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Low Power Laser Irradiation Stimulates the Proliferation of Adult Human Retinal Pigment Epithelial Cells in Culture

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Abstract

We investigated the effects of low power laser irradiation on the proliferation of retinal pigment epithelial (RPE) cells. Adult human RPE cells were artificially pigmented by preincubation with sepia melanin, and exposed to a single sublethal laser pulse (590 nm, 1 μ s, <200 mJ/cm²). DNA synthesis, cell number, and growth factor activity in irradiated RPE cells were subsequently monitored. The effect of sublethal laser irradiation on the “wound” healing response of an RPE monolayer in an in vitro scratch assay was also investigated. Single pulsed laser irradiation increased DNA synthesis in pigmented RPE cells measured 6 h post-treatment. In the scratch assay, laser irradiation increased the rates of cell proliferation and wound closure. Conditioned medium, collected 48 h following laser treatment, increased cell proliferation of unirradiated cells. Irradiation increased RPE cell secretion of platelet-derived growth factor (PDGF)-B chain, and increased mRNA levels of several growth factors and their receptors, including PDGF, transforming growth factor- β 1, basic fibroblast growth factor, epidermal growth factor, insulin-like growth factor, as well as heat shock proteins. This demonstrates, for the first time, that low power single pulsed laser irradiation stimulates the proliferation of RPE cells, and upregulates growth factors that are mitogenic for RPE cells.

Keywords

Photothermolysis; Wound healing; Heat shock; Macular degeneration

INTRODUCTION

Laser photocoagulation is one of the most effective treatments for a variety of retinal disorders, such as age-related macular degeneration (AMD), diabetic maculopathy (DMP), proliferative retinopathy (DRP), or central serous retinopathy (CSR). In these diseases, laser photocoagulation is known to have therapeutic effects, and is therefore widely used.⁴⁹ The therapeutic effects derive not from the laser burn itself, but from the subsequent biological

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reactions.^{14,22–24,32,52,54–56} Despite widespread clinical use, the exact mechanisms the beneficial effects of laser photocoagulation are poorly understood.

Low-level laser therapy (LLLT) as a therapeutic modality was introduced by the work of Mester and colleagues who noted the improvements in wound healing with the application of a ruby laser at a laser energy of 1 J/cm².²⁶ Several studies have been conducted since then to better understand LLLT's effect at the cellular level. Recently low-level laser studies have focused on the impact of laser irradiation on cell growth processes involved in wound healing, such as fibroblast proliferation, locomotion, and production of collagen.²⁵

Researchers have also examined the effects of LLLT on monocytes and endothelial cells. Scheibel *et al.* demonstrated an increase in the proliferation of human umbilical vein endothelial cells (HUVECs) following irradiation with a 670 nm diode laser using a light dose of 2 to 8 J/cm² (corresponding to 10–65 mW/cm² with a laser exposure duration around 120–400 s).⁴² Kipshidze suggested that low-power helium-neon laser irradiation promoted endothelial cell proliferation *in vitro* by enhancing the production of vascular endothelial growth factors.²⁰ Grossman *et al.* reported the stimulation of keratinocyte proliferation following irradiation to a 780 nm continuous-wave diode laser with a dose of 0 to 3.6 J/cm² (0–180 s).¹⁵

A major limitation of continuous wave laser irradiation, as was used in all of the above cited work, is the significant amount of heat diffusion through surrounding tissues. Selective photocoagulation of the pigmented retinal pigment epithelial (RPE) cells without thermal coagulation to adjacent non-pigmented cells or tissues,^{5,47} using microsecond or shorter exposures which minimize heat diffusion to the surrounding tissues, is a more attractive alternative for treatment of retinal degeneration diseases, such as drusen in early age-related macular degeneration, diabetic macular edema, and central serous retinopathy. Roider *et al.* reported the healing response of the RPE in rabbits following selective photocoagulation.³⁹ They demonstrated that the RPE was re-formed by a single sheet of hypertrophic RPE cells within two weeks with few associated inflammatory cells. RPE cells showed signs of viability, such as phagocytosis of photoreceptor outer segments. The RPE barrier was restored within four weeks with resolution of local photoreceptor and subretinal edema. To the best of our knowledge, this is the only detailed description of a wound healing response induced by low power laser stimulation of RPE cells in the literature. A systematic study of the underlying biological mechanisms is still lacking.

The aims of this work are to systematically investigate the stimulatory effects of single pulsed laser irradiation on pigmented RPE cells in a controlled *in vitro* setting, and the mediators of this response. In this study, we used an *in vitro* cell culture system developed in a prior study⁴⁷ to analyze the effects of low power laser stimulation on pigmented RPE cells. We found that single pulsed laser irradiation increased DNA synthesis, and promoted a “wound” healing response in a scratch assay. Furthermore, we report for the first time that the effect was mediated by soluble factors, and was correlated with the induction of a host of growth factors and heat shock proteins in the irradiated cells. Our results suggest a potential treatment modality for RPE-related retinal diseases such as age-related macular degeneration, in which diseased RPE cells in the macular region can be selectively targeted and adjacent healthy RPE cells stimulated to proliferate to restore an intact RPE cell layer.

MATERIALS AND METHODS

Reagents

Dulbecco's Modified Eagle Medium/F12 (DMEM/F12), fetal bovine serum (FBS), phosphate-buffered saline (PBS), 100× penicillin–streptomycin stock solution, and 0.5% trypsin–0.02% EDTA stock solution were obtained from Invitrogen Life Technologies (Carlsbad, CA). F-12K Medium (Kaighn's Modification of Ham's F12) was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Sepia melanin, urea, thiourea, 3-[(3-cholamicyclopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 1,4-dithioerythritol (DTE), ethyleneglycol-bis-(β -aminoethyl ether) *n,n,n,n*-tetraacetic acid (EGTA) and ethylenediamine-tetraacetic acid (EDTA) were obtained from Sigma–Aldrich Chemicals (St. Louis, MO). Protease inhibitors were purchased from Roche Applied Science (Indianapolis, IN). Bradford protein assay reagent was obtained from Bio-Rad Laboratories (Hercules, CA). Fluorescent live/dead viability/cytotoxicity assay was obtained from Molecular Probes (Eugene, OR). An enzyme-linked immunosorbent assay (ELISA) kit for platelet-derived growth factor (PDGF)-BB was purchased from R&D Systems Inc. (Minneapolis, MN). Nuclearspin RNA II kit was obtained from Clontech Laboratories, Inc. (Mountain View, CA). SuperScript™ III First Strand kit was purchased from Invitrogen Life Technologies (Carlsbad, CA). Preamplification System for cDNA Synthesis, and PCR core system were purchased from Promega (Madison, WI). Microcon Centrifugal filters with molecular weight cut off 3 kD were obtained from Millipore (Billerica, MA). Primary antibodies used for Western blot analysis and immunofluorescence were goat polyclonal antibodies for Hsp27 and Hsp70 (Santa Cruz Biotechnology Inc., Santa Cruz, CA). CY3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was used for immunofluorescence, and horseradish peroxidase (HRP)-conjugated antibody (Sigma–Aldrich) was used for Western blot analysis.

