Basic Research

Varying Ratios of Wavelengths in Dual Wavelength LED Photomodulation Alters Gene Expression Profiles in Human Skin Fibroblasts

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Background and Objective: LED photomodulation has been shown to profoundly influence cellular behavior. A variety of parameters with LED photomodulation can alter cellular response in vitro. The effects of one visible and one infrared wavelength were evaluated to determine the optimal ratio to produce a net increase in dermal collagen by altering the ratio of total energy output of each wavelength. The ratio between the two wavelengths (590 and 870 nm) was shifted in 25% increments.

Study Design/Materials and Methods: Human skin fibroblasts in culture were exposed to a 590/870 nm LED array with total combined energy density fixed at 4.0 mW/cm^2. The ratio of 590/870 nm tested parameters were: 100/0%, 75/25%, 50/50%, 25/75%, and 0/100%. These ratios were delivered using pulsed duty cycle of exposure (250 milliseconds "on" time/100 milliseconds "off" time/100 pulses) for a total energy fluence of 0.1 J/cm^2. Gene expression was examined using commercially available extra cellular matrix and adhesion molecule RT PCR Arrays (SA Biosciences, Fredrick, MD) at 24 hours post-exposure.

Results: Different expression profiles were noticed for each of the ratios studied. Overall, there was an average (in an 80 gene array) of 6% expression difference in up or downregulation between the arrays. The greatest increase in collagen I and decrease in collagenase (MMP-1) was observed with 75/25% ratio of 590/870 nm. The addition of increasing proportions of IR wavelengths causes alteration in gene expression profile. The ratios of the wavelengths caused variation in magnitude of expression.

Conclusions: Cell metabolism and gene expression can be altered by simultaneous exposure to multiple wavelengths of low energy light. Varying the ratios of specific wavelength intensity in both visible and near infrared light therapy can strongly influence resulting fibroblast gene expression patterns. Lasers Surg. Med. 42:540–545, 2010. © 2010 Wiley-Liss, Inc.

Key words: LED; photobiomodulation; gene expression; microarray; LILT; fibroblast; human

INTRODUCTION

It has been previously shown that photomodulation to reverse photoaging is possible with a specific array of LEDs with a specific fluence using a precise pulsing or "code" sequence with a specific wavelength. The previously reported wavelength was a visible light 590 nm LED array pulsed as 250 milliseconds on time/100 milliseconds off time with a total of 100 pulses at an irradiance of 4.0 mW/cm^2 (0.1 J/cm^2) [1]. Skin textural improvement by digital imaging and surface profilometry is accompanied by increased collagen I deposition with reduced matrix metalloproteinase (MMP)-1 (collagenase) activity in the papillary dermis was demonstrated. Prior to this study, the concept that that cell activity can be up or downregulated by low energy light was considered but evidence of consistent or impressive results were lacking [2]. Wavelengths previously examined include a 670-nm LED array [3,4] a 660 nm array [5] and higher infrared wavelengths [6]. Fluences in these studies were variable with energy as high as 4 J/cm^2 required for results [7]. The concept of much lower levels of light producing alteration of gene expression was originally introduced and presented by McDaniel et al. [8,9] at the 2002 annual meeting of ASLMS. Recently combinations of wavelengths of LEDs have been tested in treating a number of disorders including acne [10], psoriasis [11], and additional evidence for photoaging [12]. LED's of these wavelengths have been used to treat photodamaged skin as well as reduce erythema following pulsed light treatments [13,14]. Previous experiments using membrane-based microarrays (DermArray®, Integriderm, LLC, Birmingham, AL) have demonstrated varied

