

Low-Intensity Laser Irradiation Stimulates Bone Nodule Formation Via Insulin-Like Growth Factor-I Expression in Rat Calvarial Cells

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Background and Objective: We previously reported that low-intensity laser irradiation stimulated bone nodule formation through enhanced cellular proliferation and differentiation. However, the mechanisms of irradiation are unclear. Thus, we attempted to determine the responsibility of insulin-like growth factor (IGF)-I for the action observed.

Study Design/Materials and Methods: Osteoblast-like cells were isolated from fetal rat calvariae and cultured with rat recombinant (r) IGF-I, IGF-I-antibody (Ab), and/or the cells were irradiated once (3.75 J/cm²) with a low-intensity Ga-Al-As laser (830 nm). The number and area of bone nodules formed in the culture were analyzed, and IGF-I expression was also examined.

Results: Treatment with rIGF-I significantly stimulated the number and area of bone nodules. This stimulatory effect was quite similar to those by laser irradiation, and this stimulation was abrogated dose-dependently by treatment with IGF-I-Ab. Moreover, laser irradiation significantly increased IGF-I protein and gene expression.

Conclusion: The stimulatory effect of bone nodule formation by low-intensity laser irradiation will be at least partly mediated by IGF-I expression. *Lasers Surg. Med.* 39:551–559, 2007. © 2007 Wiley-Liss, Inc.

Key words: low-intensity laser; bone formation; IGF-I; bone nodule; calvarial cell; osteoblast

INTRODUCTION

The acceleration of bone regeneration by low-intensity laser treatment has been the focus of contemporary research [1,2,3,4,5] as it may hold great potential benefit for clinical therapy in orthopedics and dentistry. However, since the stimulatory mechanisms involved with the action of laser irradiation on bone are not fully elucidated, laser therapy is often carried out haphazardly in clinical use and may not always be effectively applied. Thus, it is necessary to elucidate the mechanisms involved. It has been reported that the particular properties of laser irradiation do not

act directly on osteosynthesis, but rather create a series of environmental conditions that accelerate the healing of bones [4]. However, in recent investigations using an *in vitro* bone nodule formation assay system with fetal rat calvarial cell [6,7] we found that low-intensity laser irradiation significantly stimulated bone formation via acceleration of cellular proliferation and differentiation, suggesting direct stimulatory effects on bone formation by laser irradiation. Osteoblast-like cells derived from fetal rat calvariae are known to produce osteoblast differentiation factors [8,9,10], and these factors may be presumed to act as autocrine or paracrine stimulators with these cells. Among them, insulin-like growth factor (IGF)-I is known to be a potent stimulator of bone formation *in vivo* [11,12,13] and *in vitro* [14,15,16], therefore, it is likely that laser treatment stimulates bone formation via IGF-I expression. To determine the mechanisms responsible for the action of laser irradiation, we investigated the effects on bone formation and IGF-I expression using osteoblast-like cells derived from rat fetal calvariae.

MATERIALS AND METHODS

Fetal Rat Calvarial Cell Isolation and Culture Procedures

The procedures used for osteogenic cell isolation and culture have been described by Bellows et al. [17,18]. Briefly, calvariae were dissected aseptically from 21-day-old fetuses of timed pregnant Wistar rats and adherent soft

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connective tissues were gently removed. The calvariae were minced and sequentially digested in a collagenase mixture. Five samples were obtained after digestion times of 10, 20, 30, 50, and 70 minutes, respectively. Cells retrieved from the last four steps of the five-step digestion sequence were pooled and plated in T-75 tissue culture flasks (Falcon 3110, Franklin, NJ) in α -minimal essential medium (α -MEM; Gibco, Grand Island, NY) containing 15% fetal calf serum and antibiotics comprising 100 mg/ml penicillin G (Sigma Chemical Co., St. Louis, MO), 50 mg/ml gentamicin sulfate (Sigma), and 0.3 mg/ml fungisone (Flow Laboratories, McLean, VA), supplemented with 50 mg/ml ascorbic acid (Wako, Osaka, Japan) and 10 mM Na β -glycerol-phosphate (β -GP, Wako). Cultures were maintained in a humidified atmosphere consisting of 95% air/5% CO₂ at 37°C. After 24 hours of incubation, attached cells were washed with PBS to remove the non-viable cells and debris, trypsinized with 0.05% trypsin (Gibco) in phosphate-buffered saline (PBS), and counted using a Coulter Counter (Model ZM, Electronics Ltd., Northwell drive, Luton, Beds, England). For quantitation of bone nodules, the cells were resuspended in the culture medium described above and then plated in 35-mm tissue culture dishes (Falcon) at a density of 5×10^4 cells/dish (5.2×10^3 cells/cm²). The cells were also plated in 24-well plates (Corning Inc., Corning, NY) for the IGF-I assay and 4-well Lab-Tek chamber slides (Nunc, Inc., Naperville, IL) for immunohistochemistry at the same density as described above. Medium was changed every 3 days and cultures were maintained for up to 24 days.