RPE Cell Culture and Phagocytosis of Melanin

All experiments were performed using third, fourth and fifth passage adult human retinal pigment epithelial (ARPE-19) cells. ARPE-19 cells (obtained from ATCC) were cultured in DMEM/F12 (1:1) with 10% FBS, 1% penicillin–streptomycin at 37 °C, in a 5% CO₂ balance air atmosphere. The cells were maintained in 35 mm nominal diameter tissue culture-treated dishes. ARPE-19 cells were non-pigmented in their normal growth state, and served as control non-pigmented RPE cells. To obtain pigmented ARPE-19 cells, confluent ARPE-19 cell cultures were incubated for 20 h with varying concentrations of sepia melanin as previously described.⁴⁶ Prior to incubation, sepia melanin was washed with RPE cell culture medium, and sonicated to obtain a uniform suspension.⁴⁶ Just prior to irradiation, the medium was replaced with phosphate-buffered saline (PBS) to avoid absorption of laser energy by the medium, and the cells were returned to standard culture medium following laser irradiation.

Assessment of Melanin Content in Pigmented RPE Cells

Cultured cells were detached by incubation with 1 mL of 0.25% trypsin and 0.02% EDTA for 10 min at 37 °C. An aliquot (50 μ L) was removed for counting in a hemocytometer. The remaining cell suspension was centrifuged, and the pellet was dissolved in 1N NaOH. The

melanin concentration was determined by measurement of absorption at 475 nm, and then compared with a standard curve obtained using pure melanin.⁴⁰

Laser Irradiation Setup

Irradiation of the RPE cells was performed using a pulsed dye laser (Palomar Medical Inc., Burlington, MA) emitting at 590 nm. The pulse energy was measured using an energy power meter (R-752 Universal Radiometer, Terahertz Technologies Inc., Oriskany, New York). ARPE-19 cells were irradiated at various radiant exposures (mJ/cm^2) using the experimental set-up shown in Fig. 1. A Helium-Neon laser with an output of 15 mW, coupled into the optical path, was used for the alignment of laser beam. The pulsed laser was delivered through a 1 mm diameter, 2 m long optical fiber (0.22 NA) (Thorlabs, Inc., Newton, NJ) to a beam steerer, which sat on a conventional slit lamp (SL130, Zeiss, Germany). The lens system inside the beam steerer ensured that the lasing spot had a top hat profile, which was characterized by taking video images of the laser irradiation region using a CCD camera through the slit lamp. The images were then digitized, displayed on the screen, and analyzed by MATLAB software (Fig. 2). The following parameters were used for the laser irradiation: 5.0–2550 mJ/cm^2 of laser radiant exposure, 1 μs duration, and laser exposure spots were separated by a distance of 2 mm. Three sample dishes were irradiated per experimental condition. The laser radiant exposure was calculated as the pulse energy measured by the power meter divided by the spot area with a spot radius of $500 \pm 10 \mu\text{m}$.

For the laser stimulation experiments using low powers, a pulse 110 mJ/cm^2 was used. Based on a prior study using ARPE-19 cells with a melanin content of 0.03 mg/mg protein, powers up to that level did not cause significant cell death as determined by a fluorescent live/dead viability/cytotoxicity assay using calcein acetoxymethylester (calcein-AM) and ethidium homodimer (EthD-1).⁴⁷ Note that the system in that prior study had a Gaussian beam profile while a “top hat” profile was used herein. Nevertheless, since the reported pulse energy is the average over the beam area, the peak irradiance in the center of the beam is expected to be greater in the Gaussian vs. the top hat profile. Thus, laser radiances found to be in the subthreshold range with the Gaussian system will remain in the subthreshold range with the top hat system.

DNA Synthesis Assay

Pigmented ARPE-19 cells were cultured post-confluence, where there was minimal cell proliferation, as previously described.³³ The pigmented ARPE-19 cells were seeded in 35 mm dishes in serum free F-12K medium for 24 h. The cells were irradiated in PBS and returned to cell culture medium after the laser irradiation. Six hours later, 10 μM BrdU were added to the cell culture medium, and incubated overnight at 37 °C. The cells were fixed with 70% ethanol for 45 min at room temperature, incubated with 4 M HCl for 20 min at room temperature and permeabilized with 0.2% Triton X-100 for 15 min at room temperature. Genomic DNA was denatured by adding 1 mL/dish of blocking buffer (PBS with 10% FBS) for 10 min at room temperature. 10 μL anti-BrdU-Alex594 stock solutions were added, and incubated for 60 min at 37 °C. Then, the cells were washed three times. BrdU staining was visualized on an epifluorescence microscope (Zeiss Axiovert 200M, Carl

Zeiss MicroImaging, Inc.) using excitation and emission wavelengths of 590 and 617 nm. Images were digitally captured and BrdU positive cells were counted manually.

In Vitro Scratch Wound Healing Assay

Pigmented ARPE-19 cells were cultured to post-confluence, where there was minimal cell proliferation, as previously described.³³ The pigmented ARPE-19 cells were seeded in 35 mm dishes in serum free F-12K medium for 24 h. To simulate a wound, confluent monolayers were gently stroked using a 1 mL pipette tip in the center of each grid (2 × 2 mm). This process creates a uniform lane that is devoid of cells. The width of the scratch region is about 1 mm. The area of scratch region is near 2 mm². The cells were then incubated at 37 °C for 30 min before being irradiated. The culture medium was replaced by PBS for the duration of the laser irradiation. Following the laser irradiation, PBS was replaced by the culture medium with 1% FBS. The purpose of reducing FBS from 10 to 1% is to limit RPE cell proliferation. The medium was replaced every 48 h, and the cultures were subjected to laser irradiation every 48 h. Changes in unscathed and wounded ARPE-19 cell morphologies were evaluated by light microscopy. The cell number within the scratched region was counted during the process.

Collection of Conditioned Medium

Pigmented ARPE-19 cells were grown to post-confluence. The pigmented ARPE-19 cells were irradiated using different laser radiant exposures in PBS. Immediately after laser irradiation, the cells were returned to serum free F-12K medium, incubated for 48 h at 37 °C in 5% CO₂, and the conditioned medium collected.

