Effect of a diode laser on cell proliferation, alkaline phosphatase activity, and osteopontin mRNA expression in proliferating and in differentiating osteoblastic cells

Kenichi MATSUZAKA¹, Kazumasa OHTA², Ken TAKAHASHI³, Nahoko MIYAKE⁴, Masayuki HATTORI⁵, Takashi MURAMATSU⁶, Toru SATO⁴, Yutaka ODA⁵, Masaki SHIMONO⁶ and Tatsuya ISHIKAWA³

Oral Health Science Center, ¹Department of Clinical Pathophysiology, ²Department of Biochemistry, ³3rd Department of Conservative Dentistry, ⁴Department of Crown and Bridge Prosthodontics, ⁵Department of Dental Material Science, and ⁶Department of Pathology, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan

(Received 1 April 2004; and accepted 9 June 2004)

ABSTRACT

Different types of osteoblast behavior result from external stimulation in the early and in the late stages of cell differentiation. The purpose of this study was to investigate the influence of a diode laser on osteoblasts and to compare any effects between the proliferating and the differentiating phases. In the proliferating phases, the numbers of cells greatly increased in the laser irradiated group as compared with that of the control group. The increases in the numbers of cells in the differentiating phase were no greater than were those in the proliferating phase. The alkaline phosphatase activity of cells in the proliferating phase was highest 5 days after the laser irradiation, however, the activity in the differentiating phase increased day by day. Although osteopontin mRNA levels in the proliferating phase increased in the first 3 days of culture and then decreased in both groups, this decrease of osteopontin mRNA was small in the irradiated group. In contrast, osteopontin mRNA expression in the differentiating phase increased at day 1 and then decreased in the control group, while osteopontin mRNA levels in the irradiated group did not decrease.

Control of osteoblast behavior has been attempted by surface topography (9, 10, 11), by calcium and phosphate materials (4), and by a variety of growth factors (23). One effective method of controlling osteoblast behavior is the use of low-energy lasers, which have been used for wound healing (3, 13, 19, 24, 25), improving skin flap survival (5), and bone regeneration (18). In addition, previous studies noted various biostimulatory effects of low-energy laser irradiation, including the acceleration of bone regeneration and osteogenic cell differentiation (14,

16, 21, 22, 26). It is known that the osteoblast behavior *in vitro* depends on cell density, and low-density cells predominate in cell proliferation but high-density cells predominate in cell differentiation (2, 6, 7, 15, 17). In other words, osteoblast proliferation is predominant following differentiation in the early stages of the healing of bone wounds. Conversely, the development of the osteoblast function occurs after cell proliferation in the middle to late stage. The influence of low-energy lasers on osteoblasts in either the proliferating or the differentiating phase needs to be characterized, which was the intent of this study.

Correspondence to: Dr. Kenichi Matsuzaka
Department of Clinical Pathophysiology, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan

Tel: +81-43-270-3581, Fax: +81-43-270-3583

E-mail: matsuzak@tdc.ac.jp

MATERIALS AND METHODS

Basal characteristics of diode lasers. The basal characteristics of the GaAlAs diode laser (LD15, wave-

length; 810 nm, Dentek Laser Systems Production, Austria) regarding the relationship between the laser irradiation distance and the effective irradiation range, and between the source and the effective irradiation energy, have been published in a previous report (8). To further evaluate the basal characteristics of this laser, the relationship between laser irradiation time and temperature of the medium was measured in 24-well culture plates incubated at 37°C using a digital thermometer (Disi-Thermo CT-200, Custom Co. Ltd., Japan). To provide a sufficiently wide area, the distance from the culture plate to the tip of the probe was 50 mm.

Cell culture and laser irradiation. Osteoblastic MC3T3-E1 cells in a medium with 10% fetal bovine serum were used in this study. Cells were seeded in 24-well culture plates (Falcon, Becton Dickinson Co. Ltd., USA) at a density of $3.5 \times 10^3/\text{cm}^2$ for the proliferating phase and at a density of $3.5 \times 10^4/\text{cm}^2$ for the differentiating phase. After 24 h, GaAlAs diode laser with an exclusive probe at its tip for dental whitening was used to irradiate the cells from the bottom of the culture plate. The distance from the culture plate to the tip of the probe was 50 mm at the cell layer level. The irradiation time was 60 s at an output power of 10 watts with a continuous wave, and the effective irradiation energy was equivalent to about 60% of the source energy for the laser device (8). Thus, the total energy corresponding to a 60 s exposure was about 0.0204 J/m^2 .