Procedure of Laser Irradiation

A low-intensity Ga-Al-As diode laser apparatus (model Panalas-1000, Matsushita, Inc., Osaka, Japan) with a continuous wavelength of 830 nm (maximum power output of 500 mW) was used in this study. The laser beam was delivered by an optical fiber 0.6-mm in diameter that was defocused at the tip of the fiber by a concave lens allowing for uniform irradiation in a 100 mm in diameter circular area at the cell-layer level. The power density of the laser beam was monitored with a laser power meter, and irradiation was performed at 550 mm above the cell layer. In this manner, 3 of the 35 mm-dishes, 16 wells in a square in a 24-well plate, or 4 well Lab-Tek chamber slides were irradiated simultaneously on a clean bench. The total energy corresponding to a 10-minute exposure was 3.82 J/cm², as this dose has been reported to stimulate a number of bone nodules from rat calvarial cells using an experimental model similar to the one used in the present study [7]. Laser irradiation was performed only once, 3 days after subculture at the subconfluent stage (Day 3) and just after the medium had been changed. Control dishes, plates, and chamber slides were placed on a clean bench for 10 minutes without any irradiation on Day 3.

Treatment with rIGF-I, IGF-I-Ab, and Laser Irradiation

To determine whether the stimulatory effect of bone nodule formation by laser irradiation is mediated by IGF-I,

several concentrations of recombinant (r)-rat IGF-I (0.1, 1, 10, 100 nM; GroPep Pty.Ltd., Adelaide, Australia) and anti-human IGF-I-antibody (Ab) (0.01, 0.1, 1, 10 μ g/ml; Upstate Biotechnology Inc., Lake Placid, NY) were added to separate cultures for 6 days (Days 3–9) after the cells had reached the subconfluent stage. We also investigated whether the neutralization of bone nodule formation was dependent on the specific reaction of IGF-I-Ab protein or not, IgG (10 μ g/ml) (Upstate Biotechnology) protein was added to the culture. Laser irradiation was performed once on Day 3 immediately after the medium was replaced with new medium with or without IGF-I-Ab. The concentration of rIGF-I was determined by the method of previous studies, in which rIGF-I stimulated bone and bone matrix formation [14,15,16].

After the calvarial cell cultures were maintained for 24 days *in vitro*, the contents of each well were fixed for 10 minutes in 4% paraformaldehyde in PBS and stained using the von Kossa technique. The number of bone nodules present in each 35-mm culture dish was counted at 40 \times magnification using a dissecting microscope (Olympus, Tokyo, Japan) by placing the culture dish on a transparent acetate grid that was ruled in 2 \times 3 mm rectangles, as described previously [6].

The areas of bone nodule formation were also evaluated according to the method of our previous study [6]. Briefly, bone nodules were photographed at 100 \times magnification using a dissecting microscope (Olympus, Tokyo, Japan). The outline of each bone nodule was then traced on the photographs and the traces were measured using image analysis software (Ultimage Ver. 2.0, Graftek France, Voisins-Le-Bretonneux, France). Total bone nodule area was determined by adding each area in a dish and the mean area was calculated by dividing the total area by the number of nodules in each dish. Mean \pm standard deviation (SD) results of both total area and mean area were calculated from four replicate measurements.

Assay for IGF-I and PGE₂ Production

The amount of IGF-I released into culture medium was measured on Days 3, 6, 9, 12, 15, and 18, in duplicate, by a radioimmunoassay (RIA) using a commercially available kit with a [¹²⁵I]IGF-I assay system, employing anti-human IGF-I-specific antibody (Amersham, Arlington Heights, IL). This system was previously used for measuring rat IGF-I expression [19]. The test sample in a tube was mixed with antiserum and incubated for 4 hours at room temperature. [¹²⁵I]IGF-I was then placed into the tube with a pipette and the mixture was incubated for 24 hours at 4°C. After incubation for 10 minutes with the secondary antibody, the antibody-bound fraction was separated by magnetic separation. The radioactivity present in each tube was then determined by using a gamma scintillation counter (Aloka, Tokyo, Japan).

