Cellular Responses on Anodized Titanium Discs After Laser Irradiation

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Background and Objectives: Although the laser is one of the widely used systems in dental field, literature about the biological effects of laser irradiation on the titanium surface is rare. The aim of this study was to investigate the responses of osteoblast-like cells seeded onto laser irradiated anodized titanium discs, using a CO_2 (carbon dioxide) and Er,Cr:YSGG (erbium chromium-doped yttrium scandium gallium garnet) laser, with reference to cellular proliferation and differentiation in vitro.

Study Design/Materials and Methods: Osteoblast-like HOS cells were cultured on four differently treated anodized titanium disc surfaces. Group 1, anodized (control); group 2, CO_2 laser irradiated; group 3, Er,Cr:YSGG laser irradiated (150 J/cm^2); group 4, Er,Cr:YSGG laser irradiated (300 J/cm^2). MTS-based cell proliferation assay and alkaline phosphatase (ALP) activity test were used to compare cellular responses after 1 and 3 days. Three-way analysis of variance (ANOVA) and post hoc method were carried out to determine the statistical significance of the differences.

Results: The cells proliferated actively on all substrates; greatest cellular proliferation was observed in group 4, followed by groups 2, 3, and 1, respectively ($P<0.05$). The test groups also presented significantly higher ALP activities than the control group ($P<0.05$) except group 3. For both tests, measured optical densities at 3 days were greater than that of 1 day in control and all test groups ($P<0.001$).

Conclusion: The data shows that irradiation with a CO_2 laser or Er,Cr:YSGG laser may induce a measurable positive effect on osteoblast proliferation and differentiation. Lasers Surg. Med. 40:738–742, 2008. © 2008 Wiley-Liss, Inc.

Key words: anodic oxidation; cell differentiation; cell proliferation; osteoblast; laser; titanium disc

INTRODUCTION

The interest in application of lasers for both clinical use and experimental purpose in dental fields has been growing. Various laser systems have been introduced for different aims, needs, and targets [1–3]. When laser is applied to implant therapy, new indications may be the surface modification of dental implant or the sterilization of exposed implant surfaces to rehabilitate ailing implants [4–7]. The positive effect of dental laser on osteoblasts can be used to obtain earlier and better osseointegration because initial attachment, spreading, proliferation, and differentiation of osteoblasts are crucial for successful bone healing process of dental implant [8,9].

Many research studies on the biostimulatory effect of low power semiconductor diode laser on human osteoblast-like cell have been done [10–12], while studies on the effect of high power surgical laser irradiation such as CO_2 (carbon dioxide), Nd:YAG (neodymium-doped yttrium aluminum garnet), Er:YAG (erbium-doped yttrium aluminum garnet), and Er,Cr:YSGG (erbium chromium-doped yttrium scandium gallium garnet) lasers on cellular responses is rare. Dörtbudak et al. [10] tested the effect of continuous wave diode laser irradiation on osteoblasts derived mesenchymal cells in vitro. By irradiating the cultures three times, a significant increase in bone matrix production was achieved in the lased group, compared with the non-lased control group. Khadra et al. [12] also investigated the effect of low-level laser therapy (LLLT) on the attachment, proliferation, differentiation, and production of transforming growth factor-β_1 (TGF-β_1) by human osteoblast-like cells. In this cellular model, LLLT enhanced the attachment and proliferation of cells derived from human

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mandibular bone, cultured on titanium implant material. Cells exposed to GaAlAs diode laser light of 3 J/cm^2 showed significantly increased osteocalcin and TGF-β_1 production, suggesting that LLLT stimulates differentiation of osteoblast-like cells in a dose-dependent manner.

In other recent studies, CO_2 laser or Er,Cr:YSGG laser was irradiated directly onto the titanium material surface to improve osteoblast cell behavior. Romanos et al. [8] demonstrated that osteoblasts proliferate better on hydroxyapatite (HA)-coated discs, sandblasted-surface discs, and titanium plasma sprayed (TPS)-coated discs irradiated with either a CO_2 or an Er,Cr:YSGG laser. Huang et al. [9] showed that the Er,Cr:YSGG laser treatment of titanium disc increased cellular proliferation and improved cell adhesion morphology. Currently, several surface treatments such as anodizing, HA-coating, sandblasting, TPS-coating are being applied for implant osseointegration, and there are several reports that show good results in osseointegration with anodized surfaces [13–17]. Therefore, the cellular responses on anodized titanium surfaces were evaluated after laser irradiation in the present study. The purpose of the present study was to investigate the responses of osteoblast-like cells to laser irradiated anodized titanium disc, using a CO_2 and Er,Cr:YSGG laser, with reference to cellular proliferation and differentiation in vitro.

