs	Gallium arsenide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HB-EGF	Heparin-binding epidermal growth factor-like growth factor
HeNe	Helium-neon
HGF	Hepatocyte growth factor
IGF-1	Insulin-like growth factor 1
IL-1(6, 8)	Interleukin 1(6, 8)
KGF	Keratinocyte growth factor
LED	Light-emitting diode
NASA	National Aeronautics and Space Administration
NIR	Near infrared
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
TGF-α(β)	Transforming growth factor α(β)
TNF-α	Tumor necrosis factor α
UV	Ultraviolet
VEGF-A	Vascular endothelial growth factor A

now available. Therefore, we utilized red, blue, and green narrowband LEDs and explored their effects on wound healing.

MATERIALS AND METHODS

All experiments described were approved by the Medical Ethical Committee of Osaka University and were conducted according to the Declaration of Helsinki Principles.

Cell culture

Normal human dermal fibroblasts (which originated from the normal skin of a 13-year-old male) were isolated and cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan), supplemented with 10% fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS), penicillin (50 units/mL), and streptomycin (50 μg/mL) at 37 °C under a humidified atmosphere of 95% O_2 and 5% CO_2. Cells were subcultured after treatment with 0.025% trypsin (usually 4 weeks after the beginning of primary cell culture). Human HaCat keratinocytes (a spontaneously immortalized keratinocyte cell line derived from adult human skin, kindly provided by Professor Fusenig) were cultured in DMEM with 10% FCS, penicillin (50 units/mL), and streptomycin (50 μg/mL) at 37 °C in a humidified atmosphere of 95% O_2 and 5% CO_2.

Irradiation sources

Mignon Belle LT-1 Crystalline (Mignon Belle Co., Ltd, Osaka, Japan) narrowband LED devices were used for this study. These devices produce narrower emission spectra than original LEDs and deliver uniform power of light to a large area using band-pass filters and diffusing lens. The following peak wavelengths (half-width; bandwidths at 50% power) were used: blue 456 nm (10 nm), green 518 nm (8 nm), and red 638 nm (10 nm). For the in vivo study, we used the Mignon belle LT-1 Crystalline for portable use loaded with one LED lamp with areas of 30 cm^2 to be applied over the backs of mice, with power outputs ranging from 0~2.52 W for red, 0~2.31 W for blue, and 0~2.28 W for green, and actual values of intensities on the wound surfaces (at a 10 cm distance from the light source) ranging from 0~0.65 mW/cm^2 for red, 0~0.3 mW/cm^2 for blue, and 0~0.25 mW/cm^2 for green. To irradiate the three types of LEDs at the same power, we selected 0.25 mW/cm^2 (the highest intensity of green LED) exposure for 20 minutes, resulting in 0.3 J/cm^2 at the wound surfaces. For the in vitro study, the irradiation time was fixed for each LED to avoid changes in culture conditions. The Mignon belle LT-1 Crystalline loaded with three LEDs, which can emit LEDs over 70 cm^2 enough to cover six 35-mm dishes, was used. The power output without a variable resistor was 7.56 W for red, 6.93 W for blue, and 6.84 W for green. At the irradiation site, at a 10-cm distance from the light source, the actual values of intensities were 0.75 mW/cm^2 for red, 0.25 mW/cm^2 for blue and 0.17 mW/cm^2 for green. We irradiated the cells using these LEDs for 20 minutes, delivering 0.6 J/cm^2 for red, 0.3 J/cm^2 for blue, and 0.2 J/cm^2 for green on the fibroblasts or keratinocytes. Power fluences at the irradiation sites were measured using a power meter (ADC, Tokyo, Japan) and the formula used to calculate the dose was: $E(J/cm^2)$ = time (s) × intensity (W/cm^2).

Animals

Eight-week-old male B6.V-Lep^{ob}/J mice were purchased from Charles River Japan (Tokyo, Japan). All animal experiments were carried out in accordance with a protocol approved by the Institutional Animal Care and Use Committee of Osaka University.

In vivo wound healing

After the mice were anesthetized, their dorsal skins were shaved and disinfected, and one 8-mm diameter dorsal full-thickness excisional wound was created with a sterile biopsy punch in each animal. The wound was left without suturing or dressings. The animals were randomly distributed into four groups, with four animals in each: a control group, a red LED group, a blue LED group, and a green LED group. Starting the next day, red, blue, or green LEDs were irradiated for 20 minutes from a 10-cm distance to each wound every other day, and the size of each wound area was measured before irradiation.

Analysis of mRNA by *semi-quantitative reverse transcription polymerase chain reaction* (*RT-PCR*)

After reaching subconfluency, the culture medium was replaced by DMEM without phenol red for the irradiation experiments. LEDs (red, blue, or green) were exposed to normal human dermal fibroblasts, and red or green LEDs to the HaCat keratinocytes, for 20 minutes at a clean bench. For the control sham treatment, no light was irradiated. The distance from the light source to the culture dish was fixed at 10 cm. At 0, 4, 8, and 24 hours after the LED irradiation, RNA samples were extracted from the cells to analyze mRNA expression levels of various growth factors (fibroblast growth factor [FGF]-2, hepatocyte growth factor [HGF], insulin-like growth factor 1 [IGF-1], keratinocyte growth factor [KGF], leptin, transforming growth factor [TGF]-β1, and vascular endothelial growth factor A [VEGF-A] for human fibroblasts; heparin-binding epidermal growth factor-like growth factor [HB-EGF], TGF-α, and VEGF-A for HaCat keratinocytes), inflammatory cytokines (interleukin [IL]-1α, IL-6, IL-8, and tumor necrosis factor α [TNF-α] for human fibroblasts; IL-6 and TNF-α for HaCat keratinocytes) and matrix proteins, such as procollagens type 1α2 and type 3α1, for human fibroblasts by semi-quantitative RT-PCR. Total RNAs were isolated using the acid guanidium thiocyanate-phenol-chloroform method. The recovery and purity of each RNA was calculated from the optical densities at 260 and 280 nm. cDNAs were reverse-transcribed from 1 μg total RNA from each sample using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The resultant cDNAs were amplified using a thermal cycler (Astec, Fukuoka, Japan) in a final volume of 25 μL containing 0.020 U/mL Blend Taq Plus (TOYOBO, Tokyo, Japan), Blend Taq Buffer (TOYOBO, Tokyo, Japan), 0.2 mM dNTPs, and 0.4 mmol/L of each of the forward and reverse primers. The following oligonucleotide primers were used, FGF-2:

sense 5′-CTTTGGCTGCTACTTGGAGG-3′ and antisense 5′-TGTAGAGACGGGGTTTCACC-3′; HB-EGF: sense 5′-TCCTCCAAGCCACAAGCACT-3′ and antisense 5′-CATC ATAACCTCCTCTCCTA-3′; HGF: sense, 5′-CTGGTTCCC CTTCAATAGCA-3′ and antisense 5′-GTCGGGATATCTTT CAGGCA-3′; IGF-1: sense 5′-ACGGCTGGACCGGAGA CGCTCGGGC-3′ and antisense 5′-GCAAACAGCGTCAC AGCAAC-3′; leptin: sense, 5′-CTGTGCCCATCCAAAAA GTCC-3′ and antisense 5′-TCTGTGGAGTAGCCTGAAGC-3′; VEGF-A: sense 5′-AAGGAGGAGGGCAGAATCAT-3′ and antisense 5′-GAGGCTCCAGGGCATTAGAC-3′; TGF-α: sense 5′-CGCTCTGGGTATTGTGTTGG-3′ and antisense 5′-CTCCTCCTCTGGGCTCTTCA-3′; TGF-β1: sense 5′-CCACAACGAAATCTATGACA-3′ and antisense 5′-ACTCCGGTGACATCAAAAGA-3′; IL-1α: sense 5′-TTGAAGACCTGAAGAACTGTTACAG-3′ and antisense 5′-TAAAGTTGTATTTCACATTGCTCAGGA-3′; IL-6: sense 5′-GTACATCCTCGACGGCATCTCAGC-3′ and antisense 5′-TGTGGTTGGGTCAGGGGTGGTTAT-3′; IL-8: sense 5′-TTGGCAGCCTCCTGATTTC-3′ and antisense 5′-AACTCTCCACAACCCTCTG-3′; TNF-α: sense 5′-GAGCACTGAAAGCATGATCCGG-3′ and antisense 5′-AAAGTAGACCTGCCCAGACTCGG-3′; procollagen type 1α2: sense 5′-GGTGGTGGTTATGACTTTGG-3′ and antisense 5′-GTTCTTGGCTGGGATGTTT-3′; collagen type 3α1: sense 5′-GCTCTGCTTCATCCCACTATTA-3′ and antisense 5′-TGCGAGTCCTCCTACTGCTAC-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control: sense 5′-CCCATCACCATCTTCCAG-3′ and antisense 5′-CCTGCTTCACCACCTTCT-3′. PCR amplification was performed at 26 cycles for HB-EGF, denaturation at 94 °C for 30 seconds, annealing at 54 °C for 30 seconds, and extension at 72 °C for 30 seconds, at 30 cycles for HGF, at 35 cycles for IGF, VEGF-A (fibroblasts), and IL-1α, at 27 cycles for KGF, at 24 cycles for TGF-β, and at 30 cycles for VEGF-A (HaCat), denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds, at 23 cycles for FGF-2, at 33 cycles for leptin, at 25 cycles for TGF-β1, and at 21 cycles for collagen type 1α2, collagen type 3α1, and GAPDH, denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 30 seconds, and at 30 cycles for IL-6 and TNF-α, denaturation at 94 °C for 30 seconds, annealing at 50 °C for 30 seconds, and extension at 72 °C for 30 seconds. For IL-8, amplification was performed at 45 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 40 seconds, and extension at 72 °C for 90 seconds. These cycle numbers of PCR were within the logarithmic phase. PCR products were analyzed by 2% agarose gel electrophoresis, and their sizes were compared with 100-bp molecular weight markers (Gibco-BRL, Rockville, MD).

Analysis of protein levels by enzyme-linked immunosorbent assay (ELISA)

After the culture medium was replaced with phenol red-free DMEM, red, blue, or green LEDs were irradiated to the cultured fibroblasts, and red or green LEDs to the HaCat keratinocytes, at days 1, 2, and 3 for 20 minutes. No LED was irradiated for the control sham treatment. The distance from the light source to the culture dish was 10 cm. At day 4, the culture media were harvested and subjected to ELISA to measure protein levels of growth factors and cytokines whose mRNA expression levels were induced according to the RT-PCR performed as mentioned above. We utilized human ELISA kits for each factor (R&D Systems, Inc., Minneapolis, MN), according to the manufacturer's instructions.

In vitro migration assay for HaCat keratinocytes

HaCat keratinocytes were cultured on plastic dishes until confluent in DMEM containing 10% FCS, then were starved for 24 hours, treated with 10 mg/mL mitomycin C (Sigma Aldrich Japan, Tokyo, Japan) for 2 hours to inhibit proliferation of the cells, and finally were subjected to an in vitro migration assay. A cell-free area was introduced by scraping the monolayer with a yellow pipette tip. After the culture medium was replaced with phenol red-free DMEM with 10% FCS, red, blue, or green LEDs were irradiated to the cultured HaCat keratinocytes for 20 minutes from a 10-cm distance to the culture dish. No LED was irradiated for the control sham treatment. Cell migration into the cell-free area over the next 24 hours was evaluated using photographs taken using a microscope (model IX70; Olympus, Tokyo, Japan).

Statistical analysis

Data are expressed as means ± standard deviation. Data were subjected to one-way analysis of variance with Dunnett's multiple comparison post hoc test. A p-value of less than 0.05 is considered to be statistically significant.