The amount of PGE₂ in culture medium was measured similarly on Days 3, 6, and 9 with a commercially available kit utilizing [¹²⁵I]-PGE₂ as the tracer (Amersham, Arlington Heights, IL). The cross-reactivity of the antibody at 50% B/Bo with PG was previously determined as PGE₂ = 100%;

PGE₁ = 5%; and 8-iso-PGE₂ = 62%. Cross reactivity with PGD₂, PGF₂, 6-keto-PGE₂, 6-keto-PGF₁, TXB₂, and arachidonic acid was less than 0.001%. (Inter- and intra-assay coefficients of variation for the PGE₂ assay used in these experiments were 10.0 and 6.8, respectively).

Cells growing in 24-well plates were collected by digestion with a 1:1 mixture of trypsin (0.05%) and collagenase (0.1%) solutions for up to 25 minutes to release the cells from the collagenous matrix. The number of cells in each well was determined using a Coulter Counter.

Immunocytochemistry for IGF-I

Calvarial cells cultured on 4-well Lab-Tek chamber slides (Nunc, Inc.) with or without laser treatment were fixed with 4% paraformaldehyde in PBS on Days 5 and 8. After blocking any non-specific reactions, the cells were incubated with anti-human mouse IGF-I-Ab (10 µg/ml, Upstate Biotechnology Inc., Lake Placid, NY) overnight, followed by washing with PBS. After incubations with the secondary-Ab (rhodamine conjugated goat anti-mouse IgG ×100) for 90 minutes, the expression of IGF-I was observed using a confocal laser scanning microscope (model: TCS4D, Leica, Germany). As a negative control, cells were also incubated with normal goat serum instead of the primary antibody.

RNA Preparation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA in the cells with or without laser irradiation treatment was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction [20] on Days 4, 6, and 9. cDNA synthesis and amplification by RT-PCR were carried out using a GeneAmp RNA kit (Perkin Elmer, NJ). Two micrograms of total RNA were incubated in the reaction mixture for reverse transcription (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM MgCl₂, 1 mM dNTPs, 2.5 mM oligo d(T)16 and 2.5 random hexamer, 1 unit/ml RNase inhibitor, and 2.5 unit/ml MuLV reverse transcriptase in a total volume of 20 ml) for 15 minutes at 42°C, and then the reaction was stopped by heating at 95°C for 5 minutes. The cDNA was then amplified using a PCR method, with 0.2 mM of each up and down primer used for the reaction. Further, the concentration of magnesium chloride was changed to 2 mM for PCR. The PCR primers for amplification were designed referring to the sequences of cDNA that have been reported for IGF-I and GAPDH [21,22]. The following primer sequences with the size of the amplified fragment in brackets were used: IGF-I [710bp], 5'-TGG TGG ACG CTC TTC AGT-3' and 5'-CGA TGT TTT GCA GGT TGC-3', and GAPDH [318bp] 5'-ATC ACC ATC TTC CAG GAG-3' and 5'-CTC ATG ACC ACA GTC CCA T-3'. To evaluate saturated or incomplete PCR reactions, the gene fragments were amplified simultaneously every 3 cycles from the 18th to 24th cycle at 94°C for 1 minutes, 55°C for 2 minutes and 72°C for 2 minutes. PCR fragments amplified by different cycles were electrophoresed on a 1.5% agarose gel, and subsequently stained with ethidium bromide. When the PCR products were sequenced to check

the validity of our PCR primers, each product had the same DNA sequence as reported previously (data not shown).

STATISTICAL METHODS

Values were calculated as the mean ± SD. Data were subjected to one-way analysis of variance (ANOVA) as indicated in the results. Student's-*t* test was used for analysis of the difference in some groups, and Tukey-Kramer's test was used for analysis of the difference among the various group tested.

RESULTS

Effect of IGF-I, IGF-I-Ab, and Laser Irradiation on Bone Nodule Formation

The kinetics of stimulation or inhibition were investigated by treating cultures with IGF-I, IGF-I-Ab, laser irradiation, and irradiation plus IGF-I-Ab. Well-delineated three-dimensional nodular mineralized tissue structures were formed by rat calvarial cells in all four groups. However, the number and size of bone nodules formed were found to be higher in the rIGF-I treatment and laser irradiation groups as compared to the control and IGF-I-Ab treatment groups (Fig. 1). When the number of bone nodules present in 4 replicate 35-mm dishes was quantitated (Fig. 2), significant stimulation was caused by rIGF-I treatment for 6 days (maximum 1.4-fold, *P*<0.001) as compared to the control, in a dose-dependent manner (*P*<0.001, by one-way ANOVA). In contrast, the number of nodules was significantly decreased by treatment with IGF-I-Ab (0.85-fold, *P*<0.001) in a dose-dependent manner (*P*<0.001, by one-way ANOVA). With laser irradiation, the number of nodules markedly increased (1.4-fold, *P*<0.001) and was nearly equal to the highest concentration of rIGF-I treatment (100 mM). However, this stimulatory effect was significantly diminished by IGF-I-Ab treatment in a dose-dependent manner (*P*<0.001, by one-way ANOVA), and it was completely removed by treatment with 1 µg/ml IGF-I-Ab. IgG treatment as the control of IGF-I-Ab protein, did not affect the number of bone nodules.