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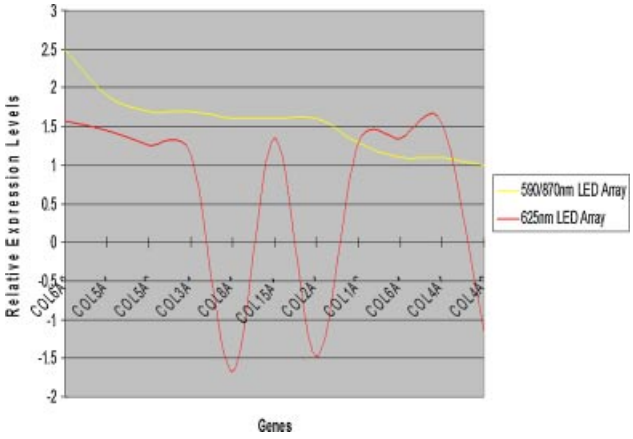

Fig. 1. Wavelength-dependent expression values of multiple collagen genes from a membrane-based microarray (DermArray®). All values were measured at 24 hours following 250 milliseconds on time/100 milliseconds off time/100 pulse exposure at 4.0 mW/cm^2 or 0.1 J/cm^2. [Figure can be viewed in color online via www.interscience.wiley.com.]

gene expression profiles based on wavelength changes, a selection of these results are presented in Figure 1. This manuscript describes subsequent experiments using RT-PCR-based microarrays (SABiosciences, Frederick, MD) that examine distinct gene expression patterns in cultured human skin fibroblasts and other cell types when wavelength of light is varied.

MATERIALS AND METHODS

The initial series of experiments was designed to determine if there was a measurable difference demonstrated through dermal matrix and adhesion molecule focused RT-PCR microarray expression values when the ratio of visible to Infrared light of two wavelengths was increased or decreased in 25% increments. The wavelengths selected were 590 nm in the visible spectrum and 870 nm in the infrared (IR) spectrum. The LED array was designed to achieve homogeneous light distribution of both types of LED (590 and 870 nm) to the cells at the desired ratio. The ratios tested were: 100% 590 nm/0% 870 nm; 75% 590 nm/25% 870 nm; 50% 590 nm/50% 870 nm; 25% 590 nm/75% 870 nm; and 0% 590 nm/100% 870 nm. The irradiance was 4.0 mW/cm^2 delivered using a 250 milliseconds on time/100 milliseconds off time/100 pulse duty cycle for a total fluence of 0.1 J/cm^2.

Study Design

Human dermal fibroblasts in culture were exposed to an LED array populated with 590 and 870 nnm LED's which could be independently controlled to vary the output ratio of each individual wavelength from a total combined output of 4.0 mW/cm^2. The ratios of visible to IR light were decreased (in the case of visible) and increased (in the case of IR) in series of 25% increments from no IR to fully IR. The RNA was extracted 24 hours later for use in a PCR-based microarray focusing on extracellular matrix and adhesion molecules. The experiment was repeated three times and an average expression level generated for each ratio. This ratio was compared to untreated/unexposed control cells of the same culture conditions as baseline.

LED Light Source

The photomodulation source was a custom built design consisting of a circuit board populated with approximately equal numbers of 590 and 870 nm surface mount LED diodes. The diodes were spaced to present a homogeneous distribution of light to the cells without loss of power from the bottom of the cell culture dish. The device had the exposure cycle (pulsed exposure 250 milliseconds on time, 100 milliseconds off time, 100 repetitions) fixed, but had independent voltage controls allowing for adjustments of power levels for each LED via linked PC and software. This was used to produce the ratios of power output by each LED type. The ratios were determined by setting the power output from each LED and dividing it by the total output of 4.0 mW/cm^2. For example, the 50/50% ratio would be 2.0 mW/cm^2 of 590 nm and 2.0 mW/cm^2 of 870 nm totaling 4.0 mW/cm^2 delivered to the cultured fibroblasts. The total power output was verified using a Newport 1835-C light meter and detector head. This same light meter and detector were used to determine the penetration of various wavelengths of light through fresh ex vivo Fitzpatrick Type II facelift preauricular skin samples that had the adipose tissue removed so only the dermis and epidermis were examined. The light measured at the detector head after passing through the ex vivo sample was recorded as a percentage of light transmitted. The 590 nm LED transmitted 20% through the ex vivo skin.