RESULTS

In vivo wound healing study

First, to examine the putative effects of LEDs on wound healing, red (638 nm/0.3 J/cm^2), blue (456 nm/0.3 J/cm^2), or green (518 nm/0.3 J/cm^2) LEDs were irradiated on circular excision wounds created surgically on the dorsal skins of 8-week-old male B6.V-Lep ^{ob}/J mice. The size of each wound was then measured every second day. No LED was irradiated for the control sham treatment. At day 7, the wound areas irradiated with the green LED were significantly reduced in size (green: 24.65 ± 16.76%, $p < 0.03$) compared with the sham-treated control (57.44 ± 13.90%) (Figure 1A and B). On the other hand, red or blue LEDs had no significant effect on wound healing in our experimental conditions (Figure 1A). Total epithelialization of the wounds irradiated with red or green LEDs was observed at day 11, while the wounds treated with the blue LED or the sham-treated control had epithelialized at day 13 (Figure 1A). These results indicate that exposure to red or green LEDs accelerates wound healing.

Effect of LEDs on normal human dermal fibroblasts

Analysis of mRNA by *semi-quantitative RT-PCR*

Next, to investigate the molecular mechanism(s) of the effect of LED irradiation on wound healing, cultured normal human dermal fibroblasts were irradiated with red (638 nm/

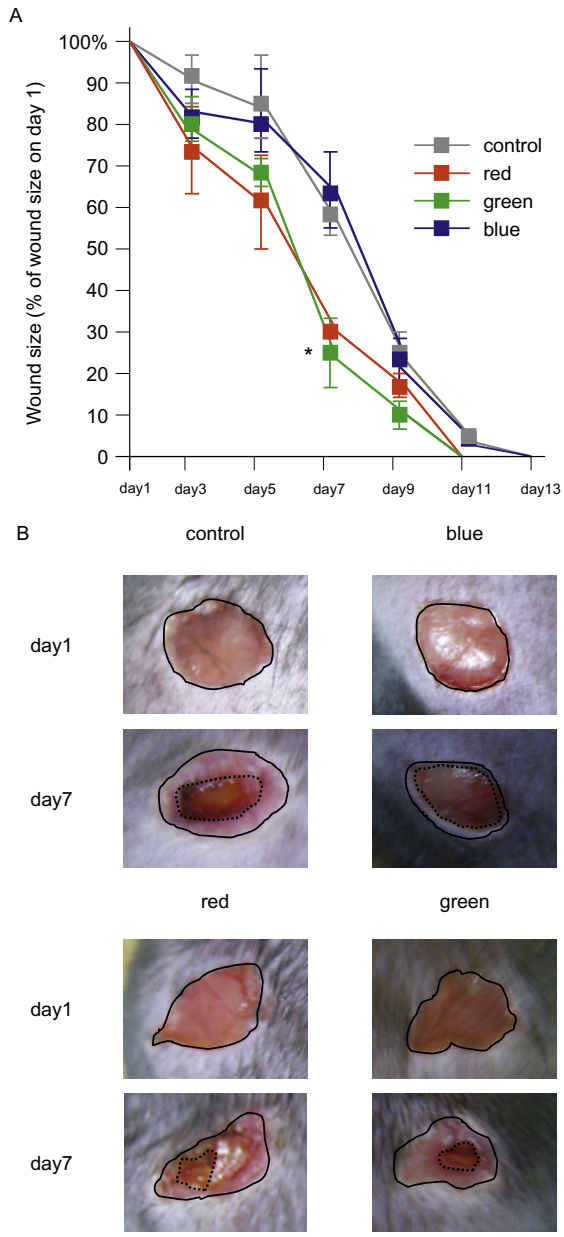

Figure 1. In vivo wound healing study. (A) One 8-mm diameter full thickness excisional wound was created with a sterile biopsy punch in the dorsal skin of each animal. The animals were randomly distributed into four groups with four animals in each: a control group, a red light-emitting diode (LED) group, a blue LED group, and a green LED group. Starting the next day (day 1), red (638 nm, 0.3 J/cm^2), blue (456 nm, 0.3 J/cm^2), or green (518 nm, 0.3 J/cm^2) LEDs were used for irradiation, and the sizes of the wound areas were measured on every second day. The wound area sizes were evaluated as relative to the size on day 1. Data are means ± SD of four independent experiments. *$p < 0.03$, as compared with the control group (one-way analysis of variance with Dunnett's analysis). (B) Representative wounds at days 1 and 7 are shown. The wound areas on days 1 and 7 are surrounded by solid lines and by dotted lines, respectively.

0.6 J/cm^2), blue (456 nm/0.3 J/cm^2), or green (518 nm/0.2 J/cm^2) LEDs; no LED was used for the sham treatment control. At 0, 4, 8, and 24 hours after irradiation, mRNAs were extracted from the cells to analyze the expression levels of potential mediators for the effects of LEDs, such as growth factors, inflammatory cytokines, and cell matrix proteins, which might be released from fibroblasts and could play important roles in wound healing. *Semi-quantitative* RT-PCR showed that HGF and KGF mRNAs were increased by exposure to red, blue, or green LEDs, and leptin and IL-8 mRNAs were increased by red or green LEDs while VEGF-A was increased only by the green LED compared to the sham treatment (Figure 2). No significant effects on mRNA levels of other factors following exposure to the LEDs tested were noted (Figure 2).

