Effects of Low-level Hard Laser Irradiation on Mineralization in Three-dimensional Cultured Rat Bone Marrow Cells

Kazuhiro Gomi[a], Akihiro Yashima[b], Satoshi Shirakawa[b], Mikimoto Kanazashi[c], Kazuyuki Kobayashi[c], Takatoshi Nagano[c], Hiroyasu Yamagichi[c], Takashi Arai[d]

[a] *Associate Professor, Tsurumi University, School of Dental Medicine, Department of Periodontics and Endodontics, Yokohama, Japan.*
[b] *PhD Student, Tsurumi University, School of Dental Medicine, Department of Periodontics and Endodontics, Yokohama, Japan.*
[c] *Research Associate, Tsurumi University, School of Dental Medicine, Department of Periodontics and Endodontics, Yokohama, Japan.*
[d] *Professor, Tsurumi University, School of Dental Medicine, Department of Periodontics and Endodontics, Yokohama, Japan.*

Purpose: It is known that the low-power irradiation of a high-output laser accelerates wound healing. However, there is little fundamental research on it, especially for cultured osteoblasts. The purpose of this study was to evaluate the effect of low-level irradiation of a hard laser on cultured osteoblasts.

Materials and Methods: Osteoblasts cultured in collagen gel were irradiated using either a CO_2 or a semiconductor laser. Expressions of OC, BSP, and HSP27 were examined at 3 days after irradiation and calcium volumes were measured after 1 week.

Results: In the CO_2 laser group, a significant difference was only observed with 2.6-W laser irradiation. There was no promoting effect of semiconductor laser irradiation. Additionally, the highest expression of HSP27 was shown with 2.6 W of CO_2 laser irradiation.

Conclusion: The CO_2 laser at 2.6 W can increase the calcification of osteoblasts in three-dimensional cultures. The mechanism seems to be related to the cascade of HSP27.

Keywords: carbon dioxide laser, semiconductor laser, calcification, heat shock protein 27, osteoblast, low-level irradiation.

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Two types of laser – low-output (soft laser) and high-output (hard laser, class 4) – are used in the dental field. It has been shown that low-output laser can promote wound healing.[1,2] It is used in dentistry for that purpose and to support bone formation in cases of bone fracture and socket healing after extraction.[3-6] Especially with regard to the influence of the low-output laser on bony regeneration, many studies have been carried out by using cultivated cells or wound recovery models. These reports show that cell proliferation, ALP activity, or nodule formation of cultured osteoblasts were increased by the irradiation.[7-9] In addition, healing and ossification was accelerated by the irradiation of experimentally fractured bones of laboratory animals.[10-14]

On the other hand, because of the photobiodestructive reaction (PDR) that causes the vaporization of cells, high-output laser has been used for high-level

laser treatment, such as incision or ablation of lesions. However, the photobioactive reaction, which causes the proliferation and differentiation of cells, was found to occur as a result of using a high-output laser at a low-power setting. Low-level laser treatment (LLLT), which uses a low-level output of a high-output laser and a shortened exposure time, is often used today to accelerate healing.[15]

It has been reported that the carbon dioxide (CO_2) laser is superior in terms of healing acceleration compared with other lasers, such as YAG laser and Er laser.[16-19] Tang[20] reported that healing of artificial bone fracture and calcification was accelerated by using low-power irradiation of CO_2 laser. Tajima[21] showed that bone regeneration begins early in the bone marrow by irradiating rat tibias with a CO_2 laser. In addition, he showed that 1/2 extracellular signal-regulated kinase (ERK1/2), which was one of the mitogen-activated protein kinases (MAPK), phosphorylated and activated around the tissue that was irradiated with a CO_2 laser, suggesting the potency of cell proliferation that is promoted by the irradiation.