When total bone nodule areas were compared (Fig. 3A), they were found to be significantly larger in the rIGF-I treatment and laser irradiation groups (2.1-, 2.0-fold, *P*<0.001) as compared to the control, however, the increased area in the laser irradiation group was significantly diminished by IGF-I-Ab treatment (0.49-fold, *P*<0.001). The mean area of bone nodules in each group was similar to the total area (Fig. 3B). It was found to be significantly larger in the rIGF treatment and laser irradiation groups (1.5-, 1.4-fold, *P*<0.001) as compared to the control, however, the increased mean area in the laser irradiation group was significantly diminished by IGF-I-Ab treatment (0.49-fold, *P*<0.001), but IgG or IGF-I-Ab treatment did not affect the area of bone nodules.

Effect of Laser Irradiation on IGF-I and PGE₂ Production

The time course of the effects of laser irradiation on IGF-I production by calvarial cells is shown in Figure 4. IGF-I was constitutively expressed in the conditioned medium. Laser

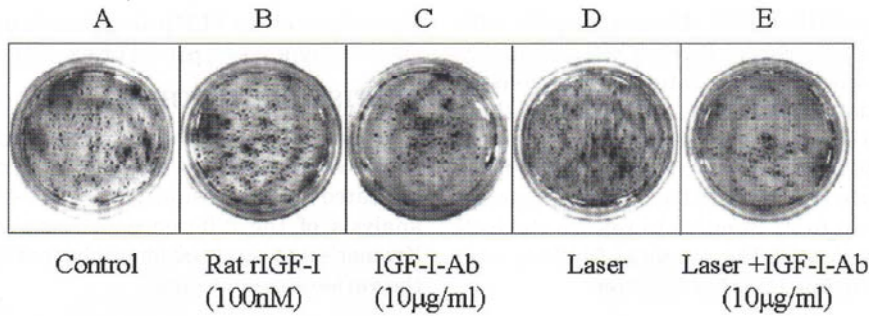


Fig. 1. Bone nodule formation in each treatment group. Three-dimensional mineralized tissue nodular structures formed on Day 24 in fetal rat calvarial cell cultures. rIGF-I (100 nM) (B) and laser irradiation (D) treatments induced a higher quantity and larger nodule formation as compared to the control (A). In contrast, IGF-I-Ab treatment (10 µg/ml) (C) and laser irradiation plus IGF-I-Ab treatment (10 µg/ml) (E) induced less nodule formation as compared to the rIGF-I treatment and laser irradiation groups.

irradiation significantly stimulated IGF-I production on Days 6 (1.5-fold, $P < 0.01$) and 9 (1.3-fold, $P < 0.05$) as compared to the corresponding controls (A). When IGF-I production was expressed as ng per 10^5 cells (B), laser irradiation sustained IGF-I production, which decreased spontaneously in the control, and a significant difference was observed on Day 6 (1.4-fold, $P < 0.01$).

PGE_2 was also constitutively expressed in the conditioned medium of the calvarial cell cultures on Day 3 in the control and laser groups (Fig. 5). However, it rapidly decreased on Days 6 and 9, and there was no difference in production between the control and laser groups.

Immunocytochemistry for IGF-I

The results of immunocytochemical staining for IGF-I in rat calvarial cells are shown in Figure 6. In both the control

and irradiation groups, IGF-I was expressed only in the center of the cell masses on Day 5, and laser irradiation seemed to increase the number of cell masses expressing IGF-I as compared to the control. No IGF-I fluorescence was observed in the negative control. The marked expression of IGF-I in the center of the cell masses was slightly decreased on Day 5 (data not shown).

Effect of Laser Irradiation on IGF-I Gene Expression

To elucidate the molecular mechanisms of stimulated bone formation by laser irradiation, IGF-I mRNA expression in both irradiated and non-irradiated cells was analyzed by RT-PCR (Fig. 7). Each band of 2 gene fragments was amplified simultaneously every 3 cycles and constantly increased as the number of cycles increased.