Human Skin Fibroblast Cell Culture and Exposures

Human skin fibroblasts in maintenance medium at subsaturation density levels were trypsinized and seeded into wells of a six-well cluster dish at a density of 2.5×10^5 cells/2.5 ml seeding medium/well in seeding medium. Cells were mixed with at least equal volume of medium after trypsinization and centrifuged for 5 minutes, 500–600*g*. After counting, cells were pipetted into wells using a repeat pipettor set to deliver 500 µl. Seeded dishes were incubated at 37°C in 5% CO_2. Two days after seeding, the wells were aspirated and fed with 2.5 ml fresh seeding medium and returned to the incubator. On the fourth day, the cells were prepared for experimental use that day, under lowest level light conditions needed for procedures. Each well was washed three times with wash medium. The washing sequence was three 2 ml washes, with the last wash remaining on the cell monolayer until after the exposure event. At this point before exposure, the dish was returned to the incubator to equilibrate for at least 30 minutes but not more than 60 minutes before it was exposed in an experiment. Black, light proof, boxes were used to transfer unexposed dishes to and from the incubator before and after exposure events. After exposure to the low level light source for the exposures mentioned above, the test well was aspirated, the monolayer fed 2.5 ml of test medium and the dish restored to the incubator. Control wells in the experiments were treated as test wells but not

exposed; the third wash was aspirated and 2.5 ml test medium was added to the control wells and the dishes returned to the incubator. Twenty-four hours later the cells were lysed and extracted for processing in an extracellular matrix and adhesion molecule pathway focused microarray from SABiosciences (PAHS-013). The RNA isolation kit was also from SABiosciences (#1027A) and designed specifically for use the PCR array system. The manufacturer's protocol was followed in all instances. The arrays were run three times per selected parameter and an average expression value and profile was generated for each ratio tested. Use of laminar flow hood and gloves to preserve RNA integrity was employed.

Measurement of Gene Expression

Upon completion of the data analysis of the three separate microarrays run per ratio tested it was determined that 80 genes were within detectable range. All threshold cycle values for every gene in the array were averaged for each tested ratio and compared against average threshold cycles for the unexposed control arrays. The resulting relative expression values were then plotted on several line graphs (Figs. 2 and 3). Each of the genes in the array comprises the x-axis, while the relative expression value of each gene is plotted on the y-axis.

RESULTS

The response of the cultured cells demonstrates that the ratio of simultaneous dual wavelengths have an effect on gene expression profiles. In the tested ratios the 100% 595 nm/0% 870 nm ratio had 77.5% of the 80 array genes altered with a positive or negative twofold induction/reduction as the minimum requirement. The remaining ratios in order percentage of genes altered were: 75% 595 nm/25% 870 nm = 58.8% genes altered; 0% 595 nm/100% 870 nm = 27.5% genes altered; 25% 595 nm/75% 870 nm = 25.0% genes altered; and 50% 595 nm/50% 870 nm = 21.3% genes altered.

When comparing cellular response across all tested ratios, 15 of 80 or 18.8% of genes shared an expression change (up or downregulation) in the same direction at all ratios, and even when the directional change was the same, the relative magnitude of the changes was not. When examining collagenases, MMP/collagenase downregulation is most affected by the visible light, as the 100% 595 nm light downregulated 69.2% of the tested MMP's with the higher IR ratios upregulating more MMPs. However, the 75% 590 nm/25% 870 nm ratio produced the most optimal combination of gene expression for the purpose of increasing net dermal collagen matrix by both upregulating collagen and downregulating MMP/collagenase, which is what the current commercially available LED array was designed to accomplish (Fig. 4).

