DOI: 10.1111/exd.12353
www.wileyonlinelibrary.com/journal/EXD

Original Article

Blue light inhibits transforming growth factor-β1-induced myofibroblast differentiation of human dermal fibroblasts

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Abstract: Transforming growth factor-β1 (TGF-β1) is the major promoter of phenotypic shift between fibroblasts and myofibroblasts accompanied by the expression and incorporation of α-smooth muscle actin (α-SMA). This differentiation is crucial during normal wound healing and wound closure; however, myofibroblasts are considered as the main effecter cell type in fibrosis, for example in scleroderma and hypertrophic scarring. As blue light has exerted antiprolific and toxic effects in several cell types, we investigated whether blue light irradiations with a light-emitting diode array (420 nm) were able to affect proliferation and differentiation of human dermal fibroblasts (HDF). We found that repeated irradiation with non-toxic doses significantly inhibits TGF-β1-induced differentiation of HDF into myofibroblasts shown by α-SMA immunocytochemistry and Western blotting. Additionally, used doses reduced proliferation and myofibroblast contractibility measured by resazurin and collagen gel contraction assays. It could be demonstrated that blue light mediates cell toxicity by oxidative stress due to the generation of singlet oxygen. We postulate that irradiations at non-toxic doses induce low-level oxidative stress and energy-consuming cellular responses, which both may effect proliferation stop and interfere with myofibroblast differentiation. Thus, targeting differentiation, proliferation and activity of myofibroblasts by blue light may represent a useful strategy to prevent or reduce pathological fibrotic conditions.

Abbreviations: HDF, human dermal fibroblasts; α-SMA, α-smooth muscle actin; TGF-β1, transforming growth factor-β1; LED, light-emitting diode; nm, nanometer; UV, ultraviolet.

Key words: blue light – differentiation – fibroblasts – fibrosis – myofibroblasts

Accepted for publication 11 February 2014

Introduction

Human skin is exposed to sunlight on a daily basis. The spectrum sunlight which reaches the earth surface comprises 50% visible light, 45% infrared and 5% ultraviolet radiation (1). The biological effects and mutagenic properties of the ultraviolet region namely UV-B (315-280 nm) and UV-A (400–315 nm) have been intensely investigated within the last decades (2–5), showing that chronic UV-A and UV-B exposure is related to increased risk of skin cancer (6) and premature skin ageing (7). In contrast to this, the effects of visible light on skin and cells are less well understood. It was demonstrated that besides laser light (8,9) also unpolaric and non-coherent light with certain wavelengths in the visible spectra can influence cell growth, metabolism, DNA synthesis and further cell functions (1,10,11). In particular, blue light (400–500 nm) reveals toxic effects and cellular dysfunction because of its relatively high energy. Furthermore, blue light is associated with malignant melanoma development in animal models and the induction of photochemical injury to the retina in the eye, called photoretinitis (12). It is hypothesized that the interaction of photons with endogenous photoreceptor molecules, such as cytochrome c oxidase (13,14), flavin-based photosensors (15) and lipofuscin (16), effects the generation of singlet oxygen or other reactive oxygen species (ROS) which are thought to be responsible for the observed effects. Blue light is used in the treatment of acne vulgaris (17) and reveals anti-inflammatory properties (18). Recently, also a suppression of dendritic cells activation by blue light could be found *in vitro* (19). In addition, irradiation with blue light (470 nm) inhibits the growth of skin tumors in mice (20) and improves wound healing in rats (21). Further studies have shown that blue light can inhibit the mitosis and proliferation in cultured cells such as pig kidney embryo cells (22), melanoma cells (23) and gingival fibroblasts (24). Recently, we could also demonstrate in human skin fibroblasts that blue light induces dose- and wavelength-dependent toxicity. In particular, shorter wavelength (410, 420 nm) showed pronounced toxicity, whereas longer wavelength (453, 480 nm) did not induce any toxic effects on fibroblasts. We observed a distinct lower proliferation using repeated blue light irradiation with lower, non-toxic doses (25).

As the major cell type in the dermis, fibroblasts play a pivotal role in skin physiology for example in the synthesis and deposition of various extracellular matrix (ECM) proteins (26). Apart from maintaining the mechanical properties of the skin, dermal fibroblasts take an important part in the process of wound healing