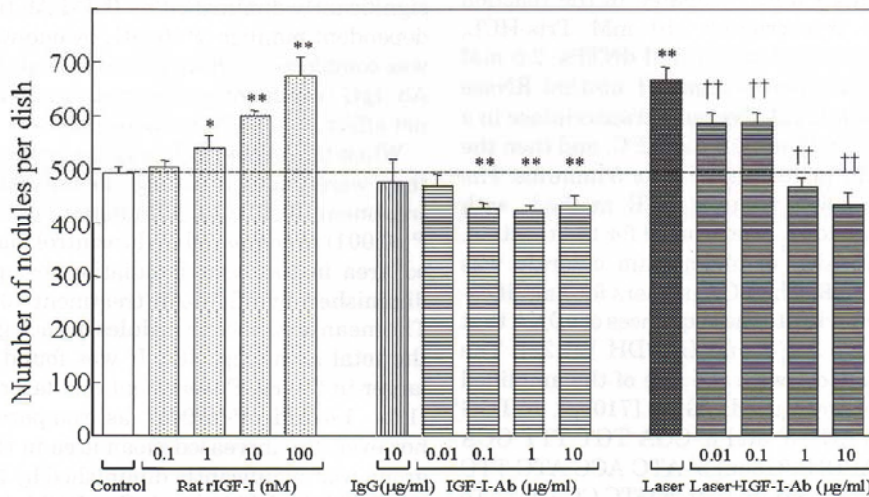


Fig. 2. Effects of rIGF-1, IGF-1-Ab, and laser irradiation on the number of bone nodules on Day 24. The number of bone nodules formed in each dish was significantly increased by rIGF-I treatment (0.1–100 nM), and significantly decreased by IGF-I-Ab treatment (0.01–10 µg/ml) as compared to the control. Laser irradiation (3.82 J/cm²) significantly increased the number of nodules to equal the maximum dose (100 nM) of

IGF-I treatment. The increase in nodule formation was significantly diminished by IGF-I-Ab treatment (0.01–10 µg/ml). IgG (10 µg/ml) did not affect bone nodule formation. Values are mean ± SD for 4 cultures. Significantly different from control: * $P < 0.01$, ** $P < 0.001$. Significantly different from laser irradiation group: †† $P < 0.001$. Similar results were obtained from two different experiments.

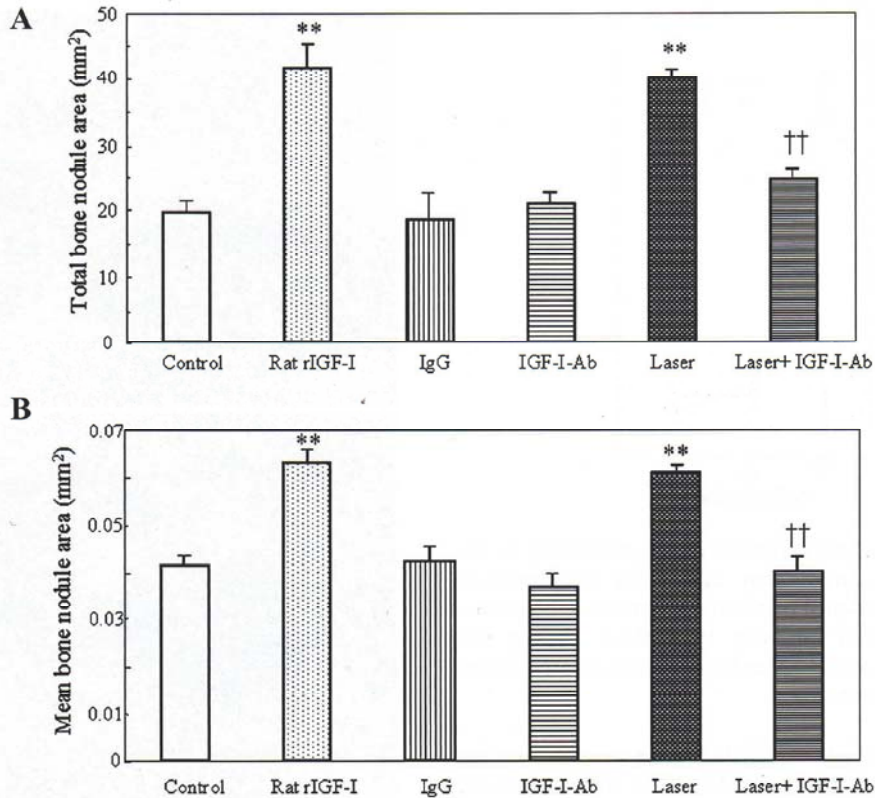


Fig. 3. Effects of rIGF-1, IGF-1-Ab, and laser irradiation on the area of bone nodules. The total bone nodule areas were significantly larger in the rIGF-I treatment (100 nM) and laser irradiation groups as compared to the control, however, the area in the laser treatment group was significantly reduced to the level of the control after IGF-I-Ab treatment (10 µg/ml) (A).