MTT Assay

To determine the RPE cell response, ARPE-19 cells were seeded onto 96-well plates at a density of 1000 cells/well. Conditioned medium, collected after 48 h of incubation on laser irradiated RPE cells, was placed on the seeded ARPE-19 cells. The conditioned cell culture medium was changed twice a week. Cell number per well was determined using a colorimetric dimethylthiazol diphenyl tetrazolium bromide (MTT) assay. For analysis, the culture medium was removed, and 100 µL of PBS containing 10 µL of MTT (5 mg/mL) were added to each well. The cells were then incubated at 37 °C for 3 h. The reaction was terminated by adding 0.04 N HCl in isopropanol. The absorbance was measured in a plate reader at 570 nm and subtracting background measured at 630 nm. Individual experiments used three replicates for each experimental condition and were repeated at least three times.

Immunofluorescence Staining of Hsp70

For immunofluorescent staining, cells were fixed in 4% paraformaldehyde for 20 min at room temperature, and permeabilized with 0.2% Triton X-100. Cells were blocked with blocking buffer (10% FBS, 0.25% DMSO and 0.1% Triton X-100 in PBS) for 60 min. Primary and secondary antibodies were also diluted in this blocking buffer. Hsp70 was detected by staining with a polyclonal rabbit antibody (Santa Cruz biotechnology, Inc.), and appropriate CY3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), Hoechst stain 33342 (Invitrogen) which labels DNA were added at 10 µM

to visualize nucleus and all fluorescent images were visualized and captured by fluorescence microscopy (Zeiss Axiovert 200M, Carl Zeiss MicroImaging, Inc.) with same exposure time and gain.

RNA Isolation and RT-PCR

ARPE-19 cells were grown to post-confluence. The pigmented ARPE-19 cells were seeded in 35 mm dishes in serum free F-12K medium for 24 h. The cells were irradiated in PBS and returned to F-12K medium. The total RNA was extracted from cultured pigmented ARPE-19 cells from seven separate dishes at 6, 24 h and 14 days after the laser irradiation, according to the manufacturer's protocol. RNA from pigmented ARPE-19 cells without laser irradiation served as the sham control. Reverse transcription was conducted using the Preamplification System (Promega, USA) for cDNA Synthesis with oligo(dT)₁₅ primers according to the manufacturer's protocol. The cDNA was added to the PCR mixture in a final volume of 50 µL containing 50 mM KCl, 10 mM Tris-HCl, 2.5 mM MgCl₂, and 10 mM each of dNTPs, and 1U heat-stable DNA polymerase from *Thermus brockianus* (Promega, Madison, WI). The following PCR parameters were used for 25–40 cycles: initial denaturation for 2 min at 95 °C, annealing at 55 °C for 1 min; polymerization at 72 °C for 1 min; followed by a final extension at 72 °C for 2 min. Parallel RT-PCR reactions without reverse transcriptase were performed for each sample to confirm that the PCR products resulted from cDNA rather than from genomic DNA. β-Actin was used as a constitutively expressed gene product for the comparison of all other mRNA abundance between samples. All products were then analyzed with 2% agarose gel electrophoresis and ethidium bromide staining, and the resulting bands were densitometrically scanned. A semiquantitative PCR amplification method was performed using β-actin as an internal control. The band intensity volume for each mRNA of interest was normalized to the β-actin signal from the same sample using Quantity One software (Bio-Rad Laboratories, Hercules, CA). Then the results were expressed as ratios to samples not exposed to light (dark controls). Each sample was run with different cycle numbers to ensure that the signal did not reach saturation. Bands below saturation levels only are shown in the “Results” section.

Real Time PCR

Quantitative real-time PCR (qPCR) was conducted by using a Mx3000P® Quantitative PCR System (Stratagene). Briefly, duplicate reactions were prepared with 10 µL of PCR master mix consisting of 5 µL master mix (Brilliant® SYBR® Green QPCR Master Mix, Stratagene), 0.5 µL cDNA template, 0.5 µL each of gene-specific primer sets (10 µM) and 4 µL RNase-free water. The reaction was denatured at 95 °C for 2 min and amplified for 50 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s. Real-time quantification of all the genes was normalized to the threshold cycle (C_T) value of β-actin where C_T equals the PCR cycle number at which the amount of amplified product reached 100 relative fluorescence units (RFU). Gene expression levels for Hsp27, Hsp70, PDGFA, PDGFB, PDGFA-receptor, and PDGFβ-receptor in laser stimulated samples were calculated relative to the expression of the untreated control samples: 2^{-C_T} , where $C_T = [(C_{T,target} - C_{T,\beta-actin})_{laser-treated} - (C_{T,target} - C_{T,\beta-actin})_{untreated}]$. A melting curve for all products was obtained immediately after amplification by increasing the temperature in 0.4 °C increments

from 65 °C for 85 cycles of 10 s each. The presence of a single melting-temperature peak per primer pair confirmed PCR products. Each real-time experiment was repeated twice.

Protein Extraction and Western Blot Analysis

To prepare protein extracts, the pigmented ARPE-19 cells were harvested, and the pellets dissolved in lysis buffer containing 20 mM Tris-HCl (pH = 8.0), 4 M NaCl, 0.5 M EDTA, 0.5 mM CHAPS. Soluble proteins were recovered by centrifugation at 22,000g for 45 min. Protein concentrations were determined by the Bradford protein assay. Thirty micrograms of protein were run on a 10% SDS-PAGE, and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) with a Mini Trans-Blot apparatus (Bio-Rad Laboratories) according to the manufacturer's instructions. The membrane was blocked for 1.5 h in 5% fat free dry milk in PBS, and then incubated overnight with a 1:5000 dilution of anti-Hsp27 goat polyclonal IgG (Santa Cruz biotechnology, Inc., CA) in 0.5% Tween 20. After washes with PBS-0.5% Tween 20, the membrane was incubated overnight with horseradish peroxidase-conjugated anti-goat antibody (Promega, Madison, WI, USA) diluted 1:100,000. The blots were developed with an enhanced precipitation method (Pierce, Rockford, IL, USA).

PDGF Detection via ELISA

Pigmented ARPE-19 cells were grown to post-confluence. Cell samples were laser irradiated in PBS and returned to serum free F-12K medium. Conditioned medium was collected at 48 h, and concentrated 10-fold using Amicon microcon centrifugal filter devices. Levels of PDGFB in the concentrated culture supernatants were determined by ELISA.

Statistical Analysis

The results are presented as a means $\pm$ standard deviations (SD), and n states the number of replicates. Statistical comparisons were made using two-tailed Student's t -tests, and $p < 0.05$ was considered statistically significant.