by synthesizing collagen and relevant cytokines such as keratinocyte growth factor (27). Fibroblasts differentiate into myofibroblasts primarily known for their key role in wound healing and physiological reconstruction of connective tissue (28). After cutaneous injury, the differentiation of fibroblasts to myofibroblasts represents a crucial step in wound granulation and tissue contraction for the re-establishment of the barrier function of the skin. During differentiation, fibroblasts transform to a proliferative and contractile phenotype with characteristics of smooth muscle cells (29). The fibroblast/myofibroblast transition begins with the appearance of protomyofibroblasts, whose stress fibres contain β-and γ-cytoplasmic actins but no α-smooth muscle actin (α-SMA), the marker of differentiated myofibroblasts. This complex process is driven by at least one cytokine (TGF-β1), extracellular component and mechanical tension (30). However, cyclic strain of fibroblast can reduce levels of α-SMA, connective tissue growth factor and endothelin-1 without affecting TGF-β1 expression (31). The contractile activity by myofibroblasts is beneficial for tissue remodelling. After completed tissue repair and wound closure myofibroblasts regularly undergo apoptosis (32). The persistence of myofibroblasts and therefore excessive contractile activity can be a detrimental pathological factor in the development of hypertrophic scars, keloids and fibrotic diseases (33,34). It could be shown that blue light irradiations inhibit proliferation and induce differentiation of keratinocytes *in vitro* (35). In a clinical trial, a notable reduction in psoriatic plaques could be observed in patients with psoriasis (36). Furthermore, the clinical efficacy of blue light full-body irradiation as treatment option of severe atopic dermatitis could be shown in another clinical trial, without the induction of Langerhans cell and T cell depletion from skin, a well-known side effect of UV treatment (37).

The possibility to modulate cell proliferation and cell differentiation in skin tissue using blue light could represent a useful tool against hyperprolific skin diseases, such as hyperkeratosis and psoriasis, but also against fibroblast-mediated hypertrophic scar formation, keloids and scleroderma. As blue light affects proliferation and differentiation of keratinocytes, we have investigated in this study whether blue light is also able to modulate proliferation and differentiation of human skin fibroblasts.

Materials and methods

Materials

Unless otherwise indicated, chemicals were from Sigma (Deisenhofen, Germany).

Skin specimen was obtained with consent from eight female patients (19–69 years, mean 46.6 ± 18.4) who had undergone reduction mammoplasty (4×) and abdominoplasty (4×) procedures.

LED arrays

We used a prototype of a narrow-band light-emitting diode (LED) device provided by Philips Research (Aachen, Germany) emitting monochromatic light with a maximum intensity at 420 nm and a full width half maximum of 10 nm. Philips Research measured the irradiances of the LED devices using an integrating (Ulbricht) sphere.

Cell culture

Primary cultures of human dermal fibroblasts (HDF) from skin specimens were prepared, cultivated, pooled and cryoconservated as described elsewhere (25).

For experiments, cryoconservated stocks of pooled HDF were thawed and further cultured in 100 mm Petri dishes under normal culture conditions. Cells were fed with fresh medium twice a week and seeded out after 7 days in 6 or 12 culture plates. All measurements were performed with HDF from passages 5. For toxicity experiments, HDF were seeded in six-well plates (1.5×10^4/well; 9.6 cm^2) and cultivated for 10 days to achieve a confluent monolayer in the plates prior to irradiation. For the determination of blue light effects on the resistance against hydrogen peroxide (H_2O_2), cells were seeded in 12-well plates (1×10^4/well; 3.9 cm^2) and cultivated for 10 days prior treatment. For the determination of cell proliferation, α-SMA^+ cells and α-SMA expression, HDF were seeded in 6-well plates (1.5×10^4/well; 9.6 cm^2) and irradiations were performed from day 3 until day 7 after seeding. By the addition of TGF-β1 (10 ng/ml; Peprotech, Hamburg, Germany) directly after seeding or 3 days prior first irradiation, myofibroblast differentiation was induced. TGF-β1 was added after each irradiation step.

Irradiation of HDF cultures

The LED device was adjusted to deliver 50 mW/cm^2 in the irradiated culture plate. During irradiation, cells or cell-loaded collagen gels were maintained in PBS. To avoid heat generation and vaporization of media during irradiation, cell culture plates were cooled by ventilation so that the temperature of the buffer never exceeded 30°C and covered by a sheet of transparent quartz glass. After irradiation PBS was replaced by fresh media. Control cells were treated the same way, but were kept in ambient light. Dependent on the experiment, viability, proliferation, oxidative stress, gel contraction, α-SMA expression or number of myofibroblasts were assessed at indicated time points after irradiation.

Toxicity, growth rates and viability

Relative cell numbers were determined by neutral red staining (38) 24 h after irradiation exactly as described elsewhere (25). To determine the effects of blue light-induced intracellular stress, H_2O_2 in increasing concentrations (0–6 mM) was added to confluent HDF cultures 30 min after irradiation, and living cells were measured 24 h later. The toxicity of low-dose irradiation in non-confluent HDF cultures was excluded by Hoechst dye H33342 (1 μg/ml) and/or propidium iodide (0.5 μg/ml) staining. At different points in time (6–24 h) after single and repeated irradiation, HDF cultures (six-well) were washed with PBS, stained with both dyes for 5 min, and nuclei and necrotic cells were visualized using a Zeiss fluorescence microscope. Viable cells were defined as cells excluding propidium iodide. Resazurin-based alamarBlue assays were performed 18 h after each irradiation to measure the proliferation of HDF according to the manufacturer′s instructions (39). Cells were washed twice after measurements, and media were added. Cells were cultivated for 6 h before further irradiations were performed. To exclude the possible interaction of intracellular remaining dye and irradiations, alamarBlue assays were not performed until the last day. No significant differences were found.