DISCUSSION

The interaction of light with cells is a multi-variable equation consisting of many known variables including: wavelength(s), bandwidth, delivery mode and duty cycle, energy fluence, and cell type(s). This data details how ratio

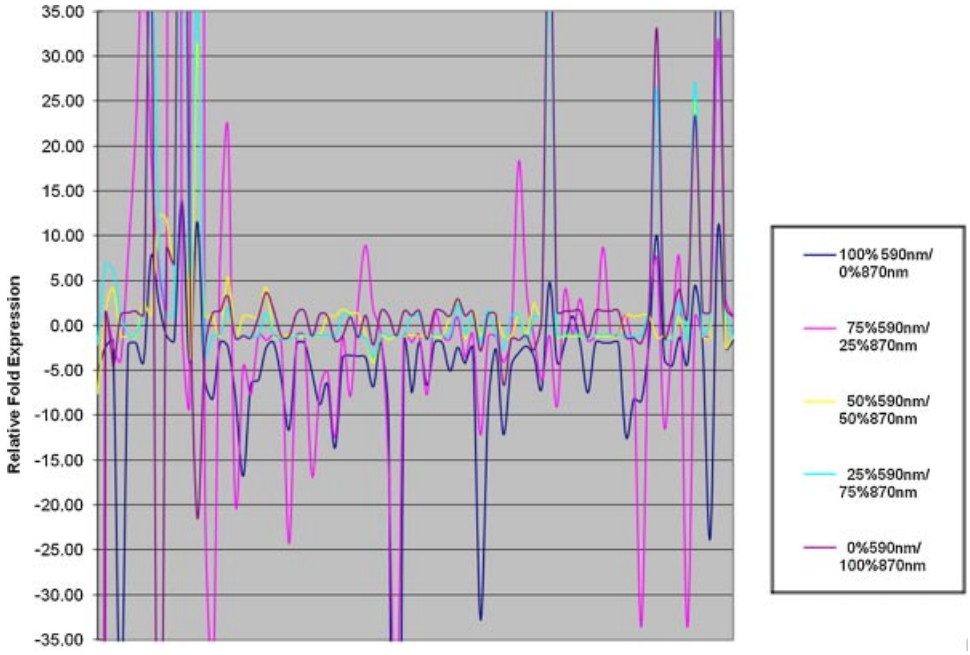

Fig. 2. Gene expression profiles of all tested ratios. x-Axis is each individual gene on the focused microarray and the y-axis is the relative expression value of that gene. When overlaid together, differences in magnitude of genetic response as well as direction can be seen when ratios of dual wavelength LED photomodulation are altered. [Figure can be viewed in color online via www.interscience.wiley.com.]

