研究課題名：自由電子レーザーの組織再生効果に関する研究

研究代表者：清水　典佳　(日本大学　歯学部歯学科)
研究従事者：石丸　透子　(日本大学　歯学部歯学研究科臨床系専攻)
馬矢原琴枝　(日本大学　歯学部歯学研究科臨床系専攻)
共同研究者：上田　祐二　(日本大学　松戸歯学部歯学科)

【研究目的】

低出力レーザーには創傷治癒作用や、骨修復促進作用があることが報告されている。我々も in vivo, in vitro の実験で低出力半導体レーザーに骨形成促進作用があることを報告している。しかし、レーザー光は照射量、照射密度、波長、パルス幅、照射時期等により生物学的作用が異なると考えられるため、このような照射条件の違いによる骨修復効果を検討し、最大の骨形成促進効果を発揮する照射条件を見出したいと考えた。さらに、レーザーの組織誘導促進メカニズムを解明することにより低出力レーザーの歯科臨床応用をはかりたい。

【研究概要】

Ga-Al-As 半導体レーザー照射器(830nm,500mW)を用いて骨芽細胞に連続波（CI）と 1Hz パルス波(PI-1)の照射を行い、レーザー照射による骨形成促進作用について検討した。妊娠２１日目のラット胎仔からコラゲナーゼを用いラット胎仔由来骨芽細胞を採取し、同密度で培養した。培養骨芽細胞に 0.48～3.84J/cm^2 レーザー照射を行い、細胞増殖、骨芽細胞分化マーカーのアルカリホスファターゼ（ALP）活性、ALP 遺伝子発現、および bone nodule 形成を検討した。その結果、対照群に比べ照射群では、培養６，９日目で細胞増殖が増大し、９，１２，１５日では ALP 活性が増大し、２１日間の実験では bone nodule の数と面積が有意に増大した。また照射量に応じて bone nodule 数も増大していた。さらに、これらの効果は CI に比べ PI-1 が有意に増大しており、間歇的な光刺激が骨形成に有効であることが示唆された[1)]。

次にラット胎仔由来骨芽細胞を用いた同様の実験で、CI, PI-1 のほかに２，８Hz のパルス波(PI-2,-8)の照射を行い比較検討した。その結果、対照群に比べすべての照射群で細胞増殖、ALP 活性および遺伝子発現、bone nodule 数、面積は増大していたが、これらは CI や PI-8 に比べ PI-1,-2 が有意に増大していることがわかった。このことから、培養骨芽細胞に対する半導体レーザー照射の骨形成促進作用は、1 あるいは 2Hz の遅いパルス波が連続波や早いパルス波より有効であることがわかり、パルス頻度が細胞の生物学的活性に影響を与えていることがわかった[2)]。

次に、レーザーの細胞感受性に関する実験を行った。分化程度の異なる２種類のラット頭蓋冠由来骨芽細胞様株化細胞、未分化型 C-26 細胞と分化型 C-20 細胞を培養し、Ga-Al-As 半導体レーザー照射 (830nm,500mW)を行い、細胞の ALP 活性を測定した。照射は 3.82J/cm^2 を１回とし数種の照射を行った。その結果、未分化型の C-26 細胞では播種後１日、３日目に１回照射を行うと非照射群に比較しその後の ALP 活性が有意に上昇したが、複数照射ではその効果は見られなかった。一方、分化型の C-20 細胞では、複数照射を含めどのような条件でも ALP 活性に変化はなかった。このことより、半導体レーザー照射は未分化な細胞に照射することで分化を促進する機能を有すると考えられ、また、至適照射量が存在し、過剰照射では効果がないことが示唆された[3)]。

さらに、半導体レーザー照射の骨形成メカニズムを検討するために、ラット胎仔由来骨芽細胞を

採取し、上記同様同密度で培養した。培養骨芽細胞に 3.82J/cm^2 1 回照射を行い、骨の成長因子である insulin-like growth factor (IGF-I)-I のタンパク及び遺伝子発現について検討した。またリコンビナント(r)IGF-I や IGF-I 抗体を作用させてレーザー照射した時の bone nodule 形成能についても検討を行った。その結果、(r)IGF-I 作用群では bone nodule 形成は顕著に促進された。レーザー照射による bone nodule 形成促進作用は IGF-I 抗体を作用させると対照群レベルまで低下した。また、IGF-I のタンパク及び遺伝子発現は照射後に顕著に増大した。以上のことから、レーザー照射による bone nodule 形成促進作用は、骨芽細胞が産生する IGF-I の遺伝子およびタンパク発現を介して起こっていることがわかった。

【まとめ】

本研究でレーザー照射による生物学的細胞応答性についていくつかの結果が得られたが、詳細についてはいまだ不明な点が多い。今後はヒト顎骨から採取した骨芽細胞を培養し、種々の条件で自由電子レーザー照射を行い、bone nodule 形成を最大に促進する条件を見出す。その条件で培養骨芽細胞に同様にレーザー照射を行い、細胞から mRNA を回収し、マイクロアレイ解析システムを用い多くの遺伝子発現レベルの変化と細胞内クロストークを検討し、レーザーの骨形成メカニズムについて解明したいと考える。このような検討を加えることで、レーザーの歯科臨床応用をはかりたい。

【研究業績等】

・発表論文

(1) Y Ueda and N Shimizu (2001) Pulse irradiation of low-power laser stimulates bone nodule formation. *J Oral Science* 43, 55-60

(2) Y Ueda and N Shimizu (2003) Effects of pulse frequency of low-power laser therapy (LLLT) on bone nodule formation in rat calvarial cells. *J of Clin Laser Med Surg* 21, 271-277

(3) 石丸透子(2003)低出力レーザー照射が骨芽細胞様株化細胞のアルカリホスファターゼ活性に与える影響 日大歯学 77, 293-300

(4) Shimizu N, Ozawa Y, Ueda Y, Ishimaru T, Mayahara K, Iwase T, Moro T, Abiko Y: Low-intensity laser irradiation stimulates bone nodule formation via insulin-like growth factor-I expression in rat calvarial cells（投稿準備中）

・著書

(1) 清水典佳(2003) 矯正とソフトレーザー, レーザーの骨形成促進作用, レーザー臨床まること大辞典 第 1 版, デンタルダイアモンド社, 東京, 130-133

・学会発表

(1) 石丸透子、中西琴枝、大山幸治、納村晉吉、清水典佳(2003.10) 低出力レーザー照射が異なる分化程度の骨芽細胞様株化細胞のアルカリホスファターゼ活性に与える影響 第 62 回 日本矯正歯科学会大会（新潟）

(2) Shimizu N, Ozawa Y, Ueda T, Ishimaru T, Iwase T, Moro I, Abiko Y, Namura S (2002) Low-intensity laser irradiation stimulates bone nodule formation via IGF-I gene expression in rat calvarial cells. The 8th International Congress in Lasers in Dentistry, (Yokohama, Japan)

・学位の取得状況

(1) 石丸透子: 博士(歯学), 低出力レーザー照射が骨芽細胞様株化細胞のアルカリホスファターゼ活性に与える影響, 2003 年 3 月, 日本大学大学院歯学研究科臨床系専攻

Journal of Oral Science, Vol. 43, No. 1, 55-60, 2001

Pulse irradiation of low-power laser stimulates bone nodule formation

Yuji Ueda[§] and Noriyoshi Shimizu[†]

[§]Department of Orthodontics, Nihon University School of Dentistry at Matsudo, Chiba 271-8587
[†]Department of Orthodontics, Nihon University School of Dentistry, Tokyo 101-8310

(Received 10 November 2000 and accepted 14 February 2001)

Abstract: Although low-power laser irradiation provides many anabolic effects such as acceleration of bone formation, the effects of different pulse frequencies used during laser irradiation on bone formation have not been elucidated. Osteoblastic cells isolated from fetal rat calvariae were irradiated once with a low-power Ga-Al-As laser (830 nm, 500 mW) in two different irradiation modes; continuous irradiation (CI), and 1 Hz pulsed irradiation (PI). We then investigated the effects on cellular proliferation, bone nodule formation, alkaline phosphatase (ALP) activity, and ALP gene expression. Laser irradiation in both groups significantly stimulated cellular proliferation, bone nodule formation, ALP activity, and ALP gene expression, as compared with the nonirradiation group. Notably, PI markedly stimulated these factors, when compared with the CI group. Since 1 Hz pulsed laser irradiation significantly stimulates bone formation in vitro, it is most likely that pulse frequency is an important factor affecting biological responses in bone formation. (J. Oral Sci. 43, 55 - 60 , 2001)

Key words: pulse frequency; low-power laser; bone nodule formation; osteoblasts; alkaline phosphatase.

Correspondence to Dr. Y. Ueda, Department of Orthodontics Nihon University School of Dentistry at Matsudo, 2-870-1 Sakaecho-Nishi, Matsudo, Chiba 271-8587, Japan
Phone: +81-473-68-6111 Fax: +81-473-64-6295
E-mail address: ueda@mascat.nihon-u.ac.jp

Introduction

Recently, various photo-biostimuratory effects of low-power laser irradiation on regeneration have been reported in skin (1), nerve (2) and skeletal muscle tissues (3,4). In particular, the acceleration of bone regeneration by laser treatment has been a focus of recent research (5-10). Since induction of bone regeneration is always accompanied by tooth and/or jaw movement with orthodontic treatment, tooth extraction, orthognathic surgery, bone fracture, and stimulation of bone regeneration by laser treatment may be of great potential benefit to shorten the treatment period.

Low-power laser irradiation has also been shown to modulate various biological responses which are affected by some factors involved with the mode of laser irradiation such as total energy dose, laser spectrum, power density, and irradiation phase. In order to apply laser therapy to clinical use, the properties and biological effects of laser irradiation should be precisely elucidated, and more effective irradiation modes and easier laser application methods should be developed. However biological effects of different pulse frequency are not well known. In the present study, we examined continuous wave and 1 Hz pulse irradiation of low-power laser on bone nodule formation using a rat calvarial osteoblastic cell culture system.

Materials and Methods

Cell isolation and culture procedures

The calvaria were dissected aseptically from 21-day-old fetuses of timed pregnant Wistar rats. The calvaria were minced and sequentially digested in a 0.3 % collagenase mixture (Wako, Osaka, Japan). Five populations were obtained after digestion times of 10, 20, 30, 50, and 70 min. 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 minimal essential medium (α-MEM; Gibco, Grand Island, NY) containing 15 % fetal calf serum, and antibiotics comprising 100 μg/ml penicillin G (Sigma Chemical Co., St. Louis, MO), 50 μg/ml gentamicin sulfate (Sigma), and 0.3 μg/ml fungisone (Flow Laboratories, McLean, VA), supplemented with 50 μg/ml ascorbic acid (Wako) and 10 mM Na β-glycerol-phosphate (β-GP, Wako) (11,12). The cultures were maintained in a humidified atmosphere consisting of 95 % air and 5 % CO_2 at 37 °C. After 24 h of incubation, attached cells were washed with phosphate-buffered saline (PBS)to the remove nonviable cells and debris, trypsinized with 0.05 % trypsin (Gibco) inPBS.

The cells were then resuspended in the culture medium described above and plated into 35-mm tissue culture dishes (Falcon) at a density of 5×10^4 cells/dish (5.2×10^3 cells/cm^2), in a 24-well culture plate (Falcon) at a density of 1×10^4 cells/well (4×10^3 cells/cm^2), as well as 100-mm tissue culture dishes (Falcon) at a density of 4×10^5 cells/dish (5.1×10^3 cells/cm^2). Medium was changed every 3 days and cultures were maintained from 2 to 21 days.

Procedure of laser irradiation

A low-energy Ga-Al-As diode laser apparatus (model Panalas-1000, Matsushita, Inc., Osaka, Japan) with a wavelength of 830 nm (maximum power put out of 500 mW) was used in this study. The laser beam was delivered by an optical fiber 0.6-mm in diameter that was deforcused at the tip by a concave lense and was irradiated uniformly in a circular area, 100 mm in diameter, when the power density of laser beam was measured by a laser power meter at the cell-layer level. Irradiation was performed at 550 mm above the cell layer (13). We used two different types of irradiation; which were continuous irradiation (CI) and 1 Hz pulsed irradiation (PI). Exposure and rest time was the same for pulsed irradiation. The total energy corresponding to CI exposures of 1.25 to 10 min was 0.48~3.84 J/cm^2, while the exposure times in the corresponding pulsed irradiation group were two-fold longer (2.5-20 min), though the same total energy was used.

Laser irradiation was carried out after subculture (day 1) on a clean bench. The cells were then maintained in a CO_2 incubator for up to 21 days after subculture without any subsequent treatment. Control dishes and plates were placed on a clean bench for corresponding periods without any irradiation.

Determination of cell number

Growing cells in the 24-well culture plates were collected by digestion with a 1:1 mixture of trypsin (0.05 %) and collagenase (0.1 %) solutions for up to 25 min at the room temperature to release the cells from the collagenous matrix. The number of cells in each well was determined with a Coulter Counter (Model ZM, Electronics Ltd., Northwell drive, Luton, Beds England).

Quantification of bone nodules

Primary rat calvarial cell cultures were maintained for 21 days after subcultivation, after which the contents of each well were fixed for 10 min in 4 % paraformaldehyde in PBS and stained using the von Kossa technique. Bone nodules in each 35 mm dish were counted at 15 × magnification using a dissecting microscope (Olympus, Tokyo, Japan).

To evaluate the bone nodule areas precisely, the 35-mm dishes were photographed at 15 × magnification. The outline of each bone nodule was traced from the photographs and the traces were measured by a personal computer using image analysis software (Ultimage Ver. 2.0, Graftek France, Voisins-Le-Bretonneux, France). The total bone nodule area was calculated by adding each area in each dish and then the mean ± S.D. was calculated from 3 replicate data.

Assay of alkaline phosphatase (ALP) activity

Calvarial cells cultured in the 24-well plates were rinsed three times with PBS,. Tris-HCl (10 mM, pH 7.4) containing 5 mM $MgCl_2$ was then added, and the cells were collected by a cell scraper and sonicated for 1 min. ALP activity was assayed on days 3 to 21 by the method described by Lowry et al. (14). The amount of *p*-nitrophenol produced was measured at 410 nm. One unit of enzyme was defined as the activity which liberated 1 mmole of product per min at 37°C, and ALP activity was shown as mU/10^5 cells.