Measurement of oxidative stress

Oxidative stress was measured using the dye 2′,7′-dichlorodihydrofluorescein diacetate (H2-DCFDA; Invitrogen, Darmstadt, Germany; 40). Cells were seeded at 2×10^4 cells/well in black 96-well plates (0.39 cm^2; Greiner, Frickenhausen, Germany). Medium was replaced by 250 μl PBS/well the following day, and the plates

were irradiated (15 and 30 J/cm^2). As standard, an unirradiated plate was treated identically. A short washing step with PBS directly followed the irradiations, before 100 μl/well of freshly prepared H2-DCFDA in PBS (10 μM) following manufacturer's instructions was added. After incubation (5 min) in the dark at 37°C/5% CO_2, wells were washed with 300 μl PBS and filled with 100 μl PBS under low light conditions, and fluorescence in each well was measured for 0.2 s with 100 flashes per well in a multiwell fluorescence plate reader (Fluostar Optima, BMG Labtech, Offenburg, Germany) using excitation (485 nm) and emission filters (520 nm).

Preparation of collagen gels

As described by Tingstrom et al. (41), collagen gels were prepared in tissue culture plates (24 well, Greiner) using PureCol collagen solution (3 mg/ml, Inamed Biomaterial, Netherlands) and HDF (50 000/gel). Gels were cultivated in 1 ml of MCDB 105 media with or without TGF-β1 (10 ng/ml). Irradiations were performed in PBS 24 h later. The measurements of gel contraction were released after 96 h of incubation. Briefly, gels were fixed with 37% formalin solution, washed once with PBS/0.2% Tween 20 and weighed on an analytical digital scale. Contraction was quantified as loss in gel weight.

Western blot analysis of α-SMA

Seven days after seeding of 1.5 × 10^4 HDF/well in six-well plates, cells were washed with 4°C cold PBS and collected by scraping them into 50 μl of RIPA lysis buffer with 14.2% protease inhibitor (7×; Roche). Extracts were further lysed by ultrasonic treatment, and supernatant was collected after centrifugation (10 min; 4°C; 20 000 g). Western blot for α-SMA was performed using monoclonal mouse anti-α-SMA antibodies, monoclonal mouse anti-α-tubulin antibodies (Abcam; Cambridge, UK) as standard and the Xcell SureLock Mini-Cell-System (Invitrogen, Karlsruhe, Germany) under reducing conditions. Prior Western blotting, protein concentrations were determined using DC Protein Assay (Bio-Rad, Munich, Germany). Samples of 30 μg were loaded on SDS-PAGE and transferred to nitrocellulose filters. The membranes were blocked in 5% non-fat milk/T-TBS (0.1% Tween 20) overnight at 4°C and then incubated with primary antibody (anti-α-SMA ab, 1:2000; anti-α-tubulin ab, 1:15 000) for 1 h. Finally, after washing (4 × 5 min T-TBS) blots, an incubation with goat anti-mouse IgG-HRP (1:5000; 1 h) followed. After washing (4 × 5 min T-TBS), enhanced luminescence reagents (SuperSignal West Pico; Pierce, Rockford, IL, USA) were applied according to manufacturer's instructions. The quantification of bands was achieved using the LAS-3000 system (Fujifilm, Düsseldorf, Germany).

Myofibroblast differentiation

To visualize the influence of blue light on the extent of myofibroblast differentiation 7 days after seeding of HDF (2.5 × 10^4/well) in six-well plates, cells were fixed by a treatment with paraformaldehyde/PBS (4%; 15 min) and permeabilized by 0.2% Triton X-100/PBS. Cells were incubated with blocking buffer (4% BSA/PBS; 30 min) and subsequently with mouse anti-α-SMA antibodies (1:400; 60 min). After three washing steps with PBS, cells were incubated for 60 min with an Alexa Fluor 488-conjugated goat anti-mouse antibody (1:1000; Invitrogen, Carlsbad, CA, USA) in blocking buffer. After three washing steps, cells were incubated with Hoechst 33342 (1 μg/ml) in PBS for 10 min. After washing, cells were visualized using an invert fluorescence microscope (DMI4000B, Leica, Wetzlar, Germany). Ten photos of each well were taken, and the total number of cells and α-SMA$^+$-cells was counted using Image J (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Values were reported as means ± standard deviations (SD). After consulting the Institute of Medical Statistics of the Medical Faculty, RWTH Aachen (Germany), data were analysed with the help of SAS V 9.2 (SAS Institute, Cary, NC, USA) using multifactorial ANOVA. $P < 0.05$ was considered to be significant.