Evaluation of cell behavior. Fig. 1 shows the experimental schedule. Cells growing in the 24-well culture plates were collected by 0.1% trypsin 1, 3, and 5 days after the laser irradiation and the numbers of cells were counted. To measure alkaline phosphatase (ALP) activity, the cells cultured in 24-well plates were rinsed three times with Phosphate buffered saline (PBS), collected using a cell scraper, and then sonicated for 5 min. ALP activity was measured 1, 3, and 5 days after laser irradiation by p-nitrophenylphosphatase assay. The amount of p-nitrophenol produced was measured at 405 nm using

a microplate reader (Biorad 450; Bio Rad, CA, USA). To determine the specific activity of ALP, protein contents in the lysates were determined using the Pierce BCA protein assay (Pierce, IL, USA). Absorbances were then measured at 562 nm using the microplate reader. The following formula was used to determine the specific activity of ALP: $\{\text{ALP specific concentration } (\mu\text{mol/ml}) / \text{protein concentration } (\mu\text{g/ml})\} \times \{1/\text{ALP incubation time (min)}\}$. For osteopontin mRNA expression, the total RNA was extracted using the Isogen reagent (Nippon Gene, Japan) according to the manufacturer's instructions. Briefly, the cultured cells were homogenized and solubilized in the Isogen / chloroform solution at 4°C. Supernatants were obtained following centrifugation at $12,000 \times g$ for 20 min at 4°C. The precipitates were obtained by decantation and washed with 75% ethanol. Finally, the RNA pellets were dissolved in RNase-free water, and preserved at -20°C until used. Using the extracted RNA as a template, reverse transcription reactions were conducted with an RT-PCR kit (RNA-PCR kit ver 2.1, Takara Biomedicals, Japan) to synthesize cDNA. Quantitative PCR was then conducted using osteopontin (forward: GTG ATT TGC TTT TGC CTG TTT G, reverse: TGA GCT GCC AGA ATC AGT CAC T), and GAPDH (forward: TGC CCA GAA CAT CAT CCC TG, reverse: TCA GAT CCA CGA CGG ACA CA) primers with a LightCyclerR using the double-stranded DNA dye SYBR Green I (Roche Diagnostics, Germany). Quantification was performed by comparing the levels obtained with standard samples. In the present study, the concentrations of cDNA in the unstimulated samples were 0.2, 0.5, 1.0, and 2.0 μl . The PCR conditions used in the LightCycler® were 40 cycles. After the PCR amplification, a melting curve analysis was also performed to confirm the absence of the primer dimer in the PCR products. The ratios of osteopontin mRNA expression were adjusted by the value of the housekeeping gene GAPDH. Four independent experiments were performed for all assays of cell behavior; two specimens of each sample type were used in each experiment. Statistical analysis was performed using t-test ($p < .01$).

Fig. 1 Experimental schedule

RESULTS

Fig. 2 shows the changes in medium temperature during the laser irradiation. The temperature of the medium increased in proportion to the irradiation time.

In the cell proliferation assay, the numbers of

Fig. 2 Relationship between the temperature of the medium and the duration of laser irradiation. The distance from the fiber tip to the culture plate is 50 mm, and the output power is 10 watts (effective power of approximately 6 watts).

cells increased in the proliferating phase with or without laser irradiation (Fig. 3a). The numbers of cells 3 and 5 days after laser irradiation were significantly higher than were those of the control group. In the differentiating phase, the numbers of cells also increased with or without the laser irradiation by day 5 of culture, but the rate of increase was not as great as that during the proliferating phase (Fig. 3b).

The ALP activity of the cells in the proliferating phase was elevated after 5 days with or without laser irradiation, and there was no difference between the groups (Fig. 4a). In the differentiating phase, however, the ALP activity of the cells increased day by day. Further, the ALP activity of the differentiating cells 5 days after the laser irradiation was significantly greater than that of the control group (Fig. 4b).

In the proliferation phase, although the osteopontin mRNA levels increased until day 3 of culture and then decreased in both groups, the degree of de-

crease in osteopontin mRNA expression on day 5 was small in the experimental group (Fig. 5a). In the differentiating phase, osteopontin mRNA expression increased on day 1 in both groups. After 3 to 5 days of culture, osteopontin mRNA expression in the control group decreased remarkably, but its expression in the laser irradiation group remained constant (Fig. 5b), and the differences between the irradiation and control groups was statistically significant.