RNA preparation and RT-PCR analysis

Total RNA was extracted on day 9 from rat osteoblastic cells by an acid guanidium thocyanate-phenol-chloroforum extraction method (15). cDNA synthesis and amplification by RT-PCR were carried out using a GeneAmp RNA kit (Perkin-Elmer-Cetus, NJ). PCR amplification was carried out using the GeneAmp PCR system 9600 (Perkin-Elmer-Cetus) for 30 cycles under the following conditions: 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 3 min. The PCR primers for amplification of ALP (16) and GAPDH (17) were designed based on the published sequences. PCR fragments were electrophoresed on 2.0 % agarose gels and subsequently stained with ethidium bromide.

Statistics

The values were calculated as mean values ± standard deviation (S.D.). Significance was determined by Student's *t*-test, Tukey test, and one-way analysis of variance (ANOVA).

Results

Laser irradiation effect on cellular proliferation

The growth curve for fetal rat calvarial cells cultured under the preceding conditions is shown in Table 1. Laser irradiation under both conditions significantly stimulated cellular proliferation as compared with the controls. However, Proliferation in PIwas significantly stimulated on days 6 ($P < 0.05$), 9 ($P < 0.01$), and 12 ($P < 0.05$), when compared with the corresponding controls, and the number of cells in PI was significantly higher ($P < 0.01$) on days 9 (1.20-fold), when compared with the corresponding CI.

Table 1 Effects of low frequency laser irradiation on cellilar proliferation. Laser irradiation at both conditions significantly stimulated cellular proliferation as compared with the controls (**$P < 0.01$, *$P < 0.05$), while PI showed grater cellular proliferation on days 9 than CI($^{\dagger}P < 0.05$). Values are mean ± S.D. for 3 cultures.

×10⁴

Irradiation mode \ Culture day	0	3	6	9	12	15	18	21
Cont	1.0	11.9±0.4	40.0±1.4	44.9±1.4	53.5±6.7	57.1±1.9	43.9±2.2	41.3±1.0
CI	1.0	13.3±0.4	* 47.2±1.9	** 54.3±1.9	55.7±1.7	55.8±1.7	41.2±1.9	42.9±1.8
PI	1.0	14.0±0.4	* 47.8±1.9	**, † 63.1±1.9	** 62.0±2.0	56.9±2.1	40.8±2.3	43.2±2.2

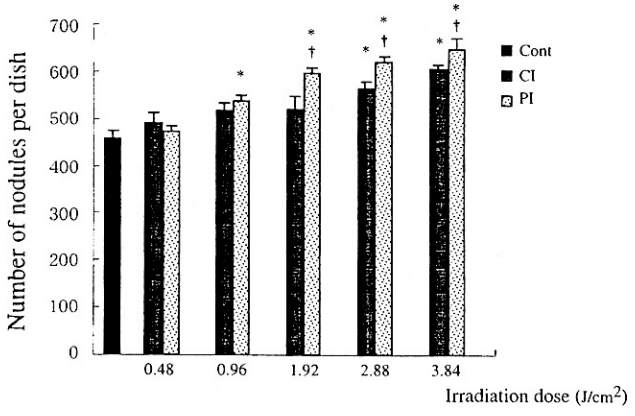


Fig. 1 Effect of laser irradiation on the number of bone nodules on day 21. Laser irradiation at both conditions significantly stimulated the number of done nodules as compared with the control, and the effects increased in a dose dependents manner. Values are mean ± S.D. fore 4 cultures. *:Significant difference from nonirradiation control ($P < 0.01$). †: Significant difference from corresponding CI ($P < 0.05$ by tukey test). Similar results were obtained from three different experiments.

Laser irradiation on bone nodule formation

The number of bone nodules present in 3 replicate 35-mm dishes was counted and significant stimulation by laser irradiation occurred under both conditions, however, the stimulatory effects were dose dependent in each groups (one-way ANOVA, $P < 0.01$) (Fig. 1). The maximal stimulation rates were 1.2-fold in CI (10 min), 1.3-fold in

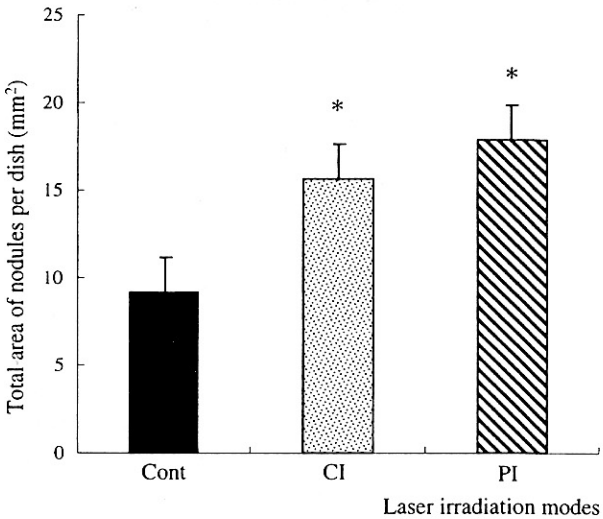


Fig. 2 Comparison of different laser irradiation modes on total area of bone nodules. Laser irradiation at both conditions significantly stimulated the total area, as compared with the control. Values are mean ± S.D. for 3 cultures.*:Significant difference from nonirradiation control ($P < 0.01$).

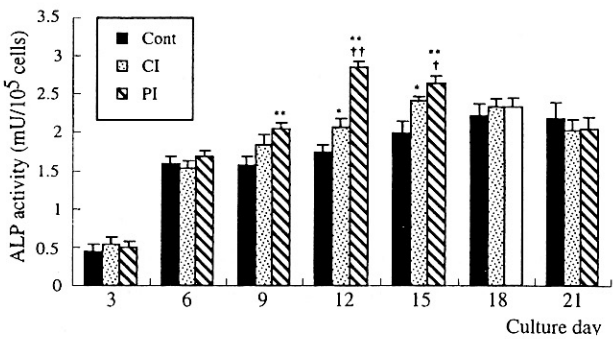


Fig. 3 Stimulatory effects of laser irradiation on ALP activity in rat calvarial cells.Laser irradiation at both conditions significantly stimulated ALP Activity on days 9,12 and15, as compared with the control (**$P < 0.01$, *$P < 0.05$),ands PI had greater increase in ALP activity than corresponding CI ($^{\dagger\dagger}P < 0.01$,$^{\dagger}P < 0.05$). Values are mean ± S.D. for 3 cultures.

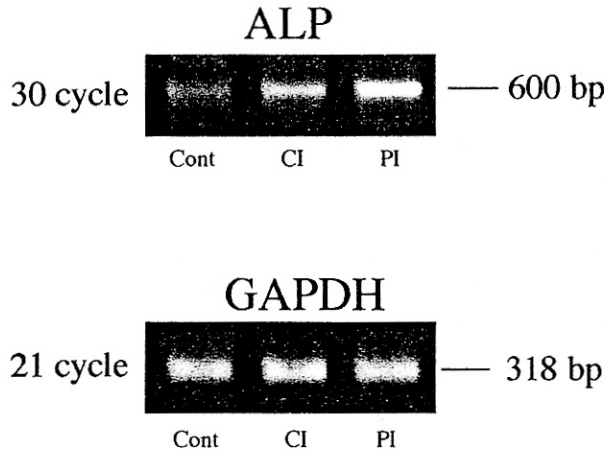


Fig. 4 Stimulatory effects of laser irradiation on ALP mRNA level in rat calvarial cells. Ethidium bromide staining patterns of simultaneously amplified PCR probucts on agarose-gel electrophoresis are shown. The gene expression of ALP on day 9 was markedly increased by laser irradiation as compared with the control. Results represent those from three similar experments.

PI (20 min), as compared with the controls ($P < 0.01$).

When the effects of dose on bone nodule formation were compared, the lowest dose (0.48 J/cm^2) did not have any stimulatory effect in the PI, while the higher doses (0.96~3.84 J/cm^2) showed stimulatory effects. In the CI, only the two highest doses (2.88 and 3.84 J/cm^2) showed stimulatory effects. Since the number of bone nodules was dependent on the dose, the stimulatory effects of bone nodule formation in each group were compared at the same dose of irradiation. The PI had significant bone nodule stimulation, as compared with the CI at 1.92 to 3.84 J/cm^2 (1.07~1.15-fold ; $P < 0.05$)(Fig. 1). When the total bone nodule areas were compared, they were found to be significantly stimulated in both irradiation groups, as compared with the controls (CI : 1.7-fold, PI : 1.9-fold, c0.01) (Fig. 2).

Effect of laser irradiation on ALP activity

We next determined ALP activity, the marker of osteoblast differentiation, in the cultures. ALP activity in the controls, CI, and PI increased late in the culture, with peak expression seen at 18 days in the controls, at 15 days in CI and at 12 days in PI-1 (Fig. 3). Laser irradiation on day 1 significantly stimulated ALP activity on days 9, 12, and 15 ($P < 0.01$) in the PI, with a maximal increase of 1.8-fold occurring on day 12. Significant activity was also seen on days 12 and 15 ($P < 0.05$) in the CI, with the maximal increase being 1.2-fold on day 15, as compared with the controls. Moreover, PI had significantly increased ALP activity as compared with the CI on day 12 (1.4-fold $P < 0.01$), and on day 15 (1.1-fold ; $P < 0.05$).

Effect of laser irradiation on ALP gene expression

To elucidate the mechanisms for the alteration of ALP activity by laser irradiation, ALP mRNA levels in both the control and laser irradiation groups were examined by RT-PCR analysis. As shown in Fig. 4, the visualized PCR products corresponding to GAPDH were the same in the three samples (control, CI, PI), therefore, it seemed that the amount of PCR products reflected ALPmRNA level. The bands of the laser irradiated cells were more intense than those from the control. Furthermore, the band for ALP mRNA from the PI was more intense than CI.

Discussion

Using a rat calvarial osteoblastic cell culture system, we clearly demonstrated that low frequency (1Hz) pulsed laser irradiation (PI) significantly stimulated cellular proliferation (1.2-fold), ALP activity (1.4-fold), and bone nodule formation (number, 1.15-fold), as compared with continuous laser irradiation (CI) at the same total energy dose. Although laser irradiation in both conditions used in this study significantly stimulated bone formation in vitro, bone formation capacity was much higher in the PI than in the CI. In the CI, the number of bone nodules was increased with only the two highest doses, while in the PI, the lower dose stimulated the number of bone nodules. These results showed that the mode of irradiation used in the PI was effective for bone nodule formation.

Various biostimulatory effects of low-energy laser irradiation have been reported such as cell proliferation (18), defferentiation (13), collagen synthesis (19), and the release of growth factors (20,21) from cells. These effects are affected by many factors including total energy dose, laser spectrum, power density, and irradiation phase. However, there are no knowledge concerning the effects of laser irradiation pulse frequency on bone formation.

Sanders et al. (22) reported that irradiation with shorter pulse duration in CO_2 laser incisions minimized wound-healing delays more effectively than continuous irradiation. Miyamoto et al. (23) also reported that the cytotoxicity ratio of HeLa cells irradiated by pulsed laser (10 Hz) was lower than that by continuous wave laser in photodynamic therapy. However the type of cell death differed between pulsed (apoptosis) and continuous wave (necrosis) irradiation, as greater amount of the sensitizer entered the cells during pulsed irradiation than continuous wave irradiation, causing a different type of DNA damage. Although these studies may not be directly comparable with our present results, because of different experimental

designs, it is most likely that pulse frequency influences biological responses. Since laser light is electromagnetic irradiation that provides physical stimulus, the biological effects of irradiation may be comparable with those of electromagnetic fields, which are known to modify some relevant physiological parameters of cell cultures, such as proliferation, protein synthesis, secretion of growth factors, transcription, and others (24). These biological responses have also been shown to be influenced by the pulse frequency of the electromagnetic field. There are some studies that special pulse frequencies such as 10 or 100 Hz from an electromagnetic field increased cellular proliferation (25, 26). In osteoblastic cells, Ochi (27) reported that a 100 Hz pulse frequency was most effective in stimulating the proliferation of mouse osteoblastic MC3T3-E1 cells, among tested frequencies between 50 and 200 Hz, and concluded that an effective electromagnetic field pulse frequency for cellular proliferation may be dependent on cell type, as cells seem to have their own peculiar sensitivity for certain pulse frequencies. In our experimental conditions, that used in the PI may be the more optimal frequency for bone formation compared with CI in rat calvarial cells.

Bone nodules found arising in cultures of osteoblastic cells originated from nodule-forming immature precursors that proliferated and differentiated to mature osteoblasts over a period of 3 weeks *in vitro* (11,13). In the present study, laser irradiation at both conditions would have increased the induction of nodule forming commitment, as compared with the control, and the conditions associated with PI may have had more capacity to increase the induction of nodule forming commitment than those with CI. Since the total area of bone nodules may show bone formation capacity, PI may be better for bone formation.

ALP activity is considered to be a marker of osteoblast differentiation (28,29). In the present experiment, ALP activity was significantly stimulated on days 12 and 15 after laser irradiation, in CI (Fig. 3). In PI it was dramatically stimulated on days 12, which was significant when compared with CI. Furthermore, an increase in ALP activity could involve transcriptional events of the bone type ALP gene, while dramatic increases in the activity may reflect stimulation of both differentiation and proliferation of cells, resulting in a significant increase in the number of differentiated cells that express differentiation markers, and finally form more and larger bone nodules.

In conclusion, low frequency pulsed laser irradiation, such as 1 Hz, significantly stimulated bone formation *in vitro*, as compared with continuous irradiation. Although the stimulatory capacity of laser irradiation is influenced by factors such as total energy dose, laser spectrum, power density, and irradiation phase, the pulse frequency of low-energy laser irradiation must also be considered as an important factor for influencing biological response.

To definite better irradiation mode, further studies such as effects of different pulse frequencies on bone formation should be examined.