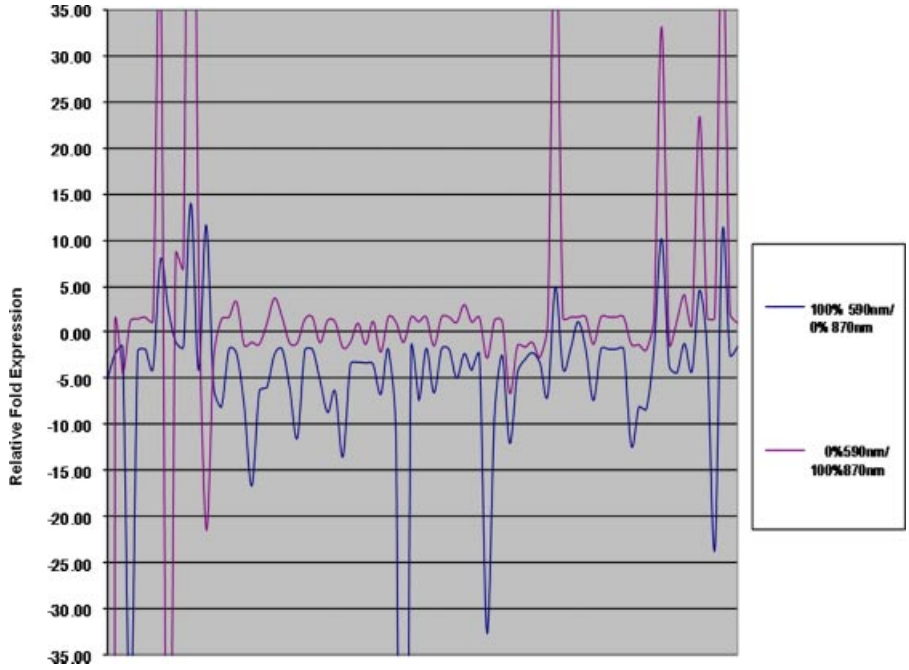

Fig. 3. Gene expression profiles of 75% visible 590 nm/25% 870 nm IR light plotted with 50% visible 590 nm/50% 870 nm IR light. [Figure can be viewed in color online via www.interscience.wiley.com.]

of two wavelengths, one visible and one infrared may alter gene expression.

The complex interaction of light and cells has already demonstrated that wavelength, fluence, exposure duty cycle, and exposure intervals can alter cellular responses. This in vitro study demonstrates that cells exposed simultaneously to distinct wavelengths of light in a homeogenous fashion can not only be affected as previously described photomodulation [15], but the ratio of wavelengths that comprise that fluence can alter the cellular response to varying degrees. In some cases the responses are unchanged based upon altered ratio, but many of the

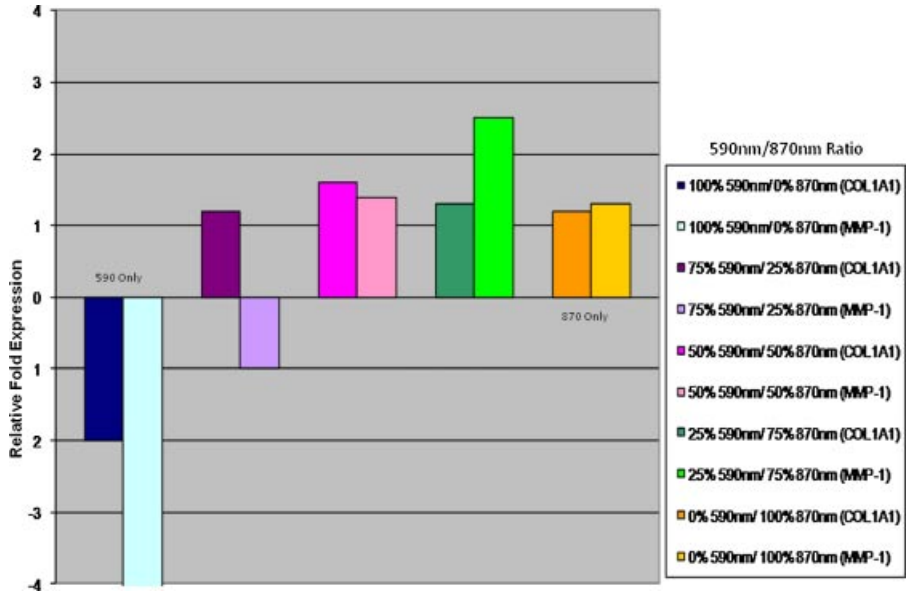

Fig. 4. Collagen 1A1 and MMP-1 expression values 24 hours post-exposure to various ratios of 590/870 nm LED photomodulation (0.1 J/cm^2; 250 milliseconds on time, 100 milliseconds off time, 100 repetitions). [Figure can be viewed in color online via www.interscience.wiley.com.]