Analysis of protein levels by ELISA

Next, to examine whether the effects of LEDs on the mRNA levels shown above are consistent with the respective protein levels, cultured normal human dermal fibroblasts were irradiated on three consecutive days after seeding with red (638 nm/0.6 J/cm^2), blue (456 nm/0.3 J/cm^2), or green (518 nm/0.2 J/cm^2) LEDs; no LED was irradiated as the sham treatment control. At 24 hours after the last irradiation, the conditioned media were harvested, and protein levels of the cytokines were measured by ELISA. ELISA of the conditioned media showed that levels of HGF and KGF were significantly increased by exposure to red, blue, or green LEDs (HGF; red: 2450 ± 452 pg/mL, $p < 0.01$, blue: 2208 ± 481 pg/mL, $p < 0.05$, green: 2857 ± 381 pg/mL, $p < 0.001$, KGF; red: 231.6 ± 11.1 pg/mL, $p < 0.01$, blue: 263.2 ± 41.9 pg/mL, $p < 0.003$, green: 201.0 ± 47.2 pg/mL, $p < 0.05$) compared with sham treatment (HGF; 1462 ± 97 pg/mL, KGF; 94.7 ± 79.2 pg/mL) (Figure 3A and B). The protein levels of leptin, IL-8, and VEGF-A were only induced by the green LED (leptin: 77.0 ± 8.7 pg/mL, $p < 0.03$, IL-8: 1629 ± 515 pg/mL, $p < 0.0001$, VEGF-A: 513.0 ± 214.1 pg/mL, $p < 0.0001$) compared with the control (leptin: 40.8 ± 16.9 pg/mL, IL-8: 55.1 ± 48.3 pg/mL, VEGF-A: 0 pg/mL) (Figure 3C–E). These results suggest that LEDs, especially green LEDs, can promote wound healing by releasing these cytokines from dermal fibroblasts.

Effect of LEDs on HaCat keratinocytes

In vitro migration assay

To explore the effects of LEDs on the migration of HaCat keratinocytes, we performed an in vitro migration assay. First, we inflicted an in vitro wound in confluent HaCat keratinocytes pretreated with mitomycin C to prevent cell proliferation. Following the wounding, closure of the cell-free area by migrating cells was observed. Red (638 nm/0.6 J/cm^2), blue (456 nm/0.3 J/cm^2), or green (518 nm/0.2 J/cm^2) LEDs were irradiated to cultured HaCat keratinocytes; no LED was irradiated for the control sham treatment. The red and the green LEDs stimulated the migration of HaCat keratinocytes over 24 hours (red: 26.73 ± 3.33 µm/hour, $p < 0.05$, green: 29.05 ± 4.69 µm/hour, $p < 0.01$) compared with the sham treatment (20.47 ± 4.89 µm/hour) (Figure 4A and B), indicating that exposure to the red or green LEDs accelerates keratinocyte migration.

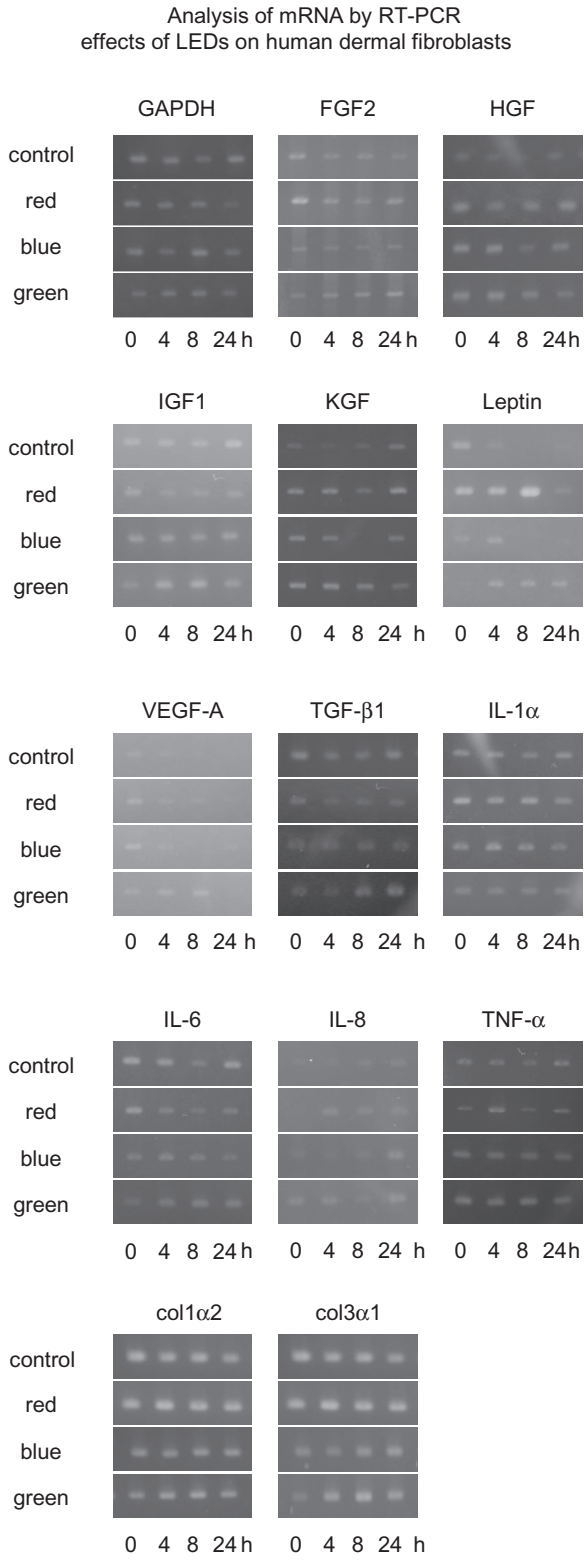

Figure 2. Effects of light-emitting diodes (LEDs) on human dermal fibroblasts: analysis of mRNA levels by *semi-quantitative reverse transcription polymerase chain reaction* (*RT-PCR*). Red (638 nm, 0.6 J/cm^2), blue (456 nm, 0.3 J/cm^2), or green (518 nm, 0.2 J/cm^2) LEDs were used to irradiate cultured normal human dermal fibroblasts; for the control sham treatment, no light was irradiated. At 0, 4, 8, and 24 hours after the LED irradiation, RNA samples were extracted from the cells, and mRNA expression levels of growth factors (fibroblast growth factor [FGF]-2, hepatocyte growth factor [HGF], insulin-like growth factor 1 [IGF-1], keratinocyte growth factor [KGF], leptin, transforming growth factor [TGF]-β1, and vascular endothelial growth factor A [VEGF-A]), inflammatory cytokines (interleukin [IL]-1α, IL-6, IL-8, tumor necrosis factor α [TNF-α]), and matrix proteins, such as procollagens type 1α2 (col1α2) and type 3α1 (col3α1), were analyzed by *semi-quantitative RT-PCR*. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. HGF and KGF mRNAs were increased by exposure to red, blue, or green LEDs, and leptin and IL-8 mRNAs were increased by red or green LEDs while VEGF-A was increased only by the green LED compared with the sham treatment. No significant effects on mRNA levels of other factors following exposure to the LEDs tested were noted.