However, there are few reports about influences on cultivated cells by low-power irradiation of a high-output laser,[23] in particular, published studies on osteoblasts in this context seem to be lacking. There is also little information on heat shock protein caused by high-output laser radiation.[24,25] In order to protect organisms from various stresses, the heat shock protein appears and, for example, raises the temperature by ca 13 to 19°C as reported by Sugata.[24]

The purpose of this study was to determine the effect of low-level laser irradiation of a high-power laser on osteoblasts, which has not been examined before. We used a CO_2 laser and a semiconductor laser as the high-output laser for this study. We examined gene expression of osteocalcin (OC), bone sialoprotein (BSP) which is dependent on calcium dosage and is a marker to differentiate osteoblasts, and heat shock protein 27 (HSP 27),[26] which is a typical heat shock protein developed in osteoblasts. Moreover, we used a three-dimensional culture system of rat bone marrow cells to simulate the in vivo condition, because the results of laser treatment are chiefly manifest in tissues.

MATERIALS AND METHODS

Laser devices

We used an Opelaser 03S (Yoshida Dental Trade Distribution, Tokyo, Japan) as the CO_2 laser and light surge 3000 (Osada Electric, Tokyo, Japan) as the high-output semiconductor laser in this experiment. The Opelaser 03S is a CO_2 gas laser, with an oscillatory wavelength of 10.6 μm and continuous oscillation. The light surge 3000 is a GaAlAs laser with an oscillatory wavelength of 810 nm and continuous oscillation.

Cell collection

The bone marrow cells were obtained from the femur of a Wistar rat (male, 125 g BW) according to the methods of Maniatopoulos.[27] Briefly, femora were excised and washed 4 times in alpha-minimal essential medium (α-MEM) that contained 1 mg/ml penicillin G, 500 μg/ml gentamicin. Epiphyses were removed and the marrow from each diaphysis was flashed out with 15 ml supplemented α-MEM containing one-tenth of the concentration of antibiotics (see above), 10% fetal bovine serum, freshly added 50 μg/ml ascorbic acid, and 10 mM Na-β-glycerophosphate. Bone marrow cell suspension was incubated in a CO_2 incubator (95% air, 5% CO_2) for one week, and re-fed three times. The first passage cells were used for this experiment.

Three-dimensional culture

A collagen culture medium of 0.21% gel was made, consisting of α-MEM, fetal bovine serum, antibiotics, ascorbic acid, Na-β-glycerophosphate, and dexamethasone, which is the same concentration of liquid culture medium described above using 0.3% collagen liquor (cellgen I-PC, KOKEN, Tokyo, Japan). Bone marrow cells were collected using 0.1% trypsin (Sigma, St Louis, MO, USA) and adjusted to 1.5×10^5 cells/ml in the collagen culture medium. One ml of collagen gel cell suspension was distributed into 24-well dishes (2 such dishes per experiment), and incubated in a CO_2 incubator for 12 h. After gelation was confirmed, 1.5 ml of FS-α-MEM was added to the gels. The liquid medium was changed 3 times a week.

Laser irradiation

Three days after gelation, the medium was aspirated off and laser radiation was performed. The irradiation parameters for each laser are shown below.

CO_2 laser group: The distance from the bottom of the well dishes to the tip of laser was 300 mm, irradiation diameter was 10.3 mm, and radiation time was

Table 1 PCR oligonucleotide primers and predicted size of PCR products

OC	sense:	5' CATATGCTCAACAATGGACTT 3'	
	Antisense:	5' CTAAACGGTGGTGCCATAGAT 3'	(size 151 bp)
BSP	sense:	5' TGTCTCAGAGATCCGACAGAC 3'	
	Antisense:	5' ACCGAGAGATGTAGCCATGTT 3'	(size 158 bp)
HSP27	sense:	5' GGAGACTTCAAATGAAGAGGA 3'	
	Antisense:	5' TCCTCGTCGCTTTCCTTCATT 3'	(size 216bp)

20 s. Five wells were irradiated under the conditions of 2.0, 2.2, 2.4, 2.6, 2.8, and 3.0 W. The power density was 2.40, 2.64, 2.88, 3.12, 3.36, 3.60 W/cm^2, respectively.

Semiconductor laser group: The distance from the bottom of the well dishes to the tip of laser was 12 mm, irradiation diameter was 14.4 mm, and radiation time was 20 s. Five wells were irradiated under the conditions of 0.1, 0.3, 0.5, 0.8, 1.0, 2.0, and 3.0 W. The power density was 0.06, 0.18, 0.31, 0.49, 0.61, 1.22, 1.83 W/cm^2, respectively.

