Photobiomodulation Therapy Promotes Expansion of Epithelial Colony Forming Units

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Abstract

Objective: This preliminary study examines the effects of low-dose light therapy, also called Photobiomodulation (PBM) therapy, on epithelial colony forming units (eCFUs) in epithelial cells from skin and mucosa to assess their potential to contribute to tissue regeneration. Also, preliminary comparison of basic PBM parameters such as wavelengths, light sources, and dose were evaluated in promoting eCFUs. **Background Data:** Regenerative medicine is at the brink of exploiting the tremendous potential offered by advances in stem cell biology. The two distinct aspects for utilization of stem cells, either resident (endogenous) or transplanted (exogenous), rely on cells amenable to expansion and being directed toward mature, functional tissues. Despite major progress in fundamental understanding of stem cell pluripotency, there remain fundamental challenges in applying these insights into clinical practice. **Methods:** PBM treatments with various devices, wavelengths, and doses were used on two epithelial cell lines and colony forming assays were performed. **Results:** This study noted a dose-dependent effect of 810 nm laser on increasing eCFUs, either in terms of size or numbers. Comparisons of different wavelengths and light sources noted better efficacy of collimated and coherent lasers compared to LEDs and broad-band light. **Conclusions:** PBM therapy promotes expansion of eCFUs that represent progenitors and stem cell populations capable of contributing to tissue repair and regeneration. Further exploration of the precise mechanisms would allow optimization of PBM clinical protocols to harness the regenerative potential of stem cells for wound healing and other clinical regenerative applications.

Keywords: colony forming units, laser, photobiomodulation, stem cells

Introduction

THE ABILITY OF ORGANISMS to repair and regenerate tissues after injury is dependent on an evolutionary conserved trait involving endogenous, resident (non-embryonic) stem cells. The characteristics of stem cells to self-renew and differentiate into specialized functional tissues have profound clinical implications.¹ A major limitation in using stem cells is their naturally low numbers limiting their clinical use. The regenerative potential of stem cells is being harnessed through several modalities such as transplanting exogenous stem cells (auto- or xenotransplantation) and administration of small molecules and biomolecules to mobilize and/or differentiate these cells. The use of low-dose light has been shown to stimulate wound healing and tissue regeneration. This process has been called low level light/laser therapy or, more appropriately, Photobiomodulation (PBM) therapy.²

Mechanisms of PBM therapy have been attributed to absorption of photons by specific chromophores within (intra-

cellular) and outside (extracellular) the cell that results in generation of reactive oxygen species (ROS). Cytochrome C oxidase in the mitochondria is among the most well-studied intracellular PBM targets that result in modulation of the electron transport chain, increased adenosine triphosphate formation, and release of nitric oxide along with generation of other ROS. This leads to activation of diverse downstream signaling pathways that activate potent biological processes such as cell proliferation, migration, and differentiation.^{3–6} More recently, we demonstrated an extracellular pathway involving low-dose laser-generated ROS, which acts on a ubiquitous, multifaceted latent growth factor complex, TGF- β 1.⁷ This distinct PBM growth factor mechanism was noted to be capable of directing differentiation of dental and mesenchymal stem cells.^{7,8} The therapeutic benefits of PBM therapy on adipose-derived stem cells and bone marrow mesenchymal stem cells have been documented previously.^{9,10} Moreover, mouse embryonic fibroblasts and human skin fibroblasts have been shown to respond to PBM therapy with clonal expansion

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and differentiation of stem cells using the colony forming unit (CFU) assays.¹¹

This preliminary study addresses the effect of PBM treatments on epithelial colony forming units (eCFUs) as a measure of changes in epithelial progenitors and stem cell populations. We used two human epithelial cell lines derived from skin and oral mucosa with the CFU assays to assess effects of various PBM parameters on expansion of stem cell populations. Cells were treated with different light doses, sources, and wavelengths. It was observed that PBM treatment with specific parameters appears to have a positive effect on promoting eCFUs.

Materials and Methods

Cell lines

Two epithelial cell lines, human dermal keratinocyte and human normal oral keratinocyte, spontaneously immortalized (NOKSI) were cultured in DMEM supplemented with 10% fetal bovine serum along with 100 U/mL penicillin and 100 µg/mL streptomycin (all from Invitrogen; ThermoFisher Scientific). Cells were maintained at 37°C in a humidified chamber with 5% CO₂.

Light treatments

Light treatments were performed using three different sources, namely, Laser (AMD Lasers), LED (QBMI Photomedicine, Inc.), and broad-band light (Photo Medtech, Inc.) with different wavelengths (600–850 nm). Cells were treated with 0.003 and 0.01 W/cm² (irradiance) for 300 sec, which corresponds to 1 and 3 J/cm² (fluence), respectively. For the laser treatments, six-well plates or 60-mm dishes were treated at a distance of 14.5 cm (beam divergence 15°) to ensure that spot size covered complete treatment surfaces. Target surface irradiance was measured with a power meter (Thorlabs) and details are presented in Table 1. During light treatment, cells were covered with 2 or 4 mL media for six-well plates and 60-mm dishes, respectively.