Results

Blue light induces the generation of reactive species and toxicity in HDF

In our experiments, a single irradiation of blue light at a dose of 30 J/cm² caused no significant toxicity in HDF (Fig. 1a). In the presence of deuterium blue at 30 J/cm², a significant toxicity could be observed. Higher blue light doses (60 and 90 J/cm²) led to a significant decrease in the number of live cells (55.8 ± 4.5% and 30.1 ± 5.8%). Here, deuterium enhanced the toxicity at 60 J/cm² (31.9 ± 4.4%), but not at 90 J/cm² (32.9 ± 2.5%). By the addition of sodium azide, no blue light-induced toxicity was induced at any light dose. A significant induction of intracellular oxidative stress was measurable at blue light doses of 15 and 30 J/cm² (Fig. 1b). Furthermore, blue light treatment at non-toxic doses prior to the addition of hydrogen peroxide enhanced toxicity (Fig. 1c). For example, at non-toxic hydrogen peroxide concentrations (1 mM), 30 J/cm² led to a significant reduction (0.47 ± 0.21 vs 0.21 ± 0.12) of neutral red absorbance. At moderate toxic hydrogen peroxide concentrations (2 mM), enhancement of toxicity could be observed with prior blue light irradiations at 15 and 30 J/cm² (0.29 ± 0.10 vs 0.12 ± 0.09 or 0.05 ± 0.03).

Non-toxic doses of blue light inhibit proliferation of HDF

Daily applied, low doses of blue light inhibit significantly the proliferation of HDF under standard cell culture conditions (Fig. 2a). A significant inhibition of proliferation could be observed in HDF cultures, which were treated with TGF-β1 (Fig. 2b). In both cases, inhibition was already significant at day 2 that is, after two irradiations. Staining with Hoechst 33342 and propidium iodide virtually did not showed any toxic cells during control experiments. For comparison, alamarBlue values on day 4 for TGF-β1 treated and non-treated HDFs are given in Fig. 2c. Here, a significant increase in fluorescence signal could be observed in unirradiated HDF, when TGF-β1 was used (19520 ± 1681 vs 15913 ± 1888). Blue light irradiations on a daily basis could significantly decrease the fluorescence signal in TGF-β1 treated (12335 ± 3958 at 15 J/cm²; 10981 ± 3216 at 30 J/cm²) and untreated HDF (10520 ± 4421 at 15 J/cm²; 9372 ± 4015 at 30 J/cm²) compared with unirradiated controls. Manual cell counting confirmed these observations (Fig. 2d), demonstrating a good correlation between the found fluorescence values found and the number of Hoechst 33342 stained nuclei.

Under condition with reduced FCS (5%), the addition of TGF-β1 could not significantly affect fluorescence signal or cell number (Fig. 3). Generally, fluorescence values were somewhat lower without TGF-β1; in TGF-β1-treated cultures, values were significantly lower in comparison with HDF cultured with 10% FCS. HDF cultivated with 5% FCS proliferation was inhibited by daily blue light irradiations (Fig. 3a). However, the reduction in

