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ORIGINAL ARTICLE

The effects of low-level diode laser irradiation on differentiation, antigenic profile, and phagocytic capacity of osteoblast-like cells (MG-63)

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Abstract Previous in vivo and in vitro studies have reported that low-level diode laser therapy induces a biostimulatory effect, such as cell proliferation. The aim of the present study was to evaluate whether the laser irradiation of osteoblast-like cells (MG-63) can modify alkaline phosphatase activity (ALP), antigenic profile, and phagocytic capacity. The MG-63 cell line was exposed to diode laser (ezLase) of 940 nm at 1–1.5 W/cm^2 and 3–4 J. ALP was evaluated by a spectrophotometric technique and antigenic expression analysis (CD 54, CD80, CD86, HLA-DR), and phagocytic activity was analyzed by flow cytometry. At 24 h, the treated groups showed an increased ALP, and the highest increase versus controls (P=0.002) was at the dose of 1 W/cm^2 and 3 J; this modulation of the antigenic profile translated into a reduced expression of CD54, CD86, and HLA-DR and a slightly decreased phagocytic capacity with respect to the nonirradiated control group at the different intensities and fluencies assayed. These results demonstrate that laser therapy can exert a biostimulatory effect on osteoblastic cells at different levels, which may be clinically useful in the regeneration of bone tissue.

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Introduction

Laser therapy has proven highly useful in biomedicine, with the use of different laser types and energies for distinct purposes [1, 2]. Thus, low-level laser can have anti-inflammatory, analgesic, and biostimulatory effects and is therefore used clinically for wound healing and tissue regeneration [3–7].

Various in vivo and in vitro studies have demonstrated the biostimulatory effect of low-level laser energy on cell populations of diverse origin [8–10], but the underlying mechanisms are not fully understood. Numerous studies have suggested that low-level laser therapy increases the regenerative potential of biological tissues by modulating cellular metabolic processes [11], and it has been reported to induce proliferation and differentiation in osteoblasts [12, 13]. In a previous study, we observed a positive correlation between the energy density applied and the growth rate, which reached a maximum value at 3 J and decreased with higher fluency values [14]. Pyo et al. [15] attributed the biostimulatory effect of low-level laser on osteoblasts to an increased expression of type 2 bone morphogenetic protein (BMP-2) and transforming growth factor (TGF)-β1, among other factors. BMP-2 and TGF-β1 are autocrine factors that regulate osteoblast proliferation and differentiation [16, 17].

Bone is a complex tissue under continuous remodeling, and osteoblasts are the cells responsible for bone formation and regeneration. Osteoblasts have also been attributed with immunological functions, including the expression of markers of antigen-presenting capacity (CD54,

CD80, CD86, HLA-DR), the allogeneic stimulation of T cells, phagocytic activity, and cytokine synthesis [16, 18–20]. It has been found that the presence of certain growth factors, such as TGF-β1, fibroblast growth factor (FGF)-bb, and platelet-derived growth factor (PDGF), decreases the expression of antigen presentation markers in osteoblasts [21].

The objective of this study was to determine the effect of low-level diode laser on osteoblast differentiation, antigenic expression, and phagocytic capacity, using the osteoblast-like MG-63 cell line for this purpose.

Material and methods

Cell culture

The human MG-63 osteosarcoma cell line was purchased from American Type Cultures Collection (ATCC, Manassas, VA) and maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen Gibco Cell Culture Products, Carlsbad, CA) with 100 IU/mL of penicillin (Lab Roger SA, Barcelona, Spain), 50 μg/mL of gentamicin (Braum Medical SA, Jaen, Spain), 2.5 μg/mL of amphotericin B (Sigma, St Louis, MO), 1 % glutamine (Sigma, St Louis), and 2 % HEPES (Sigma, St Louis) and supplemented with 10 % of fetal bovine serum (FBS) (Gibco, Paisley, UK). Cultures were kept at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. Cells were detached from the culture flask with a solution of 0.05 % trypsin (Sigma, St Louis) and 0.02 % ethylene diamine tetraacetic acid (EDTA) (Sigma, St Louis) and then washed and suspended in a complete culture medium with 10 % of FBS.

Laser irradiation

We used a GaAlAs diode laser (ezLase™, Biolase Technology, Inc. 4 Cromwell Irvine, USA), which operates in the near-infrared spectrum at a wavelength of 940 nm, with power output of 70 mW and spot diameter of 400 μm. Cells were seeded in 24-well plates at 1×10^4 cells/mL per well and at an adequate distance from each other to avoid overlapping or scattered irradiation. After 24 h, the cultures were pulse-irradiated at doses and intensities of 1 W/cm^2-3 J (14.7 s), 1.5 W/cm^2-3 J (10.6 s), 1 W/cm^2-4 J (19.31 s) or 1.5 W/cm^2-4 J (12.96 s), with the probe tip held at 1 cm from the cell layer. The plates were not covered during the irradiation procedure, which was performed at room temperature. All experiments included a control group of cells grown under the same conditions but not irradiated.

