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Superpulsed Laser Irradiation Increases Osteoblast Activity Via Modulation of Bone Morphogenetic Factors

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Background and Objective: Laser therapy is a new approach applicable in different medical fields when bone loss occurs, including orthopedics and dentistry. It has also been used to induce soft-tissue healing, for pain relief, bone, and nerve regeneration. With regard to bone synthesis, laser exposure has been shown to increase osteoblast activity and decrease osteoclast number, by inducing alkaline phosphatase (ALP), osteopontin, and bone sialoprotein expression. Studies have investigated the effects of continuous or pulsed laser irradiation, but no data are yet available on the properties of superpulsed laser irradiation. This study thus aimed to investigate the effect of superpulsed laser irradiation on osteogenic activity of human osteoblast-like cells, paying particular attention to investigating the molecular mechanisms underlying the effects of this type of laser radiation.

Study Design/Materials and Methods: Human osteoblast-like MG-63 cells were exposed to 3, 7, or 10 superpulsed laser irradiation (pulse width 200 nanoseconds, minimum peak power 45 W, frequency 30 kHz, total energy 60 J, exposure time 5 minutes). The following parameters were evaluated: cell growth and viability (light microscopy, lactate dehydrogenase release), calcium deposits (Alizarin Red S staining), expression of bone morphogenetic factors (real-time PCR).

Results: Superpulsed laser irradiation decreases cell growth, induces expression of TGF-β2, BMP-4, and BMP-7, type I collagen, ALP, and osteocalcin, and increases the size and the number of calcium deposits. The stimulatory effect is maximum on day 10, that is, after seven applications.

Conclusions: Reported results show that superpulsed laser irradiation, like the continuous and pulsed counterparts, possesses osteogenic properties, inducing the expression of molecules known to be important mediators of bone formation and, as a consequence, increasing calcium deposits in human MG-63 cells. Moreover, the data suggest a new potential role for PPARγ as a regulator of osteoblast proliferation. Lasers Surg. Med. 41:298–304, 2009.

Key words: BMPs; calcium deposits; MG-63 cells; superpulsed laser; TGF-β2

INTRODUCTION

Laser therapy is a new approach in different medical fields, including orthopedics and dentistry, when bone loss occurs, that is, in cases of bone fracture and tooth extraction [1]. Recent studies have reported the benefits of low level laser therapy (LLLT), which has been used to induce soft-tissue healing, for pain relief, bone, and nerve regeneration [2], although the molecular mechanisms triggered are not yet fully clear.

With regard to bone synthesis, in vivo experiments on rat femur have shown that pulsed laser irradiation with high peak power stimulates bone formation by increasing osteoblast activity and decreasing osteoclast numbers [3]. Beneficial properties have been ascribed to LLLT's anti-inflammatory effect, postulating that the treatment modulates transcription factors and regulates the expression of pro-inflammatory cytokines [4]. A recent study on the subplantar tissue of rat's paw evidenced that LLLT decreased mRNA content of TNF-α, IL-1β, and IL-6 [5]; it was suggested that an early target of radiation was TNF-α which, in turn, activates other cytokines. These authors also reported a decreased expression of kinin receptors in the same experimental model [6].

It has also been reported that LLLT induces the formation of small amounts of reactive oxygen species (ROS), which can trigger cell stimulation via increased mitochondrial respiration and ATP formation [7].

The stimulatory effect of LLLT has also been confirmed in vitro in different cell lines. In osteoblast-like cells isolated from fetal rat calvariae, LLLT stimulated proliferation and differentiation, inducing alkaline phosphatase (ALP), osteopontin (OP), and bone sialoprotein expression [8]. Similar results have been obtained in cultured human SaOS-2 cells, where early induction of ALP, type I collagen,

Contract grant sponsor: Regione Piemonte, Italy; Contract grant sponsor: University of Turin, Italy; Contract grant sponsor: Heraeus Kulzer GmbH, Division Dentistry, Hanau, Germany.
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Accepted 19 February 2009
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/lsm.20762

and OP was observed in cells irradiated with 670 nm diode laser [9,10]. Moreover, treatment with LLLT of human osteoblast-like cells, cultured on titanium, induced cellular activity in a dose-dependent manner, through increased osteocalcin and TGF-β_1 production [11]. TGF-β is one of the most abundant regulatory factors stored in the bone matrix [12]; it drives the cascade of events leading to new bone formation. In case of bone resorption, TGF-β is released early, and favors bone growth; however, once the process of bone formation is started, it acts as an inhibitor of mineralization and suppresses markers of osteoblast activity; this suggests that TGF-β affects osteoblast precursors but not differentiated osteoblasts [13,14].