Acknowledgments

We are indebted to professors Yoshimitsu Abiko, Department of Biochemistry, Kazutaka Kasai Department of Orthodontics, Nihon University School of Dentistry at Matsudo and professor Shinkichi Namura, Department of Orthodontics, Nihon University School of Dentistry, for their advice.

We also thank Mr. Akira Kaneda, senior engineer of Matsushita Industrial Equipment Company Limited, for providing the laser therapy apparatus.

This research was supported in part by Grants-in-Aid for Scientific Research from The Ministry of Education, Science, Sports and Culture, C-2 (12672018), Grants-in-Aid for Research for Frontier Science, A-1 (11307051), an Uemura Grant to N.S., and a Nihon University General Individual Research Grant (00-099).

References

1. Conlan, M.J., Rapley, J.W. and Cobb, C.M. (1996) Biostimulation of wound healing by low-energy laser irradiation. A review. J. Clin. Periodontol. 23, 492-496
2. Assia, E., Rosner, M., Belkin, M., Solomon, A. and Schwartz, M. (1989) Temporal parameters of low energy laser irradiation for optimal delay of post-traumatic degeneration of rat optic nerve. Brain Res. 476, 205-212
3. Weiss, N. and Oron, U. (1992) Enhancement of muscle regeneration in the rat gastrocnemius muscle by low energy laser irradiation. Anat. Embryol. (Berl.) 186, 497-503
4. Bibikova, A. and Oron, U. (1993) Promotion of muscle regeneration in the toad (Bufo virdis) gastrocnemius muscle by low-energy laser irradiation. Anat. Rec. 235, 374-380
5. Chen, J. and Zhou, Y. (1989) Effect of low level carbon dioxide laser radiation on biochemical metabolism of rabbit mandibular bone callus. Laser Ther. 1, 83-87
6. Motomura, K., Nakajima, M., Ihara, A. and Atsumi, K. (1984) Effects of various laser irradiation on callus formation after osteotomy. Nippon Reza Igakkaishi 4, 195-196
7. Nagasawa, A., Kato, K. and Negishi, A. (1991)

Bone regeneration effect of low level lasers including argon laser. Laser Ther. 3, 59-62
8. Tang, X.M. and Chai, B.P. (1986) Effect of CO_2 laser irradiation on experimental fracture healing: a transmission electron microscopic study. Lasers Surg. Med. 6, 346-352
9. Trelles, M.A. and Mayayo, E. (1987) Bone fracture consolidates faster with low-power laser. Lasers Surg. Med. 7, 36-45
10. Kawasaki, K. and Shimizu, N. (2000) Effects of low-energy laser irradiation on bone remodeling during experimental tooth movement in rats. Lasers Surg. Med. 26, 282-291
11. Bellows, C.G. and Aubin, J.E. (1989) Determination of numbers of osteoprogenitors present in isolatted fetal rat calvaria cells in vitro. Dev. Biol. 133, 8-13
12. Bellows, C.G., Aubin, J.E. and Heersche, J.N.M. (1987) Physiological concentrations of glucocorticoids stimulate formation of bone nodules from isolated rat calvaria cells in vitro. Endocrinology 121, 1985-1992
13. 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
14. Lowry, O.H., Roberts, N.R., Wu, M.L., Hixon, W.S. and Crawford, E.J. (1954) The quantitative histochemistry of brain. II. Enzyme measurements. J. Biol. Chem. 207, 19-37
15. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156-159
16. Noda, M., Yoon, K., Thiede, M., Buenaga, R., Weiss, M., Henthorn, P., Harris, H. and Rodan, G.A. (1987) cDNA cloning of alkaline phosphatase from rat osteosarcoma (ROS 17/2.8) cells. J. Bone Miner. Res. 2, 161-164
17. Tokunaga, K., Nakamura, Y., Sakata, K., Fujimori, K., Ohkubo, M., Sawada, K. and Sakiyama, S. (1987) Enhanced expression of glyceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. Cancer Res. 47, 5616-5619
18. Yu, W., Naim, J.O. and Lanzafame, R.J. (1997) Effects of photostimulation on wound healing in diabetic mice. Lasers Surg. Med. 20, 56-63
19. Balboni, G.C., Brandi, M.L., Zonefrati, R. and Repice, F. (1986) Effects of He-Ne/I.R. laser irradiation on two lines of normal human fibroblasts in vitro. Arch. Ital. Anat. Embriol. 91, 179-188
20. Yu, W., Naim, J.O. and Lanzafame, R.J. (1994) The effects of photo-irradiation on the secretion of TGF & PDGF from fibroblasts in vitro. Lasers Surg. Med. Suppl. 6, 8 (abstract)
21. Yu, W., Naim, J.O. and Lanzafame, R.J.(1994) The effect of laser irradiation on the release of bFGF from 3T3 fibroblasts. Photochem. Photobiol. 59, 167-170
22. Sanders, D.L. and Reinisch, L. (2000) Wound healing and collagen thermal damage in 7.5-μsec pulsed CO_2 laser skin incisions. Lasers Surg. Med. 26, 22-32
23. Miyamoto, Y., Umebayashi, Y. and Nisisaka, T. (1999) Comparison of phototoxicity mechanism between pulsed and continuous wave irradiation in photodynamic therapy. J. Photochem. Photobiol. B. 53, 53-59
24. Goodman, E.M., Greennebaum,B. and Marron, M. T.(1995) Effects of electromagnetic fields on molecules and cells. Int. Rev. Cytol. 158, 279-338
25. Takahashi, K., Kaneko, I., Date, M. and Fukada, E. (1986) Effect of pulsing electromagnetic fields on DNA synthesis in mammalian cells in culture. Experientia 42, 185-186
26. Date, M., Kaneko, I. and Fukada, E. (1986) Pulsed magnetic stimulator for biological systems. Sci. Papers I.P.C.R. 80, 11-16
27. Ochi, M. (1993) Effect of pulsing electromagnetic fields on the MC3T3-E1 osteogenic cell line. Nihon Koku Inpuranto Gakkaishi 6, 82-96(in Japanese)
28. Gundberg, C.M., Hauchka, P.V., Lian, J.B. and Gallop, P.M. (1984) Osteocalcin: isolation, characterization, and detection. Methods Enzymol. 107, 516-544
29. Owen, T.A., Aronow, M., Shalhoub, V., Barone, L.M., Wilming, L., Tassinari, M.S., Kennedy, M.B., Pockwinse, S., Lian, J.B. and Stein, G.S. (1990) Progressive development of the rat osteoblast phenotype in vitro:reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J. Cell. Physiol. 143, 420-430

Journal of Clinical Laser Medicine & Surgery
Volume 21, Number 5, 2003

Pp. 271–277

Effects of Pulse Frequency of Low-Level Laser Therapy (LLLT) on Bone Nodule Formation in Rat Calvarial Cells

YUJI UEDA[1] and NORIYOSHI SHIMIZU[2]

ABSTRACT

Objective: **The purpose of this study was to determine the effect of pulse frequencies of low-level laser therapy (LLLT) on bone nodule formation in rat calvarial cells *in vitro*. *Background Data:* Various photo-biostimulatory effects of LLLT, including bone formation, were affected by some irradiation factors such as total energy dose, irradiation phase, laser spectrum, and power density. However, the effects of pulse frequencies used during laser irradiation on bone formation have not been elucidated. *Materials and Methods:* Osteoblast-like cells isolated from fetal rat calvariae were irradiated once with a low-energy Ga-Al-As laser (830 nm, 500 mW, 0.48–3.84 J/cm^2) in four different irradiation modes: continuous irradiation (CI), and 1-, 2-, and 8-Hz pulsed irradiation (PI-1, PI-2, PI-8). We then investigated the effects on cellular proliferation, bone nodule formation, alkaline phosphatase (ALP) activity, and ALP gene expression. *Results:* Laser irradiation in all four groups significantly stimulated cellular proliferation, bone nodule formation, ALP activity, and ALP gene expression, as compared with the non-irradiation group. Notably, PI-1 and -2 irradiation markedly stimulated these factors, when compared with the CI and PI-8 groups, and PI-2 irradiation was the best approach for bone nodule formation in the present experimental conditions. *Conclusion:* Since low-frequency pulsed laser irradiation significantly stimulates bone formation *in vitro*, it is most likely that the pulse frequency of LLLT an important factor affecting biological responses in bone formation.**

INTRODUCTION

RECENTLY, various photo-biostimulatory effects of low-energy laser irradiation on regeneration have been reported in skin,[1,2] nerve,[3] and skeletal muscle tissues.[4,5] In particular, the acceleration of bone regeneration by laser treatment has been a focus of recent research.[6–10] Since bone regeneration induction is always accompanied by tooth movement with orthodontic treatment, tooth extraction, orthognathic surgery, bone fracture, and the like in the fields of dentistry and orthopedic surgery, stimulation of bone regeneration by laser treatment may be of great potential benefit to abbreviate the treatment period.

Low-level laser therapy (LLLT) has also been shown to modulate various processes in different biological systems[11] that involve cellular proliferation,[12] differentiation,[13] collagen synthesis,[14] and the release of growth factors,[15,16] from cells. Further, these biological responses are affected by the mode of laser irradiation such as total energy dose, laser spectrum, power density, and irradiation phase. We previously demonstrated that LLLT at an earlier stage of bone formation was more effective than irradiation at a later stage,[13,17] and that stimulation of bone formation by LLLT was dependent on the total energy dose.[17,18] Further stimulatory effects of bone formation have been obtained by repeated irradiation with a small-energy dose for a certain period rather than irradiation once at the same total energy dose.[17,18] In order to apply LLLT to clinical use, the properties and biological effects of laser irradiation should be precisely elucidated, and more effective irradiation modes and easier laser application methods should be developed.

Recently, Miyamoto et al.[19] reported that 10-Hz frequency pulsed laser irradiation induced apoptosis, while continuous wave laser irradiation induced necrosis in photodynamic therapy. Since biological responses differ between pulsed and continuous wave irradiation, we speculated whether the frequency

[1]Department of Orthodontics, Nihon University School of Dentistry at Matsudo Chiba, Japan.
[2]Department of Orthodontics, Nihon University School of Dentistry, Tokyo, Japan.

used during laser irradiation affects bone formation capacity and what mode of frequency most promotes bone formation. In the present study, we examined the effects of irradiation pulse frequencies of LLLT on bone nodule formation using a rat calvarial cell culture system.

MATERIALS AND METHODS

Cell isolation and culture procedure

The procedures of osteogenic cell isolation and culture used in the present study have been described by Bellows et al.[20,21] Briefly, calvariae were dissected aseptically from 21-day-old fetuses of timed pregnant Wistar rats, and the adherent soft connective tissues were loosely removed. The calvariae were minced and sequentially digested in a collagenase mixture. Five populations were obtained after digestion times of 10, 20, 30, 50, and 70 min. 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 minimal essential medium (α-MEM; Gibco, Grand Island, NY) containing 15% fetal calf serum, and antibiotics, 100 μg/mL penicillin G (Sigma Chemical Co., St. Louis, MO), 50 μg/mL gentamicin sulfate (Sigma), and 0.3 μg/mL fungisone (Flow Laboratories, McLean, VA), supplemented with 50 μg/mL ascorbic acid (Wako, Osaka, Japan) and 10 mM Na β-glycerol-phosphate (β-GP, Wako). The cultures were maintained in a humidified atmosphere consisting of 95% air and 5% CO_2 at 37°C. After 24 h of incubation, attached cells were trypsinized with 0.05% trypsin (Gibco) in phosphate-buffered saline (PBS), and then counted using a Coulter Counter (model ZM, Electronics Ltd., Luton, Beds, U.K.).

The cells were then resuspended in the culture medium described above and plated onto 35-mm tissue culture dishes (Falcon) at a density of 5×10^4 cells/dish (5.2×10^3 cells/cm^2), in a 24-well culture plate (Falcon) at a density of 1×10^4 cells/well (4×10^3 cells/cm^2), as well as 100-mm tissue culture dishes (Falcon) at a density of 4×10^5 cells/dish (5.1×10^3 cells/cm^2). Medium was changed every 3 days, and cultures were maintained from 3 to 21 days.

Procedure of laser irradiation

A low-energy Ga-Al-As diode laser apparatus (model Panalas-1000, Matsushita, Inc., Osaka, Japan), which has a 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 de-focused at the tip by a concave lens to provide a uniform circle of irradiation, 100 mm in diameter, at the cell-layer level. The power density of the laser beam was measured by a laser power meter. We used four different modes of irradiation: continuous irradiation (CI) and 1-, 2-, and 8-Hz pulsed irradiation (PI-1, PI-2, PI-8). Exposure and rest time was the same for each pulsed irradiation (50% duty cycle). The total energy corresponding to CI exposures of 1.25–10 minutes was 0.48–3.84 J/cm^2, while the exposure times in the corresponding pulsed irradiation groups were twofold longer (2.5–20 min), though the same total energy was used.

The laser dose chosen was previously reported to stimulate a number of bone nodules from rat calvarial cells using an experimental model similar to the one in the present study.[13] Laser irradiation was carried out after subculture (day 1) on a clean bench. The cells were then maintained in a CO_2 incubator for up to 21 days after subculture without any subsequent treatment. Control dishes and plates were placed on a clean bench for corresponding periods without any irradiation.

Determination of cell number

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

Quantification of bone nodules

The number of bone nodules present in the 35-mm culture dishes was quantified, as described previously.[13] Primary rat calvarial cell cultures were maintained for 21 days after subcultivation *in vitro,* after which the contents of each well were fixed for 10 min in 4% paraformaldehyde in PBS and stained using the von Kossa technique. Bone nodules in each dish were counted at ×15 magnification using a dissecting microscope (Olympus, Tokyo, Japan) by placing the culture dish on a transparent acetate grid ruled in 2-mm squares.