RESULTS

Laser Irradiation Stimulates DNA Synthesis in RPE Cells

In order to test whether single pulsed laser irradiation can stimulate quiescent pigmented ARPE-19 cells to enter the cell cycle, DNA synthesis was measured using a BrdU uptake assay. In our experiments, we cultured the pigmented RPE cells well beyond confluence (4–8 weeks) to ensure that they reached a quiescent non-proliferating status. Under such conditions, RPE cells do not take up BrdU. Cells were incubated in BrdU overnight starting 6 h after laser irradiation, and stained with an anti-BrdU antibody. BrdU positive cells were counted manually. There were more BrdU positive cells in laser treated samples compared to un-irradiated control samples. Few BrdU positive cells were observed in un-irradiated control samples, as expected for cells that were not proliferating (Fig. 3a). Low power laser irradiation increased BrdU staining, demonstrating a significantly enhanced DNA synthesis of the pigmented RPE cells compared to the unirradiated cells (Fig. 3b). The response to all doses of laser irradiation was similar. Note that we also investigated irradiances below 27

mJ/cm², and there appeared to be no stimulatory effects (data not shown). However, the laser set up was not very reproducible at such low power settings; thus, it was not possible to accurately map out the dose–response curve.

Low Power Laser Irradiation Promotes Wound Healing Responses

Next, we used a scratch assay to mimic a wound-like damage to the RPE cell layer and determine whether low power laser irradiation can stimulate the repair of the RPE monolayer. The healing responses of irradiated and non-irradiated cells were compared (Fig. 4). The wound margins remained clearly defined in the unirradiated sham control (Fig. 4b), even after 3 days of culturing. In contrast, cells that were treated with laser irradiation at energy levels varying from 27 to 110 mJ/cm² showed an increase in cell density around the wound margin, and displayed a greater rate of cell migration and proliferation across the wound thus leading to faster closure of the wound (Figs. 4c and 4d). Cell numbers as a function of laser radiant exposures and time post irradiation during the *in vitro* wound healing assay are shown in Fig. 4e. In a similar experiment, non-pigmented cells did not exhibit any increase in cell proliferation in response to laser irradiation (data not shown).

Laser Irradiation Increases Gene Expression and Protein Levels of Heat Shock Proteins

We hypothesized that heat shock proteins (Hsps) could play a role in the altered growth factor signaling induced by single pulsed laser irradiation. Laser stimulation increased gene expression of Hsp27, Hsp70, and Hsp90 (Fig. 5a). The corresponding temporal expression profiles are shown in Fig. 5b. Hsp27 and Hsp90 reached their maximum values 6 h post treatment, where the effect was more significant for Hsp90 compared to Hsp27 at both the treatment intensities. For both Hsp27 and Hsp90, the expression values had returned to baseline within 24 h to 14 days post-treatment, with slower dynamics for Hsp27. The dynamic profile of Hsp70 was different, having a more delayed response peaking at 24 h post treatment. Following this, the expression level slowly decreased, reaching baseline in the case of low intensity exposure (laser radiant exposure of 55 mJ/cm²), but remaining significantly elevated in the case of high intensity exposure (laser radiant exposure of 110 mJ/cm²).

In order to corroborate some of the end-point RT-PCR data, we also performed quantitative real-time PCR (qPCR) measurements of a subset of genes. Based on RT-PCR, mRNA levels of Hsp27 and Hsp70 were increased by 1.17 and 1.29 fold at 6 h after an exposure to 55 mJ/cm², whereas the qPCR data demonstrated a more significant increase, namely 2.75 and 2.65 fold, respectively (Table 3).

In order to verify that the upregulation of Hsps at the mRNA level translated into higher protein levels, Hsp27 protein level changes were measured in cell extracts by Western blot analysis. Cell extracts were harvested 12 h after laser treatment. Figure 5c shows that Hsp27 levels were increased in the laser irradiated pigmented ARPE-19 cells compared to the unirradiated controls. Quantification of the protein bands by image analysis showed increases of ~15% after 55 mJ/cm², and ~40% after 110 mJ/cm². The upregulation of Hsp70 protein expression was assessed by immunofluorescence staining, which was also

conducted 12 h after laser treatment. Figure 6 shows that Hsp70 protein expression was higher in the laser irradiated pigmented RPE cells compared to the unirradiated controls.

Conditioned Medium by Laser Irradiated Cells Stimulates ARPE-19 Cell Proliferation

To determine whether the increased “wound” healing response following laser irradiation was due to increased production of soluble growth factors, we collected conditioned medium from laser-treated cell cultures and tested its effect on unirradiated ARPE-19 cells. The conditioned medium was collected 48 h after laser treatment. The results are shown in Fig. 7a, where one can see a dose-dependent increase in cell density that was significantly higher than in parallel cultures incubated in conditioned medium collected from non-irradiated cells. Furthermore, a dose response study of intensity of laser irradiation showed increased cell proliferative response as a function of laser irradiation dose.

Laser Irradiation Increases PDGFB Secretion and Gene Expression of PDGFA, PDGFB, and Their Receptors

We hypothesized that the observed biological activity was due to the PDGF pathway since PDGF is a growth factor commonly involved in wound healing.³⁰ Consistent with this notion, we found a ~2-fold increase in the levels of PDGFB protein in the conditioned medium at laser radiant exposure of 55 mJ/cm² vs. non-irradiated samples, and another ~2-fold increase at laser radiant exposure of 110 mJ/cm² (Fig. 7b). Gene expression (as measured by RT-PCR) of PDGFA, PDGFB, PDGF α -receptor, and PDGF β -receptor was significantly up-regulated in irradiated pigmented ARPE-19 cells, as illustrated in Fig. 8a. Figure 8b shows the dynamic profile of gene expression for PDGFA and PDGF α receptor over time. The dynamics in response to 55 and 110 mJ/cm² were qualitatively similar, while gene expression increased as a function of laser intensity. The PDGFA profile reached a peak value 8 h after laser treatment, after which it started decreasing, almost reaching the pre-treatment level by 24 h, with no further change up to 14 days after treatment. PDGF α receptor expression exhibited dose response as well, although a slower dynamics than PDGFA, taking 24 h to reach its peak value. In addition, the increase in PDGF α was more sustained, as it was still significantly elevated at the 14 day time point. Figure 8c shows the dynamic profile of gene expression for PDGFB and PDGF β receptor over time. PDGFB had significantly increased at 8 h, although the light dose-dependent increase was more muted than PDGFA, but unlike PDGFA, it continued to remain elevated until 14 days. The light dose-dependent PDGF β profiles were similar, although more elevated, compared to PDGF α . A subset of PDGF-related genes were corroborated by qPCR (Table 4). The changes measured by RT-PCR and qPCR were in qualitative agreement in all cases except for PDGFB, where no change occurred from 8 to 24 h with RT-PCR while a further increase in mRNA was observed by qPCR. In most cases, the changes reported by qPCR were greater in magnitude than those reported by RT-PCR, but showed the same trends.