Analysis of mRNA by *semi-quantitative RT-PCR*

To explore potential mediators involved in the stimulatory effect of LED irradiation on keratinocyte migration, cultured HaCat keratinocytes were irradiated with red (638 nm/0.6 J/cm^2) or green (518 nm/0.2 J/cm^2) LEDs. After 0, 4, 8, and 24 hours, mRNAs were extracted from the cells to analyze the expression levels of potential mediators, such as HB-EGF, VEGF-A, TGF-α, IL-6, and TNF-α, which reportedly stimulate keratinocyte migration in autocrine and/or paracrine manners. Among them, HB-EGF and VEGF-A mRNAs were increased by exposure to the green LED, while TGF-α mRNA was increased by the red or green LEDs compared with the sham treatment (Figure 5). There were no significant effects on other factors following exposure to any of the LEDs.

Analysis of protein levels by ELISA

Next, to examine whether the effects of LEDs on the mRNA levels shown above are consistent at the protein levels, cultured HaCat keratinocytes were irradiated on three consecutive days with red (638 nm/0.6 J/cm^2) or green (518 nm/0.2 J/cm^2) LEDs; no LED was irradiated as a control. Four days later, conditioned media were harvested and subjected to ELISA to measure protein levels of growth factors whose mRNA levels were increased by the LEDs as noted above. ELISA of the conditioned media showed that protein levels of HB-EGF and VEGF-A were significantly increased by green LEDs (HB-EGF: 35.35 ± 6.97 pg/mL, $p < 0.003$, VEGF: 10.21 ± 2.74 ng/mL, $p < 0.003$) compared with the sham control treatment (HB-EGF; 19.65 ± 1.61 pg/mL, VEGF; 4.87 ± 0.17 ng/mL) (Figure 6A and C). On the other hand, the protein level of TGF-α was not significantly different following exposure to any of the LEDs (Figure 6B).