Control group: The medium was aspirated from 24-well dishes which were then left on a clean laboratory bench during the irradiation of the test groups.

Calcium measurement

One week after laser irradiation, collagen gels were dissolved by 0.5 ml of 0.2% collagenase (WAKO, Osaka, Japan) with 4 h of incubation. Samples were assessed using a calcium C test Waco kit (WAKO).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using RNAzol B (Tel-Test, TX, USA) from the rat bone marrow cells that were irradiated after 3 days. Single strand cDNA was synthesized from 3 μg of the total RNA using an oligo-dT primer and the You-primed First-Strand Beads kit (Amersham-Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions. PCR primers were designed based upon rat and mouse mRNA sequences (Table 1). A primer set amplifying glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Clontech, Palo Alto, CA, USA) was used as a control. PCR conditions (Perkin-Elmer/GeneAmp PCR system 9700) were as follows: PCR started with a 5-min denaturation at 94°C, followed by 30 cycles with denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and product extension at 72°C for 30 s; in the final cycle, the 72°C extension lasted 10 min. PCR products were analyzed by 4.5% polyacrylamide gel electrophoresis in TBE buffer (pH 8.0). Gels were stained with ethidium bromide, and the bands were visualized under UV light.

RESULTS

A morphological alteration of gel culture media was not found after semiconductor laser or CO_2 laser irradiation.

In the CO_2 laser irradiated group, a higher calcium quantity was shown compared to the control at 2.0, 2.2, and 2.4 W, but a significant difference was not found. The formation of a significantly higher calcium quantity was found at the 2.6 W compared to the control group (Fig 1). However, the calcium formation significantly decreased at 2.8 and 3 W compared to the control. Power density of 2.6-W irradiation was 3.12 W/cm^2, and the applied dose rate per area was 62.4 J/cm^2. In the semiconductor laser irradiation group, the quantity of calcium decreased according to the increase in irradiation energy from 0.1 W (Fig 2). A significant difference was particularly evident between the control and semiconductor groups at 0.8, 1.0, 2.0, 3.0 W.

PCR was performed 3 days after laser irradiation. In the group treated with CO_2 laser, strong gene expres-

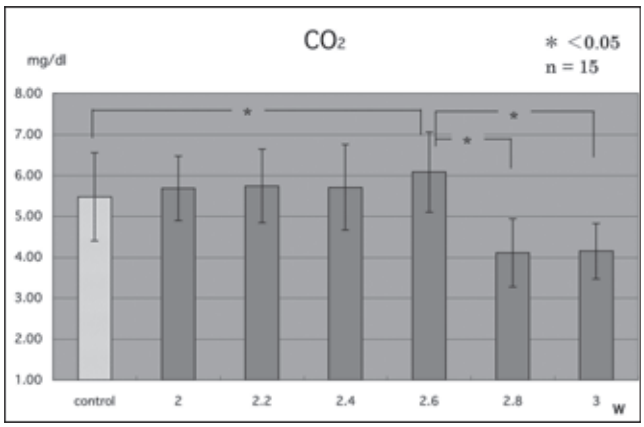

Fig 1 Calcium quantity formed in gel after irradiating with CO_2 laser. Higher calcium quantity was shown compared to the control at 2.0, 2.2, 2.4 W, but a significant difference was not found. The formation of a significantly higher calcium quantity was found at 2.6 W compared to the control group.