Laser Irradiation Modulates Transforming Growth Factor β 1 (TGF β 1), Basic Fibroblast Growth Factor (bFGF), Insulin-Like Growth Factor (IGF), Epidermal Growth Factor (EGF), and Their Receptors

We also investigated the effect of low power laser irradiation on a host of growth factors involved in wound healing responses, including TGF β 1, bFGF, IGF, and EGF, and their

receptors. The effects of laser treatment on these genes was quite transient (Figs. 9 and 10), reaching the highest expression level around 6 h post-treatment, and regaining baseline levels by 24 h, with no further change until the 14 day time point. The peak values reached by these genes were, compared to the unirradiated controls: 30 and 138% increases for TGF β 1 using low and high irradiation levels, respectively, 66 and 105% increases for the TGF receptor, 70 and 93% increases for bFGF, 73 and 172% increases for the FGF receptor. Of the above genes, the dynamics of the TGF receptor was slightly different at the high laser intensity condition, as it exhibited a more sustained response, remaining 50% above baseline after 14 days.

IGF1 and IGF2 reached peak gene expression level at 6 h. For IGF1, the peak was 45 and 86% above baseline for low and high levels of irradiation, respectively. The corresponding IGF receptors also reached their peak value at 6 h. For the IGF1 receptor, the peak was 22 and 127% above baseline, and for the IGF2 receptor, the peak was 39 and 162% above baseline. The expression levels returned to nearly baseline levels by 14 days, although they remained statistically significantly elevated.

With respect to EGF, both laser intensities initially increased the gene expression levels, peaking at 24 h. The peak values were 87 and 145% above baseline for the low and high intensity irradiation, respectively. Interestingly, the higher intensity laser exposure retained a long term effect (still 117% above baseline) while the low intensity did not. Low level of laser irradiation did not significantly change EGF receptor expression. In contrast, the higher intensity of irradiation caused the EGF receptor level to be increased 3 fold within 6 h of laser exposure. Following this peak value, although the level exhibited a decreasing trend, it was still 50% above baseline 2 weeks after laser irradiation.

DISCUSSION

We hypothesized that low power single pulsed laser irradiation can stimulate a wound healing response in the RPE layer. This hypothesis was tested in an *in vitro* system consisting of a confluent ARPE-19 cell mono-layer to mimic the RPE layer *in vivo*. The RPE layer was pigmented by incubation with exogenous melanin, and then exposed to a single pulse of laser irradiation. The intact RPE layer responded to irradiation by a significant increase in DNA synthesis (Fig. 3). Furthermore, irradiation led to faster wound closure in a scratch assay (Fig. 4). Taken together, these results show that low power single pulsed laser irradiation triggers the proliferation of pigmented ARPE-19 cells, and that this response likely contributes to the faster wound healing response after mechanical injury to the RPE monolayer. We observed that irradiation induced the expression of heat shock proteins as well as several growth factors and their receptors. Experiments using medium conditioned with cells exposed to irradiation strongly suggest that soluble factors are involved (Fig. 7a). There were detectable amounts of PDGFB in the conditioned medium (Fig. 7b), and RPE cells are known to proliferate in response to this growth factor.⁶ This is the first report that systematically investigates the mechanisms of single pulsed laser irradiation-mediated induced wound healing responses in RPE cells.

Melanin is the strongest absorbing chromophore of visible and near-infrared light in the retina. The RPE is a 10 μm thick monolayer of epithelial cells, heavily loaded with melanin granules as the only absorbers. Approximately 53% of the incident 514 nm light delivered by an argon laser is absorbed by the RPE, compared to less than 10% absorption by the photoreceptors.¹³ A study has shown that the RPE damage observable after a train of repetitive 5 μs pulses is dominated by the temperature peaks inside the RPE, and the temperature increase in a melanin granule can be calculated to be 40 °C above the surroundings with a single 5 μs pulse at the lethal threshold.³⁸ Although the current study used exogenous melanin because cultured RPE cells do not express endogenous melanin, we expect the thermal effects of laser absorption to be similar to those of the RPE with endogenous melanin.

Melanin concentration in the RPE of 61 postmortem normal human eyes from donors of ages ranging from 14 to 97 ranges from 0.095 mg/mg dry weight in the 14–50 years old group to 0.022 mg/mg dry weight in the group of over 70 years old.⁴³ Based on an analysis of bovine RPE, protein accounts for 43.5% of the dry weight,³ thus these values fall in the range of 0.22 to 0.051 mg/mg protein. Melanin content in this study was 0.03 mg/mg protein, which is slightly below the physiological range estimated above. Thompson *et al.* used a diameter of 1 μm for melanin granules when modeling thermal effects of laser radiation.⁵¹ Exogenous melanin is thought to primarily end up in endosomes and lysosomes, and these structures have size scales of 1 μm as well. Thus, it is reasonable to assume that the thermal effects of laser irradiation on endogenous and exogenous melanin will be very similar.

We did not measure intracellular temperatures during and after laser irradiation because it is beyond our capabilities. A temperature increase of 80 K was estimated at the surface of melanosomes in RPE cells for a 1 μs pulse of 100 mJ/cm²,⁴ which is close to the higher values of exposure used herein. Furthermore, this peak temperature is expected to decrease to body temperature within a few hundred microseconds by thermal diffusion. Such thermal effects are more likely to affect noncovalent bonds leading to protein denaturation, for example, than covalent bond breakage. Such a transient temperature increase is likely to induce heat shock protein expression, but not necessarily thermal necrosis.¹ Literature data do mention the upregulation of HSPs in retinal epithelial cells as a result of laser irradiation and “thermotherapy”.^{11,27} In addition, heating or cooling corneal tissue as a preconditioning treatment induces HSPs.¹⁹ Thus, it is reasonable to hypothesize that laser irradiation may transiently increase the temperature inside pigmented RPE cells due to melanin absorption of laser energy, thereby leading to heat shock protein induction.