The size of each bone nodule (mean bone nodule area) showed similar results to those of the total bone nodule area (B). Values are mean ± SD for four cultures. Significantly different from control: ***P*<0.001. Significantly different from laser irradiation group: ††*P*<0.001.

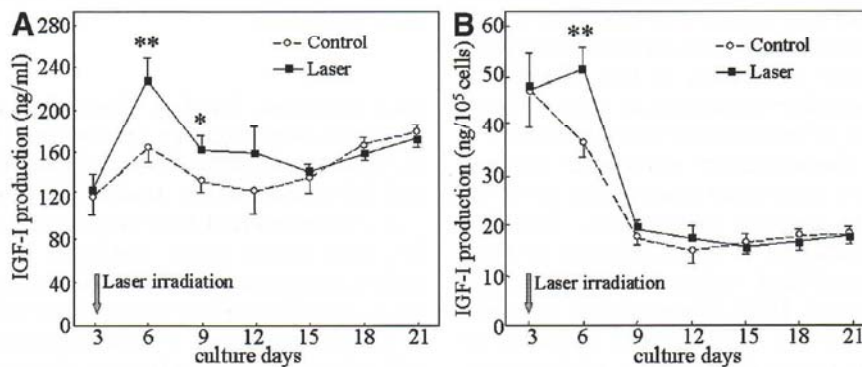


Fig. 4. Effect of laser irradiation on IGF-I production in rat calvarial cells. IGF-I production was markedly stimulated soon after laser irradiation (A). When the production was expressed as per 10⁵ cells (B), laser irradiation sustained IGF-I production until Day 6, whereas it was significantly

decreased in the control. Values are mean ± SD for 4 cultures. Significantly different from corresponding control: **P*<0.05, ***P*<0.01. Similar results were obtained from two different experiments.

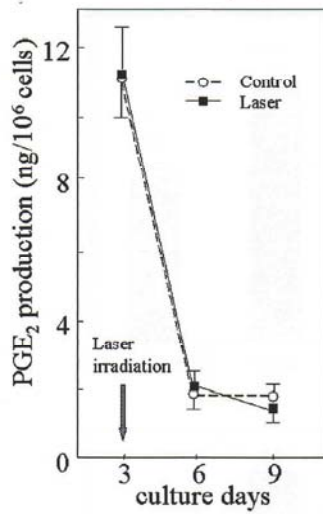


Fig. 5. Effect of laser irradiation on PGE₂ production in rat calvarial cells. PGE₂ production was found constitutively expressed in the conditioned medium of calvarial cell cultures that were not affected by laser irradiation. Values are mean \pm SD for 4 cultures. Similar results were obtained from two different experiments.

Thus, we considered that the PCR reactions were neither saturated nor incomplete. Bands for IGF-I mRNA in both irradiated and non-irradiated cells were visible on the 18th cycle, while IGF-I gene expression in the irradiated cells was more intense than that in the control cells on Day 4. The intensity of gene expression in both irradiated and control cells was decreased on Day 6, though with a slight difference, and there was no difference on Day 9.

DISCUSSION

The stimulatory effects of low-intensity laser irradiation on bone formation have been reported from based on non-quantitative observations [3,4]. Recently, some studies [23,24] have used biochemical and quantitative histomorphometrical methods in *in vivo* rat osteotomy experiments and found that low-intensity laser irradiation caused a twofold enhancement of bone repair with increases in ALP activity and calcium accumulation. We have also noted [25] that low-intensity laser irradiation to the mid-palatal suture during rapid maxillary expansion in rats produced a 1.4-fold enhancement of bone regeneration in the area of the suture. However, the regulatory mechanism involved with low-intensity laser irradiation toward bone is not yet understood clearly. In *in vitro* experiments, Kusakari et al. [26] reported promotion of bone formation by laser irradiation, stimulation of DNA and protein synthesis, and elevation of ALP activity in UMR 106 osteoblast-like cells. We also reported stimulatory effects of bone formation by laser irradiation with increase in cellular proliferation, ALP activity, osteocalcin expression, and Ca accumulation using a quantitative bone nodule formation assay [6,7]. These results suggest that laser irradiation may stimulate both proliferation and differentiation of osteoblastic cells *in vivo* and *in vitro*, resulting in an enhancement of