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily, known to play an important role in bone formation [15]. Of the BMPs, BMP-2, 6, and 9 have been shown to be the most potent agents in inducing osteoblast differentiation of mesenchymal stem cells. On the contrary, BMP-3 exhibits no osteogenic activity and antagonizes the osteogenic activity of BMP-2, 4, 6, 7, and 9 [16].

Recent clinical trials have shown that BMP-7 is effective in stimulating ALP production in relatively mature osteoblasts [17]. Taken together, these findings suggest that interactions among BMPs are fundamental in regulating and driving the bone regeneration process.

Based on the fact that osteoblasts and adipocytes share a common progenitor (multipotential mesenchymal stem cells in bone marrow), and since peroxisome proliferated-activated receptor (PPAR) γ is a key regulator of adipocyte differentiation, the possible role of this nuclear receptor in bone metabolism has been investigated. It has been reported that low PPARγ expression enhances formation of bone mass by stimulating osteoblastogenesis [18]. In cultured primary osteoblasts, PPARγ ligands have been shown to decrease mineralized bone nodules and ALP activity, suggesting that activation of PPARγ inhibits the expression of mediators involved in bone formation, such as BMP-2 and osteocalcin [19], and that the appropriate antagonists of PPARγ could provide a new approach to treating some bone disorders [20].

Laser irradiation can also be used in the superpulsed modality, although no reports on its use have yet been published. The present study thus investigated the effects of superpulsed laser irradiation on osteogenic activity of human osteoblast-like cells, paying particular attention to investigating the molecular mechanisms underlying those effects.

MATERIALS AND METHODS

Materials

MG-63 human osteoblast-like cells were from ATCC (Rockville, MD); L-glutamine was from (Gibco Life Technologies, Paisley, UK); Trypan Blue (TB) was from Carlo Erba Reagenti (Milano, Italy); all the other reagents were from Sigma (St. Louis, MO).

Cell Culture

Cells were seeded $5\times10^4/cm^2$ in modified eagle medium (MEM) supplemented with 2 mM L-glutamine, 1% (v/v) antibiotic/antimycotic solution, 1 mM sodium pyruvate, and 10% (v/v) fetal bovine serum (FBS. Cells were maintained at 37°C in a 5% CO_2 atmosphere.

Laser Treatment

Twenty-four hours after seeding, cells were exposed to superpulsed laser irradiation, with the following protocol: every 24 hours for the first 5 days, then every 48 hours until day 20. A Lumix 2 HFPL Dental device IR (904–910 nm) Gallium Arsenide laser (Fisioline s.n.c., Verduno, Cuneo, Italy) was used, with the following experimental parameters: pulse width 200 nanoseconds, minimum peak power 33 W, average out power 200 mW, frequency 30 kHz, exposure time 5 minutes, total energy 60 J for each well (illuminated surface size: 9 cm^2). The administered dose was 6.7 J/cm^2. Control cells were not exposed to laser irradiation. Both control and laser-treated cells were harvested by trypsinization at days 4 (3 exposures to laser irradiation), 10 (7 exposures to laser irradiation), and 20 (12 exposures to laser irradiation), and used for the determinations reported below.

Cell Growth and Viability

Cells present in monolayer and culture medium were counted using a Burker chamber. Cell viability was determined by the TB exclusion test and through lactate dehydrogenase (LDH) activity in the culture medium, as marker of necrotic death. TB exclusion was evaluated on cells suspended in the presence of the dye (0.8 mg/ml); 400 cells were counted for each sample and results expressed as percentages of TB-positive cells. LDH activity was assessed spectrophotometrically on clear culture supernatants after centrifugation at 600*g* for 10 minutes and expressed as nmoles NADH oxidized//minute/ml of culture medium [21].