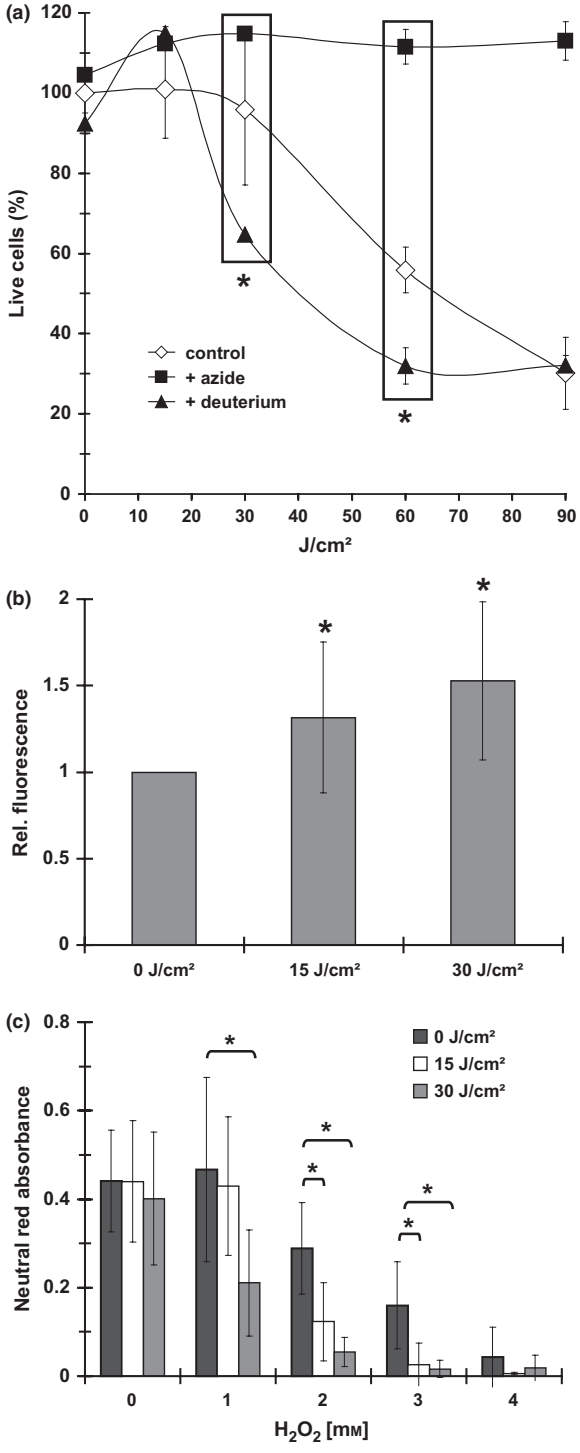

Figure 1. Impact of blue light on cell viability, intracellular oxidative stress and antioxidative capacity. (a) Confluent primary human dermal fibroblast (HDF) cultures were irradiated with light-emitting diode devices emitting light at 420 nm with and without deuterium or sodium azide (1 mM). Cell number was assessed by neutral red staining 24 h after irradiation and normalized to the unirradiated control. (b) Intracellular stress was measured directly after irradiation by staining with the fluorescence dye 2′,7′-dichlorodihydrofluorescein diacetate. (c) Confluent HDF cultures were treated with hydrogen peroxide (0–4 mM) 30 min after irradiation. The relative number of cells was assessed by neutral red staining 24 h after irradiation and normalized to the untreated and unirradiated control. The mean ± SD of 3–5 independent cell cultures experiments is shown. $*P < 0.05$.

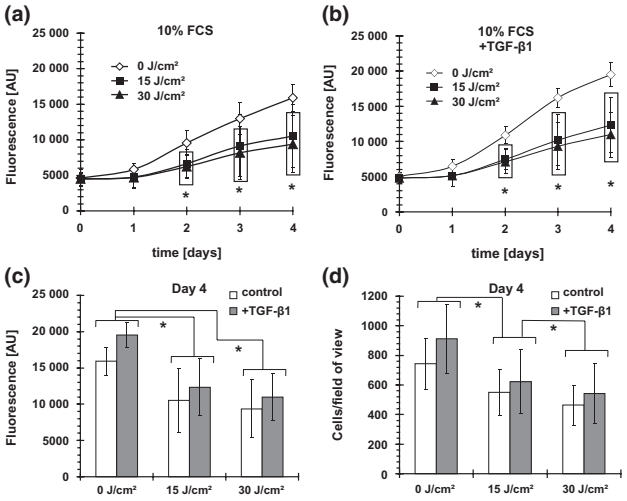

Figure 2. Effects of non-toxic blue light irradiations on proliferation of human dermal fibroblasts (HDF) cultivated with 10% FCS and transforming growth factor-β1 (TGF-β1). (a) Three days after seeding, non-confluent HDF cultures cultivated in media supplemented with 10% FCS were irradiated daily for 4 days with blue light (420 nm). Relative cell number was assessed by alamarBlue assay 6 h prior to and 18 h after each irradiation, and fluorescence values are presented. (b) TGF-β1 (10 ng/ml) was added after seeding and after each irradiation. (c) AlamarBlue fluorescence values and (d) mean of counted nucleoli/sight field at day 4 after a total of four irradiations. Virtually no propidium iodine$^+$ cells could be observed at any day. The mean ± SD of 3–5 independent cell culture experiments is shown. $*P < 0.05$.

fluorescence values observed was not even significant on day 4 (Fig. 3c). Analogously, a slight but not significant reduction in fluorescence values by blue light could be observed, when TGF-β1 was added (Fig. 3b). Interestingly, manual cell counting gave controversial results. First, HDF cultivated in 10% FCS gave a number of 743 ± 171 cells and alamarBlue fluorescence value of 15913 ± 1888 in the control on day 4 (Fig. 2c,d). In comparison, HDF cultured in 5% FCS 426 ± 134 cells gave a fluorescence signal of 13909 ± 3064 (Fig. 3c,d). Thus, as shown in Fig. 3f, the ratio of fluorescence signal per number of cells was roughly a third higher when HDF were cultured in media containing 5% FCS compared with 10% FCS (32.65 vs 21.41). Second, although no significant differences between alamarBlue values of irradiated and unirradiated HDF could be observed using 5% FCS, a significant reduction in cell number by blue light irradiations was found in TGF-β1-treated (285 ± 90 at 15 J/cm², 226 ± 101 at 30 J/cm² cells vs 419 ± 140 cells/sight field) or TGF-β1-untreated (275 ± 75 at 15 J/cm², 220 ± 89 at 30 J/cm² cells vs 426 ± 134 cells/sight field) HDF cultures (Fig. 3d). The ratios of alamarBlue values to cell numbers were constant in HDF cultivated in media containing 10% FCS (approximately 20) and were not affected by TGF-β1 and or blue light irradiation (Fig. 3f). In contrast, using 5% FCS led to a higher ratio (>30) in unirradiated HDF, which could be further increased by blue light. Here, the addition of TGF-β1 slightly increased the ratio in unirradiated and irradiated HDF.