To evaluate the bone nodule areas precisely, the 35-mm dishes were photographed at ×100 magnification using a dissecting microscope (Olympus, Tokyo, Japan). The outline of each bone nodule (outside edge of light brown stained area) was traced from the photographs, and the traces were measured by a personal computer using image analysis software (Ultimage Ver. 2.0, Graftek France, Voisins-Le-Bretonneux, France). The total bone nodule area was calculated by adding each area in each dish, and then the mean ± standard deviation (SD) was calculated from five replicate data. The mean area of bone nodules was then determined by dividing the total area by the number of nodules in each dish, and the mean ± SD was calculated from five replicate data.

Assay of alkaline phosphatase (ALP) activity

Calvarial cells cultured in the 24-well plates were rinsed three times with PBS. Tris-HCl (10 mM/L, pH 7.4), containing 5 mM/L $MgCl_2$, was then added, and the cells were collected by a cell scraper and sonicated for 1 min. ALP activity was assayed on days 3–21 by the method described by Lowry et al.[22] The amount of *p*-nitrophenol produced was measured at 410 nm. One unit of enzyme was defined as the activity that liberated 1 mmole of product per min at 37°C, and ALP activity was shown as mU/10^5 cells.

RNA preparation and RT-PCR analysis

Total RNA was extracted on day 9 from rat osteoblastic cells cultured in 100 mm dishes by an acid guanidinium thiocyanate–phenol–chloroform extraction method.[23] cDNA synthesis and amplification by RT-PCR were carried out using a Gene Amp RNA kit (Perkin-Elmer-Cetus, NJ). PCR amplification was carried out using the Gene Amp PCR

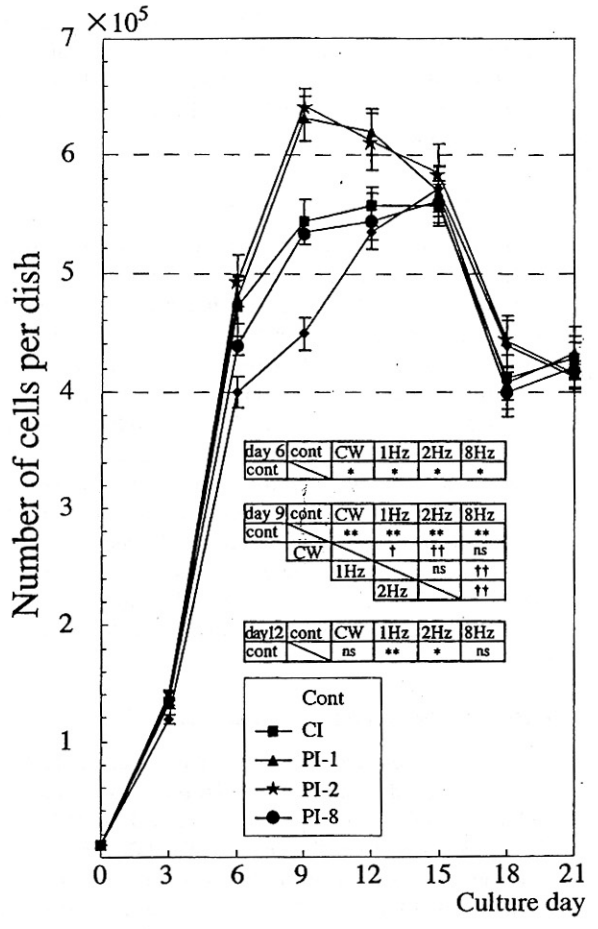


FIG. 1. Effects of different modes of laser irradiation on cellular proliferation. Laser irradiation at all conditions (3.84 J/cm², 10 or 20 min) significantly stimulated cellular proliferation as compared with the controls (**$p < 0.01$, *$p < 0.05$), while PI-1 and -2 showed grater cellular proliferation on days 9 than CI or PI-8 (†$p < 0.05$, ††$p < 0.01$). Values are mean ± SD for four cultures. Similar results were obtained from two different experiments.

system 9600 (Perkin-Elmer-Cetus) for 21–33 cycles under the following conditions: 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. The PCR primers for amplification of ALP[24] and GAPDH[25] were designed based on the published sequences. The primers were as follows: 5′–GAA AGA GAA AGA CCC CAG–3′ (forward primer for ALP); 5′–ACC ACC CAT GAT CAC ATC–3′ (reverse primer for ALP); 5′–ATC ACC ATC TTC CAG GAG–3′ (forward primer for GAPDH); 5′–ATG GAC TGT GGT CAT GAG–3′ (reverse primer for GAPDH). PCR fragments were electrophoresed on 2.0% agarose gels and subsequently stained with ethidium bromide.

Statistics

The values were calculated as mean values ± SD. Significance was determined by Student's *t*-test, Tukey's test, and two-way analysis of variance (ANOVA).

RESULTS

Laser irradiation effect on cellular proliferation

The growth curve for fetal rat calvarial cells cultured under the preceding conditions is shown in Figure 1. Laser irradiation under all modes significantly stimulated cellular proliferation as compared with the controls. Although the total dose was the same in all groups (3.84 J/cm², 10 or 20 min), the stimulatory effects were different in each. CI and PI-8 showed similar effects, as they had significant cellular proliferation on days 6 ($p < 0.05$) and 9 ($p < 0.01$), as compared with the corresponding controls. However, PI-1 and -2 had more effective proliferation, as cellular proliferation was significantly stimulated on days 6 ($p < 0.05$), 9 ($p < 0.01$), and 12 ($p < 0.05$) when compared with the corresponding controls, and the number of cells in both groups were significantly higher ($p < 0.01$) on day 9 (1.20-fold) when compared with the corresponding CI and PI-8 groups. In contrast, final saturation densities on day 21 were the same in all groups.

Laser irradiation on bone nodule formation

The number of bone nodules present in five replicate 35-mm dishes was counted, and significant stimulation by laser irradiation occurred under all modes (Fig. 2). The interaction between the two independent factors (irradiation dose and mode) was significant ($p < 0.0001$) by two-way ANOVA. The maximal stimulation rates in each group were 1.33-fold in CI (10 min), 1.43-fold in PI-1 (20 min), 1.49-fold in PI-2 (20 min), and 1.31-fold in PI-8 (20 min) as compared with the controls ($p < 0.01$).

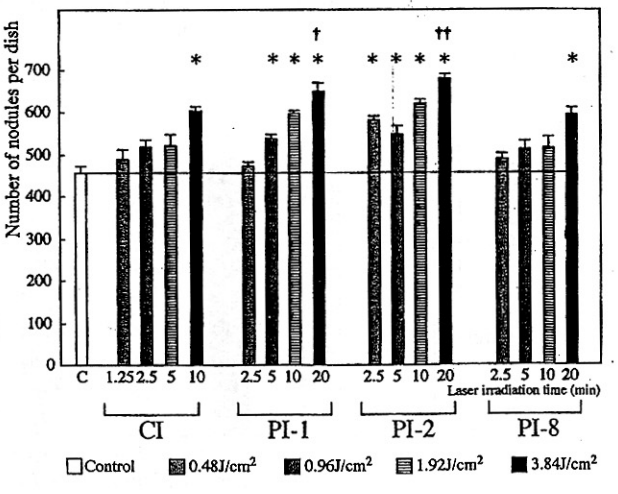


FIG. 2. Effect of different modes of laser irradiation on the number of bone nodules. Laser irradiation at all conditions significantly stimulated the number of bone nodules as compared with the controls, and the effects increased in a dose-dependent manner. In the PI-2 group, the lowest dose of irradiation still had the capacity to stimulate bone nodule formation. Values are mean ± SD for five cultures. Significant difference from non-irradiation control (*$p < 0.01$). Significant difference from CI and PI-8 (††$p < 0.01$, †$p < 0.05$ by Tukey test).

When the effects of dose on bone nodule formation were compared, irradiation in the PI-2 group was considered the most effective, because the lowest dose (0.48 J/cm²) still had stimulatory effect. In the PI-1 group, however, the lowest dose (0.48 J/cm²) did not have any stimulatory effect, while the higher doses (0.96–3.84 J/cm²) showed stimulatory effects. In the CI and PI-8 groups, only the highest doses (3.84 J/cm²) showed stimulatory effects. When the effects of bone nodule formation in each group were compared at their maximal dose of irradiation, the PI-1 and -2 groups had significant bone nodule stimulation, as compared with the CI and PI-8 groups (PI-1, $p < 0.05$; PI-2, $p < 0.01$). When the total bone nodule areas were compared (Fig. 3A), they were found to be significantly stimulated in all irradiation groups, as compared with the controls (CI, 1.73-fold; PI-1, 1.94; PI-2, 2.18; PI-8, 1.69; $p < 0.01$). Furthermore, the total area of bone nodules in PI-2 group was significantly greater, as compared with all other groups ($p < 0.05$ by Tukey test). To determine the size of each bone nodule, the mean area of bone nodules was evaluated and found to be significantly greater in all irradiation groups (1.29–1.45-fold; $p < 0.01$), as compared with the controls; however, there were no significant differences among the irradiation groups (Fig. 3B).

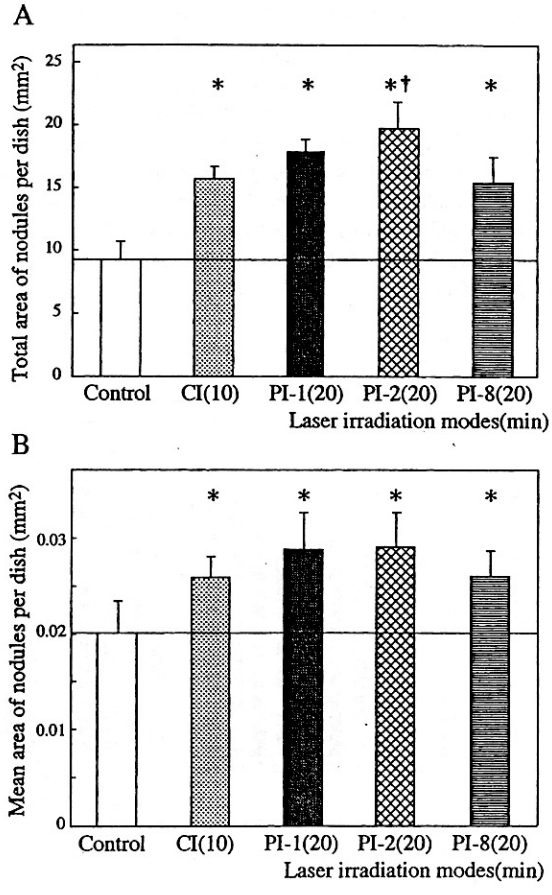


FIG. 3. Effect of different modes of laser irradiation on total area (A) and mean area (B) of bone nodules at the same laser dose. Laser irradiation at all modes significantly stimulated the total area (A) and mean area (B) of bone nodules as compared with the controls, and PI-2 had the significantly greater amount of total area than other groups. Values are mean ± SD for five cultures. Significant difference from non-irradiation control (*$p < 0.01$). Significant difference from CI and PI-8 (†$p < 0.05$ by Tukey test).

Laser irradiation on ALP activity

We next determined ALP activity, the marker of osteoblast differentiation, in the cultures. ALP activity in the controls, CI, and all pulsed irradiation groups increased late in the culture, with peak expression seen at 18 days in the controls, at 12 days in PI-1 and PI-2, and at 15 days in CI and PI-8 (Fig. 4). Laser irradiation on day 1 significantly stimulated ALP activity on days 9, 12, and 15 ($p < 0.01$) in the PI-1 and PI-2 groups, with a maximal increase of 1.8-fold occurring on day 12. Significant activity was also seen on days 12 and 15 ($p < 0.05$) in the CI and PI-8 groups, with the maximal increase being 1.2-fold on day 15, as compared with the controls. Moreover, PI-1 and PI-2 had significantly increased ALP activity as compared with CI and PI-8 on day 12 (1.4-fold; $p < 0.01$), and in PI-2 the effect remained on day 15, in contrast to CI and PI-8 (1.1-fold; $p < 0.05$).

Laser irradiation on ALP gene expression

To elucidate the mechanisms for the alteration of ALP activity by laser irradiation, ALP mRNA levels in both the control and laser irradiation groups were examined by RT-PCR analysis. As shown in Figure 5A, the visualized PCR products corresponding to GAPDH were the same in the five samples (control, CI, PI-1, PI-2, PI-8); therefore, it seemed that the amount of PCR product reflected each mRNA level. The bands for ALP mRNA from rat calvarial cells were visible after 27, 30, and 33 cycles, though the bands of the laser irradiated cells were more intense than those from the control. Furthermore,

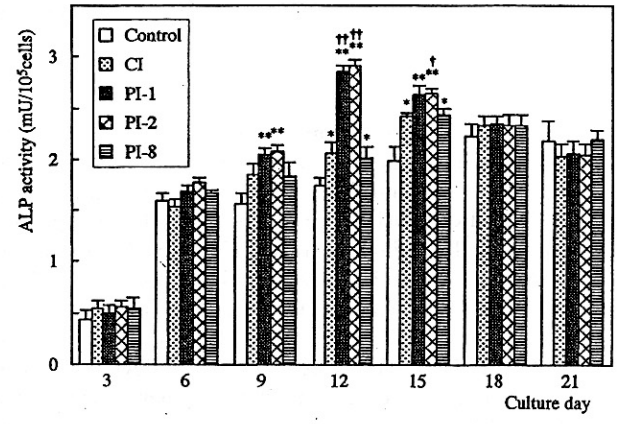


FIG. 4. Effect of different modes of laser irradiation on ALP activity. Laser irradiation at all conditions significantly stimulated ALP activity on days 12 and 15, as compared with the controls, while PI-1 and -2 had greater increases in ALP activity than corresponding CI or PI-8. Values are mean ± SD for four cultures. Significant difference from non-irradiation control (**$p < 0.01$, *$p < 0.05$). Significant difference from corresponding CI and PI-8 (††$p < 0.01$, †$p < 0.05$ by Tukey test).