Calcium Deposition

Alizarin Red S staining was used to evaluate the formation of calcium deposits and mineral matrix: detached cells were fixed in 70% ethanol, stained with 1% Alizarin Red solution for 2 minutes, washed with Tris-buffered saline solution and observed under a light microscope to evaluate Alizarin positivity.

Real-Time Polymerase Chain Reaction (Real-Time PCR)

Total RNA was isolated from MG-63 cells with a RNEasy® Mini Kit (Qiagen, GmbH, Germany) following the manufacturer's protocol.

One microgram of RNA was reverse transcripted using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). PCR was performed using IQ™ SYBR-Green Supermix (Bio-Rad, Hercules, CA) in a iCycler system (Bio-Rad).

Each sample was tested three times, and the threshold cycle (C_t) values from each reaction were averaged.

TABLE 1. Human Primer Sequences for Real-Time PCR

Gene	Forward primer 5′-3′	Reverse primer 5′-3′	bp
GAPDH	GTCGGAGTCAACGGATTTGG	GGGTGGAATCATATTGGAACATG	142
ALP	CTCCCAGTCTCATCTCCT	AAGACCTCAACTCCCCTGAA	120
BMP-4	CTCGCTCTATGTGGACTT	ATGGTTGGTTGAGTTGAGG	130
BMP-7	GTGGAACATGACAAGGAAT	GAAAGATCAAACCGGAAC	65
OCN	GTGACGAGTTGGCTGACC	CAAGGGGAAGAGGAAAGAAGG	129
PPARα	GGATGTCACACAACGCGATT	GCCAGAGATTTGAGATCTGCAGTT	127
PPARγ	GCCGAGAAGGAGAAGC	TGGTCAGCGGGAAGG	150
TGF-β2	GAG TAC TAC GCC AAG GAG GTT TAC A	CGA ACA ATT CTG AAG TAG GGT CTG T	104
COLL I	GAG GAA ACT GTA AGA AAG G	GTT CCC ACC GAG ACC	150

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ALP, alkaline phosphatase; BMP-4, bone morphogenetic protein 4; BMP-7, bone morphogenetic protein 7; OCN, osteocalcin; PPARα, peroxisome proliferators-activated receptor α; PPARγ, peroxisome proliferators-activated receptor γ; TGF-β2, transforming growth factor β2; COLL I, type I collagene; bp, product length.

The change was quantified as the relative expression compared to that at time 0 (before the first exposure to laser irradiation), calculated as $2^{-\Delta\Delta DC_t}$, where $\Delta C_t = C_t\text{sample} - C_t\text{GAPDH}$ and $\Delta\Delta C_t = \Delta C_t\text{sample} - \Delta C_t\text{T0}$. Human primer sequences used for real-time PCR are reported in Table 1.

Statistical Analysis

Data are expressed in all cases as means ± SD. The significance of differences between group means was assessed by variance analysis, followed by the Newman–Keuls test ($P<0.05$).

RESULTS

The effect of super-pulsed laser irradiation on cell proliferation and viability, and on the expression of some parameters involved in bone synthesis was studied in human osteoblast-like cells MG-63.

Figure 1 reports proliferation, showing a decrease of cell numbers in laser-treated cells versus controls. The decrease started from the fourth day and reached a maximum on day 10 after seven applications, at which point the number of laser-treated cells was 75% of the control value. No induction of cell death was shown by the TB exclusion test, nor by counting cells floating in the medium or determining LDH release in the medium, at any time studied (data not reported).