MATERIALS AND METHODS

Forty-eight titanium discs were fabricated using commercially pure titanium (Warantec Co., Seoul, Korea), with dimensions of 25 mm diameter and 1 mm thickness. Prior to use, degreasing and acid prepickling of all discs were done by washing them in acetone, processing through 2% ammonium fluoride/2% hydrofluoric acid/10% nitric acid solution at 55°C for 30 seconds, and pickling in 2% hydrofluoric acid/10% nitric acid at room temperature for 30 seconds. The pretreated discs were further processed to produce an anodized surface. The anodic oxidation treatment of the titanium discs was performed at 300 V in an aqueous electrolytic solution of 0.02 M/L calcium glycerophosphate ($CaC_3H_7O_6P$) and 0.15 M/L calcium acetate. All procedures were done at room temperature, and the total time for anodization of one disc was 3 minutes [14,16]. The anodized discs were washed with distilled water, dried, and then sterilized in ethylene oxide (E.O.) gas before the experiment.

The 48 discs were divided into 4 groups according to the laser used. Group 1 was non-irradiated which served as a control. Group 2 was irradiated using a CO_2 laser (Panalas CO5Σ; Panassonic, Kasnagawa, Japan). The CO_2 laser was used with 700 μmΦ tip (2A tip; Panassonic). The power output was set at 2 W with a frequency of 20 Hz in repeat (RPT) mode according to the manufacturer's advice. The calculated energy of 300 J/cm^2 was applied per titanium disc. Groups 3 and 4 were irradiated with the Er,Cr:YSGG laser (Waterlase MD; Biolase Technology Inc., Santa Clemente, CA). This laser system was used with 600 μmΦ tip (MZ6 tip; Biolase Technology Inc.) in hard tissue (H)-mode, air pressure setting at 15%, and the water spray at 15% according to the manufacturer's advice. In groups 3 and 4 two different laser energy densities, calculated 150 and 300 J/cm^2, were applied. The power of 1.5 W, 30 Hz was applied to group 3 and 2.5 W, 30 Hz to group 4. During the lasers application, the distance between the laser tip and specimen surface was controlled at about 1 mm.

The osteoblast-like human osteogenic sarcoma (HOS) cells (ATCC, Rockville, MD) were used in these experiments. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were seeded onto the control and the irradiated anodized titanium discs in plates at a density of 5×10^5 cells/ml and cultured at 37°C in 5% CO_2. Cells were cultured for 1 and 3 days. After 1 day, three discs in the culture medium for each group were treated with MTS-based cell proliferation assay (CellTiter 96® AQ_{ueous} One Solution Cell Prolifertion Assay, Promega Corp., WI) and another three discs were used for ALPase activity test. After 3 days, the same procedure was done. The sample number used for each group was three. The MTS-based cell proliferation assay is based on the reduction of a tetrazolium compound to a colored formazan product that is soluble in tissue culture medium by viable cells (or metabolic activity) [18]. Twenty microliters of CellTiter 96® AQ_{ueous} One Solution reagent was directly added into each well containing the samples in 100 μl of culture medium, and incubated for 3 hours in a humidified incubator with 95:5 air/CO_2, and then the absorbance was recorded at 490 nm. For alkaline phosphatase (ALP) activity test, cells cultured for 1 and 3 days were rinsed three times with PBS and extracted with 0.5% Triton-X in 25 mM glycine–NaOH. One hundred microliter aliquots of the extracts were added to 50 μl ALP solution (pNPP; Sigma, Steinheim, Germany) in a 96-well culture plate for 30 minutes at 37°C [19,20]. After development of color, the time was recorded and the reaction was stopped by adding 50 μl of 2N NaOH, and the final absorbance was read at 405 nm using a microplate reader.

Statistical Data Evaluation

Three-way analysis of variance (ANOVA) and Student–Newman–Keuls test as post hoc method were carried out to determine the statistical significance of the differences among observed groups.