Non-toxic doses of blue light reduce number of α-SMA$^+$ cells in HDF cultures

Apart from cell number, repeated blue light treatments reduced the number of α-smooth muscle actin positive (α-SMA) cells in HDF cultures. In HDF cultures, on the average <1 (0.8 ± 0.7)

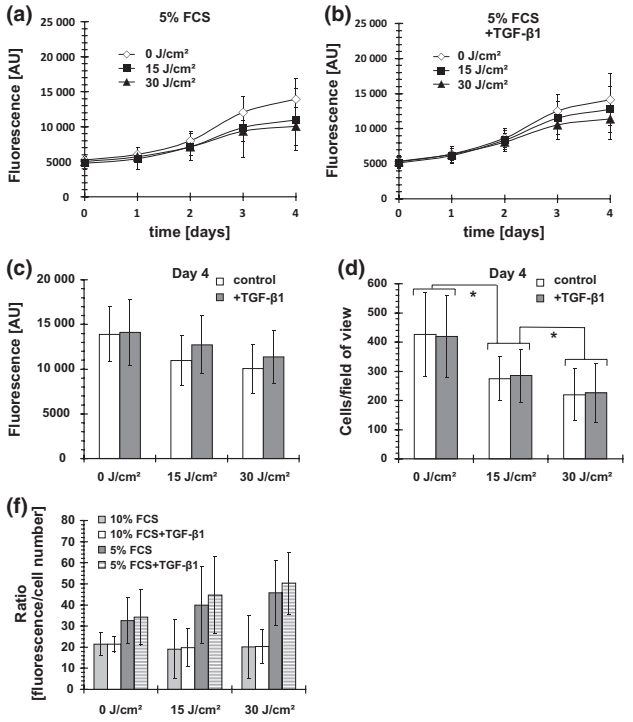

Figure 3. Effects of non-toxic blue light irradiations on proliferation of human dermal fibroblasts (HDF) cultivated with 5% FCS and transforming growth factor-β1 (TGF-β1). (a) Three days after seeding, non-confluent HDF cultures cultivated in media supplemented with 5% FCS were irradiated daily for 4 days with blue light (420 nm). Relative cell numbers were assessed by alamarBlue assay 6 h prior to and 18 h after each irradiation and fluorescence values are presented. (b) TGF-β1 (10 ng/ml) was added to cell cultures after seeding and after each irradiation. (c) AlamarBlue fluorescence values and (d) mean of counted nuclei/sight field at day 4 after a total of four irradiations. Virtually no propidium iodide$^+$ cells could be observed at any day. The mean ± SD of 3–5 independent cell culture experiments is shown. *P < 0.05.

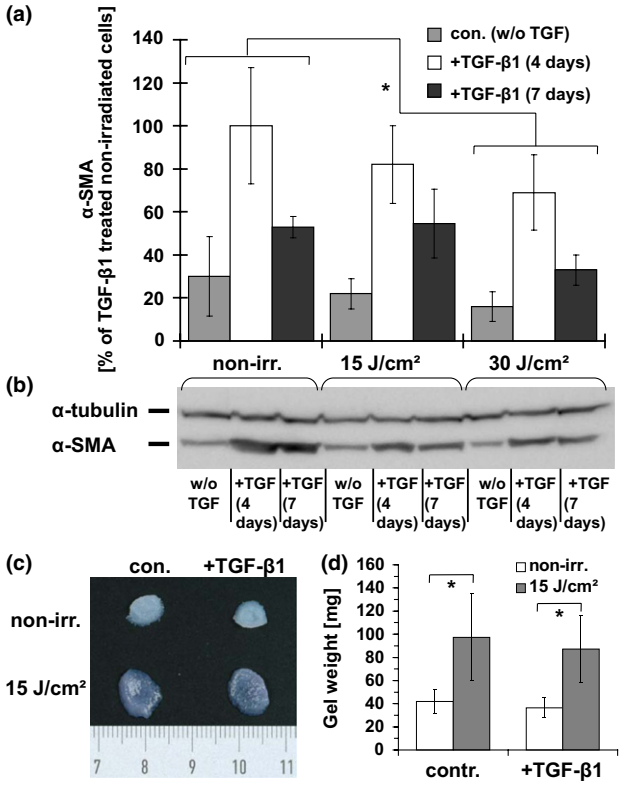

Figure 4. Non-toxic blue light irradiations affect α-smooth muscle actin (α-SMA) expression levels and contractibility. Human dermal fibroblasts (HDF) were daily irradiated with blue light (420 nm) from day 3 until day 6. Transforming growth factor-β1 (TGF-β1; 10 ng/ml) was added to cell cultures directly (7 days) or 3 days after seeding (4 days**)** and each irradiation. (a) Western blot analyses of α-SMA were performed on day 7 (shown are mean ± SD α-SMA densities normalized to respective α-tubulin bands. Mean of raw values was compared to TGF-β1-treated non-irradiated cells = 100%). (b) Representative Western blot. (c) Representative photographs of collagen gels incubated with HDF and TGF-β1 for 5 days irradiated once on day 2. (d) Mean values of gel weights (day 5). Contraction of a gel is antiproportional to its gel weight. (n = 5; *P < 0.05).