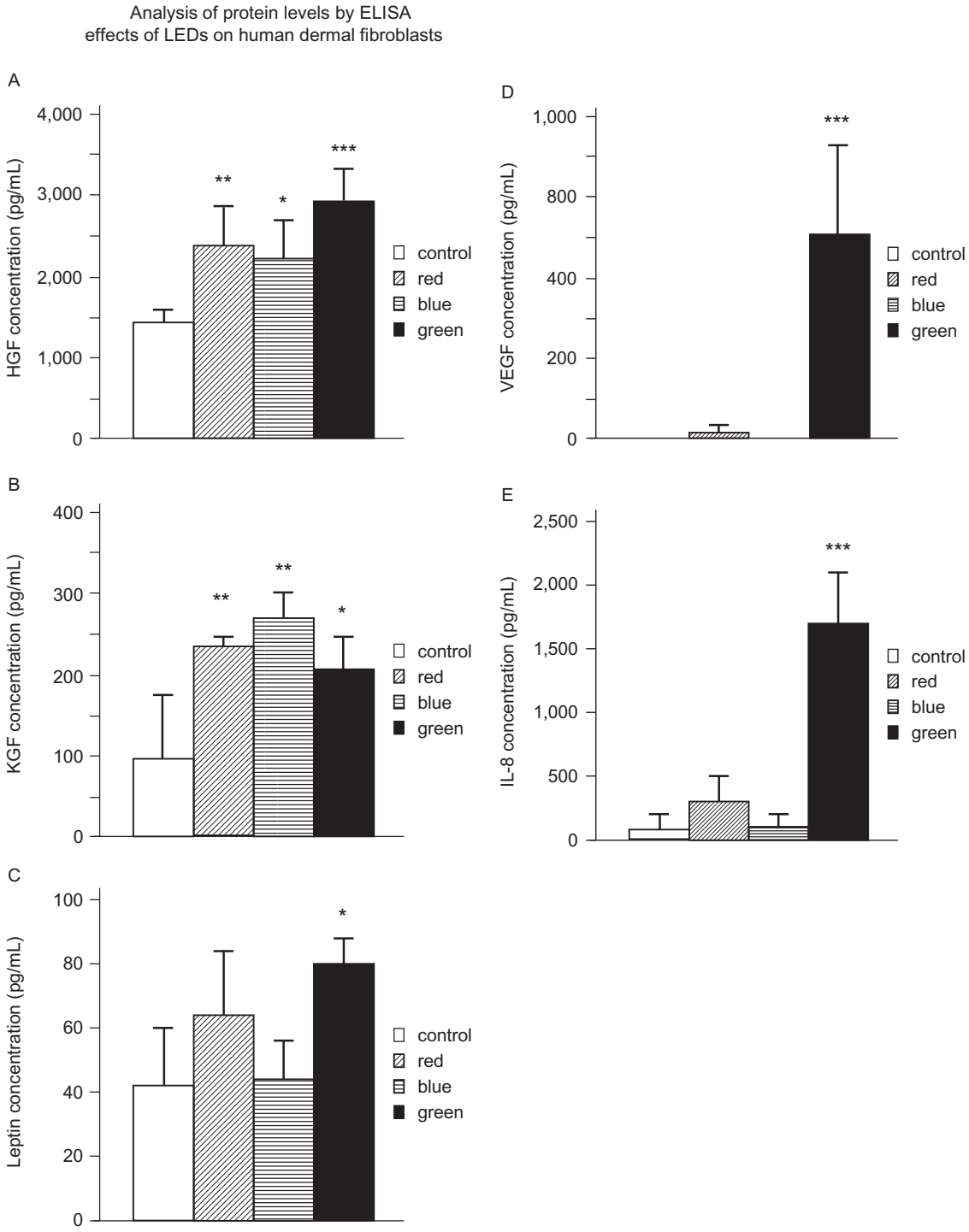

Figure 3. Effects of light-emitting diodes (LEDs) on human dermal fibroblasts: analysis of protein levels by enzyme-linked immunosorbent assay (ELISA). Red (638 nm, 0.6 J/cm^2), blue (456 nm, 0.3 J/cm^2), or green (518 nm, 0.2 J/cm^2) LEDs were used to irradiate cultured fibroblasts at days 1, 2, and 3; no LED was irradiated for the control sham treatment. At day 4, the culture media were harvested, and protein levels of growth factors and cytokines whose mRNA expression levels were increased by LEDs in the RT-PCR experiments were measured. We utilized human ELISA kits for each factor as mentioned in the Materials and Methods. (A) Hepatocyte growth factor (HGF); (B) keratinocyte growth factor (KGF); (C) leptin; (D) vascular endothelial growth factor (VEGF); (E) interleukin (IL)-8. Data are means ± SD of four independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ as compared with the control group (one-way analysis of variance with Dunnett's analysis).

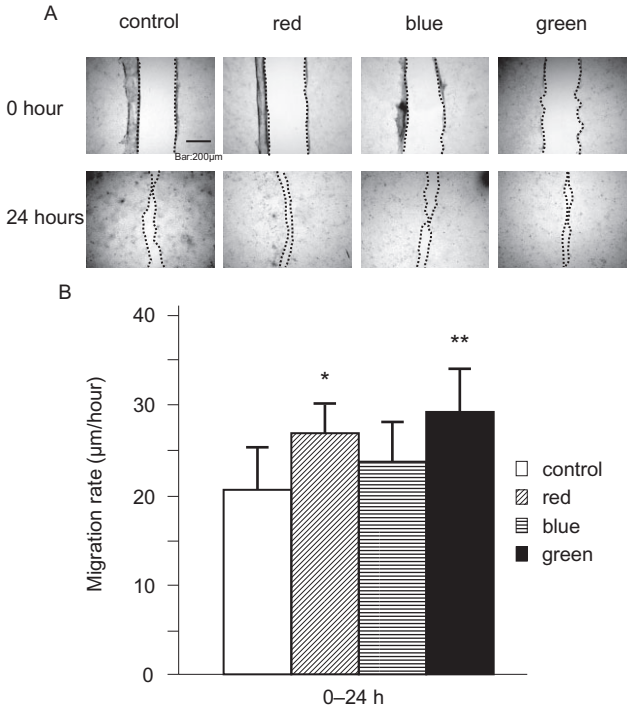

Figure 4. In vitro migration assay for HaCat keratinocytes. (A) HaCat keratinocytes were pretreated with mitomycin C to inhibit proliferation. After a cell-free area was introduced by scraping the monolayer, red (638 nm, 0.6 J/cm^2), blue (456 nm, 0.3 J/cm^2), or green (518 nm, 0.2 J/cm^2) light-emitting diodes (LEDs) were used to irradiate cultured HaCat keratinocytes; no LED was irradiated for the control sham treatment. Cell migration to the cell-free area over 24 hours was evaluated using photographs taken with a microscope. The photographs show the cell migration at 0 and 24 hours after LED exposure. The dashed lines indicate the wound edges. Scale bar: 500 μm. (B) Analysis of the migration rate from 0 to 24 hours expressed as migration distance/time (μm/hour). Data are means ± SD of six independent experiments. $*p < 0.05$, $**p < 0.01$ as compared with the control group (one-way analysis of variance with Dunnett's analysis).

DISCUSSION

In this study, we demonstrate that green and red LEDs accelerate wound healing in ob/ob mice. Many reports have suggested that light irradiation, such as GaAs lasers, helium-neon (HeNe) lasers, argon lasers, and ruby lasers, as well as red LEDs, stimulate wound healing.[2,3,5,7,8] However, only a few studies have reported the effects of green wavelengths of light (470–550 nm) on wound healing in a rodent model and increased fibroblast proliferation and collagen deposition.[9–12]

Regarding the effects of phototherapy on wound healing at the cellular level, various studies have shown an increase in proliferation of various types of cells, such as human dermal fibroblasts and keratinocytes, and increased collagen deposition in the dermis following exposure to various wavelengths of light,[2,5,13–15] which indicates that keratinocytes and fibroblasts are the primary cellular targets to study wound healing mechanisms. On the other hand, considering the in vivo implication of the LED effect, the longer the wavelength, the deeper the penetration into tissue in general. In addition, at wavelengths less than 600 nm, blood hemoglobin is a major obstacle to photon absorption. Thus, red LEDs can penetrate more deeply than blue and green LEDs. The penetration depth is less than 1 mm at 400 nm, 1–2 mm at 514 nm, and 1–6 mm at 630 nm, depending on the type of tissue.[16,17] Regarding wound healing, the penetration depths of all three LEDs are enough to stimulate cells on the wound surface; furthermore, green and red LEDs can activate cells within the dermis.

Hence, to delineate the cellular and molecular mechanism(s) by which LEDs stimulate wound healing, we investigated their effects on fibroblasts and keratinocytes.