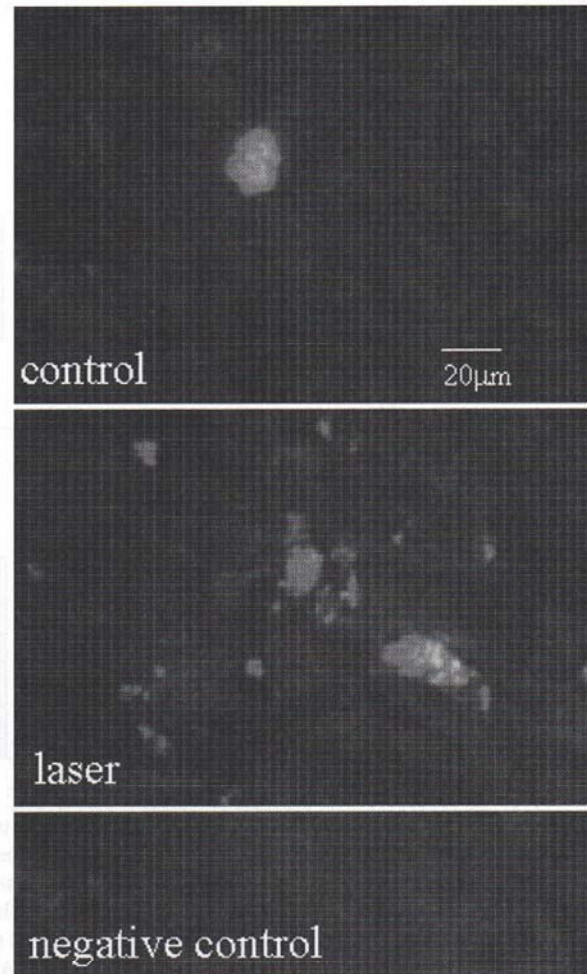


Fig. 6. Immunocytochemical staining for IGF-I in rat calvarial cells cultures. IGF-I was found expressed only in the center of the cell masses on Day 5 in both the control and laser groups. Laser irradiation seemed to increase the number of cell masses expressing IGF-I as compared to the control. Laser treatment group without incubation of IGF-I-Ab used as a negative control. Similar results were obtained from the cells on Day 8 (data not shown).

bone formation. Further, these phenomena appear to be the direct effects of laser irradiation to the cells. However, the question remains regarding why cellular proliferation and differentiation are stimulated by laser irradiation.

It is reported that local regulation of bone cell function has been shown to be regulated by cytokines, growth factors, and prostaglandins [27]. Among these, prostaglandin E₂ [28,29], and IGF [13,30] have been demonstrated to be potent stimulators of bone formation and produced by cells with an osteoblast lineage. Therefore, it is likely that the stimulatory mechanism of laser irradiation toward bone formation may be mediated by these factors, as they have been shown to have proliferation and differentiation-inducing properties and are produced from cells of osteoblast lineage by laser irradiation.

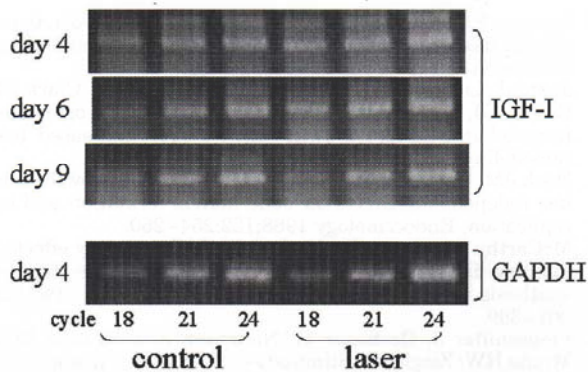


Fig. 7. Stimulatory effects of laser irradiation on IGF-I gene expression in rat calvarial cells. Ethidium bromide staining pattern of simultaneously amplified PCR products using agarose-gel electrophoresis. IGF-I mRNA was constitutively expressed in calvarial cells and increased by laser irradiation on Days 4 and 6. Levels returned to the control level on Day 9. Similar results were obtained from three different experiments.

In the present study, bone nodule formation from calvarial cell cultures was significantly increased by rIGF-I treatment and significantly decreased by IGF-I-Ab treatment. These results suggest that calvarial cells constitutively produce IGF-I, which may act as an autocrine or paracrine stimulator on calvarial cells and partly supports bone nodule formation in the control cultures, and neutralization of constitutively produced IGF-I decreased the number of nodules but did not the size of nodules suggesting this IGF-I may stimulate differentiation, but not replication of calvarial cells.