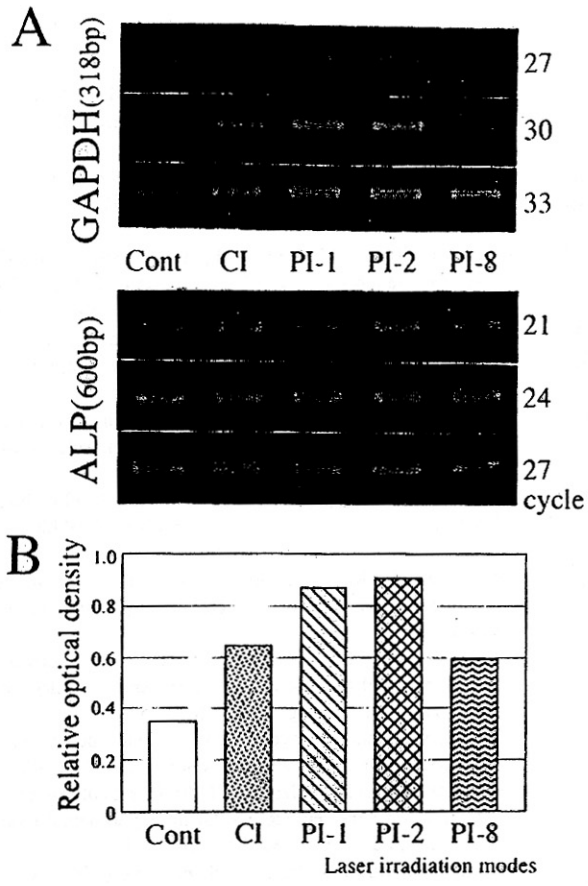


FIG. 5. Effect of different modes of laser irradiation on ALP mRNA levels in rat calvarial cells. Ethidium bromide staining patterns of simultaneously amplified PCR products on agarose–gel electrophoresis are shown. The gene expressions of ALP on day 9 was markedly increased by laser irradiation as compared with the control (**A**). When ALP gene expressions were compared in each group by densitometric readings, the order was PI-2, PI-1, CI, PI-8, and control. The expressions were normalized to equivalent amounts of GAPDH housekeeping mRNA (**B**).

the bands for ALP mRNA from the PI-1 and PI-2 cells were significantly more intense than the other laser irradiation groups.

Relative optical density measurements indicated that ALP mRNA in the PI-2 cells was highest, as compared with the other irradiation groups, and the rate of increase in the PI-2 group was approximately 2.6-fold of the value seen in the control, and 1.5-fold in PI-8, 1.4-fold in CI, and 1.05-fold in PI-1 (Fig. 5B).

DISCUSSION

Various photo-biostimulatory effects of LLLT have been reported, such as cell proliferation,[12] differentiation,[13] collagen synthesis,[14] and the release of growth factors[15,16] from cells, and these effects are affected by many factors, including total energy dose, irradiation phase, laser spectrum, and power density. We previously demonstrated that laser irradiation stimulated bone formation in a mid-palatal suture during rapid maxillary expansion in rats[17] and bone nodule formation *in vitro* in a rat calvarial cell culture[18] in an irradiation-dependent manner. Moreover, these stimulatory effects were much greater with laser irradiation at an earlier stage of maxillary expansion[17] or of calvarial cell culture,[13] as compared with irradiation at a later stage. Karu[12] has elucidated an action spectrum for the biostimulation of DNA synthesis by laser irradiation in HeLa cells. Hans et al.[26] reported that the power density and exposure time of He-Ne laser irradiation were more important than total energy dose for stimulation of fibroblast proliferation and collagen production. However, there are no known publications concerning the effects of pulse frequency of laser irradiation on bone formation.

In the present study, using a rat calvarial cell culture system, we clearly demonstrated that low frequency (1- or 2-Hz) pulsed laser irradiation (PI-1 and PI-2 groups) significantly stimulated cellular proliferation (1.2-fold), ALP activity (1.4-fold), and bone nodule formation (number, 1.1-fold; total area, 1.3-fold only in PI-2), as compared with continuous laser irradiation (CI) or higher frequency (8-Hz) pulsed laser irradiation (PI-8). In the CI and PI-8 groups, the number of bone nodules was increased with only the highest dose (3.84 J/cm^2), while in the PI-1 group the lower doses (1.92 and 0.96 J/cm^2) stimulated the number of bone nodules, and even the lowest dose (0.48 J/cm^2) was effective in the PI-2 group. These results showed that the mode of irradiation used in the PI-2 group was effective for bone nodule formation, even though it had only one-eighth the energy dose of that in the CI and PI-8 groups.

Sanders et al.[27] reported that irradiation with shorter pulse durations in CO_2 laser incisions minimized wound-healing delays more effectively than continuous irradiation. Miyamato et al.[19] also reported that the cytotoxicity ratio of HeLa cells irradiated by pulsed laser (10-Hz) was lower than that by continuous wave laser. However, the type of cell death differed between pulsed (apoptosis) and continuous wave (necrosis) irradiation, as a greater amount of the sensitizer entered the cells during pulsed irradiation than continuous wave irradiation, causing a different type of DNA damage.

Although these studies may not be directly comparable with our present study, it is very likely that pulse frequency influences biological responses. Since laser light is electromagnetic irradiation that provides a physical stimulus, the biological effects of irradiation may be comparable with those of electromagnetic fields, which are known to modify some relevant physiological parameters of cell cultures, such as proliferation, synthesis, secretion of growth factors, and transcription.[28] These biological responses have also been shown to be influenced by the pulse frequency of the electromagnetic field. Takahashi et al.[29] reported that DNA synthesis in Chinese hamster lung fibroblast V79 cells was significantly enhanced when exposed to an electromagnetic field with 10- and 100-Hz pulse frequencies. Date et al.[30] also reported, using HeLaS3 cells and IMR 90 cells, that 100-Hz pulse frequencies increased cellular proliferation. In osteoblastic cells, Ochi[31] reported that a 100-Hz pulse frequency was the most effective in stimulating the proliferation of MC3T3-E1 cells, and concluded that an effective electromagnetic field pulse frequency for cellular proliferation might be dependent on cell type, as cells seem to have

their own peculiar sensitivity for certain pulse frequencies. With regard to our laser irradiation modes, low-frequency pulsed laser irradiation, especially PI-2, may be the most optimal mode for bone formation.

In the present study, bone nodules found arising in cultures of osteoblastic cells originated from nodule-forming immature precursors that proliferated and differentiated to mature osteoblasts over a period of 3 weeks *in vitro*.[13,20] Therefore, the significant increase in the number of nodules caused by LLLT may have been the result of an increased induction of nodule-forming commitment in the uncommitted precursors. The conditions associated with PI-1 and especially PI-2 may have had more capacity to increase greater induction of nodule forming commitment than those with CI or PI-8. Since the total area of bone nodules may strongly show bone formation capacity, it was most likely that PI-2 mode of laser irradiation had 1.3-fold greater capacity of bone formation compared with CI or PI-8 mode.

ALP activity is considered to be a marker of osteoblast differentiation.[32] It has been reported that early progenitor cells do not express ALP activity, but differentiate through a defined number of cell divisions to ultimately express a mature osteoblast phenotype, which is a postmitotic cell expressing this marker that is capable of bone formation.[33] In the present experiment, ALP activity was dramatically stimulated on days 12 and 15, which was significant when compared with CI and PI-8. Furthermore, an increase in ALP activity could involve transcriptional events of the bone type ALP gene, while dramatic increases in activity may reflect stimulation of both differentiation and proliferation of cells, resulting in a significant increase in the number of differentiated cells, and finally form more and larger bone nodules.

The stimulatory mechanisms of LLLT on bone are not fully understood. It was recently shown that osteoblast-like cells may simultaneously secrete osteoblastic differentiation factors *in vitro*,[34–37] and that local regulation of bone cell functions is known to be regulated by cytokines, growth factors, and prostaglandins.[38] Since Yu et al.[15,16] reported that LLLT can cause the release of growth factors from cultured fibroblasts, further studies of the effect of LLLT on the production of these growth factors may be necessary to clarify in detail the stimulatory mechanisms of bone formation by laser treatment.

In conclusion, low-frequency pulsed laser irradiation, such as 1- and 2-Hz, significantly stimulated bone formation *in vitro*, as compared with continuous irradiation or higher frequency pulsed irradiation (8 Hz). It is suggested that the pulse frequency of LLLT must be considered as an important factor for influencing bone formation.

ACKNOWLEDGMENTS

We wish to express gratefulness to Akira Kaneda, senior engineer of Matsushita Industrial Equipment Company Limited, for providing the laser therapy apparatus. This research was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, C-2 (12672018), grants from the Ministry of Education, Culture, Sports, Science and Technology to promote a multidisciplinary research project, 2001, and the Satoh Fund grant to N.S.

REFERENCES

1. Conlan, M.J., Rapley, J.W., and Cobb, C.M. (1996). Biostimulation of wound healing by low-energy laser irradiation. A review. J. Clin. Periodontol. 23, 492–496.
2. Yu, W., Naim, J.O., and Lanzafame, R.J. (1997). Effects of photostimulation on wound healing in diabetic mice. Lasers Surg. Med. 20, 56–63.
3. Assia, E., Rosner, M., Belkin, M., et al. (1989). Temporal parameters of low-energy laser irradiation for optimal delay of post-traumatic degeneration of rat optic nerve. Brain Res. 476, 205–212.
4. Weiss, N., and Oron, U. (1992). Enhancement of muscle regeneration in the rat gastrocnemius muscle by low-energy laser irradiation. Anat. Embryol. 186, 497–503.
5. Bibikova, A., and Oron, U. (1993). Promotion of muscle regeneration in the toad (*Bufo virdis*) gastrocnemius muscle by low-energy laser irradiation. Anat. Rec. 235, 374–380.
6. Chen, J., and Zhou, Y. (1989). Effect of low-level carbon dioxide laser radiation on biochemical metabolism of rabbit mandibular bone callus. Laser Ther. 1, 83–87.
7. Nagasawa, A., Kato, K., and Negishi, A. (1991). Bone regeneration effect of low-level lasers including argon laser. Laser Ther. 3, 59–62.
8. Tang, X.M., and Chai, B.P. (1986). Effect of CO_2 laser irradiation on experimental fracture healing: a transmission electron microscopic study. Lasers Surg. Med. 6, 346–352.
9. Trelles, M.A., and Mayayo, E. (1987). Bone fracture consolidates faster with low-power laser. Lasers Surg. Med. 7, 36–45.
10. Kawasaki, K., and Shimizu, N. (2000). Effects of low-energy laser irradiation on bone remodeling during experimental tooth movement in rats. Lasers Surg. Med. 26, 282–291.
11. Belkin, M., Zatrunsky, B., and Schwartz, M. (1988). A critical review of low-energy laser bioeffects. Lasers Light Ophthalmol. 2, 63–71.
12. Karu, T. (1989). Photobiology of low-power laser effect. Health Physics 56, 691–704.
13. Ozawa, Y., Shimizu, N., Kariya, G., et al. (1998). Low-energy laser irradiation stimulates bone nodule formation at early stages of cell culture in rat calvarial cells. Bone 22, 347–354.
14. Balboni, G.C., Brandi, M.L., Zonefrati, R., et al. (1986). Effects of He-Ne/I.R. laser irradiation on two lines of normal human fibroblasts *in vitro*. Arch. Ital. Anat. Embryol. 91, 179–188.
15. Yu, W., Naim, J.O., and Lanzafame, R.J. (1994). The effects of photoirradiation on the secretion of TGF-β and PDGF from fibroblasts *in vitro*. Laser Surg. Med. Suppl. 6, 8(abst).
16. Yu, W., Naim, J.O., and Lanzafame, R.J. (1994). The effect of laser irradiation on the release of bFGF from 3T3 fibroblasts. Photochem. Photobiol. 59, 167–170.
17. Saito, S., and Shimizu, N. (1997). Stimulatory effects of low-power laser irradiation on bone regeneration in mid-palatal suture during expansion in rat. Am. J. Orthod. Dentfac. Orthop. 111, 525–532.
18. Ozawa, Y., Shimizu, N., Mishima, H., et al. (1995). Stimulatory effects of low-power laser irradiation on bone formation *in vitro*. Proc. SPIE 1984, 281–288.
19. Miyamoto, Y., Umebayashi, Y., and Nisisaka, T. (1999). Comparison of phototoxicity mechanism between pulsed and continuous wave irradiation in photodynamic therapy. J. Photochem. Photobiol. B 53, 53–59.
20. Bellows, C.G., and Aubin, J.E. (1989). Determination of numbers of osteoprogenitors present in isolatted fetal rat calvarial cells *in vitro*. Dev. Biol. 133, 8–13.
21. Bellows, C.G., Aubin, J.E., and Heersche, J.N.M. (1987). Physiological concentrations of glucocorticoids stimulate formation of bone nodules from isolated rat calvaria cells *in vitro*. Endocrinology 121, 1985–1992.