Concerning the parameters of the LED irradiation, such as wavelength, dose, intensity, and irradiation time, many reports have suggested that biological effects depend on them,

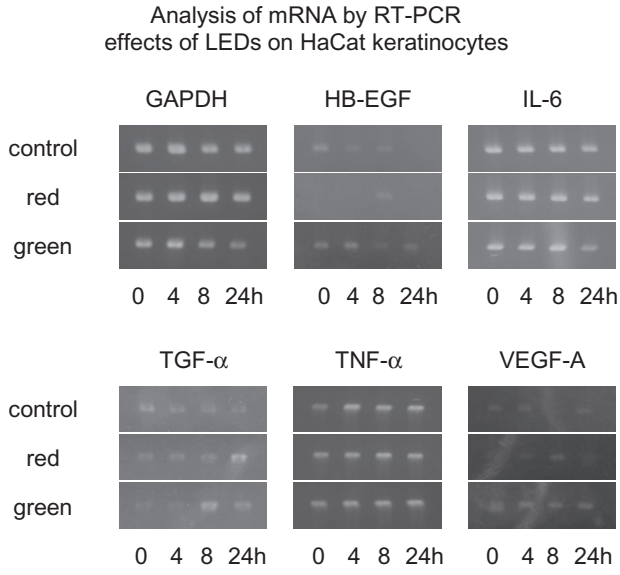

Figure 5. Effects of light-emitting diodes (LEDs) on HaCat keratinocytes: analysis of mRNAs by *semi-quantitative reverse transcription polymerase chain reaction* (*RT-PCR*). To explore potential mediators involved in the stimulatory effect of LED irradiation on keratinocyte migration, cultured HaCat keratinocytes were irradiated with red (638 nm/0.6 J/cm^2) or green (518 nm/0.2 J/cm^2) LEDs. After 0, 4, 8, and 24 hours, mRNAs were extracted from the cells to analyze the expression levels of potential mediators, such as heparin-binding epidermal growth factor-like growth factor (HB-EGF), vascular endothelial growth factor A (VEGF-A), transforming growth factor (TGF)-α, interleukin (IL)-6, and tumor necrosis factor α (TNF-α), which stimulate keratinocyte migration in autocrine and/or paracrine manners, using *semi-quantitative RT-PCR*. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. HB-EGF and VEGF-A mRNAs were increased by the exposure to green LEDs, while TGF-α mRNA was increased by red or green LEDs compared with the sham treatment. There were no significant effects on other factors following exposure to any of the LED. h represents hours after irradiation with the LEDs.

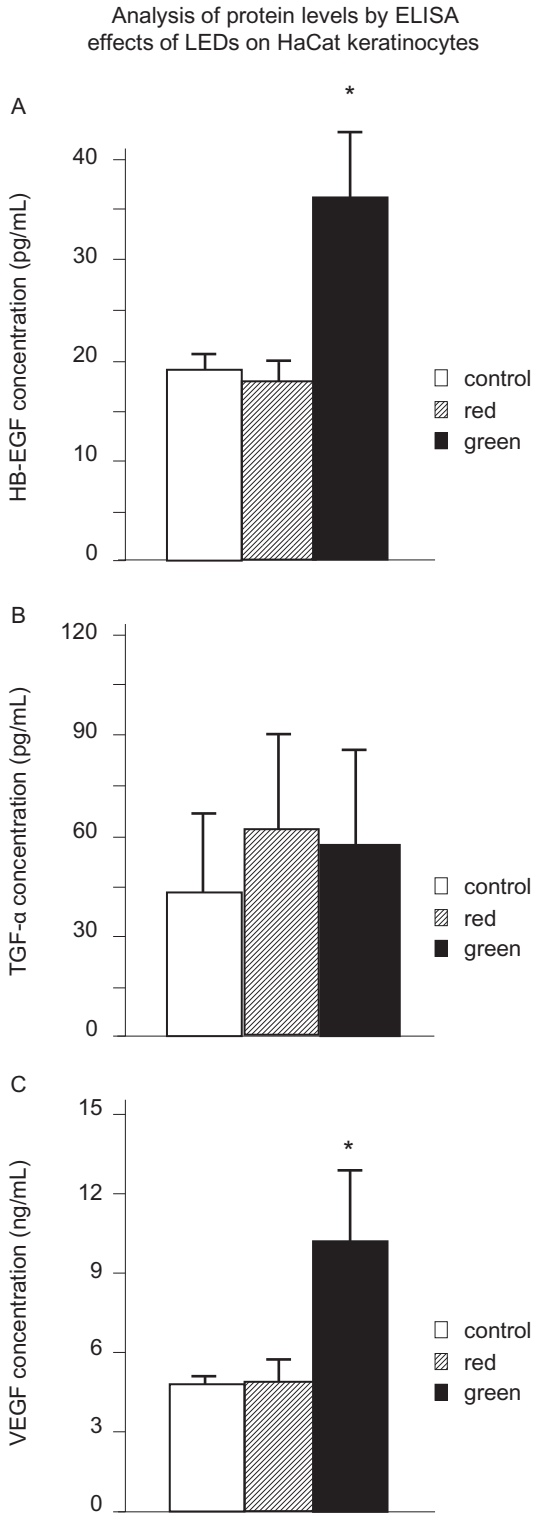

Figure 6. Effects of light-emitting diodes (LEDs) on HaCat keratinocytes: analysis of protein levels by enzyme-linked immunosorbent assay (ELISA). Cultured HaCat keratinocytes were irradiated on 3 consecutive days with red (638 nm/0.6 J/cm^2) or green (518 nm/0.2 J/cm^2) LEDs; no LED was irradiated as the control. Four days later, conditioned media were harvested and subjected to ELISA for growth factors such as heparin-binding epidermal growth factor-like growth factor (HB-EGF) (A), transforming growth factor (TGF-α) (B), and vascular endothelial growth factor A (VEGF-A) (C), whose mRNA expression levels were increased by LEDs in the above reverse transcription polymerase chain reaction (RT-PCR) results. Data are means ± SD of four independent experiments. $*p < 0.03$ as compared with the control group (one-way analysis of variance with Dunnett's analysis).