Figures 2–6 show the stimulatory effect of super-pulsed laser irradiation on osteoblast activity. Figure 2 reports the

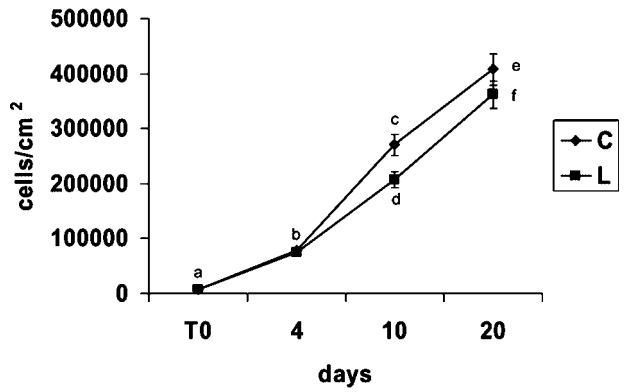

Fig. 1. Growth of cells exposed or not to superpulsed laser irradiation. Data refer to cells present in the monolayer and are means ± SD of five experiments. Means with different letters are significantly different from one another ($P<0.05$) as determined by analysis of variance followed by post hoc Newman–Keuls test. C, control cells; L, laser-treated cells.

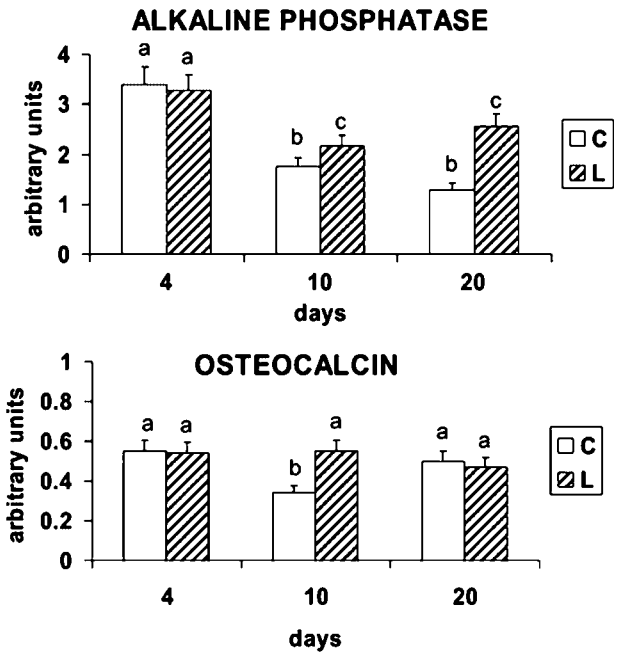

Fig. 2. mRNA content of alkaline phosphates (ALP) and osteocalcin in cells exposed or not to superpulsed laser irradiation. Data are means ± SD of five experiments. Means with different letters are significantly different from one another ($P<0.05$) as determined by analysis of variance followed by post hoc Newman–Keuls test. C, control cells; L, laser-treated cells.

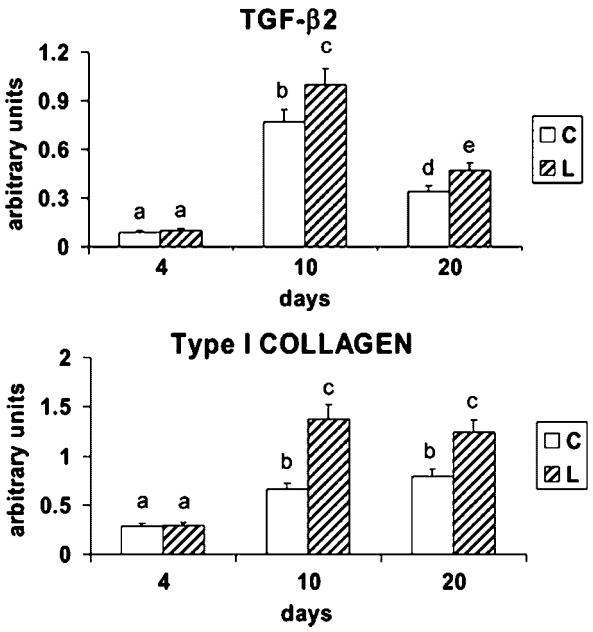

Fig. 3. mRNA content of TGF-β2 and type I collagen in cells exposed or not to superpulsed laser irradiation. Data are means ± SD of five experiments. Means with different letters are significantly different from one another ($P<0.05$) as determined by analysis of variance followed by post hoc Newman–Keuls test. C, control cells; L, laser-treated cells.