The broad-band light was similarly adjusted at a distance from the culture dishes to deliver desired irradiance. The LED device consists of a large (4×6") array enabling uniform illumination of the treatment field. Power of both devices was adjusted to generate the desired irradiance measured with a power meter at the cell treatment surface. Time was kept constant at 300 sec for all treatments.

Colony formation assay

Following PBM treatments, cells were incubated for 24 h, trypsinized, counted (Moxi Z mini automated cell counter; ORFLO Technologies), and 500 or 1000 cells were plated in six-well plates. After 2 weeks, colonies were fixed with 1 mL

of 10% neutral buffered formalin solution per well for 10 min. Cell colonies were stained with 1 mL of 0.4% (w/v) crystal violet for 5 min. Stained colonies were washed repeatedly with distilled water to remove excess crystal violet and allowed to dry. Images were taken using FluorChem E System (ProteinSimple). For quantitation, images were imported into ImageJ and converted to eight bit images, and contrast-brightness was adjusted to highlight stained areas. Circular ROIs were used to select the well, and individual colony size and total colonies were analyzed with the particle tool option.

Statistics

The statistical software GraphPad Prism (GraphPad Software, Inc.) was used to perform paired Student's *t*-test, where *p* < 0.05 was considered statistically significant.

Results

Effect of PBM dose on eCFUs

Based on our previous work with the 810 nm laser on dental and mesenchymal stem cells, epithelial cells were treated with two different intensities, 1 and 3 J/cm², and assessed with CFU assays. The lower dose laser treatments appear to form more epithelial colonies, either in number or size, compared with the higher dose (Fig. 1A–D). It has been suggested that lower number of colonies could result from merging of smaller colonies, and hence, both colony size and numbers were assessed at low (500 cells) and high (1000 cells) cell numbers to interpret observations in this study. The skin eCFUs appear to be fewer in number but larger in size compared with those derived from the oral mucosa. These observations suggest that optimal PBM laser treatments can facilitate expansion of eCFUs potentially contributing to their epithelial repair and regenerative benefits.

Effect of different sources of light on eCFUs

To assess the effects of different light sources on stem cells, treatments were performed using broad-band light, LED, and laser at visible and near-infrared wavelengths at 1 J/cm². Treatments with all three light sources were noted to be capable of increasing eCFUs compared to nontreated controls. Interestingly, laser treatment appears to have a slightly better efficacy compared with LED and broad-band light treatments (Fig. 2A–D). These data suggest the monochromatic, collimated, and coherent biophotonic light sources that lasers represent are more efficient at eliciting eCFUs compared with other light sources that have fewer or lack these attributes.

Effect of wavelengths on CFUs

To assess the effect of wavelengths on colony formation, the two epithelial cell lines were treated with light sources at

TABLE 1. TABLE SHOWING VARIOUS PARAMETERS USED FOR PHOTOBIOMODULATION STUDY

Power (W) display	Irradiance (W/cm ²) at nozzle	Spot size diameter (cm) cell surface	Target surface irradiance (W/cm ²)	Power (W) cell surface	Fluence (J/cm ²) cell surface	Time (sec)
0.1	0.2598	3.8	0.003	~0.04	1	300
0.5	1.2992	3.8	0.01	~0.19	3	300

FIG. 1. Differential effect of laser dose on eCFUs. (A) Representative eCFU images of skin epithelial cell line, HaCaT, treated with different doses of 810 nm laser. (B) Quantitation of images for CFU number and size of individual colonies. (C) Representative eCFU images for oral mucosal epithelial cell line, NOKSI, treated with different doses of 810 nm laser. (D) Quantitation of images for CFU number and size of individual colonies. CFU, colony forming unit; eCFU, epithelial colony forming unit; HaCaT, human dermal keratinocyte.

different wavelengths, namely, 660, 700, 810, and 850 nm. It was observed that among all the different wavelengths used, 810 nm appears to be the most efficient wavelength followed by 660 and 850 nm for eCFUs, while 700 nm appears to be least efficient (Fig. 2A–D). This supports prior observations that putative wavelength-specific biological targets (chromophores) aid in the direct transference of radiant energy to evoke discrete biological responses.