and various parameters have been used in experiments studying photobiomodulation. However, optimal parameters are still unknown. In this study, to irradiate uniform fluences of light focused on the irradiation sites, we fixed the LED light sources at a 10-cm distance from the bottom of the dishes, resulting in different values of intensity at the irradiation site, 0.75 mW/cm^2 for red, 0.25 mW/cm^2 for blue, and 0.17 mW/cm^2 for green. In addition, because fibroblasts or HaCat keratinocytes were moved from the CO_2 incubator to the laminar flow cabinet for LED exposure, they must be under the same conditions to compare the LED irradiation groups with the sham treatment group. That was the reason why the time of irradiation was the same among all groups. As a result, we irradiated three wavelengths of LEDs with different doses in vitro; however, it was enough to compare each LED irradiation group with the control group. In our investigations on cytokines, the cultured cells were irradiated for 3 days for the ELISA study and 1 day for the RT-PCR study. The reason for that is as follows: In a preliminary experiment, we irradiated LEDs every day and investigated the expression levels of mRNAs and proteins after 1, 2, and 3 days. Although the expression of mRNAs varied within 1 day, protein levels changed most significantly after 3 days, which is why we irradiated cells for 3 days to analyze protein levels by ELISA and 1 day for RT-PCR. This result suggests that responses of mRNA expression can be observed within 24 hours after irradiation, whereas other responses, such as synthesis and secretion of proteins, required 3 days before the effects were observed. In addition, the protein level of IL-8 wasn't increased by red LED exposure, while the mRNA of IL-8 was induced by the red LED irradiation. That discrepancy between mRNA and protein levels may be due to posttranscriptional regulation.

Our data show that the production of HGF and KGF by fibroblasts is significantly increased by red and green LEDs, while leptin, IL-8, and VEGF are increased only by the green LED. Those cytokines have effects on fibroblasts, keratinocytes, endothelial cells, and neutrophils to promote their proliferation, migration, and release of growth factors, and they improve wound healing.[18–20] Indeed, HGF stimulates endothelial cells, fibroblasts, and keratinocytes, and is widely involved in the angiogenic process, in reepithelialization, and in other processes, such as priming neutrophils and increasing matrix synthesis and deposition.[21,22] KGF induces the proliferation and migration of keratinocytes, and leads to accelerated reepithelialization.[23] Leptin exerts a specific mitogenic response on keratinocyte and fibroblast proliferation, on collagen synthesis, and it also acts as a potent angiogenic factor for endothelial cells.[24–29] IL-8 stimulates human keratinocytes and endothelial cells and leads to reepithelialization and

angiogenesis.[18,30–32] VEGF stimulates multiple components of the angiogenic cascade, but likely also promotes collagen deposition and epithelialization.[33] Collectively, the induction of those factors by exposure to LEDs triggers cell networks to accelerate wound healing. In addition, because our data show that leptin, IL-8, and VEGF are significantly induced in fibroblasts only by the green LED, these are specific or preferential mediators for green LEDs compared with red LEDs. Regarding the effects of phototherapy on keratinocytes, although recent reports noted that HeNe irradiation of human keratinocytes in vitro induced their motility, and that a 780-nm diode laser stimulated their proliferation,[1] no study has shown the effects of exposure to green LEDs on keratinocytes. Thus, to investigate the effects of LEDs on keratinocytes, we examined HaCat cell migration, and found significant stimulation of HaCat motility following exposure to red or green LEDs. Moreover, our data show that exposure to green LEDs increases the production of HB-EGF and VEGF, which reportedly promotes the migration of keratinocytes,[34] while the production of those cytokines was unaltered by the red LEDs. This suggests that green LEDs accelerate the motility of HaCat keratinocytes by stimulating the release of HB-EGF and VEGF, while red LEDs promote migration by another as yet unknown pathway. Green LEDs did not significantly stimulate the proliferation of HaCat keratinocytes or fibroblasts (data not shown).

Light must be absorbed by a photoacceptor to exert effects on living biological systems. Indeed, recent studies identified mitochondrial photoacceptor-cytochrome c oxidase as a photoacceptor for visible/near infrared (NIR) spectral light,[35] and porphyrin-containing enzymes and flavoproteins as photoacceptors for the blue wavelength range, linking the mitochondrial respiratory chain to photostimulation.[36–38] Because visible/NIR spectral light increases ATP synthesis,[7,15,39,40] we examined the effect of green LEDs on cytochrome c oxidase activity and on ATP production by human fibroblasts. However, we could not observe any significant effect of green LEDs (data not shown) on either of those molecules. This may be due to differences in experimental settings, including the irradiation energy, density, exposure time, and/or the cell types used. Another reason is the presence of another specific photoacceptor for green LEDs. In this study, because we reduced the bandwidth with a band-pass filter and diffusion by a diffusing lens to deliver narrower wavelengths and a uniformly larger area of light, the levels of intensities were comparatively lower than the previous report while our studies suggest that green LED could promote wound healing with lower doses. Previous studies suggested that different wavelengths can have different effects,[9,12] and in fact, our results show that some cytokines secreted by fibroblasts and HaCat keratinocytes are significantly induced only by green LED irradiation, although the dose of green LEDs was the lowest among the 3 types of LEDs. This suggests the possibility that these cytokines are specific and preferential mediators for green LEDs compared with red and blue LEDs, and/or that the physiological threshold value of green LEDs is lower than red and blue LEDs. We do not know whether the wavelength of 518 nm used in this study caused specific effects, because previous reports suggested the effects of green wavelengths of light (470~550 nm) on wound healing and fibroblasts.[9,10,12] To resolve this question, further investigation is needed. However, it is very important to first examine the possibility that green LEDs promote wound healing by inducing migratory and proliferative mediators that red LEDs do not induce, suggesting that not only red LEDs but also green LEDs can be a new powerful therapeutic strategy for wound healing.

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