22. Lowry, O.H., Roberts, N.R., Wu, M.L., et al. (1954). The quantitative histochemistry of brain. II. Enzyme measurements. J. Biol. Chem. 207, 19–37.
23. Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.
24. Noda, M., Yoon, K., Thiede, M., et al. (1987). cDNA cloning of alkaline phosphatase from rat osteosarcoma (ROS 17/2.8) cells. J. Bone Miner. Res. 2, 161–164.
25. Tokunaga, K., Nakamura, Y., Sakata, K., et al. (1987). Enhanced expression of glyceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. Cancer Res. 47, 5616–5619.
26. Hans, H.F.I., Breugel, V., and Bar, D. (1992). Power density and exposure time of He-Ne laser irradiation are more important than total energy dose in photo-biomodulation of human fibroblasts *in vitro*. Lasers Surg. Med. 12, 528–537.
27. Sanders, D.L., and Reinisch, L. (2000). Wound healing and collagen thermal damage in 7.5-μsec pulsed CO_2 laser skin incisions. Lasers Surg. Med. 26, 22–32.
28. Goodman, E.M., Greennebaum, B., and Marron, M.T. (1995). Effect of electromagnetic fields on molecules and cells. Int. Rev. Cytol. 158, 279–338.
29. Takahashi, K., Kaneko, I., Date, M., et al. (1986). Effect of pulsing electromagnetic fields on DNA synthesis in mammalian cells in culture. Experientia 42, 185–186.
30. Date, M., Kaneko, I., and Fukuda, E. (1986). Pulsed magnetic stimulator for biological system. Sci. Papers I.P.C.R. 80, 11–16.
31. Ochi, M. (1993). Effect of pulsing electromagnetic fields on the MC3T3-E1 osteogenic cell line. J. Jpn. Soc. Oral Implant. 6, 82–96.
32. Owen, T., Aronow, M., Shalhoub, V., et al. (1990). Progressive development of the rat osteoblast phenotype *in vitro:* reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J. Cell. Physiol. 143, 420–430.
33. Hughes, F.J., Collyer, J., Stanfield, M., et al. (1995). The effects of bone morphogenetic protein–2, –4, and –6 on differentiation of rat osteoblast cells *in vitro*. Endocrinology 136, 2671–2677.
34. Birnbaum, R.S., Bowsher, R.R., and Wiren, K.M. (1995). Changes in IGF-I and -II expression and secretion during the proliferation and differentiation of normal rat osteoblasts. J. Endocrinol. 144, 251–259.
35. Ellies, L.G., Heersche J.N.M., Vadas, P., et al. (1991). Interleukin-1α stimulates the release of prostaglandin E_2 and phospholipase A_2 from fetal rat calvarial cells *in vitro:* relationship to bone nodule formation. J. Bone Miner. Res. 6, 843–850.
36. Harris, S.E., Sabatini, M., Harris, M.A., et al. (1993). Expression of bone morphogenetic protein messenger RNA in prolonged cultures of fetal rat calvarial cells. J. Bone Miner. Res. 9, 389–394.
37. Nair, S.P., Meghji, S., and Henderson, B. (1993). Osteoblastic-like cells respond to mechanical stress by secretion of osteolytic and osteoblastic differentiation factors. Calcif. Tissue Int. Suppl. 52, S31.
38. Marks, S.C., and Popoff, S.N. (1988). Bone cell biology: the regulation of development, structure and function in the skeleton. Am. J. Anat. 183, 1–44.

Address reprint requests to:
Dr. Noriyoshi Shimizu
Department of Orthodontics
Nihon University School of Dentistry
1–8-13, Kanda-Surugadai, Chiyoda-ku
Tokyo 101–8310, Japan

E-mail: shimizu-n@dent.nihon-u.ac.jp

日大歯学, Nihon Univ Dent J, 77, 293-300, 2003

低出力レーザー照射が骨芽細胞様株化細胞のアルカリホスファターゼ活性に与える影響

石　丸　透　子

日本大学大学院歯学研究科歯科臨床系専攻

（指導：納村晉吉 教授，清水典佳 助教授）

要旨：低出力レーザーの骨形成促進作用を検討する一環として，最適なレーザー照射条件を見出す必要がある。そこで，2種類のラット頭蓋冠由来骨芽細胞様株化細胞，未分化型C26細胞（C-26）と分化型C20細胞（C-20）に低出力レーザー（Ga-Al-As半導体レーザー，出力500 mW）を照射し，細胞増殖とアルカリホスファターゼ（ALPase）活性値を測定した。そして非照射群と比較することでC-26，C-20に対するレーザー感受性の細胞特異性，照射時期，照射量の特定を行った。低出力レーザーの照射条件は3.82 J/cm^2，10分間を1回とし，細胞播種後1日目に1回照射，3日目に1回照射，5日目に1回照射と，播種後1日目に3回連続照射，播種後1，2，3日目に各1回，計3回照射の計5種類で行った。その結果，未分化型C-26においては，細胞増殖に変化は認められなかったが，ALPase活性値で，播種後1日目に1回照射したときと，3日目に1回照射を行ったときに非照射群に対し，その後のALPase活性値が有意に増大していた。その他の条件では有意差は認められなかった。分化型C-20の照射では，いずれの条件でも細胞増殖およびALPase活性において，有意差は認められなかった。以上の結果より，低出力レーザー照射は，未分化な細胞に照射することで，細胞分化を促進する機能を有すると考えられる。また，至適照射量が存在し，過剰照射では効果が認められなかった。

キーワード：低出力レーザー，骨形成，培養骨芽細胞，アルカリホスファターゼ

緒　言

歯科矯正治療では動的治療を終了するまで2～3年を要し，さらに保定を必要とすることが多く，長い治療期間は患者にとっても大きな負担となっている。近年，圧迫側の骨吸収を促進し歯の移動促進を図るため，移動歯周囲にプロスタグランディンE_2[1]，活性型ビタミンD_3[2]，パラサイトホルモンなどが局所投与されている。しかしこれらの薬剤投与は，局所，全身への悪影響が問題となり，いまだ臨床応用には至っていない。歯科矯正治療は，骨の形成や吸収を伴う治療であり，これらを促進できれば治療期間や保定期間を短縮できると思われる。

近年，低出力レーザーの生体組織に対する光刺激作用が注目され，創傷治癒効果[3]や骨の形成促進作用[4-11]が報告されている。低出力レーザー照射により骨形成を促進できれば，歯科矯正治療のみならず，他の歯科領域での有効な治療法になると考えられる。これまでの*in vivo*の研究では，歯根嚢胞による骨欠損部へのレーザー照射が骨形成を促進するとされている[7]。しかし照射法等の詳細については検討されていない。*in vitro*の研究では，ラット胎仔頭蓋冠から採取した骨芽細胞様細胞に低出力レーザー照射し，骨様結節の形成が増加することが報告されている[12]。しかしながら，この研究ではラット胎仔頭蓋冠から初代培養した細胞を用いているため，種々の分化程度の異なる細胞が存在しており，レーザーの標的細胞や作用機序を検討するには適していない。そこで，同様にラット由来で異なった分化程度を示す，骨芽細胞様株化細胞を用いることでレーザーの効果を検討することを計画した。

ROB細胞は新生ラット頭蓋冠由来で多くの亜株を有し，その中でもROB-C26細胞（C-26）は，最も未熟な細胞で，筋細胞や脂肪細胞への分化能をも保有する骨芽細胞の前駆細胞である[13]。また，ROB-C20細胞（C-20）は骨芽細胞への分化程度が高く，筋細胞や脂肪細胞への分化能を失っており[13-15]，そのアルカリホスファターゼ（ALPase）活性はC-26のそれと比較すると3倍ほど高い[16]。

本研究では，これまで明らかにされていなかった，骨形成を促進するための標的細胞への最適なレーザーの照射条件を特定するために，分化程度の異なったC-26とC-20を使用し，低出力レーザー照射による，細胞増殖とALPase活性値について検討を行った。

材料および方法

1．細胞

実験に用いた細胞は，骨芽細胞の前駆細胞で未分化なC-26および成熟骨芽細胞に近く分化したC-20である[13,17]。これらの細胞は，長崎大学歯学部の山口 朗教授から，日本大学歯学部解剖学教室第1講座の高城 稔教授を通して提供されたものである。

（受付：平成15年1月28日）

〒101-8310 東京都千代田区神田駿河台1-8-13

2. 細胞培養

細胞は，10%ウシ胎児血清(FBS, JRH BIOSCIENCES)，抗生物質〔100 μg/ml ペニシリンG(萬有製薬)，100 μg/ml カナマイシン(明治製菓)，0.3 μg/ml ファンギゾン(Gibco Laboratories)〕を含むα-Minimum Essential Medium (Gibco Laboratories)で通法にしたがって37°C，5%炭酸ガス気相下で培養した。細胞がコンフルエントになった時点で，0.05%トリプシン(Gibco Laboratories)含有PBSで細胞を分離し，遠心分離によって細胞を回収したあと，1.0×10^4 個/cm^2 の密度で3～4代継代培養を行い，実験に用いた。なお，細胞増殖の状態は，位相差顕微鏡で経日的に観察し，3日ごとに培養液の交換を行い，10日間培養を続けた。C-26およびC-20を，それぞれ96穴マルチプルウェルプレート(Corning Science Products)の各ウェルに 9×10^3 個/cm^2 となるよう播種し培養した。各プレートには，各群5ウェル(n＝5)となるように，またレーザーが均等に照射されるように，プレート中央のみに播種し，実験を行った。

3. 低出力レーザー照射

低出力レーザー照射は，Ga-Al-As半導体レーザー治療器(パナラス1000，出力500 mW，波長830 nm，松下産業機器)を用い，クリーンベンチ内で培養細胞に照射した。レーザー光は0.6 mmの光ファイバーで誘導され，先端のレンズで拡散され，各ウェルの細胞に均一に照射される。96穴ウェルプレート上に培養された細胞に，プレート中央真上からプレート底面まで240 mmの距離から照射した。本研究条件では，直径100 mmの均一な照射野を得ることができる。照射量は，Ozawaら[12]の研究結果を参考に，1回につき10分間(3.82 J/cm^2)とした。本実験でのC-26およびC-20両細胞への照射条件は，1) 1日目1回照射群(細胞播種後1日目に1回照射)，2) 3日目1回照射群，3) 5日目1回照射群，4) 1日目3回照射群および5) 1，2，3日目1回照射群とした。

C-26，C-20それぞれについて上記の5種の条件で，計10通りの照射を行った。なお，照射時は黒色紙上にプレートを置いてレーザー照射を行い，レーザー光の乱反射を防止した。それぞれの照射群の対照として，レーザー照射群と同様に細胞培養し，各群に対応するようにレーザー照射中にプレートの蓋をあけ，クリーンベンチ内に放置した非照射群を作った。

4. 細胞数の測定

細胞数は，細胞播種日を0日目とし，cell counting kit (同仁化学研究所)を用いた比色定量法により測定した。測定は，毎日各々のウェルから培養液を取り除いた後，PBSで洗浄し，10%の発色液を加えた培養液を100 μlずつ加え，37°C，5% CO_2 気相下で2時間加温した。その後，マイクロプレートリーダー(Multiskan MS，大日本製薬)を用いて，波長450 nmで吸光度を測定，照射条件ごとに非照射群とレーザー照射群を比較した。測定は，細胞播種後1日目にレーザーを照射する直前に測定し，その後毎日1回，10日間測定した。測定値は5ウェルの細胞数の平均値と標準偏差で表した。

5. ALPase活性値の測定

ALPase活性値の測定は，マイクロプレート上で直接酵素反応を行うOshimaら[18]の方法に準じて行った。細胞数の測定と同様，細胞播種日を0日目とし，測定日にPBSでウェルを洗浄した後，8 mMパラニトロフェニルリン酸(和光純薬工業)と10 mM塩化マグネシウム(和光純薬工業)および0.1 mM塩化亜鉛(和光純薬工業)含有の0.1 Mグリシン(和光純薬工業)緩衝液(pH 10.5)を100 μlずつ加え，37°C，20分間の酵素反応を行った。その後，1.0 M水酸化ナトリウム(和光純薬工業)溶液を100 μlずつ加え，酵素反応を停止させた。さらに，マイクロプレートリーダー(MultiskanMS，大日本製薬)を用いて，吸光度405 nmで測定した。ALPase活性値の1 unit(U)は，37°Cで1分間に1 μmolのパラニトロフェニルリン酸を加水分解させる酵素量とし，細胞数10^4個当たりの値(mU)に換算し，5ウェルの平均値と標準偏差で表した。

6. 統計

統計処理には，Student's t-testを用いた。

結　果

1. C-26におけるレーザー照射群と非照射群の細胞増殖の比較

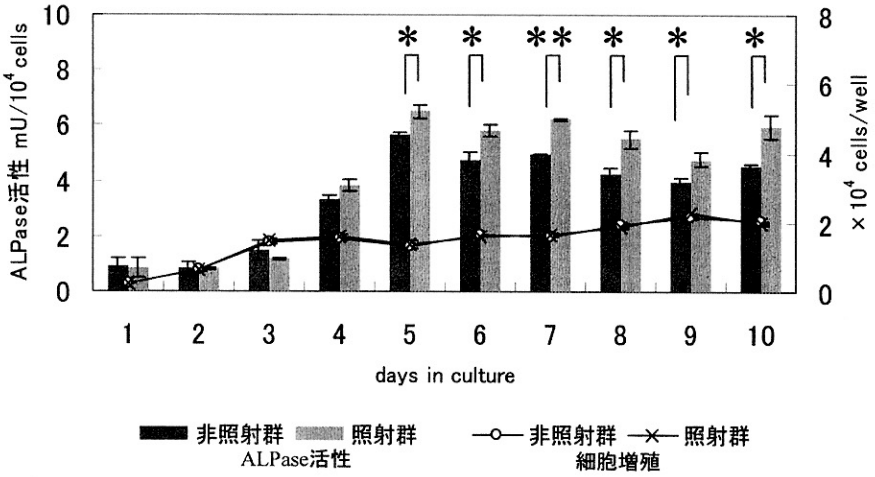
C-26播種後約3日目に鏡検下でコンフルエントとなり，その細胞数は約2×10^4 cells/wellであった(第1～5図)。1～5）のどのようなレーザー照射条件においても，照射群と非照射群で有意差は認められなかった。

2. C-26におけるレーザー照射群と非照射群のALPase活性の比較

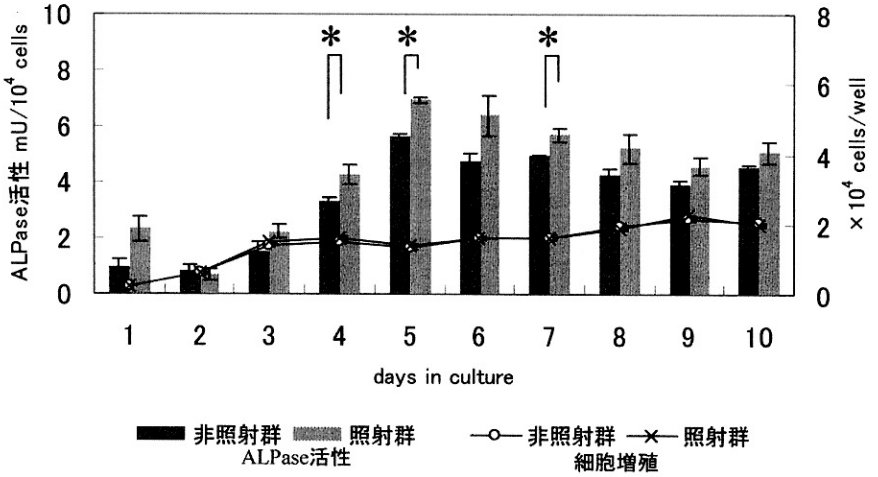
C-26のALPase活性は，3日または4日目から上昇し，5日あるいは6日目で最大値に達し，その後徐々に減少した。1日目1回照射群では，照射後5日目から実験終了10日目まで，レーザー照射群でALPase活性が有意に高かった。その値はレーザー照射群が非照射群に対し，7日目に最大約1.3倍，平均約1.2倍高かった(第1図)。3日目1回照射群では，4，5，7日目にレーザー照射群でALPase活性が有意に高かった。その値は5日目で最大約1.3倍，平均で約1.2倍であった(第2図)。5日目1回照射群では，非照射群との間に有意差は認められず，また，1日目3回照射群，1，2，3日目1回照射群も同様に有意差は認められなかった(第3～5図)。