genes studied in this experimental series have large variances in either the positive or negative stimulation of the gene or in the relative expression value of the gene stimulated either positively or negatively. This information has not been previously described by multiple sources and may lead to new combinations of LED therapies for a wide range of medical conditions beyond photoaging and acne treatment. Further study of the basic mechanisms of photomodulation accompanied by profiling multiple wavelengths and combinations/ratios of multiple wavelengths of light on cells, skin equivalent cultures and in vivo skin should continue to yield valuable information. Focused microarrays are an efficient and reproducible method for obtaining data when performed as described. They are cost effective and are available off the shelf for many common pathways while maintain the flexibility for fully customized gene lists. The primary advantages to this method is a rapid turnaround time and the ability to study multiple pathways using a single experimental exposure, since the RNA/cDNA requirements for each sample can be as low as 1 ng, with most experiments generating several micrograms of RNA. The arrays are not specific to in vitro cultures, and can be used from in vivo samples as well. The primary limitation of this methodology is that the generation of an increased/decreased gene signal does not always correlate to increased/decreased production of protein in culture. Validation of the end result of the genetic signal (i.e., the production of the related protein) should be done by ELISA or histological staining or other accepted methods.

Photomodulation with a 590/870 nm LED array is effective for altering gene expression, collagen synthesis, and reduction of MMP-1 expression in vitro as described herein and in vivo by the testing of a device similar to the 75/25:590/870 nm ratio in a human clinical trial. This trial involved clinical evaluations and histology [1,8,9] and demonstrated through immunoflorescent staining an increase in collagen I and a decrease in MMP-1 following LED photomodulation treatment [16]. By manipulation of various light parameters, the genetic activity of fibroblasts in vitro can be altered from an untreated state. As previously reported the end clinical result is smoother skin texture without inherent risks of thermal photorejuvenation devices [1]. Our study demonstrates the importance of wavelengths as a significant variable. Wavelength plus other variables including pulse duration, fluence, and total number of pulses determines the influence of photomodulation for gene expression by fibroblasts in culture. Further studies involving skin equivalent models and in vivo testing should be conducted to further establish the link between in vitro culture models and clinical changes.

The data presented several other genes that while not achieving statistical significance, demonstrated changes suggesting future study is desirable. The genes were:

(1) *NCAM1*: Role in cell–cell and cell–matrix adhesion; mediates adhesion between neurons and muscle. Generally downregulated across the ratios.
(2) *COL11a1*: Important in fibrillogenesis with *COL5a1*; essential for normal formation of cartilage collagen fibrils and cohesive properties. Mix of up/dpwnregulation dependant on the ratios.
(3) *COL6a1*: Involved in cell migration and differentiation; localize close to cells, nerves, blood vessels and large collagen fibrils and considered to have an anchoring function. Generally downregulated across the ratios.
(4) *CD44*: An integral cell membrane glycoprotein with a postulated role in matrix adhesion lymphocyte activation and lymph node homing. Mix of up/downregulation dependant on the ratios.
(5) *MMP11*: Important in ECM remodeling during embryonic development, tissue repair, and tumor progression. Generally downregulated across the ratios.
(6) *CTGF*: Has been implicated in extracellular matrix remodeling in wound healing, scleroderma, and other fibrotic processes, as it is capable of upregulating both MMPs and their inhibitors (TIMPs). Mix of up/downregulation dependant on the ratios.

TABLE 1. Relative Gene Expression Values of Selected Genes for all Tested 590/870 nm Ratios

Gene symbol	590/870 nm ratio (%)				
	100/0	75/25	50/50	25/75	0/100
NCAM1	−274.4	−137.2	−111.4	−97.0	−131.0
COL11A1	−5.7	1.2	1.5	2.6	−1.7
COL6A1	−4.9	−2.3	1.2	−6.5	−2.9
CD44	−68.6	1.2	−1.0	1.7	1.1
MMP11	−12.1	−1.7	−1.1	−1.9	−7.1
CTGF	−18.4	1.1	1.2	1.2	−1.1

The expression values, by ratio, for these genes and selected other genes are included in Table 1.

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