DISCUSSION

Diode lasers are one type of low-energy laser, which have been used for wound healing (19, 24), improving skin flap survival (5), bone regeneration (18), and for accelerated intracellular metabolism (1, 12, 20). Various biostimulatory effects of low-energy laser irradiation have been reported that involve the acceleration of bone regeneration by laser treatment (16). There are many reports that low-level laser therapy is an important agent affecting biological re-

Fig. 3 Cell proliferation rate. a: proliferating phase of osteoblasts, b: differentiating phase of osteoblasts. *: significant difference ($p < .01$)

Fig. 4 Alkaline phosphatase activity. a: proliferating phase of osteoblasts, b: differentiating phase of osteoblasts. *: significant difference ($p < .01$)

Fig. 5 Quantitative analysis of osteopontin mRNA. a: proliferating phase of osteoblasts, b: differentiating phase of osteoblasts. Data are shown as fold expressions of the corresponding values at day 0 just before the laser irradiation. *: significant difference ($p < .01$)

sponses and accelerating bone formation (13, 14, 21). Furthermore, Dortbudak *et al.* reported the effects of a low-power laser on bony implant sites, and obtained positive effects on the integration of bony implants (3).

The basal characteristics of this diode laser system have been published in a previous report (8). To further evaluate the basal characteristics of this laser, the relationship between laser irradiation time and temperature of the medium was measured. It is known that medium temperature affects the cell behavior, so this is the reason the decision of 60 seconds of laser irradiation.

The different behaviors of osteoblasts in the proliferating phase and in the differentiating phase are well known (2, 6, 7, 17). Quarles *et al.* reported that cells displayed a time-dependent increase in ALP activity that was independent of the initial plating density (17). In addition, Katagiri *et al.* designated the behavior of osteoblasts at a low cell density as the

differentiation phase and at a high cell density as the proliferating phase (6). In other words, the proliferation of osteoblasts is predominant following differentiation in the early stages of the healing of bone wounds. In contrast, the development of the osteoblast function occurs in the middle to late stages after the cells have proliferated. Ozawa *et al.* studied osteoblasts treated with low-energy lasers and concluded that laser irradiation plays an important role in both cellular proliferation and cellular differentiation (16). In this study, after 3 to 5 days of culture osteopontin mRNA expression in the control group decreased remarkably. This result was the anticipated because the protein secretes following the expression of mRNA. Further, the degree of decrease in osteopontin mRNA expression on day 5 was small in the experimental group. This result means that laser irradiation keeps the osteoblast's function in mRNA level, and supports Ozawa's report (16). This study investigated the effects of a low-energy

laser on osteoblasts distinguished between the proliferating phase and the differentiating phase. The numbers of cells 3 days after laser irradiation were higher than those in the control group in the proliferating phase, but the numbers of cells with and without laser irradiation after 3 days were not different in the differentiating phase. Furthermore, although the ALP activity and osteopontin mRNA expression of cells with and without laser irradiation in the proliferating phase were not different, there were a significantly higher number of cells in the laser irradiated group than in the control group in the differentiating phase. Thus, our data suggest that low-energy lasers are effective in inducing osteoblasts to proliferate in the proliferating phase and to differentiate in the differentiating phase.

Acknowledgment

This study was supported by the Oral Health Science Center HRC5A03, Tokyo Dental College.