3. C-20におけるレーザー照射群と非照射群の細胞増殖の比較

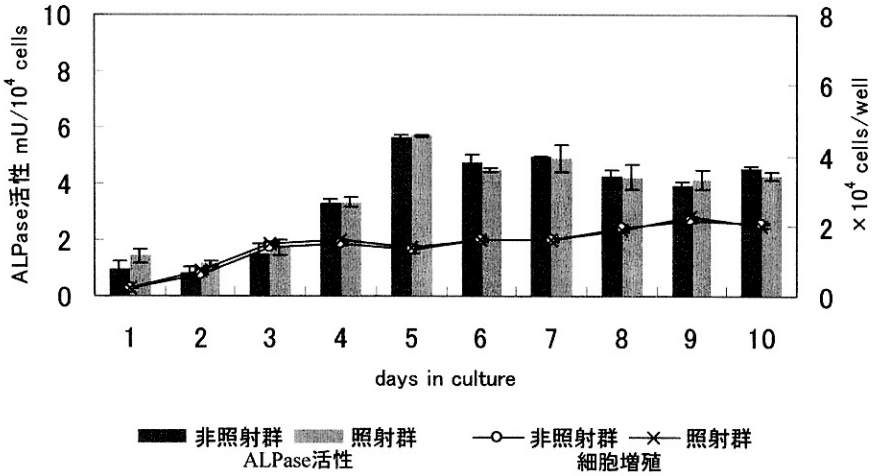
C-20播種後，5日あるいは6日目にコンフルエントとなり，その細胞数は約1×10^4 cells/wellであった(第6～



第1図　C-26の1日目1回照射群
細胞播種後，1日目に1回レーザー照射したときのALPase活性(mU/10^4cells)と細胞増殖(×10^4cells/well)を示す。ALPase活性は5～10日目まで非照射群よりも有意に増加している(*: $p < 0.05$, **: $p < 0.01$)。細胞増殖は，3日目にほぼ定常値を示している。(n = 5)



第2図　C-26の3日目1回照射群
細胞播種後，3日目に1回レーザー照射したときのALPase活性(mU/10^4cells)と細胞増殖(×10^4cells/well)を示す。ALPase活性は4,5,7日目で非照射群よりも有意に増加している(*: $p < 0.05$)。細胞増殖は，3日目にほぼ定常値を示している。(n = 5)



第3図　C-26の5日目1回照射群
細胞播種後，5日目に1回レーザー照射したときのALPase活性(mU/10^4cells)と細胞増殖(×10^4cells/well)を示す。ALPase活性は4日目に上昇し，その後ほぼ定常値を示す。細胞増殖は，3日目にほぼ定常値を示している。(n = 5)

10図)。どのようなレーザー照射群いずれにおいても，細胞数では非照射群との有意差は認められなかった。

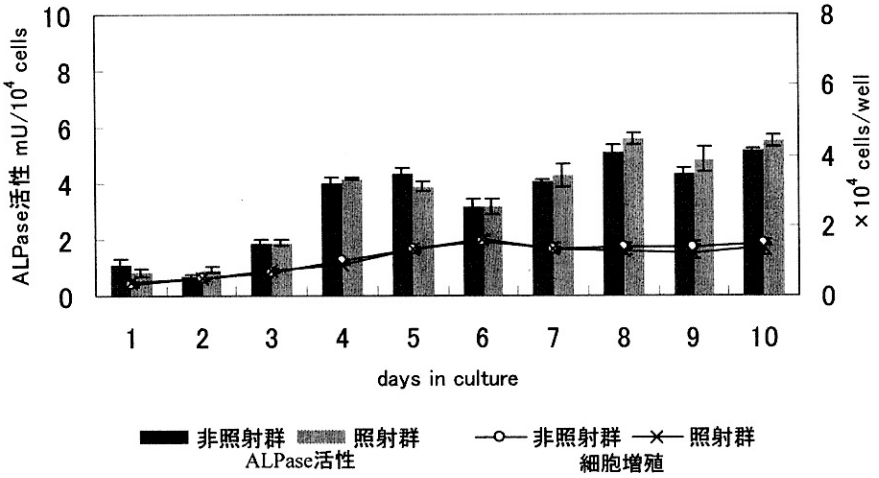
4. C-20におけるレーザー照射群と非照射群のALPase活性の比較

C-20のALPase活性は，培養後徐々に増大し，6日目でピークに達し，その後軽度に減少した。また，どの照射条件においてもレーザー照射群と非照射群との間では有意差が認められなかった(第6～10図)。

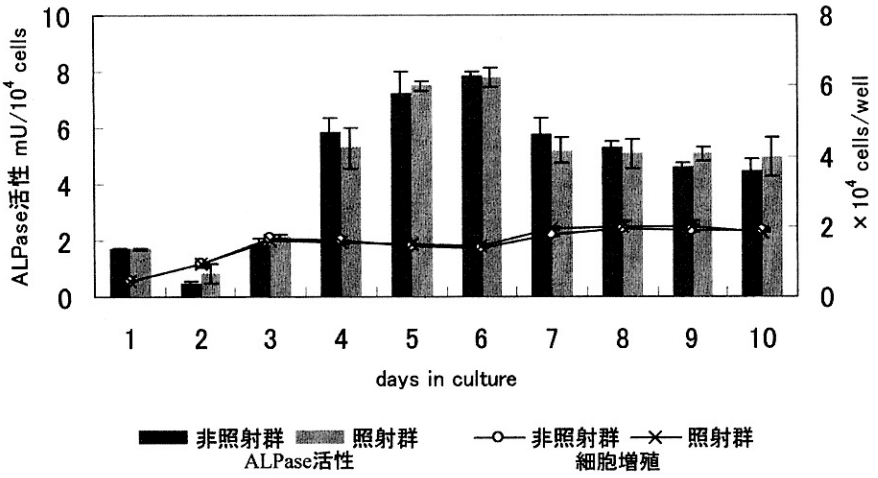
考　察

近年，低出力レーザーの骨形成促進作用がウサギ橈骨骨折[4]，マウス大腿骨骨折[5]，ヒト根尖歯槽骨形成[7]などで報告されているが，骨芽細胞への直接作用ではなく，局所の循環や温度変化などの環境要因によるものとしている。一方，Ozawaら[12]は，骨芽細胞様細胞にレーザー照射し，骨様結節形成促進作用を見い出し，レーザー照射の骨芽細胞への直接作用を報告した。しかし，これらの研究では促進効果を向上させるための照射条件等について十分な検討がなされていない。したがって，低出力レーザーを歯科矯正治療に応用するためには，骨形成を最大に発揮するレーザー照射条件を詳細に検討する必要がある。

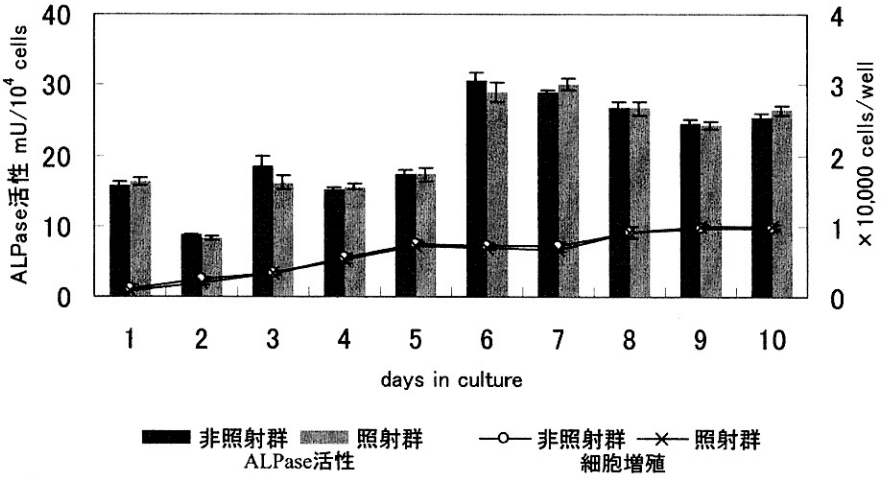
SaitoとShimizu[19]は，*in vivo*の実験でラット正中口蓋縫合急速拡大時の縫合部への毎日1回のレーザー照射が，縫合部への骨形成を1.4倍増大させたが，拡大初期のレーザー照射のみ有効であり，後期の照射は無効であったと報告している。また，Ozawaら[12]はラット胎仔頭蓋冠から採取した骨芽細胞様細胞に1回レーザー照射のみで，ALPase活性の上昇を伴う最大3.4倍の骨様結節の形成が促進されたことを報告している。さらに，レーザーの骨様結節形成促進作用は，培養初期の照射が最も効果的で，培



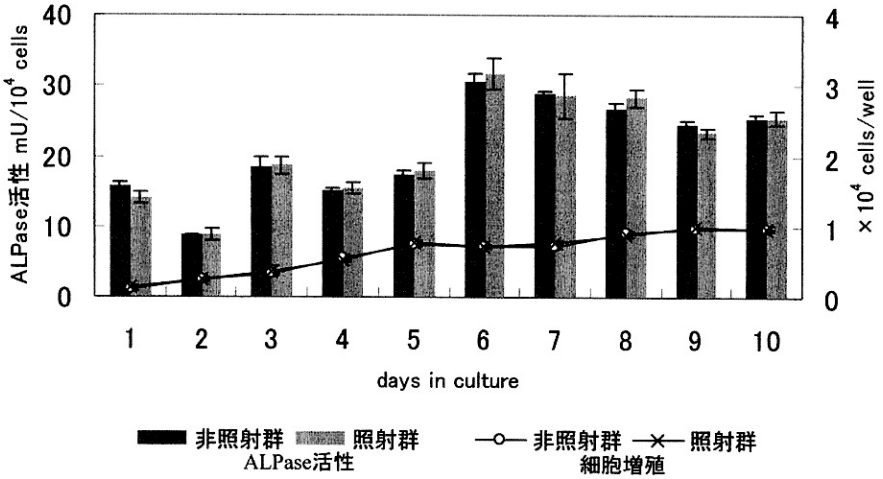
第4図　C-26の1日目3回照射群
細胞播種後，1日目に3回レーザー照射したときのALPase活性(mU/10^4cells)と細胞増殖(×10^4cells/well)を示す。ALPase活性は4日目に上昇し，その後ほぼ定常値を示す。細胞増殖は，4～5日目にほぼ定常値を示している。(n＝5)



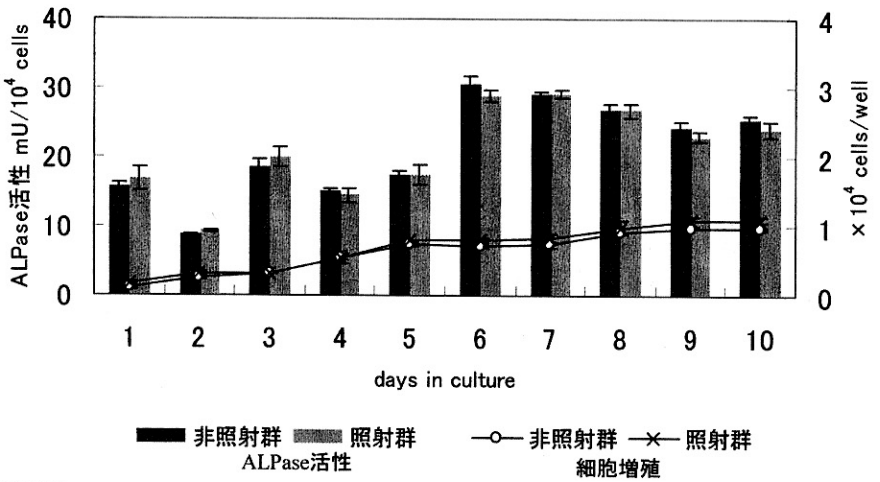
第5図　C-26の1,2,3日目1回照射群
細胞播種後，1，2，3日目に1回ずつレーザー照射したときのALPase活性(mU/10^4cells)と細胞増殖(×10^4cells/well)を示す。ALPase活性は4日目に上昇し，その後ほぼ定常値を示す。細胞増殖は，3日目にほぼ定常値を示している。(n＝5)



第6図　C-20の1日目1回照射群
細胞播種後，1日目に1回レーザー照射したときのALPase活性(mU/10^4cells)と細胞増殖(×10^4cells/well)を示す。ALPase活性は6日目に上昇し，その後ほぼ定常値を示す。細胞増殖は，4～5日目にほぼ定常値を示している。(n＝5)



第7図　C-20の3日目1回照射群
細胞播種後，3日目に1回レーザー照射したときのALPase活性(mU/10^4cells)と細胞増殖(×10^4cells/well)を示す。ALPase活性は6日目に上昇し，その後ほぼ定常値を示す。細胞増殖は，4～5日目にほぼ定常値を示している。(n＝5)