Alkaline phosphatase activity

The effect on osteoblast-like cell differentiation was assessed by evaluating the alkaline phosphatase activity (ALP) activity of MG-63 cells cultured in an osteogenic medium [22] and treated with diode laser. ALP activity was quantified by using a colorimetric assay (Diagnostic kit 104-LL, Sigma, St. Louis, MO, USA) to determine early osteoblast differentiation.

This assay measures the conversion by ALP enzyme of the colorless substrate *p*-nitrophenyl phosphate to the yellow product *p*-nitrophenol: the rate of color change corresponds to the amount of enzyme present in the solution. Standard solutions of *p*-nitrophenol (0–250 μM) were prepared from dilutions of a 1,000-μM stock solution and assayed in parallel. The ALP assay was performed as described by Sandrini et al. [23]. Cells were seeded into 24-well plates at 1×10^4 cells/mL per well and cultured in the osteogenic medium under standard conditions for 7 days. The culture medium was then replaced by a fresh medium, and the cells were irradiated with diode laser, followed by their culture for 48 h under standard conditions. Untreated cells were used as control group. Finally, the cells were lysed with 0.1 % (*v*/*v*) Triton X-100, at 37 °C. Samples were then centrifuged at 1,500 rpm, and the supernatants were stored at −70 °C until their use. ALP activity was determined by using *p*-nitrophenol phosphate as substrate, as follows: the cell lysate solution (50 μL) was added to 50 μL of ALP substrate (Sigma, St Louis, MO, USA) and incubated at 37 °C for 45 min in darkness.

The enzymatic reaction was stopped by adding 50 μL of 0.1 M NaOH, and finally, the absorbance was measured at 405 nm with a spectrophotometer (Biotek EL×800). The total protein content was also estimated with a protein assay kit from Bio-Rad Laboratories (Nazareth-Eke, Belgium), based on Bradford's method. All samples were run in triplicate, and specific ALP activity was expressed in U/mg cellular protein.

Antigenic phenotype

Antigenic phenotype was studied by flow cytometry at 24 h of culture after diode laser treatment. Untreated cells were used as controls.

Cells were then detached from the cultured flask by treatment with 0.4 % (*w*/*v*) EDTA solution, washed, and suspended in phosphate-buffered saline (PBS) at 2×10^4 cells/mL. Cells were labeled by direct staining with the monoclonal antibodies (MAbs) CD54, CD80, CD86, and HLA-DR (CD54/IOL1b, CD80, CD86, and OKDR, respectively from Invitrogen Corp, Carlsbad, CA, USA). Aliquots of 100 μL cell suspension were incubated with 10 μL of the appropriate MAb for 30 min at 4 °C in darkness. Cells were washed, suspended in 1 mL PBS, and immediately analyzed in a flow cytometer with diode laser (FASC Canton II, SE Becton Dickinson, Palo Alto, CA, USA) at a wavelength of 488 nm

to determine the percentage of fluorescent cells. Untreated cells were used as controls. The percentage of antibody-positive cells was calculated from counts of 2,000–3,000 cells. At least three experiments were run for each antigen in all cultures.

Phagocytic activity

Phagocytic activity was studied by flow cytometry at 24 h of culture after diode laser treatment. Untreated cells were used as controls. Human MG-63 osteosarcoma cells were detached from the culture flask by treatment with 0.04 % EDTA solution, washed, and then suspended in a complete culture medium with 10 % FBS at 1×10^6 cells/mL. Cells were labeled by direct staining with labeled latex beads, incubating 100 μL of cell suspension with 200-μL carboxylated FICT-labeled latex beads with a diameter of 2 μm (Sigma, St Louis) for 30 min at 37 °C in darkness. Cells were washed, suspended in 1 mL PBS, and immediately analyzed in a flow cytometer (FASC Canton II, SE Becton Dickinson, Palo Alto, CA). Control assays were carried out at 4 °C. Results were obtained as the percentage of cells positive for phagocytosis.