Heat shock proteins, first reported to be induced by elevated temperature,³⁶ are also induced by a wide range of other nonlethal stresses, and protect against a subsequent insult. Hsps are ubiquitous and maintain basic cellular functions both under basal conditions and various stresses.³¹ Transcriptional and translational activation of Hsp27 and extensive membrane blebbing caused by oxidative injury were observed in ARPE-19 cells, and a high level of Hsp27 protein was detected within membrane blebs.⁵⁰ These results indicate that Hsp27 may be an important component of the RPE response to injury or stress. Hsp70 plays a central role in protecting cells from lethal stresses. There is considerable evidence that

HSP70 functions as an anti-apoptotic molecule by down-regulating several apoptotic pathways.^{2,41} HSP70 can also prevent apoptosis by blocking the release of apoptosis inducing factor.³⁴ Hsp70 has also been demonstrated to stimulate the proliferation of human CD4+ memory T cells,¹⁶ epithelial cells,²⁹ and natural killer cells.²⁸ Hsp90 functions as a molecular chaperone which binds to a specific subset of cellular proteins, including transcription factors, several tyrosine, serine-threonine protein kinases,⁹ and components of the cytoskeleton.¹⁰ Previous studies have shown that continuous wave laser irradiation induced heat shock proteins in the RPE²⁷ and choroids.¹¹ Our study is the first one to demonstrate that a single microsecond pulse of laser irradiation induces heat shock proteins in pigmented RPE cells. Dynamic gene expression profiles showed that Hsp27 and Hsp90 reached their peak values earlier than Hsp70. Western blot analysis and immunofluorescence staining also demonstrated that the upregulation of Hsp27 and Hsp70 mRNA levels translated into higher protein levels. Thus, Hsp27 and Hsp90 are more likely involved in the earlier response and Hsp70 in the later response following laser irradiation.

Kim *et al.* demonstrated that overexpression of Hsps as a result of preconditioning by heating or cooling, resulted in more rapid wound healing and less corneal opacity.¹⁹ Another study reported that Hsp70 stimulates epithelial proliferation.²⁹ Based on those studies, we hypothesized that the heat shock response induced by laser irradiation may induce the secretion of growth factors. PDGF, TGF, bFGF, IGF and EGF have previously been shown to stimulate bovine RPE cell migration and proliferation.⁴⁸ Cell migration and proliferation are important components to wound healing responses; therefore, we focused on those growth factors for our gene expression studies. To better understand the dynamics of the expression profiles, and the long-term and short-term effects of laser exposure on these cells, gene expression levels were assessed at different time points after the treatment, starting from 6 h to 14 days. In general, higher laser intensity exposure led to a greater change in the expression of heat shock proteins, growth factors and the corresponding receptors, as would be expected. Based on the response time and longevity of the effect, two types of responses were observed. Type I was characterized by a fast response, reaching the peak expression levels by around 6 h post treatment, irrespective of the intensity of laser exposure. The only effect of the laser intensity was in the magnitude of the change. These fast responding genes also rapidly returned to the original pre-treatment exposure levels. Type II genes had a much slower response, reaching their peak value at 24 h post treatment, irrespective of the intensity of laser irradiation. However, the laser irradiation dose seemed to impact on the late stage dynamics, as the cells exposed to the lower intensity slowly returned to near initial pre-treatment level by 2 weeks, while the cells exposed to the higher intensity retained significantly elevated expression levels even 2 weeks after the treatment.

Members of the PDGF family are known to be involved in wound healing, extracellular matrix regulation, and certain pathologic conditions.¹⁷ Exogenous, as well as endogenous PDGF stimulate RPE cell proliferation.^{6,7} We observed that PDGFA peaked earlier than PDGFB mRNA levels (8 h vs. 2 weeks). Interestingly, the PDGF receptors PDGFA and PDGFB reached their maximal values between 24 h and 2 weeks. PDGFB receptor mRNA and protein have been reported to increase in skin wounds with similar time scales.³⁵ Since PDGFA can bind both PDGFA and PDGFB receptors, while PDGFB only binds PDGFB,¹⁷ it

is more likely that the PDGFB–PDGF β interaction is the major player in the later stages of the response after laser irradiation, while PDGFA may be more important in the early phases.

Prior studies have shown that several common growth factors are produced by RPE cells and that RPE cells respond to these factors as well, including TGF- β ,⁸ bFGF,⁴⁵ insulin-like growth factors IGF-1 and IGF-II,^{21,53} and EGF.²¹ We found that single pulsed laser irradiated pigmented RPE cells express higher levels of TGF- β 1, bFGF, and their receptors than non-irradiated quiescent RPE cells, as previously reported in studies using continuous wave laser irradiation.^{18,57} Gene expression changes for the TGF β 1, bFGF, IGF, and EGF families were of “Type I”, i.e. fast transient dynamics, thus suggesting that these growth factors are more likely to contribute to the stimulation of RPE cell proliferation in the early stages of the wound healing response. Upregulation of IGFs and their receptors in pigmented RPE cells following the single pulsed laser irradiation indicates that IGFs may act as autocrine growth stimulators in RPE cells.

Overall, for the first time, this study demonstrates that single pulsed laser irradiation stimulates pigmented RPE cells’ proliferation and “wound” closure via the induction of growth factors of the PDGF, TGF, bFGF, IGF and EGF families. Furthermore, we show that a single microsecond pulse of laser irradiation induces heat shock proteins in pigmented RPE cells. The relationship between laser-induced heat shock protein expression, growth factor expression, and increased cell proliferation warrants further study.

Schuele and co-workers have investigated the RPE damage thresholds and mechanisms for laser exposure in the microsecond-to-millisecond time regimen. They found that bubble formation is the main damage mechanism following laser pulses shorter than 50 μ s and determined the threshold irradiation (ED₅₀) for RPE cell damage.⁴⁴ They reported a porcine RPE damage threshold of ED₅₀ = 252 mJ/cm² for single 5 μ s laser pulse.⁴⁴ Carsten *et al.* reported ophthalmoscopically an ED₅₀ = 312 mJ/cm² and angiographically an ED₅₀ = 123 mJ/cm² when using 10 pulses of 1.7 μ s each.¹² Roeder *et al.* while performing microperimetry in 17 patients, reported an ED₅₀ = 298 mJ/cm² for 500 pulses of 1.7 μ s each.³⁷ The threshold for microbubble formation following single 1 μ s laser exposure on isolated pig melanosomes is 280 mJ/cm² (R. Brinkmann, personal communication). The laser intensities applied in our study are below these threshold values, and therefore could be used safely in a clinical setting.

Selective targeting of RPE cells is of important clinical significance. A variety of retinal diseases such as age-related macular degeneration (ARMD) is associated with a degeneration of the RPE. The selective targeting of the RPE can spare adjacent photoreceptors, the neural retina and choroids which are located very close to RPE layer and thus avoid scotoma. This approach could therefore be potentially very useful within the macular area in clinical practice. The validity of selective photocoagulation has also been demonstrated *in vivo*.⁵ As reported, RPE defects in patients were visualized by fluorescein angiography in the range of 450–800 mJ/cm² per pulse. With the laser parameters used in this study, neither bleeding nor scotoma was observed as proved by microperimetry, which indicated no adverse effects to the choroid and the photoreceptors, respectively. During and

after irradiation, it was shown that irradiated areas were ophthalmoscopically invisible, suggesting that the thermal effects were very limited and confined. Wavelengths used to perform selective RPE targeting are readily transmitted by the cornea; thus, selective RPE cell targeting could be performed clinically using a gonioscopic approach with a slit-lamp laser delivery system. Our findings herein suggest a potential treatment modality for RPE-related retinal diseases such as age-related macular degeneration, in which diseased RPE cells in the macular region can be selectively targeted and subsequently, adjacent healthy RPE cells in peripheral regions are stimulated to regenerate. The regenerated RPE cells could then replace the targeted diseased RPE cells.