RESULTS

Table 1 shows the overall measured values of MTS assay and ALP activity test for the control and test groups. The MTS tetrazolium compound reduction as an indicator of cell vitality has been used to quantify the proliferation capacity of the osteoblast-like HOS cells seeded onto the different substrates. The cells proliferated actively on all substrates; the smallest proliferation rate however was measured on group 1. Compared to group 1, cell proliferation increased on all of the treated samples at day 1 and 3. In particular,

TABLE 1. MTS and ALPase Optical Density Values of Osteoblast-Like HOS Cells Grown on Anodized as Control (Group 1), CO_2 Irradiated (Group 2), 150 J/cm^2-Er,Cr:YSGG Irradiated (Group 3) and 300 J/cm^2-Er,Cr:YSGG Irradiated (Group 4) Discs After 1- and 3-day Culture

Test	Day	Optical density (OD) ± standard deviation				Statistical significance
		Group l	Group 2	Group 3	Group 4	
MTS	1	0.241 ± 0.022	0.368 ± 0.022	0.309 ± 0.019	0.536 ± 0.054	Day 1 <Day 3[a]
	3	1.181 ± 0.083	1.637 ± 0.040	1.503 ± 0.079	1.661 ± 0.028	Group l <Group 3 <Group 2 <Group 4[b]
ALP	1	1.775 ± 0.024	1.948 ± 0.063	1.838 ± 0.105	2.098 ± 0.094	Day 1 <Day 3[a]
	3	2.096 ± 0.095	2.181 ± 0.054	2.102 ± 0.057	2.337 ± 0.082	Group l, Group 3 <Group 2 <Group 4[b]

The values correspond to the assessed relative values of the optical density after MTS assay and ALPase activity test. Results are mean ± SD (standard deviation).

[a]Indicates a significant difference between day 1 and 3 ($P < 0.001$).

[b]Indicates the significantly higher OD value ($P < 0.05$) by Student–Newman–Keul post hoc test.

greatest cellular proliferation was observed in group 4 (300 J/cm^2; Er,Cr:YSGG laser irradiated), followed by group 2 (CO_2 laser irradiated), group 3 (150 J/cm^2; Er,Cr:YSGG laser irradiated), and group 1 ($P<0.05$).

The test groups also presented significantly higher ALP activities than the control group ($P<0.05$) except group 3. The ALPase optical density value on group 3 was not significantly different from that observed on the control group. Specifically, group 4 showed the greatest cellular differentiation, followed by groups 2, 3, and 1, respectively. For both tests, measured optical density increased significantly during the 3-day cell culture period in the control and all test groups ($P<0.001$).

DISCUSSION

In recent years, laser light has been increasingly used for the treatment of injuries of oral and maxillofacial soft and hard tissues and for the application to implant dentistry [1,3]. Literature has revealed the effect of laser irradiation on soft tissue wound healing, osteoblast proliferation, and bone healing [9,21,22]. In this study, two different laser systems were used. The exact mechanism of effectiveness of laser irradiation of these systems is still unclear, but many researches have demonstrated that the laser was effective for enhanced osteoblast cell adhesion and proliferation [8,9,23]. Hao et al. [23] found that CO_2 laser surface treatment enhanced human osteoblast cell adhesion and proliferation on metal surface. Laser irradiated surface had a lower surface roughness than mechanically roughened sample but showed not only greater cell adhesion but also considerable increase in cell proliferation. They observed an increase in the wettability and surface energy of CO_2 laser irradiated samples and stated that increased surface energy was a more important surface characteristic than surface roughness for cellular adhesion and proliferation, and it could be achieved by CO_2 laser irradiation. Huang et al. [9] investigated an initial cell proliferation index (CPI) of human osteosarcoma cells and surface spreading morphology of the attached cells after using Er,Cr:YSGG laser on titanium discs with different laser energy densities, 125 and 190 J/cm^2. The Er,Cr:YSGG laser-treated titanium had a higher initial CPI and better cell spreading morphology than the untreated titanium. The specimen with higher applied laser energy had somewhat better biocompatibility. After laser treatment, locally melted morphology was observed on the specimen surface and increasing the applied energy density from 125 to 190 J/cm^2 led to a slight increase in both the melted area and surface roughness.