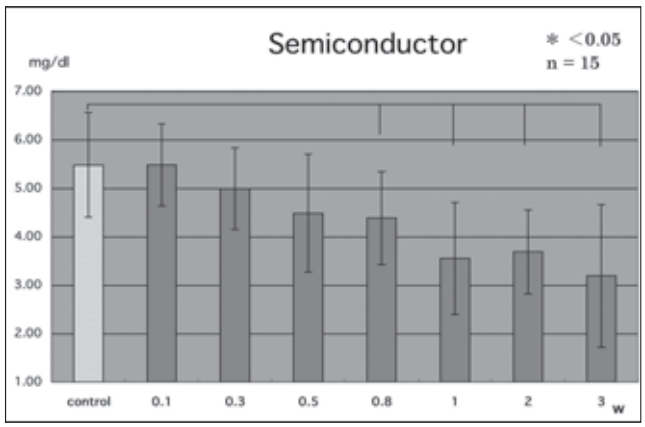

Fig 2 Calcium quantity formed in gel after irradiating with semiconductor laser. The quantity of calcium decreased according to the increase in irradiation energy from 0.1 W. A significant difference was particularly evident between the control and test groups at 0.8, 1.0, 2.0, 3.0 W.

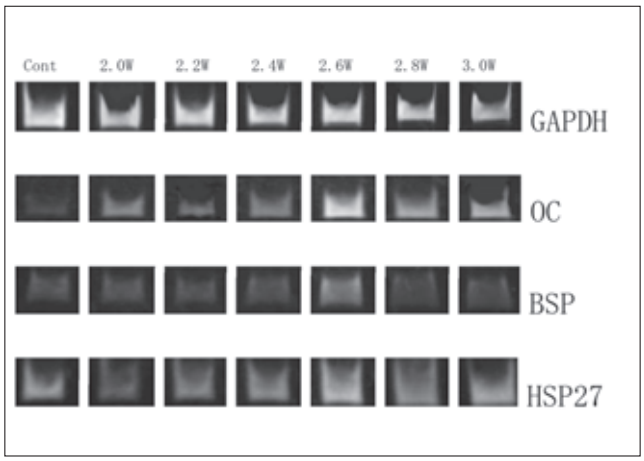

Fig 3 Detection of mRNAs encoding GAPDH, osteocalcin (OC), bone sialoprotein (BSP) and heat shock protein 27 (HSP 27) by RT-PCR in 4.5% polyacrylamide gel electrophoresis after irradiation with CO_2 laser. Strong gene expression of HSP27, BSP and OC were found at 2.6 W.

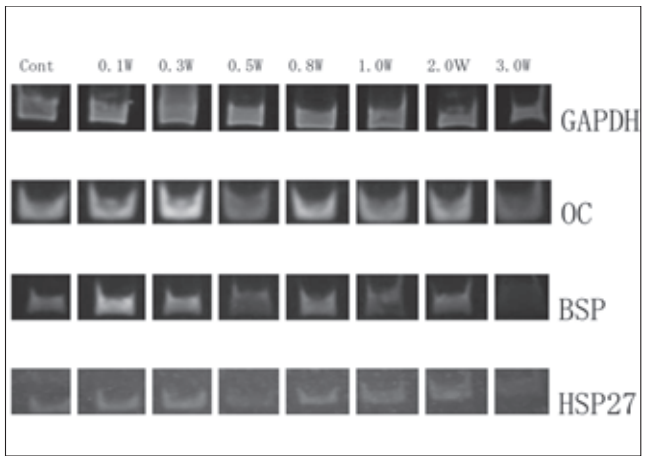

Fig 4 Detection of mRNAs encoding GAPDH, osteocalcin (OC), bone sialoprotein (BSP) and heat shock protein 27 (HSP 27) by RT-PCR in 4.5% polyacrylamide gel electrophoresis after irradiation with semiconductor laser. The gene expression of HSP27, BSP and OC showed a tendency to decrease as irradiation energy increased.

sion of HSP27, BSP and OC were found at 2.6 W with a considerable quantity of calcium (Fig 3). In the semiconductor laser group, however, the gene expression of HSP27, BSP, and OC showed a tendency to decrease as irradiation energy increased (Fig 4).

