Brief Report

Effect of Wavelength on Low-Intensity Laser Irradiation-Stimulated Cell Proliferation In Vitro

Pete Moore, MS, DDS,1 Tisha D. Ridgway, MS,3 Russell G. Higbee, DVM, PhD,3 Eric W. Howard, PhD,2 and Michael D. Lucroy, DVM, MS, DACVIM3*

1University of Oklahoma College of Dentistry, Oklahoma City, Oklahoma 73104
2Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104
3Oklahoma State University College of Veterinary Medicine, Stillwater, Oklahoma 74078

Background and Objectives: There exist contradictory reports about low-intensity laser light-stimulated cell proliferation. The purpose of this study was to determine the effect of wavelength on proliferation of cultured murine cells.

Study Design/Materials and Methods: Proliferation of primary cell cultures was measured after irradiation with varying laser wavelengths.

Results: Fibroblasts proliferated faster than endothelial cells in response to laser irradiation. Maximum cell proliferation occurred with 665 and 675 nm light, whereas 810 nm light was inhibitory to fibroblasts.


Key words: biostimulation; endothelial cell; fibroblast; wound healing

INTRODUCTION

Low-intensity laser irradiation has been explored as an alternative, non-invasive method to encourage chronic wounds to heal. The use of laser irradiation to speed wound healing first appeared in the human literature in 1971 [1]. Although several decades have passed since this report, low-intensity laser therapy is still controversial. Much of this controversy is the result of considerable research being published in non-peer-reviewed journals, a lack of consistency in reporting irradiation protocols, and the omission of appropriate experimental controls [2]. However, interest in low-intensity laser therapy persists.

Various mechanisms for the mitogenic effects of low-intensity laser irradiation have been proposed, including absorption of light by mitochondrial enzymes with localized heating [3], photon absorption by flavins and cytochromes in the mitochondrial respiratory chain affecting electron transfer [4], production of singlet oxygen by excitation of endogenous porphyrins [5], and photoactivation of calcium channels resulting in increased intracellular calcium concentration and cellular proliferation [6]. Studies of cultured fibroblasts reveal that 860 nm laser light stimulates cellular proliferation [7], 812 nm laser light increases DNA synthesis [8], 660 nm laser light up-regulates production of basic fibroblastic growth factor [9], and 632.8 nm laser light can transform fibroblasts into myofibroblasts [10]. Studies of cultured keratinocytes reveal that 632.8 nm laser light increases cellular proliferation [11], stimulates release of IL-1 and IL-8 [12], increases motility rate [13], and that 780 nm laser light stimulates cellular proliferation [14]. Macrophages are activated by 632.8 nm laser light, and various laser wavelengths are reported to increase growth factor secretion from cultured macrophages [15,16]. There are relatively less data on the effects of low-intensity laser irradiation on vascular endothelial cells; however, increased vascular endothelial cell proliferation has been described in vitro [17].

As with the in vitro studies of low-intensity laser irradiation, a variety of wavelengths have been evaluated in animal models of wound healing with compelling results. Laser wavelengths ranging from 524 to 904 nm have been reported to increase the rate of wound healing, increase collagen production, improve epithelial differentiation, and increase dermal vascularity in a variety of animal wound...
models [18–21]. A chronic wound in a dog was successfully treated with 630 nm laser light [22]. Likewise, chronic wounds in people, including recalcitrant radiation ulcers and diabetic neuropathic and microangiopathic ulcers, have been successfully treated with low-intensity laser irradiation [23–25].

However, not all reports of low-intensity laser irradiation support its efficacy. Low-intensity laser irradiation from a gallium-aluminum-arsenide laser failed to increase proliferation, migration, or adhesion of cultured keratinocytes or fibroblasts [26]. Low-intensity laser irradiation (890 nm) of wounds created in X-ray-treated skin failed to improve wound healing and inhibited healing with increasing fluence [27]. Likewise, visible red laser light (660 nm) failed to improve wound healing in X-ray-treated mouse skin [28]. In a study of low-intensity laser irradiation of experimental skin and tendon wounds in horses, no histologic differences were found between the treatment and control groups [29]. Likewise, a controlled study of low-intensity laser irradiation of minor surgical wounds in humans revealed no difference between the treatment and control groups with respect to rate of wound healing or pain levels [30].