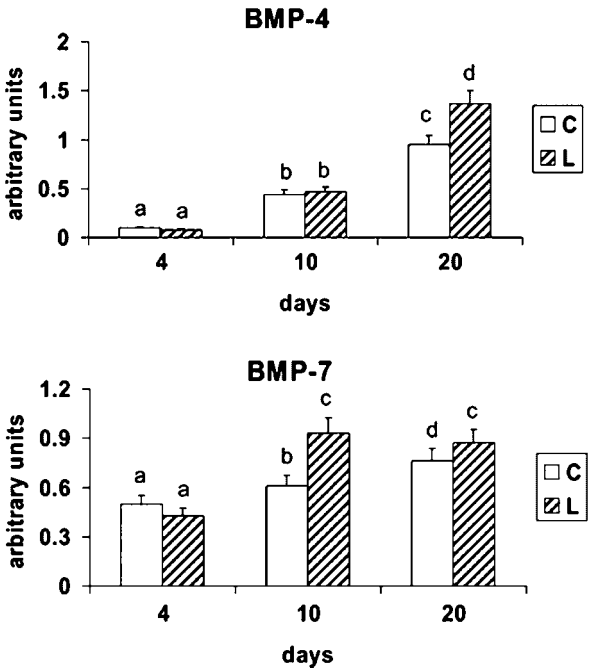

Fig. 4. mRNA content of BMP-4 and BMP-7 in cells exposed or not to superpulsed laser irradiation. Data are means ± SD of five experiments. Means with different letters are significantly different from one another ($P<0.05$) as determined by analysis of variance followed by post hoc Newman–Keuls test. C, control cells; L, laser-treated cells.

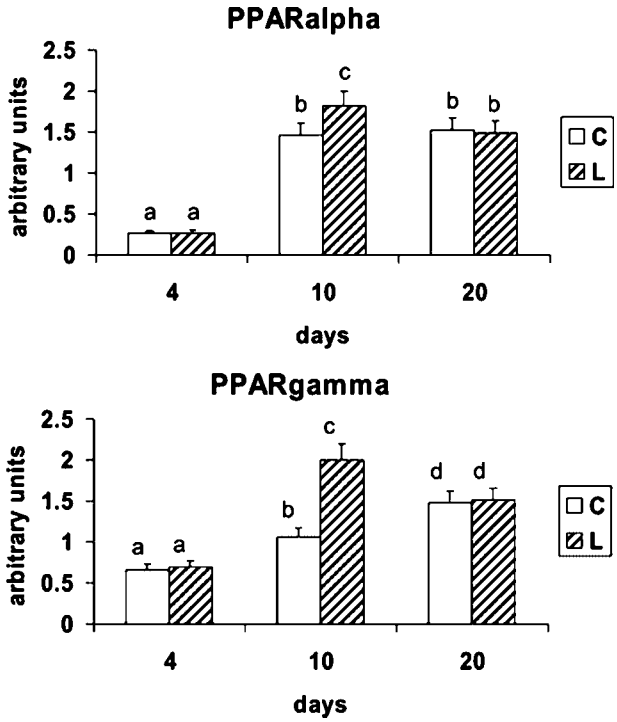

Fig. 5. mRNA content of PPARα and γ in cells exposed or not to superpulsed laser irradiation. Data are means ± SD of five experiments. Means with different letters are significantly different from one another ($P<0.05$) as determined by analysis of variance followed by post hoc Newman–Keuls test. C, control cells; L, laser-treated cells.

mRNA content of ALP and osteocalcin. In laser-treated cells, the ALP values were higher than those in control cells at days 10 and 20, being the increase versus control 23% and 98%, respectively, whereas no significant difference was present at day 4 between treated and control cells. As regards osteocalcin, in laser-treated cells, mRNA content was higher than in control cells only at day 10 (+60%), and was similar to the other experimental times.

Figure 3 shows the mRNA content of TGFβ2 and type I collagen. In laser-treated cells, the TGFβ2 mRNA content was higher on days 10 and 20 (+30%, +40% vs. controls), whereas it was similar on day 4. Type I collagen showed significant changes on days 10 and 20 (+109%, +58% vs. controls).