REFERENCES

- Atrang R and Carmichael A (1988) Soft lasers- have they a place in dentistry? *Br Dent J* **165**, 221–225.
- Beck GR Jr, Sullivan EC, Moran E and Zerler B (1998) Relationship between alkaline phosphatase levels, osteopontin expression, and mineralization in differentiating MC3T3-E1 osteoblasts. *J Cell Biochem* **68**, 269–280.
- Dortbudak O, Haas R and Mailath-Pokorny G (2002) Effect of low-power laser irradiation on bony implant sites. *Clin Oral Implants Res* **13**, 288–292.
- Ikumoto H, Matsuzaka K, Inoue T, Uchiyama T and Yoshinari M (2003) The behavior of osteoblast-like cells on different crystal systems of calcium phosphate ceramics *in vitro*. *Biomater Res* **24**, 239–248.
- Kami T, Yoshimura Y, Nakajima T, Ohshiro T and Fujino T (1985) Effects of low-power diode lasers on flap survival. *Ann Plast Surg* **14**: 278–283.
- Katagiri T, Lee T, Takeshima H, Suda T, Tanaka H and Omura S (1990) Transforming growth factor- β modulates proliferation and differentiation of mouse clonal osteoblastic MC3T3-E1 cells depending on their maturation stages. *Bone Mineral* **11**, 285–293.
- Li IWS, Cheifetz S, MacCulloch CAG, Sampath KT and Sodek J (1996) Effect of osteogenic protein-1 (OP-1, BMP-7) on bone matrix protein expression by fetal rat calvarial cells are differentiation stage specific. *J Cell Physiol* **169**, 115–125.
- Matsuzaka K, Miyake N, Takahashi K, Ohta K, Hattori M, Muramatsu T, Sato T, Oda Y, Shimono M and Ishikawa T (2004) Transformation of hydroxyapatite surface characteristics during diode laser irradiation. *Biomater Res* **25**, 105–108.
- Matsuzaka K, Walboomers XF, de Ruijter JE and Jansen JA (1999) The effect of poly-L-lactic acid with parallel surface micro groove on osteoblast-like cells *in vitro*. *Biomaterials* **20**, 1293–1301.
- Matsuzaka K, Yoshinari M, Shimono M and Inoue T (2004) Effects of multi-grooved surfaces on osteoblast-like cells *in vitro*: scanning electron microscopic observation and mRNA expression of osteopontin and osteocalcin. *J Biomed Mat Res* **68**, 227–234.
- Matsuzaka K, Walboomers XF, Yoshinari M, Inoue T and Jansen JA (2003) The attachment and growth behavior of osteoblast-like cells on microtextured surfaces. *Biomaterials* **24**, 2711–2719.
- Mezawa S, Iwata K, Naito K and Kamogawa H (1988) The possible analgesic effect of soft-laser irradiation on heat nociceptors in the cat tongue. *Arch Oral Biol* **33**, 693–694.
- Nicola RA, Jorgetti V, Rigau J, Pacheco MT, dos Reis LM and Zangaro RA (2003) Effect of low-power GaAlAs laser (660 nm) on bone structure and cell activity: an experimental animal study. *Lasers Med Sci* **18**, 89–94.
- Ninomiya T, Miyamoto Y, Ito T, Yamashita A, Wakita M and Nishisaka T (2003) High-intensity pulsed laser irradiation accelerates bone formation in metaphyseal trabecular bone in rat femur. *J Bone Miner Metab* **21**, 67–73.
- Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, Kennedy MB, Pockwinse S, Lian JB and Stein GS (1990) Progressive development of the rat osteoblast phenotype *in vitro*: Reciprocal relationship in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. *J Cell Physiol* **143**, 420–430.
- Ozawa Y, Shimizu N, Kariya G and Abiko Y (1998) Low-energy laser irradiation stimulates bone nodule formation at early stages of cell culture in rat calvarial cells. *Bone* **22**, 347–354.
- Quarles LD, Yohay DA, Lever LW, Caton R and Wenstrup RJ (1992) Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: an *in vitro* model of osteoblast development. *J Bone Miner Res* **7**, 683–692.
- Saito S and Shimizu N (1997) Stimulatory effects of low-power laser irradiation on bone regeneration in midpalatal suture during expansion in the rat. *Am J Orthod Dentofacial Orthop* **111**, 525–532.
- Schindl A, Heinze G, Schindl M, Pernerstorfer-Schon H and Schindl L (2003) Systemic effects of low-intensity laser irradiation on skin microcirculation in patients with diabetic microangiopathy. *Microvasc Res* **64**, 240–246.
- Smith PW (1988) The soft laser: therapeutic tool or popular placebo? *Oral Surg Oral Med Oral Pathol* **66**, 654–658.
- Ueda Y and Shimizu N (2003) Effects of pulse frequency of low-level laser therapy on bone nodule formation in rat calvarial cells. *J Clin Laser Med Surg* **21**, 271–277.
- Ueda Y and Shimizu N (2001) Pulse irradiation of low-power laser stimulates bone nodule formation. *J Oral Sci* **43**, 55–60.
- Ueno T, Kagawa T, Kanou M, Fujii T, Fukunaga J, Mizukawa N, Sugahara T and Yamamoto T (2003) Immunohistochemical observations of cellular differentiation and proliferation in endochondral bone formation from grafted periosteum: expression and localization of BMP-2 and -4 in the grafted periosteum. *J Craniomaxillofac Surg* **31**, 356–361.
- Webb C and Dyson M (2003) The effect of 880 nm low level laser energy on human fibroblast cell numbers: a possible role in hypertrophic wound healing. *J Photochem Photobiol B* **70**, 39–44.
- Whelan HT, Smits RL Jr, Buchman EV, Whelan NT, Turner SG, Margolis DA, Cevenini V, Stinson H, Ignatius R, Martin T, Cwiklinski J, Philippi AF, Graf WR, Hodgson B, Gould L, Kane M, Chen G and Caviness J (2001) Effect of NASA

- light-emitting diode irradiation on wound healing. *J Clin Laser Med Surg* **19**, 305–314.
26. Yamamoto M, Tamura K, Hiratsuka K and Abiko Y (2001) Stimulation of MCM3 gene expression in osteoblast by low level laser irradiation. *Lasers Med Sci* **16**, 213–217.