Moreover, higher concentrations of IGF-I demonstrated an ability to form more bone nodules. Laser irradiation also significantly increased bone nodule formation at the same level as the highest concentration of rIGF-I (100 nM), and the stimulatory effects of irradiation were neutralized by IGF-I-Ab treatment and completely abolished by 1 μ g/ml of IGF-1-Ab. This neutralized effect will be a specific effect of IGF-I-Ab protein, because IgG treatment as a control of IGF-I-Ab protein showed no effects of bone nodule formation. Therefore, it is most likely that the stimulatory effect of laser irradiation on bone nodule formation was mediated by an increase in IGF-I production by the calvarial cells and IGF-I may be the major factor of bone nodule formation enhanced by low-intensity laser irradiation in this experimental condition. This is strongly supported by the results showing that irradiation significantly stimulated IGF-I protein as well as gene expression soon after the dose was applied (Figs. 4 and 7). Considering the fact that only the center cells of the cell masses, which may have grown into three-dimensional bone nodules later, had an ability to produce IGF-I, laser irradiation may have stimulated proliferation and differentiation of calvarial cells, and some of these differentiated cells may have obtained the ability to produce IGF-I earlier than the control cells.

IGF-I belongs to a family of growth factors, however, its effect on bone formation is known to be different from that of other factors, for example, epidermal growth factor and fibroblast growth factor, which stimulate cell replication but inhibit differentiated function in skeletal tissue [31]. In contrast, IGF-I had a stimulatory effect on both replication and differentiation of bone cells in cultured rat calvaria [14,15,32] and osteoblast-like cells obtained from rat calvaria by enzymatic digestion [33], which had the same origin as the cells in the present study, as well as in MC3T3-E1 cells [34]. The stimulatory effects by IGF-I described in these studies are similar to those of our previous studies [6,7], which showed that a low-intensity laser irradiation significantly increased the number of bone nodules, cellular proliferation, ALP activity, collagen secretion, and calcification employing rat calvarial cells. Although the stimulatory effect of laser irradiation on bone nodule formation was comparable to that by the highest concentration of rIGF-I (100 nM, 770 ng/ml), laser-induced endogenous IGF-I production detected by radioimmunoassay on Day 3 was about 235 ng/ml. This difference may be explained by the finding that some of the endogenous IGF-I produced from laser stimulated cells was spontaneously degraded during the 3 days before assay. Further, not only IGF-I but other factors also stimulated by laser irradiation may have acted as mediators for bone nodule formation. Prostaglandin E is also known to be a potent stimulator of bone formation and this is constitutively produced by cultured osteoblasts [35] and it stimulates IGF expression [36]. Because PGE₂ production was not affected by laser irradiation, it may not have been a mediator of the stimulated bone nodule formation by laser irradiation seen in the present experimental condition. However, it is likely that except PGE₂, there are some mediators related to bone formation stimulated by low-intensity laser irradiation *in vivo* and/or *in vitro*, and they may form the signal transduction pathways of bone formation. The study to find out these pathways is now in progress.

Low-intensity laser irradiation is very similar to low-intensity pulsed ultrasound, in regards to the physical stress on the cells. Recently, low-intensity pulsed ultrasound has been reported to have stimulatory effects on bone-forming response *in vivo* [37,38] and *in vitro* [39–41] studies. Some *in vitro* results indicate that the stimulatory effects were mediated by increases in PGE₂ and/or COX-2 [39,41] or IGF-I [40] expressions. Although IGF-I and not PGE₂ was a mediator of the laser stimulated bone formation in the present experiment, an understanding of these differences between the effects of laser and pulsed ultrasound may be important to elucidate the action mechanisms of physical stress on cell.

In addition, osteoblast-like cells, like several other cell types known to secrete IGFs, produce IGF-binding proteins [42–44], which modulate the biological effects of IGFs on skeletal cells. These IGF binding proteins may also have important roles in stimulating bone nodule formation by laser irradiation. It was recently reported that p44/p42MAP kinase among MAP kinase super family plays a role in the IGF-1 stimulated ALP activity in MC3T3-E1

cells [45]. Considering our previous study [6,46] that laser irradiation stimulated ALP activity in calvarial cells, these kinase may also mediate laser stimulated bone nodule formation.

In conclusion, our results provide the first known evidence that low-intensity laser irradiation stimulates IGF-I protein and gene expression in rat calvarial osteoblasts. Since IGF-I is known to be a potent stimulator of bone formation, the stimulatory effect of bone nodule formation by low-intensity laser irradiation is at least partly mediated by IGF-I expression.

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