As regards BMPs, a significant increase in laser-treated cells was evident in BMP-4 mRNA only on day 20 (+44% vs. control); whereas BMP-7 was induced on both day 10 and day 20, the larger increase being on day 10 (+50% vs. control; Fig. 4).

Figure 5 shows PPARα and γ mRNA contents; both isotypes increased in laser-treated cells at day 10 in comparison with control cells, although the increase in PPARγ was larger (90% vs. 25% of PPARα); at the other times, no significant difference was observed between laser-exposed cells and controls.

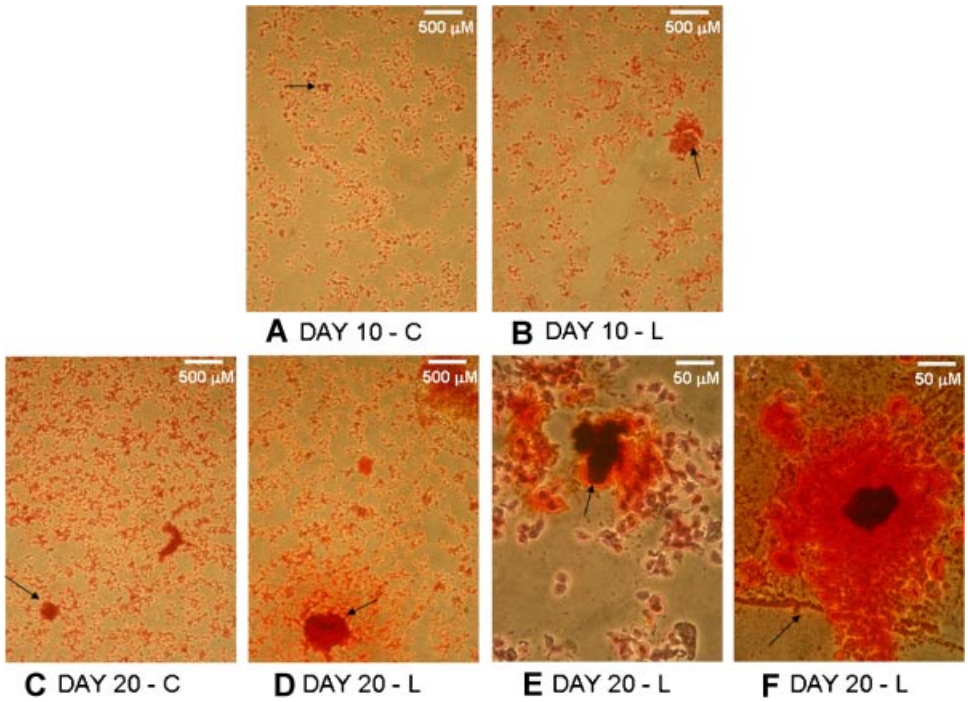

Fig. 6. Calcium deposits evidenced with Alizarin S staining in cells exposed or not to superpulsed laser irradiation. MG-63 harvested from the monolayer were stained with Alizarin red S and observed under the light microscope. Cells were analyzed on days 10 and 20. The black arrow shows the presence of calcium deposits. **Panel A**: DAY 10-C, control cells after 10 days of experimental time. **Panel B**: DAY 10-L, laser-treated cells after 10 days of experimental time. **Panel C**: DAY 20-C, control cells after 20 days of experimental time. **Panel D**: DAY 20-L, laser-treated cells after 20 days of experimental time (magnification of panel. **Panels E** and **F**: DAY 20-L, laser-treated cells after 20 days of experimental time (magnification of panel D).

Calcium deposits were investigated by staining cells with Alizarin red S (Fig. 6). Especially at 20 days (panel D vs. panel C), larger and more numerous calcium deposits were present in laser-treated cells compared to controls. Panels E and F are magnification of nodules present in laser-treated cells at 20 days.

DISCUSSION

The study constitutes the first report investigating the effect of superpulsed laser irradiation on human osteoblastic-like cells. To date, evidence of the osteogenic properties of continuous or pulsed laser irradiation has been reported, both in vitro and in vivo [22–26]. The present results show that repeated super-pulsed laser irradiation inhibits cell proliferation in human osteoblast-like cells and, especially, that it increases the expression of proteins essential for bone formation, that is, TGF-β2, BMP-4, and BMP-7, ALP, type I collagen, and osteocalcin. The stimulatory effect becomes evident on day 10, after seven laser irradiation exposures.