Statistical analysis

SPSS version 21.0 (IBM, Chicago, IL) was used for the data analysis. Analysis of variance (ANOVA) was performed to examine the effects on ALP synthesis as a function of the energy dose (1 or 1.5 W/cm^2) and fluence (3 or 4 J) in comparison to the control group. The antigenic profile and phagocytic activity were compared by using the Student's *t* test. $P<0.05$ was considered significant in all tests. Before applying the Student's *t* test, we used the Shapiro-Wilk test to calculate the normal distribution of these values ($P>0.05$ in all groups). At least three experiments were performed in all assays. Data were expressed as means ± standard deviation (SD).

Results

Effect of diode laser irradiation on ALP of MG-63 cells

All radiation modalities assayed showed an increase in the ALP activity of osteoblast-like cells cultured in the osteogenic medium, although the difference versus controls was not significant at a dose of 1.5 W/cm^2 in intensity and 4 J.

The dose with the greatest effect on ALP activity was 1 W/cm^2 and 3 J, which produced a 95.6 % increase in treated cells versus control cells ($P=0.002$) (Table 1).

Table 1 Alkaline phosphatase activity in MG-63 cell line after 48 h of pulsed laser irradiation at doses of 3 or 4 J and intensities of 1 or 1.5 W/cm^2. Data are expressed as U/mg protein

	ALP activity	SD	*P*
Control	1.82	0.06	–
1 W/cm^2-3 J	3.56	0.18	0.002*
1 W/cm^2-4 J	2.79	0.15	0.004*
1.5 W/cm^2-3 J	2.55	0.09	0.001*
1.5 W/cm^2-4 J	1.92	0.07	0.146

SD standard deviation

*$P<0.05$ significant difference

Effect of diode laser irradiation on antigenic phenotype of MG-63 cell line

The flow cytometry results in Table 2 show that the expression of CD54, CD86, and HLA-DR membrane antigens was reduced versus nonirradiated controls in the MG-63 cells cultured for 24 h after diode laser irradiation at the different intensities and fluencies assayed. However, no effects on CD80 antigens were observed.

Effect of diode laser irradiation on the phagocytic activity of MG-63 cells

Results of flow cytometry with fluorescent latex beads in Table 3 show a slight reduction in phagocytic activity versus nonirradiated controls in cells cultured for 24 h after irradiation with diode laser at the different intensities and fluencies assayed.

Discussion

Therapies that more effectively promote osteoblast growth and maturation would benefit numerous patients who require bone tissue regeneration, including those with periodontal disease, bone necrosis, bone traumatisms, or those undergoing repair with implants, among others. The results of this study demonstrate that irradiation with low-level diode laser in pulsed mode not only stimulates osteoblast growth, as recently reported by Medina-Huertas et al. [14], but also exerts an action on other cell parameters. Thus, in regard to cell differentiation, irradiation at doses of 1 W/cm^2/3 J, 1 W/cm^2/4 J, or 1.5 W/cm^2/3 J produced a major and significant increase in ALP activity, suggesting a favorable effect on the maturation process. The most effective irradiation dose in this respect was 1 W/cm^2/3 J.

Osteoblast lineage differentiation is a complex process characterized by the sequential and defined expression of tissue-specific genes that permit the identification of three

Table 2 Percentage expression of different antigens in MG-63 cell line after 24 h of treatment with diode laser irradiation. Treatment groups: 1 W/cm^2/3 J, 1 W/cm^2/4 J, 1.5 W/cm^2/3 J, and 1.5 W/cm^2/4 J. Control group: not treated with diode laser

	CD54 (%)			CD80 (%)			CD86 (%)			HLA-DR (%)		
	Mean	SD	$P \leq$	Mean	SD	$P \leq$	Mean	SD	$P \leq$	Mean	SD	$P \leq$
Control	75.83	0.75	–	19.96	2.21	–	12.93	0.28	–	5.26	0.37	–
1 W/cm^2-3 J	56.40	0.62	0.001*	17.33	3.69	0.362	9.86	0.92	0.005*	2.76	1.35	0.037*
1 W/cm^2-4 J	58.10	2.59	0.001*	16.06	1.56	0.074	9.96	0.68	0.002*	3.0	0.26	0.001*
1.5 W/cm^2-3 J	53.80	1.30	0.001*	15.93	3.70	0.196	9.166	0.49	0.001*	2.56	0.25	0.001*
1.5 W/cm^2-4 J	55.76	3.64	0.001*	14.23	3.48	0.085	9.50	0.20	0.000*	2.73	0.20	0.001*

SD standard deviation

*$P<0.05$ significant differences

phases: proliferation, maturation, and extracellular matrix synthesis/mineralization. Specific genes for the differentiated osteoblast phenotype are expressed in the second phase, including genes that encode for alkaline phosphatase (AP) and osteocalcin, producing a high level of AP synthesis [24].