DISCUSSION

It is known that low-energy laser accelerates the healing of tissues[1,2] and enhances cellular functions. In particular, there are many studies about osteogenic cells.[7-9] However, there are few reports about the effects of low-power irradiation of a high-output laser on cultured cells. The PGE2 production of cultured fibroblasts when irradiated with a low power CO_2 laser has been studied,[23] but there is apparently little information on laser's influence on osteoblasts. However, it seems that calcification in cultured osteoblasts can be accelerated by low-power CO_2 laser irradiation, because it has been reported that bone formation was accelerated by this type of irradiation in rat tibias.[21] Until

now, ALPase activity has been examined mainly as an index of calcification, but ALPase activity does not measure calcification in itself. In addition, there are methods to evaluate calcification extent by measuring the number of bone nodules formed in plate culture, but these methods do not reflect calcification extent directly. Therefore, we evaluated the degree of calcification induced by laser radiation by measuring the quantity of calcium. Moreover, it seems that the laser showed the effect in tissue, so we used collagen gel to mimic the internal part of an organism and performed a simulation using 3-dimensional culture models. Furthermore, some authors have postulated that the action of a CO_2 laser resembles mechanical stress.[23] It is thought that by using a 3-dimensional culture model, it is easier to demonstrate the effect of a laser compared to using a 2-dimensional culture. Rat bone marrow cells were cultured in a 3-dimensional culture system of collagen gel, and low-power irradiation of a high-output laser was performed 3 days after gelation. We measured the quantity of calcium formed in the gel 1 week after laser irradiation, and evaluated the quantity of calcification. In the CO_2 laser irradiated group, there was a higher quantity of calcium compared to the control at 2.0, 2.2, 2.4 W, but a significant difference was not found. However, a significantly higher calcium quantity was found at 2.6 W compared to the control group. Conversely, the quantity of calcium significantly decreased at 2.8 and 3 W compared to the contol. These results indicate that CO_2 laser accelerates the calcification of osteoblasts, and it is thought that the optimum energy dosage is at 2.6 W. Moreover, our results suggested that the CO_2 laser caused damage to cells at more than 2.6 W. The PCR results, which showed the highest expression of OC and BSP at 2.6 W, also supported the results of the calcium examination.

In contrast, the semiconductor laser and control groups showed approximately equal quantities of calcium at 0.1 W. Whenever the quantity of energy increased, the quantity of calcium decreased. The expression of OC and BSP was the highest at 0.1 W, and expression decreased according to the increase in the quantity of energy. These results showed that low-power semiconductor laser irradiation did not accelerate calcification of osteoblasts, and that the increase of the exposure dose may inhibit the calcification.

With this experiment, we confirmed the acceleration of calcification, and also discovered a high expression of HSP27 using CO_2 laser radiation when an energy dosage of 2.6 W is used. Tajima et al[22] confirms that extracellular signal-regulated kinase 1/2 (ERK1/2), which is a mitogen-activated protein kinase (MAPK), was phosphorylated by CO_2 laser irradiation. It was activated in the skeletal muscle of a rat auricle, where the evaporation locus circumference obtained the effect of low-power irradiation by a laser. In addition, Kawamura[28] reports that HSP 27 influences p38 activity in osteoblasts. These reports show that laser irradiation is associated with the MAPK cascade, which is a factor of intracellular signal transduction, and promotes calcification. In this experiment, we showed that gene expression of HSP 27 was the highest at 2.6 W, which was also associated with the highest quantity of calcium in the CO_2 laser irradiation group. This agrees with another report, which showed that HSP 27 was highly expressed in bone-forming osteoblasts.[29]

These two findings lead us to postulate the following:

The calcification of an osteoblast was accelerated by laser irradiation, but not in the smad pathway through BMP. It was induced by the potency of the MAPK cascade through HSP 27. HSP27 is considered to activate p38 by inhibiting SB-203580, which is an inhibiting factor of p38, and to be associated with calcification. However, another system to activate ERK1/2, passing through MNK from p38 MAPK, may also exist. It is necessary to conduct further research on the acceleration pathway of calcification by laser irradiation in the future.

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Contact address: Kazuhiro Gomi, Tsurumi University, School of Dental Medicine, Department of Periodontics and Endodontics, 2-1-3 Tsurumi, Tsurumi-ku, Yokohama, 230-8501, Japan. Tel: +81-45-581-1001, Fax: +81-45-583-8401. e-mail: gomi-k@tsurumi-u.ac.jp