α-SMA$^+$ cell within a microscopic sight field could be found after 7 days of cell culture using media containing 10% FCS (Figure S1A). By the addition of TGF-β1 (10 ng/ml), around 10 ± 4.7 α-SMA$^+$ cells were found. Under these conditions, four irradiations reduced the number of α-SMA$^+$ cells by half at light doses of 15 J/cm² (5.0 ± 3.1) and 30 J/cm² (4.4 ± 2.7). A reduction in α-SMA$^+$ cells could also be found (0.5 ± 0.5 at 15 J/cm²; 0.2 ± 0.4 at 30 J/cm² vs 0.8 ± 0.7) without TGF-β1. Normalized to total cell number within the microscopic sight field, only around 1% were α-SMA$^+$ cells using 10% FCS and TGF-β1 (Figure S1C) and only at doses of 30 J/cm² was a significant reduction found (0.1 ± 0.2% vs 1.3 ± 0.1%).

After 7 days, around two (1.8 ± 1.5) α-SMA$^+$ cells within a microscopic sight field could be found in HDF cultured in 5% FCS media. By the addition of TGF-β1, around 17.5 ± 8.0 α-SMA$^+$ cells could be identified. Under these conditions, four repeated blue light irradiations within the last 4 days significantly reduced the number of α-SMA$^+$ cells at light doses of 15 J/cm² (9.8 ± 5.4) and 30 J/cm² (4.6 ± 4.1). Without TGF-β1, a reduction in α-SMA$^+$ cells could be found (1.5 ± 1.0 at 15 J/cm²; 0.8 ± 0.8 at 30 J/cm² vs 1.8 ± 1.5). Normalized to total cell number within the microscopic sight field, around 4.5 ± 1.9% of counted cells were α-SMA$^+$ when using 5% FCS and TGF-β1 (Figure S1D), and only at doses of 30 J/cm² was a significant reduction recorded (0.1 ± 0.2% vs 1.3 ± 0.1%). Herein, irradiations with blue light at doses with 15 J/cm² and 30 J/cm² significantly decreased the percentage of α-SMA$^+$ cells in TGF-β1-treated HDF cultures (3.5 ± 1.6%; 2.1 ± 1.5% vs 4.6 ± 1.9%).

Non-toxic doses of blue light reduce α-SMA expression of HDF and HDF-mediated gel contraction

Western blot analysis revealed a reduction in intracellular α-SMA content in HDF cultures on day 7 after four blue light irradiations at non-toxic doses. As shown in Fig. 4a, TGF-β1 led to a fourfold increase in α-SMA concentration. By blue light treatment with 15 J/cm² 82 ± 18% and with 30 J/cm², only 69 ± 17% α-SMA compared to unirradiated but TGF-β1-treated HDF could be detected. Without TGF-β1 incubation prior to irradiation (TGF-β1 was added after first irradiation), an increase in α-SMA expression compared with HDF (w/o TGF-β1) was found (53 ± 5% vs 30 ± 18%). Under these conditions, irradiations with 30 J/cm² significantly decreased α-SMA content down to 33 ± 7%. Furthermore, a single blue light treatment reduced the fibroblast-/myofibroblast-mediated contraction of collagen gels (Fig. 4c,d). Here, a dose of 15 J/cm² significantly increased the weights collagen gels 4 days later (97.2 ± 37.6 mg vs 42.0 ± 10.2 mg w/o TGF-β1; 87.3 ± 28.8 mg vs 36.5 ± 8.5 mg with TGF-β1).

Discussion

Myofibroblast differentiation is a crucial step in normal wound healing for the re-establishment of the barrier function of the skin (28,42).