These varied results may be due to many factors, including laser irradiation parameters (e.g., wavelength, power density, and fluence), cell type irradiated, or the underlying wound healing defect of in vivo systems. The purpose of this study was to determine the effect of laser wavelength on proliferation of non-transformed primary cultures of mouse vascular endothelial cells and fibroblasts.

MATERIALS AND METHODS

Cell Isolation and Culture

Two adult male C3H mice were obtained from the Oklahoma State University Lab Animal Resources Program immediately after euthanasia by CO2 asphyxiation. Endothelial cells and fibroblasts were isolated and characterized from the aorta and skin, respectively, as previously described [31]. Briefly, the mouse carcasses were submerged in 70% ethanol for 1 minute to decrease the microorganism population on the skin. Specimens from the aorta and skin were dissected aseptically from the mice and placed into 1% type II collagenase (Worthington Biochemical Corp., Lakewood, NJ) in HBSS (Invitrogen, Inc., Carlsbad, CA) and incubated in 37°C water bath with periodic agitation for 1 hour. Following incubation, the tissues were washed with 10 volumes of DMEM high glucose (Sigma-Aldrich, Inc., St. Louis, MO) supplemented with 20% FBS (Invitrogen, Inc.), 2 mM L-glutamine (Invitrogen, Inc.), and 25 μg/ml gentamycin (Sigma-Aldrich, Inc.), then centrifuged at 200 × g for 10 mins. The supernatant was discarded and the pellet was re-suspended in 10 ml wash media and seeded into 100-mm tissue culture dishes. After 24 hour the plates were washed with HBSS to remove non-adherent cells and the media replenished. Upon establishment of cell growth, the FBS concentration in the media was gradually decreased to 10% and endothelial cell or fibroblast colonies were isolated with cloning penicylinders (Corning, Inc., Corning, NY). The contents of several cylinders were transferred to 35-mm dishes and growth media added. Cells were sequentially expanded to 60-mm then to 100-mm tissue culture dishes. Cultures were grown at 37°C in 95% air with 5% CO2 in a humidified incubator. To confirm their identity, cells were stained immunocytochemically with antibodies directed against vimentin, CD34, factor VIII, and α-smooth muscle actin. The non-transformed primary fibroblast cultures became quiescent after approximately eight passages. Mouse endothelial cells grew for up to 20 passages.

Laser Irradiation

Primary cultures were maintained as a monolayer in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 25 μg/ml gentamycin and grown at 37°C in 95% air with 5% CO2 in a humidified incubator. For laser irradiation experiments, low passage (ca. p3) cells were seeded at 250 cells/well into black-walled, clear bottom 96-well tissue culture plates (BD Falcon–Optilux; BD Biosciences, Bedford, MA). Twenty-four hours after plating, immediately before laser irradiation, the culture media was removed and replaced with 150 μl of 10 mM HEPES in HBSS without phenol red in order to force most cells into the G0/G1 phase of the cell cycle [32]. Cells were irradiated in this media. The bottoms of the control wells were occluded with black tape, whereas the bottoms of the treatment wells were unobstructed.

The 96-well plates were supported on a clear polycarbonate platform and irradiated from below. Laser light was directed at the 96-well plates through a 400-μm quartz optical fiber terminated in a micro lens (Pioneer Optics, Windsor Lock, CT). Visible red laser light was generated by a KTP-pumped tunable dye laser (Series 600 XP, LaserScape, Inc., San Jose, CA) and the near-infrared laser light was from an 810-nm diode laser (Diomed Ltd., Cambridge, UK). The wavelength was verified with a charge coupled device (CCD) spectrometer (WaveStar-V, Ophir Optronics, Ltd., Boston, MA), and the power output was measured with a thermopile power meter (Vector H410, Scientech, Inc., Boulder, CO). Cells were exposed once to a single wavelength of laser light at a power density of 5 mW/cm2 and an energy density of 10 J/cm2. Immediately after irradiation, the HBSS was removed and replaced with the aforementioned culture media.