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FIGURE 1.

Schematic representation of the experimental setup. Laser irradiation was reflected by a mirror to the stage where RPE cells were seated. The slit lamp was used to capture the video images of laser irradiation spots. Laser irradiation was performed under visual control.

FIGURE 2.

Pulsed dye laser profile analysis. (a) Light micrograph of laser spot. Slight asymmetry is caused by imaging optics. Scale bar is 500 μm . The box showing around the spot is a portion of the grid in the cell culture dish. The size of each grid is 2×2 mm. This information has been added to the figure legend. (b) Laser Intensity profile analyzed by MATLAB6.5. X and Y are positions of the laser spot and Z is the laser intensity profile in arbitrary units.

FIGURE 3.

Effect of light irradiation on DNA synthesis characterized by BrdU uptake. (a) Sham dark control. (b) Laser irradiated sample at laser radiant exposure of 110 mJ/cm². (c) Phase image of sham dark control. (d) Phase image of laser irradiated sample at laser radiant exposure of 110 mJ/cm². (e) BrdU positive cell numbers per field (manually counted) of laser irradiated samples compared to non-irradiated samples. Scale bars of (a) and (b) are 200 μ m. Scale bars of (c) and (d) are 100 μ m.

FIGURE 4.

Wound healing response to light irradiation *in vitro*. A scratch wound was created in a confluent RPE monolayer, and the cells were exposed to a single laser pulse at 30 min, 48 h, and 96 h. Cell morphology is shown at 72 h, and cell counts were obtained at 96 h. (a) Scratch model prior to laser irradiation. (b) Sham dark control. (c) Cell cultures irradiated at 27 mJ/cm². (d) Cell culture irradiated at 110 mJ/cm². (e) Cell number per field as function of time. The dotted region delimits the area of quantification, and is about 2 mm². Scale bar

is 100 μm . p -Values refer to comparisons to sham dark control at the same time point.
* $p<0.05$. ** $p<0.01$, *** $p<0.001$. $N=3$.

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FIGURE 5.

Effect of laser irradiation on heat shock protein expression. (a) Hsp27, Hsp70, and Hsp90 gene expression 6 h, 24 h and 14 days after laser irradiation. (b) Semi-quantification of the levels shown in panel (a). The density of bands was normalized to the sham dark control band: $\circ$ Hsp27 (55 mJ/cm²), $\bullet$ Hsp27 (110 mJ/cm²), $\square$ Hsp70 (55 mJ/cm²), $\blacksquare$ Hsp70 (110 mJ/cm²), $\diamond$ Hsp90 (55 mJ/cm²), $\blacklozenge$ Hsp90 (110 mJ/cm²). $N=3$. (c) Western blot and semi-quantification of Western blot intensities of Hsp27 normalized to the sham dark control band. $N=3$. Statistics are shown in Tables 1 and 2.

FIGURE 6.

Effect of light irradiation on Hsp70 protein expression visualized by immunofluorescence staining. (a) Sham control without laser irradiation. (b) Laser irradiated sample at laser radiant exposure of 55 mJ/cm² without primary antibody control. (c) Laser irradiated sample at laser radiant exposure of 55 mJ/cm². (d) Laser irradiated sample at laser radiant exposure of 110 mJ/cm². (e) Phase image of sham control without laser irradiation. (f) Phase image of laser irradiated sample at laser radiant exposure of 55 mJ/cm² without primary antibody. (g) Phase image of laser irradiated sample at laser radiant exposure of 55 mJ/cm². (h) Phase image of laser irradiated sample at laser radiant exposure of 110 mJ/cm². Scale bar is 100 μm.

FIGURE 7.

(a) Cell proliferation induced by conditioned medium. Conditioned medium collected 48 h after laser irradiation. $N = 9$. (b) PDGFB secretion amount from conditioned medium, collected 48 h after laser irradiation. $N = 3$. p -Values refer to comparisons to sham dark control. ** $p < 0.01$, *** $p < 0.001$.

FIGURE 8.

Effect of laser irradiation on PDGF and PDGF receptor gene expression. (a) RT-PCR bands for PDGFs and their receptors 8 h, 24 h and 14 days after laser irradiation. (b) Semi-quantification of PDGFA and PDGFα receptor gene expression. ○ PDGFA (55 mJ/cm²), ● PDGFA (110 mJ/cm²), □ PDGFα (55 mJ/cm²), ■ PDGFα (110 mJ/cm²). (c) Semi-quantification of PDGFB and PDGFβ receptor gene expression following laser irradiation. ○ PDGFB (55 mJ/cm²), ● PDGFB (110 mJ/cm²), □ PDGFβ (55 mJ/cm²), ■ PDGFβ (110 mJ/cm²). The density of bands was normalized to the sham dark control band. *N*= 3. Statistics are shown in Tables 1 and 2.

FIGURE 9.

Effect of laser irradiation on gene expression of TGFβ1, bFGF and their receptors. (a) RT-PCR bands for TGFβ1 and their receptors 6 h, 24 h and 14 days after laser irradiation. (b) Semi-quantification of TGFβ1 and TGFβ receptor gene expression. ○ TGFβ1 (55 mJ/cm²), ● TGFβ1 (110 mJ/cm²), □ TGFR (55 mJ/cm²), ■ TGFR (110 mJ/cm²). (c) Semi-quantification of bFGF and bFGF receptor gene expression. ○ bFGF (55 mJ/cm²), ● bFGF (110 mJ/cm²), □ FGFR (55 mJ/cm²), ■ FGFR (110 mJ/cm²). The density of bands was normalized to the sham dark control band. *N* = 3. Statistics are shown in Tables 1 and 2.

FIGURE 10.

Effect of laser irradiation on gene expression of IGF, EGF and their receptors. (a) RT-PCR bands for IGF, EGF and their receptors 6 h, 24 h and 14 days after laser irradiation. (b) Semi-quantification of the IGF1 and IGF1 receptor gene expression following laser irradiation. ○ IGF1 (55 mJ/cm²), ● IGF1 (110 mJ/cm²), □ IGF1R (55 mJ/cm²), ■ IGF1R (110 mJ/cm²). (c) Semi-quantification of IGF2 and IGF2 receptor gene expression following the laser irradiation. ○ IGF2(55 mJ/cm²), ● IGF2(110 mJ/cm²). □ IGF2R (55 mJ/cm²), ■ IGF2R (110 mJ/cm²). (d) Semi-quantification of EGF and EGF receptor gene expression following laser irradiation. ○ EGF (55 mJ/cm²), ● EGF (110 mJ/cm²), □ EGFR (55 mJ/cm²), ■ EGFR (110 mJ/cm²). The density of bands was normalized to the sham dark control band. *N* = 3. Statistics are shown in Tables 1 and 2.