The inverse correlation between cell growth and osteogenic activity in MG-63 cells is not surprising since it is well known that cell differentiation process is characterized by a reduction of proliferation coupled with an increased tissue-specific gene expression, as also recently confirmed [27].

At present, few studies have addressed the molecular mechanisms underlying the osteogenic effect of continuous or pulsed laser irradiation. It has been suggested that this type of electromagnetic radiation influences biochemical activities, inducing a transient heating of some chromophores [28] and/or changing the redox status, with consequent production of ROS. It is well know that, when produced in large amounts, ROS are cell destructive, but that in small amounts they can act as "secondary messengers" via different pathways [29]. An effect of ROS is to induce lipid peroxidation and, as consequence, to increase production of aldehydes such as 4-hydroxynonenal, which induce cell differentiation at low concentrations [30,31]. Aldehydes derived from lipid peroxidation may also affect the activity of important membrane enzymes, such as ATPase, 5′-nucleotidase, phospholipase C [31].

The possibility that the effects of laser irradiation are in part mediated by changes in plasmamembranes may be supported by observations that shock waves, another kind of mechanical biostimulation, increase substance uptake and delivery via membrane modulation [29,32].

It is likely that, in our experimental conditions, a transient but stimulating heating occurs, since the diode laser equipment used is within the infrared wavelength band (904–910 nm). In turn, the laser-induced transient

heating could increase ROS production and lipid peroxidation [33].

It has been reported very recently that "in vitro" low-power far-infrared laser irradiation activated latent TGF-β1 immediately, and subsequently TGF-β3, possibly via induction of a conformational change in the latent complexes. These changes render TGF-β more amenable to the usual activation by altered pH or proteases [34]. These results suggest that the TGF-β pathway might be a key molecular mechanism triggered by laser irradiation. In our research, superpulsed laser-treated cells showed a significant increase of TGF-β2 mRNA versus controls, on both day 10 and day 20, suggesting the possibility that this signal transduction pathway may also be activated by superpulsed laser irradiation.

Other important changes observed in the superpulsed laser-treated cells concern proteins and transcription factors involved in regulating bone formation. An early increase of BMP-7 was observed, followed by the increase of BMP-4. This temporal sequence could be in agreement with the observation that BMP-7 is an early factor involved in committing mesenchymal stem cells to osteoblast differentiation and, especially, with reports that both BMP-7 and BMP-4 increase expression of ALP and osteocalcin [35].

For the first time, our research showed that laser irradiation affects the expression of PPARs. PPARα and γ were examined because they are closely connected with the reduction of cell viability and proliferation and with the increase in differentiation [36,37]. In laser-exposed cells, the strongest increase occurred in the isotype γ. The observation regarding the PPARγ increase is in disagreement with some reports indicating an anti-osteoblastogenic role for this nuclear receptor [38–41]; these studies principally analyzed the role of PPARγ in determining the destiny of multipotent mesenchymal stem cells in bone marrow.

In the light of these observations we hypothesize that, in differentiated MG-63 cells, the increased PPARγ is mainly involved in decreasing cell proliferation, as has been reported for other types of both normal and tumor cells [42–44]. The true role of PPARγ in modulating laser effect is now under investigation in experiments using specific antagonists.

The bulk of the results obtained shows that superpulsed laser irradiation, like the continuous and pulsed counterparts, possesses osteogenic properties, inducing the expression of molecules known to be important mediators of bone formation and, as a consequence, increasing the size and the number of calcium deposits in human MG-63 cells. Moreover, a new potential role may be suggested for the nuclear receptor PPARγ as a regulator of osteoblast proliferation.

ACKNOWLEDGMENTS

This research was supported by grants from Regione Piemonte, Italy, University of Turin, Italy, and Heraeus Kulzer GmbH, Division Dentistry, Hanau, Germany. We thank Mrs Frances Cooper for revising the English.

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