Osseointegration of titanium implants can be achieved by direct bone-to-implant contact at microscopic level [24]. For this direct contact, initial cell adhesion and growth is important [25]. High level of osteoblast attachment, proliferation, and differentiation on titanium surface may enhance new bone formation. The functional activity of the cells close to the implant surface is influenced by the properties of the implant surface [26]. The surface quality of the implant depends on the chemical, physical, mechanical, and topographic properties of the surface and the different properties may change the response of osteoblasts to the titanium surface [13,16,17,27,28]. The physical and/or chemical variations, including the three dimensional change in the surface topography through laser treatment on titanium substrate might play an important role in the initial biocompatibility [9]. Low power lasers directly stimulate the cellular function of irradiated cells whereas high power lasers change the surface properties of the irradiated material so that cells can attach and spread easier. In this study, the surface's property could be changed very slightly after irradiation with the high power system laser, which implies that the laser influenced at cellular level. Romanos et al. [8] examined osteoblast attachment on laser irradiated titanium discs using scanning electron microscopic analysis. Osteoblasts grew on titanium surfaces with different patterns after CO_2 or Er,Cr:YSGG laser irradiation, and SEM analysis demonstrated the formation of filipodia, representing cell maturation. In the machined group, both disc surfaces lased by CO_2 and Er,Cr:YSGG laser presented a higher cellular density than in the non-irradiated area. In the groups of HA-coated discs, sandblasted-surface discs, and

TPS-coated discs, the osteoblasts of test group surfaces (those irradiated by the CO_2 or Er,Cr:YSGG laser) presented a spread of cells with good maturation.

The osseointegration of anodized titanium was increased compared to turned implant in many articles [14–17,29]. Nevertheless, this study showed that the laser-irradiated specimens are more biocompatible. The majority of the laser irradiated groups showed improved cellular responses such as proliferation and differentiation, and group 4 demonstrated the highest values. The power output set of CO_2 laser group (Group 2) was 2 W and total energy applied was 300 J/cm^2. 1.5 W (Group 3) and 2.5 W (Group 4) of power was used in Er,Cr:YSGG laser and total energy, 150 and 300 J/cm^2, was applied respectively. The total energy applied on the titanium discs was higher at groups 2 and 4 than group 3, and the power output was greatest in order of group 4, group 2, and group 3. The greatest power output setting and total energy of group 4 resulted in highest optical density values for MTS assay and ALP activity.

If the laser is used with a power over the "tissue cutting mode", it could influence unwanted areas. When applying the laser, factors such as power, energy, density, time, and interaction should be considered. Park [30] researched the surface change, roughness change, and surface composition change after irradiation with high energy density. Melting and surface changes were observed through SEM analysis after irradiation with a power over 3 W. An excessive change in the surface can influence the mechanical properties, therefore in this study the power was decreased using low watt energy. Moreover, the energy (dose) was set in order to provide enough laser doses on the surface. In this study, different power outputs of 2 W, 1.5 W, and 2.5 W were selected. Normally, in laser therapy for biostimulation, low power laser is used (under 2 W). High power laser transmits high power to soft and hard tissues and can generate unwanted results. In this study, for clinical application, a harmless power (watt) was selected, and in order to provide laser therapy effect on the titanium surface, the energy (dose) was selected. Therefore it is considered that using a high power laser at a low power allows an adequate dose to be irradiated on the titanium surface and concomitantly it avoids undesirable results and positively influences the attachment and spread of the cells. Based on these results, further studies should be carried on about the correlation between the power output setting and the specific cellular activity.

In this study, cellular proliferation and differentiation were measured after laser irradiation in vitro. MTS-based cell proliferation assay is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays [18]. ALP activity is widely used as an osteoblast marker, and an increase in ALP activity is associated with osteoblastic differentiation, bone formation, and matrix mineralization [31,32]. Therefore, the ALP specific activity of the osteoblast-like HOS cells was measured to determine effect on the differentiation of osteoblast cells. The results of this study shows that the optical densities of MTS assay were increased in time for all groups and showed significant differences among the groups. The specific activity of ALP was also found to be increased in time for all groups and showed differences between some groups. This means that cells not only grow and proliferate, but also differentiate better on laser irradiated specimens. Further studies for considering specific markers for differentiation such as osteocalcin to confirm if the osteoblasts differentiate to generate, and for confirming the results such as surface spreading morphology are needed.

Various studies have already demonstrated that LLLT by soft laser such as diode laser was effective on osteoblast attachment, proliferation and differentiation [8,10,12,33,34]. However, the effectiveness of CO_2 laser or Er,Cr:YSGG laser on osteoblast proliferation and differentiation is not well known yet. The results of this study suggest that both types of laser irradiation are effective and favorable for osteoblast proliferation and differentiation. Extrapolated to in vivo conditions, these findings might suggest that CO_2 and Er,Cr:YSGG laser irradiation could enhance osteoblastic activity, reduce healing time, and promote early osseointegration, and also might help to explain the effect of laser irradiation on peri-implantitis area for new bone formation. Further studies on the exact mechanism of both laser systems and clinical applications are needed.

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