By secreting extracellular proteins, growth factors and cytokines, they take an important part in the modulation of the extracellular matrix (42,43). Furthermore, by their contractile potential, myofibroblasts ensure a fast and efficient closure of dermal wounds (44). Transforming growth factor-β1 (TGF-β1) is known to be a key cytokine in physiological wound healing but also in the context of fibrosis (45). It is able to induce α-SMA (46,47), which is a major component of contractile cytoplasmatic microfilaments of differentiated myofibroblasts (48). Chronic wounds are often associated with abnormal cytokine profiles, which may interfere with α-SMA induction by TGF-β1 (49). In addition, chronic wounds are often ischaemic and hypoxic, conditions by which myofibroblast differentiation is impaired (50). On the other hand, in Dupuytren's disease, a common fibroproliferative disorder with a prevalence of over 7% in the United States (51), deregulated myofibroblasts have been made responsible for observed progressive flexion deformities of the digits of the hand by contraction of the palmar fascia (52). In all fibrotic conditions, myofibroblasts are considered to be the final effecter cell responsible for scarring, contraction and excessive collagen production (53). For example, hypertrophic scarring is often associated with thermal injuries. Although the underlying mechanism remains unknown, the pro-fibrotic growth factor TGF-β1 and overactive myofibroblasts seem to play a major role in the pathology of hypertrophic scarring, for which any definitive reduction treatment or prevention remains controversial (54). Another example of myofibroblast-mediated pathological skin condition is scleroderma. Here, effective treatment for observed fibrosis has not been found either (55). Thus, blocking myofibroblast differentiation, proliferation and activity may represent a useful strategy for the prevention of or reduction in pathological fibrotic conditions. In our study, we were able to demonstrate as key result that myofibroblast differentiation can be blocked *in vitro* by blue light. Repeated irradiations of non-toxic doses of blue light led to a significant reduction in α-SMA-positive cells (Figure S1A) as well as intracellular α-SMA levels in TGF-β1-treated and TGF-β1-untreated cultures of human dermal fibroblasts (Fig. 4a). Functionally, we observed a reduction in myofibroblast- /fibroblast-mediated contractibility of collagen gels by blue light (Fig. 4c,d). This reduction could be a result of the inhibition of myofibroblast differentiation and/or reduced cell number. In addition, using media containing 10% FCS simulating prolific conditions, non-toxic doses of blue light were able to significantly reduce the proliferation of TGF-β1-treated and TGF-β1-untreated HDF (Fig. 2).

Using only 5% FCS in media, more differentiated myofibroblasts in HDF cultures can be induced by TGF-β1 addition (Fig. 4), accompanied by lower cell numbers or reduced proliferation rates compared with HDF cultured in 10% FCS media (Fig. 3). Here, blue light irradiations also cause reduction in total cell number (Fig. 3d). However, indirect measurement of cell numbers by alamarBlue assay based on the reduction in resazurin (56) did not correlate well with the counted cell numbers when using media supplemented with 5% FCS (Fig. 3c,d). This result suggests that these kinds of assays require validation for any cell culture and that under certain conditions, the achieved values may reflect rather cell activity and metabolism than number of cells. Interestingly, although blue light reduced the number of cells and inhibited myofibroblast differentiation, the ratios between alamarBlue fluorescence values and number of cells were significantly elevated in irradiated HDF cultures (Fig. 3f), which indicates a higher cellular metabolic rate.

We demonstrated that higher doses of blue light at 420 nm lead to toxicity in HDF by the induction of intracellular oxidative stress (Fig. 1a) and, in particular, the generation of singlet oxygen as shown by using its scavenger sodium azide or its lifetime enhancer deuterium (57). Lower blue light doses induced subtoxic levels of intracellular oxidative stress (Fig. 1b), which in turn resulted in an enhanced sensitivity to hydrogen peroxide (Fig. 1c). Therefore, we postulate that repeated non-toxic blue light irradiations may induce energy-consuming cellular responses against oxidative stress, which effect a proliferation stop, interfere with myofibroblast differentiation and increase cell metabolism.

Although the exact mechanism of blue light interaction with cells or the participating cellular stress responses still remain unclear, the observed effects are promising for a clinical use of blue light in the treatment or prevention of myofibroblast-mediated pathological condition such as tissue fibrosis in scleroderma or hypertrophic scarring. However, clinical trials for proving efficiency and safety of blue light treatment at specific doses and wavelengths as well as more experimental studies for the clarification of the underling mechanism are necessary, before blue light therapy may be utilized any further than in the treatment of acne.

Acknowledgements

LT and ED performed experiments and wrote the manuscript. JK and PCF developed methodology and contributed to manuscript preparation. MB performed LED characterization and contributed to manuscript preparation. CVS and CO designed experiments, headed all data analysis, reviewed the manuscript and supervised the project. This work was supported by CICATRIX, a German Community for people with burns and scars.

Conflict of interest

The authors state no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Non-toxic blue light irradiations reduce TGF- β1-induced α-SMA expression in human dermal fibroblasts (HDF). α-SMA and Hoechst 33342 stained HDF cultures were counted 7 days after seeding. Daily blue light (420 nm) treatments started 3 days after seeding for 4 days. TGF-β1 (10 ng/ml) was added after seeding and each irradiation. Presented are the average number of α-SMA$^+$ cells in a single microscopic sight field of HDF cultures maintained in media with **A** 10% or **B** 5% FCS as well as the percentage of α-SMA$^+$ cells (**C** 10%; **D** 5% FCS). Mean values ± SD of 5 independent experiments are shown. *$P < 0.05$. **E** Representing micrographs of Hoechst 33342- and α-SMA-stained HDF cultures. Fluorescence micrographs were inverted in grey scale for better visualization. Bars = 200 μm.