TABLE 1

Mean values of gene expression levels in laser-treated samples normalized to dark control samples.

Gene	6 h (55 mJ/cm ²)	6 h (110 mJ/cm ²)	24 h (55 mJ/cm ²)	24 h (110 mJ/cm ²)	14 days (55 mJ/cm ²)	14 days (110 mJ/cm ²)
Hsp27	1.17 ± 0.04	1.65 ± 0.07	1.14 ± 0.04	1.51 ± 0.07	1.05 ± 0.03	1.16 ± 0.04
Hsp70	1.29 ± 0.02	1.42 ± 0.03	1.40 ± 0.08	1.82 ± 0.02	1.25 ± 0.06	1.68 ± 0.09
Hsp90	1.44 ± 0.08	1.98 ± 0.10	1.08 ± 0.03	1.22 ± 0.08	1.05 ± 0.03	1.18 ± 0.06
TGFβ1	1.30 ± 0.06	2.38 ± 0.11	1.10 ± 0.01	1.50 ± 0.05	1.10 ± 0.06	1.30 ± 0.07
TGFR	1.66 ± 0.04	2.05 ± 0.09	1.29 ± 0.005	2.02 ± 0.08	1.03 ± 0.05	1.50 ± 0.11
bFGF	1.69 ± 0.01	1.93 ± 0.04	1.08 ± 0.02	1.23 ± 0.02	1.01 ± 0.04	1.04 ± 0.03
FGFR	1.73 ± 0.02	2.72 ± 0.04	1.27 ± 0.01	1.46 ± 0.19	1.00 ± 0.04	1.24 ± 0.02
IGF1	1.45 ± 0.07	1.86 ± 0.09	1.38 ± 0.06	1.56 ± 0.09	1.03 ± 0.04	1.31 ± 0.08
IGF1R	1.22 ± 0.05	2.27 ± 0.07	1.05 ± 0.06	1.51 ± 0.06	1.04 ± 0.04	1.18 ± 0.08
IGF2	1.33 ± 0.03	1.73 ± 0.05	1.13 ± 0.06	1.65 ± 0.05	1.11 ± 0.02	1.21 ± 0.05
IGF2R	1.39 ± 0.04	2.62 ± 0.05	1.29 ± 0.05	1.89 ± 0.09	1.02 ± 0.01	1.41 ± 0.07
EGF	1.17 ± 0.03	1.56 ± 0.05	1.87 ± 0.06	2.45 ± 0.06	1.07 ± 0.05	2.17 ± 0.05
EGFR	1.11 ± 0.02	1.98 ± 0.08	1.10 ± 0.005	1.67 ± 0.09	1.15 ± 0.09	1.49 ± 0.01

All values are significantly higher than the dark controls ($p < 0.05$; $N = 3$).

TABLE 2

Mean values of PDGF family gene expression levels in laser-treated samples normalized to dark control samples.

Gene	8 h (55 J/cm ²)	8 h (110 mJ/cm ²)	24 h (55 J/cm ²)	24 h (110 J/cm ²)	14 days (55 mJ/cm ²)	14 days (110 J/cm ²)
PDGFA	1.36 ± 0.005	2.11 ± 0.09	1.12 ± 0.04	1.41 ± 0.04	1.11 ± 0.06	1.28 ± 0.02
PDGFB	1.23 ± 0.02	1.44 ± 0.11	1.22 ± 0.02	1.45 ± 0.04	1.24 ± 0.04	1.85 ± 0.05
PDGF α	1.06 ± 0.03	1.37 ± 0.17	1.31 ± 0.08	1.76 ± 0.03	1.22 ± 0.06	1.73 ± 0.03
PDGF β	1.27 ± 0.02	1.60 ± 0.06	1.91 ± 0.17	2.97 ± 0.19	2.04 ± 0.2	3.00 ± 0.04

All values are significantly higher than the dark controls ($p < 0.05$; $N = 3$).

TABLE 3

Comparison of mRNA levels for Hsp27 and Hsp70 using real time PCR (qPCR) and end point RT-PCR.

Transcript	FCq (6 h)	FCq (24 h)	FCe (6 h)	FCe (24 h)
Hsp27 (55 mJ/cm ²)	2.75 ± 0.26		1.17 ± 0.04	
Hsp27 (110 mJ/cm ²)	5.78 ± 0.61		1.65 ± 0.07	
Hsp70 (55 mJ/cm ²)	2.65 ± 0.18	5.74 ± 0.01	1.29 ± 0.02	1.40 ± 0.06
Hsp70 (110 mJ/cm ²)	3.84 ± 0.27	6.92 ± 0.61	1.42 ± 0.03	1.82 ± 0.09

FCq, fold changes from qPCR experiments; FCe, fold changes from end-point semi-quantitative RT-PCR experiments.

TABLE 4

Comparison of mRNA levels for PDGF and its receptors using real time PCR (qPCR) and end point RT-PCR.

Transcript	FCq (8 h)	FCq (24 h)	FCe (8 h)	FCe (24 h)
PDGFA (55 mJ/cm ²)	2.76 ± 0.21	1.19 ± 0.12	1.36 ± 0.04	1.12 ± 0.04
PDGFA (110 mJ/cm ²)	9.60 ± 1.12	1.83 ± 0.22	2.11 ± 0.1	1.41 ± 0.04
PDGFB (55 mJ/cm ²)	1.69 ± 0.13	12.2 ± 0.4	1.22 ± 0.02	1.22 ± 0.02
PDGFB (110 mJ/cm ²)	4.22 ± 0.54	14.9 ± 0.39	1.44 ± 0.11	1.45 ± 0.04
PDGFα (55 mJ/cm ²)	2.59 ± 0.14	7.14 ± 0.88	1.06 ± 0.03	1.31 ± 0.08
PDGFα (55 mJ/cm ²)	5.53 ± 0.95	12.1 ± 1.49	1.37 ± 0.17	1.76 ± 0.03
PDGFβ (55 mJ/cm ²)	1.71 ± 0.08	3.48 ± 0.45	1.27 ± 0.03	1.90 ± 0.17
PDGFβ (55 mJ/cm ²)	2.36 ± 0.01	7.20 ± 0.94	1.59 ± 0.06	2.97 ± 0.19