Discussion

Regenerative medicine uses stem cells for repair and regeneration of malfunctioning tissues or organs caused by physical damage, pathological conditions, or senescence. Stem cells are particularly attractive for many diseases that currently have limited or no effective therapies. Several of the skin and oral mucosal lesions such as pemphigus vulgaris, ulcerative lesions, and mucositis cause damage to skin or mucosa that could rely on the regenerative capabilities of stem cells.¹² Noninvasive manipulations with photonic sources of stem cells or their niche are particularly

attractive compared to administration of pharmaceutical agents (small molecules) or transplanting exogenous cells. However, a thorough understanding of the physical and biological interactions of these photonic devices is necessary before their clinical application. The physical parameters of these devices include the source (coherence and collimation), peak wavelength, dose (irradiance, time, and fluence), among others, need to be optimized to ascertain therapeutic effects, while concurrently avoiding detrimental phototoxicity.¹³

In this study, two keratinocyte cell lines were used from the skin and mucosa. Colony formation assay was used to assess expansion (increase in number) of epithelial stem cells following light treatment. The eCFUs represent the number of stem cells in a given population that are capable of forming individual colonies by self-renewal and differentiation.¹⁴ However, CFUs are also used to assess cancer stem cells, and PBM therapy has been shown to have distinct effects on transformed cells and does not cause mutagenesis indicating it would be safe to use clinically.^{13,15} This study first established a therapeutic dose that had a

FIG. 2. Effect of photonic source and wavelengths on eCFU. (A) Representative images of eCFU on skin epithelial cell line, HaCaT treated by different wavelengths of broad-band light, LED, and laser. (B) Quantitation performed on colony formation assay with 500 and 1000 cells plated using ImageJ. Quantitation is represented as number and average size of colonies. (C) Representative images of eCFU on mucosal epithelial cell line, NOKSI, treated by different wavelengths of broad-band light, LED, and laser. (D) Quantitation of mucosal eCFUs in terms of colony size and numbers.

distinct beneficial effect on epithelial stem cells' CFU and observed that a low level of light energy is beneficial while higher levels are detrimental. These observations highlight the importance of dose with PBM therapy that follows the Arendt-Schultz curve supporting hormetic responses with radiant energy delivery.¹⁶ In agreement with these principles, we had previously noted that low-dose NIR laser treatments generate low amounts of ROS that act on various biological molecules, including latent TGF- β 1.⁷ However, higher doses of the NIR laser induce phototoxicity by generating excessive ROS and heat, resulting in prominent unfolded protein response and endoplasmic reticulum stress leading to excessive autophagy, apoptosis, and tissue damage.¹³ These responses indicate the critical importance of clinical dosing for PBM treatments where the therapeutic biological responses can be optimally harnessed for clinical therapy.

We next examined the effect of various light sources and wavelengths of light on epithelial colony formation. It was noted that treatments with laser were slightly better compared to LED and broad-band light perhaps indicating that effects of coherent sources are more efficient than noncoherent light sources in evoking certain biological responses. It should also be noted that these effects were noted in an *in vitro* system in cell monolayer, the device parameters will likely vary in an *in vivo* scenario where scattering and diffusion will be more relevant within biological tissues. Finally, we also examined the effect of wavelengths but in a limited manner due to lack of access to a comprehensive set of photonic devices. It was noted that the ability of 660 and 810 nm, despite the source, was most effective at promoting eCFUs compared to other wavelengths. This is consistent with prior literature on the robustness of these wavelengths for clinical therapy as well as reported absorption spectra of chromophores attributed to contribute to PBM therapy.³ A major lack of consistency in the critical parameters defining PBM therapy dosing, namely, fluence—J/cm², irradiance—W/cm², and time—sec, is a lack of attention to the individual photon energies at given wavelengths (eVs or energy per photon). We believe attention to these details as well as clinical delivery (continuous wave versus pulsing, steady beam versus moving probe during delivery, among others) will enable development of robust PBM treatment protocols in the near future.

The presence of multipotent stem cells in the epithelium, as in other tissues, allows self-renewal throughout one's life to maintain tissue homeostasis. However, besides the capacity to maintain its own populations by limited expansion, it must maintain its ability to differentiate to particular functional lineages. Various cellular pathways such as Wnt/ β -catenin, BMPs, and FGF signaling among others have been attributed to perform specification, maintenance, and activation of stem cells while Notch and TGF- β signaling controls selective cell fate determination.¹⁷ Molecular mechanism of PBM has been shown to involve many of these pathways and it would be interesting to explore them in the context of the epithelial stem cell expansion noted in this study. While this is a preliminary study that demonstrated an interesting phenotype warranting further investigation, some shortcomings of this study are the limited analyses of dose and wavelengths as well as the lack of comprehensive molecular

characterization of stem cells, which will be addressed in the future. Also, cell lines cannot be assimilated with stem cells and there is a need to perform these studies with sorted cells (based on stem cell markers) in the future. In summary, this study notes the ability of PBM therapy to promote expansion of epithelial stem cells from skin and mucosa. This suggests that the biological responses of PBM clinical therapies for wound healing, skin and mucosal rejuvenation, promoting hair growth, among other applications, could harness the regenerative potential of resident, endogenous stem cells.

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Author Disclosure Statement

No competing financial interests exist.

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