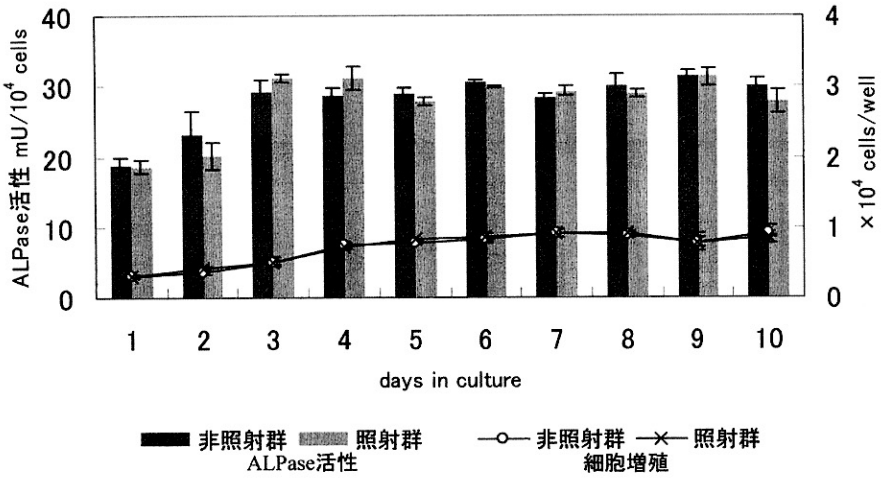


第8図　C-20の5日目1回照射群
細胞播種後，5日目に1回レーザー照射したときのALPase活性(mU/10^4cells)と細胞増殖(×10^4cells/well)を示す。ALPase活性は6日目に上昇し，その後ほぼ定常値を示す。細胞増殖は，4～5日目にほぼ定常値を示している。(n＝5)

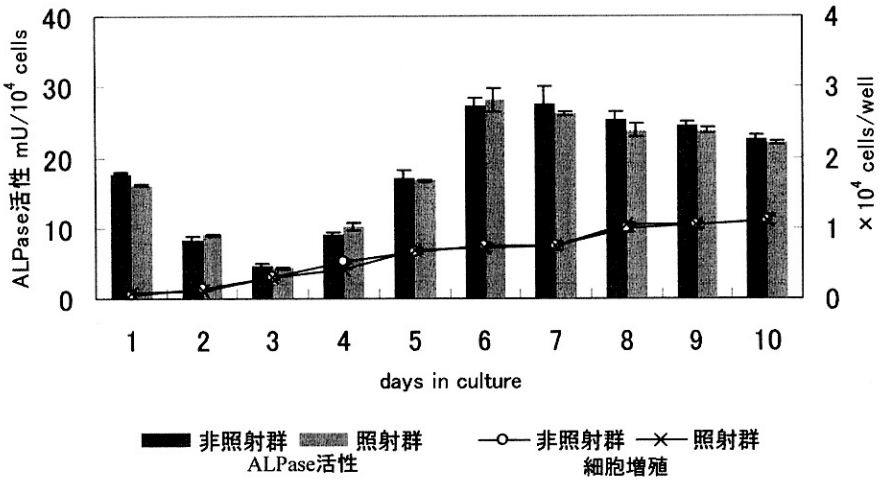
養日数の増加に伴い効果が減少し，培養13日目以後では効果がなくなったことも報告している。

胎生21日のラット胎仔頭蓋冠から採取された骨芽細胞様細胞には，種々の分化段階の細胞が存在する[13]ため，本研究ではどのような分化段階の細胞にレーザー照射の効果があるのかを検討した。そこで今回，分化程度の異なったC-26およびC-20を用いた[13]。C-26は未分化な骨芽細胞前駆細胞(osteoblast precurser cell)であるのに対して，C-20は，ALPase活性およびその遺伝子発現が顕著に高く，より分化した成熟骨芽細胞に近い細胞であると報告されている[14,17,20]。

本実験でこれらの細胞にレーザー照射を行ったところ，未分化型のC-26では，培養1，3日目の1回照射によりALPase活性は有意に増大し，またその効果は培養初期(1日目)の方がより高い持続効果が得られた。しかし，5日目の照射ではレーザー照射の効果がなくなっていたため，これより以前の分化段階の細胞が標的細胞と考えられる。C-26の静置培養では，培養7日目にデコリン遺伝子が発現することが報告されており[16]，デコリン遺伝子が発現する以前でないと効果がないと考えられる。さらにC-20は分化が進み，ALPase遺伝子やその活性を強く発現することから，レーザー照射の効果が全く現れなかったと考えられる。すなわち，細胞分化が進むと感受性が低下すると考えられ，レーザーの標的細胞は，骨芽細胞前駆細胞(osteoblast precurser cell)と考えられる。C-26，C-20にBMP-2，BMP-6を作用させた研究では，C-26のみにALPase活性や遺伝子発現促進作用を伴う細胞分化の亢進が認められている[14,21]。このことからレーザー照射群照射はBMPの骨芽細胞分化亢進機能に類似した機能を有している可能性も考えられる。



第9図　C-20の1日目3回照射群
細胞播種後，1日目に3回レーザー照射したときのALPase活性(mU/10^4cells)と細胞増殖(×10^4cells/well)を示す。ALPase活性は3～4日目に上昇し，その後ほぼ定常値を示す。細胞増殖は，4～5日目にほぼ定常値を示している。(n＝5)



第10図 C-20の1，2，3日目1回照射群
細胞播種後，1，2，3日目に1回レーザー照射したときのALPase活性(mU/10^4cells)と細胞増殖(×10^4cells/well)を示す。ALPase活性は4～5日目に上昇し，その後ほぼ定常値を示す。細胞増殖は，4～5日目にほぼ定常値を示している。(n＝5)

さらに，SaitoとShimizu[19]の研究では，ラット正中口蓋縫合急速拡大時の縫合部へのレーザー照射による骨形成促進作用が，拡大前期のレーザー照射のみ有効で，後期では全く無効であったとしている。これは拡大刺激で縫合部辺縁に存在する未分化間葉系細胞から骨芽細胞前駆細胞，前骨芽細胞へと分化する過程でレーザーが作用したものと考えられ，これらの細胞が成熟型の骨芽細胞へ分化してしまうとレーザーの効果は失われるものと思われる。

一方，レーザーの照射量を検討した実験では，レーザー照射の細胞効果は，総照射量より，照射密度と時間に影響されるという結果[22]を参考に，本実験ではALPase活性を1回照射群，培養1日目に3倍量照射群，培養1，2，3日目に各1回照射群間でレーザー照射の効果を比較した。その結果，レーザー照射の効果は1回照射群のみで有効であり，他の照射群では，非照射群に比べALPase活性の増大は認められなかった。このことから，レーザー照射のALPase活性上昇効果には至適照射量や，至適照射方法が存在し，過剰な照射量では効果は見られないと考えられる。現在のところレーザー光の細胞刺激メカニズムは解明されていないが，細胞分化による細胞の性質変化がレーザー光の感受性に強く影響していると考えられる。

C-26およびC-20は，*in vitro*で石灰化せず，ディッシュ内に形成する骨様結節を定量評価することができないため，ALPase活性を測定することにより，レーザーの効果を評価した。ALPase活性は無機リン酸の濃度を局所的に上昇させ石灰化を促進すると考えられており[23]，骨形成に重要な役割を演じていると考えられている[24]。また，骨芽細胞の分化が進むほどALPase活性が増大することから，骨芽細胞の分化マーカーと考えられており[25,26]，骨形成の指標に用いることが多い。Kusakariら[27]は，UMR-106細胞にレーザー照射を行い，DNAおよびタンパク合成とともにALPase活性の増大を報告している。また，Ozawaら[12]もラット頭蓋冠細胞へのレーザー照射でALPase活性の増大を伴った骨様結節の形成促進を報告している。レーザー照射によるALPase活性の上昇は骨芽細胞の分化促進を意味するとともに，骨の石灰化や形成を強く示唆する指標と考えられる。

本研究では，培養細胞を用いて検討を行ったが，レーザーの臨床応用にあたっては，レーザーが粘膜を進達し，骨形成サイトへ到達することが必要である。山岸ら[28]は，Ga-Al-As半導体レーザー（波長790 nm，出力60 mW）の連続波を，厚さ約1 mmのヒト新鮮皮質骨に照射すると，50%透過することを報告し，またGa-Al-As半導体レーザーは水分，ヘモグロビンによる吸収が少なく，組織透過性が高いとしている。今回用いたGa-Al-As半導体レーザーは，他の多くのレーザー光と比較して，組織透過力があり臨床的に応用できる可能性が高い[29]。今後，粘膜による光減衰等の研究を用い，到達した骨組織で最大の効果を発揮する照射方法を検討していく必要があると考えられる。

稿を終えるにあたり，ご指導ご校閲を賜りました日本大学歯学部納村晉吉教授，茂呂　周教授，高城　稔教授に厚く感謝の意を表します。また，本研究の遂行に直接ご指導，ご助力を賜りました本学矯正学教室，清水典佳助教授に心から感謝いたします。さらに，本研究を行うにあたりご協力頂きました本学病理学教室，解剖学教室第1講座，および矯正学教室員各位ならびにレーザーのご提供を賜りました松下産業機器株式会社，金田　明様に厚く御礼申し上げます。

本研究の一部は，学術フロンティア推進事業の一環として行われた。

文　献

1) Yamasaki K, Shibata Y, Fukuhara T (1982) The effect of prostaglandins on experimental tooth movement in monkeys (*Macaca fuscata*). J Dent Res 61, 1444-1446
2) Takano-Yamamoto T, Kawakami M, Kobayashi Y, Yamashiro T, Sakuda M (1992) The effect of local application of 1,25-dihydroxycholecalciferol on osteoclast numbers in orthodontically treated rats. J Dent Res 71, 53-59
3) 清水　光，田口直幸，草刈　玄(1999)口腔インプラントへのレーザー応用．日レ医誌 20, 11-19
4) Tang XM, Chai BP (1986) Effect of CO_2 laser irradiation on experimental fracture healing: a transmission electron microscopic study. Lasers Surg Med 6, 346-352
5) Trelles MA, Mayayo E (1987) Bone fracture consolidates faster with low-power laser. Lasers Surg Med 7, 36-45
6) Abe T (1990) Diode laser LLLT-enhanced bone fusion of femoral shaft fracture complicated by chronic osteomyelitis : a case report. Laser Therapy 2, 175-178
7) Nagasaka A, Kato K, Negishi A (1991) Bone regeneration effect of low level lasers including argon laser. Laser Therapy 3, 59-62
8) 紺　健一(1992)低出力レーザー照射による骨組織形成促進作用に関する実験的研究．日本レーザー歯学会誌 3, 35-44
9) 目澤修二(1996)低出力レーザーの鎮痛効果及び骨組織形成促進効果に関する検討．日レ医誌 17, 43-50
10) Kawasaki K, Shimizu N (2000) Effects of low-energy laser irradiation on bone remodeling during experimental tooth movement in rats. Lasers Surg Med 26, 282-291
11) Ueda Y, Shimizu N (2001) Pulse irradiation of low-power laser stimulates bone nodule formation. J Oral Sci 43, 55-60
12) Ozawa Y, Shimizu N, Kariya G, 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
13) Yamaguchi A, Kahn AJ (1991) Clonal ostogenic cell lines express myogenic and adipocytic developmental potemtial. Calcif Tissue Int 49, 221-225
14) Yamaguchi A, Katagiri T, Ikeda T, Wozney JM, Rosen V, Wang EA, Kahn AJ, Suda, Yoshiki S (1991) Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation in vitro. J Cell Biol 113, 681-687

15) 山口　朗(1995)骨芽細胞の分化. 実験医 13, 415-419
16) 森谷良行(2002)培養骨芽細胞前駆細胞と骨芽細胞における非コラーゲン性タンパクの遺伝子発現について. 日大歯学 76, 447-452
17) Yamada T, Kamiya N, Harada D, Takagi M (1999) Effects of transforming growth factor-β1 on the gene expression of decorin, biglycan, and alkaline phosphatase in osteoblast precursor cells and more differentiated osteoblast cells. Histochem J 31, 687-694
18) Ohshima M, Kuwata F, Otsuka K, Saito R, Sato K, Shioji S, Suzuki K (1988) Alkaline phosphatase activities of cultured human periodontal ligament cells. J Nihon Univ Sch Dent 30, 208-217
19) Saito S, 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
20) Takagi M, Yamada T, Kamiya N, Kumagai T, Yamaguchi A (1999) Effects of bone morphogenetic protein-2 and transforming growth factor-β1 on gene expression of decorin and biglycan by cultured osteoblastic cells. Histochem J 31, 403-409
21) Ebisawa T, Tada K, Kitajima I, Tojo K, Sampath TK, Kawabata M, Miyazono K, Imamura T (1999) Characterization of bone morphogenetic protein-6 signaling pathways in osteoblast differentiation. J Cell Sci 112, 3519-3527
22) van Breugel HH, Bar PR (1992) Power density and exposure time of He-Ne laser irradiation are more important than total energy dose in photo-biomodulation of human fibroblasts in vitro. Lasers Surg Med 12, 528-537
23) 小沢英浩, 山田まりえ, 山本敏男, 高野吉郎, 内田　隆, 岩久文彦, 森田修一(1979)アルカリホスファターゼの電顕細胞化学. 骨代謝 12, 6-15
24) De Bernard B (1982) Glycoproteins in the local mechanism of calcification. Clin Orthop 162, 233-244
25) Gundberg CM, Hauschka PV, Lian JB, Gallop PM (1984) Osteocalcin: isolation, characterization, and detection. Methods Enzymol. Osteocalcin: isolation, characterization, and detection. Meth Enzymol 107, 516-544
26) Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, Kennedy MB, Pockwinse S, Lian JB, Stein GS (1990) Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J Cell Physiol 143, 420-430
27) Kusakari H, Orikasa N, Tani H (1992) Effect of low power laser on wound healing of gingiva and bone. In Laser Application in Medicine and Surgery, Galletti G, Bolognani L, Ussia G eds, Monduzi Editore, Bologna, 49-52
28) 山岸久也, 篠原　親, 斉藤　茂, 佐々木洋, 鐘ヶ江晴秀, 柴崎好伸(1994)半導体レーザーの組織透過性に関する基礎的研究. 日本レーザー歯学会誌 5, 13-22
29) Hillenkamp F (1980) Interaction between laser radiation and biological systems. In Lasers in biology and medicine, Hillenkamp F, Pratesi R, Sacchi CA eds, Plenum Press, New York, 37-68