Cell Proliferation Assay

Seventy-two hours after laser irradiation, cell proliferation was measured using a colorimetric assay (CellTiter 96-AQ, Promega Biosciences, San Luis Obispo, CA) for viable cells, as previously described [33,34]. Briefly, cells were incubated for 2 hours after addition of the assay reagent, and absorbance (A490) was determined using an automated plate reader (HTS 7000, PerkinElmer, Inc., Shelton, CT). Absorbance was compared to a standard curve of a known number of cells. Experimental wells (quadruplicates) were averaged and data are expressed as percent difference from adjacent untreated control wells in
the same plate. Data are expressed as the mean (± SD) of five replicates unless otherwise noted.

RESULTS

Increased fibroblast proliferation was observed with all visible red laser wavelengths studied (Fig. 1). The proliferation of fibroblasts increased with longer wavelengths, and the maximum proliferation occurred with 665 and 675 nm light. Exposure to 810 nm laser light was inhibitory to fibroblasts. Endothelial cells, however, demonstrated increased proliferation after exposure to all wavelengths studied. The maximum proliferation of endothelial cells was observed at 655 nm and did not increase with longer visible red wavelengths. Endothelial cell exposure to

Fig. 1. Mean proliferation of actively growing cultured murine cells 72 hours after irradiation with laser light (5 mW/cm²; 10 J/cm²). The error bars represent the SD of the mean.

Fig. 2. Mean proliferation of quiescent murine fibroblasts 72 hours after irradiation with laser light (5 mW/cm²; 10 J/cm²). The error bars represent the SD of the mean.
810 nm light produced the smallest increase in cell proliferation compared to untreated controls.

When quiescent fibroblasts were irradiated with low intensity laser light, cell proliferation resumed (Fig. 2). All laser wavelengths studied produced measurable increases in cell proliferation, with the maximum observed after exposure to 665 and 675 nm light.

**DISCUSSION**

The experiments herein demonstrate that primary cultured murine endothelial cells and fibroblasts are both capable of responding to the mitogenic signals from low-intensity laser irradiation. However, not every wavelength of light studied produced the same degree of proliferative response, and proliferation inhibition occurred after near infrared light irradiation. This wavelength effect on cellular proliferation and the differences in response between endothelial cells and fibroblasts may explain some of the contradictory in vitro and in vivo results of laser biostimulation described in the literature. In the current study, power density and fluence were held constant; however, these are two additional variables that may affect cellular response to laser irradiation.

Our results demonstrate that visible red wavelengths of > 665 nm cause maximal increases in both fibroblast and endothelial cell proliferation, whereas 645 nm light preferentially stimulates fibroblasts. Although previous reports [14] suggest near infrared laser irradiation stimulates keratinocytes, we observed that 810 nm light is inhibitory to fibroblast proliferation. If the same differences in proliferative responses to various laser wavelengths exist in vivo, then low-intensity laser protocols could be designed for optimal wound healing based on whether the underlying wound-healing defect was a contraction problem or an angiogenesis issue. These results also suggest that certain wavelengths of light might be used to inhibit fibroblasts and possibly decrease abnormal collagen deposition in individuals at risk for keloid formation. Because several pathogenic bacteria have shown differential responses to varying wavelengths of low-intensity laser irradiation [35], it might be possible to select a wavelength of light for biostimulation of wound healing that minimizes the risk of infection.

Because endothelial cells and fibroblasts responded differently to some wavelengths of light, it is unclear whether the same mitogenic stimulus is created by each wavelength or if there is difference in mechanisms between cells. Molecular biology studies are warranted to determine if there are differences in molecular responses by cells exposed to various wavelengths of laser light. Recently, microarray analysis has identified the up-regulation of more than 100 genes in human fibroblasts exposed to 628 nm light [36]. These types of studies will help elucidate the mechanism(s) of laser biostimulation, and may help to identify new mitogenic pathways allowing for the development of novel wound healing therapies. Similarly, further study of quiescent cultured fibroblasts that begin proliferating after irradiation with low-intensity laser light and of fibroblasts inhibited by exposure to 810 nm light may provide insight into how non-dividing cells move from G0 to the G1 in the cell cycle.

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**REFERENCES**