Osteoblast growth and differentiation are controlled by local and systemic factors, including BMPs, growth factors (FGF, IGF, TGF), hormones, cytokines, and even mechanical forces, which in turn regulate the activity of specific transcription factors. These factors can show different and even opposite effects on the cellular metabolism as a function of the maturation degree and phenotype of the cell [25]. Multiple exogenous factors of a highly diverse nature have been reported to modulate osteoblast differentiation or maturation [26–29].

The ability of laser irradiation to modulate gene expression and release growth factors and cytokines from cells in culture was previously demonstrated [30]; the authors proposed that biomolecules released in response to irradiation may function as autocrine factors on the osteoblasts through mitogenic properties and/or the ability to induce their differentiation or maturation.

Table 3 Flow cytometry results for percentage of phagocytic cells in MG-63 cell line at 24 h of diode laser treatment. Treatment groups: 1 W/cm^2/3 J, 1 W/cm^2/4 J, 1.5 W/cm^2/3 J, and 1.5 W/cm^2/4 J. Control group: not treated with diode laser

	Phagocytic cells (%)	SD	*P*
Control	98.50	0.26	–
1 W/cm^2-3 J	97.63	0.37	0.031*
1 W/cm^2-4 J	96.20	0.10	0.000*
1.5 W/cm^2-3 J	97.50	0.10	0.023*
1.5 W/cm^2-4 J	97.43	0.58	0.045*

SD standard deviation

*$P<0.05$ significant difference

TGF-β1 plays an important role in regulating and stimulating the differentiation of osteoprogenitors during fracture repair [31]. Numerous authors have described this growth factor, among others, as responsible for the biostimulatory effect observed in osteoblast cells in response to shock waves [32, 33]. It was previously reported that low-level laser therapy induces the expression of BMP-2, osteocalcin, and TGF-β1 [15]. Basic fibroblast growth factor (FGFb) has also been implicated in osteoblast proliferation and differentiation [25, 34, 35]. Saygun et al. [36] observed that the laser irradiation of cultured osteoblasts increased their proliferative capacity and the expression of FGFb, insulin-like growth factor (IGF-I), and IGF-I receptor (IGFBP3); these authors suggested that the biostimulatory effect on the osteoblast may be attributable to increased growth factor synthesis.

Laser irradiation treatment produced a decrease in the expression of CD54, CD86, and HLA-DR antigens in the present study. These markers modulate their expression in the presence of different substances, notably cytokines, growth factors, platelet-rich plasma, bacterial lipopolysaccharide (LPS), and even certain pharmaceuticals [21, 37–39]. In this regard, in vitro treatment with TGF-β1 of human osteoblasts obtained by primary culture from bone samples is known to significantly reduce their expression of CD54 and CD86 without altering their expression of CD80 or HLA-DR. However, treatment with FGFb, PDGF-BB, or IL-2 does not modulate the expression of the molecules involved in antigen presentation. Nonetheless, there is a significant increase in the expression of these markers in response to IL-1β, IFNγ, and LPS [21]. These data, alongside previous findings on cytokine (IL-4, IL-12, IL-15, IL-18, and IFNγ) expression in the osteoblast and their modulation by different factors (FGF, TGF-β1, and PDGF) and cytokines (IL-1 and IFNγ) [21], suggest that the functional capacity of osteoblasts is modulated during their differentiation and maturation, with a gain in their bone-forming function at the expense of their immunological function. The present results support this hypothesis, with observations of an increase in AP activity and a parallel

decrease in the expression of CD54, CD86, and HLA-DR antigens, as well as a slight decrease in phagocytic capacity.

A paper recently published by Davies et al. [40] reported an absorption peak of 560 nm for DMEM with phenol red. Also, these authors recommended investigating the energy loss from other light sources, especially those with a longer wavelength, such as pulsed diode laser with a wavelength of 910 nm. In this sense, we have not yet established whether there is absorption at 940 nm, the wavelength used in the present study.

We do not know the mechanisms underlying the biostimulation produced by laser irradiation. The aim of our study was to determine its effects on different parameters of the osteoblast; however, research on the underlying mechanisms should be subject of future studies. We do not rule out that the thermal effect of laser may be one mechanism of its action. Nevertheless, it should be borne in mind that the maximum temperature reached was below 60 °C in order to prevent the denaturation of proteins and hence cell death; indeed, the laser treatment produced an increase in the number of living cells.

These findings indicate that low-level diode laser irradiation may be useful in the treatment of bone regeneration through a biostimulatory effect on osteoblasts that favors their growth and maturation. This effect appears to be mediated by the autocrine action of growth factors released by the cells themselves in response to the laser treatment, as recently proposed [15].

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