

Effects of Smads and BMPs induced by Ga-Al-As laser irradiation on calcification ability of human dental pulp cells

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Abstract: We investigated the effects of Ga-Al-As laser irradiation on the mineralization ability of human dental pulp (HDP) cells and on Smads and bone morphogenetic protein (BMP) production as one mechanism for the transmission of laser photochemical energy to cells. HDP cells *in vitro* were irradiated once with a Ga-Al-As laser at 1.0 W for 500 s, and calcified nodule formation was assessed by Alizarin red S staining. The laser irradiation was greater in the laser-irradiated group than in the non-irradiated group. Both calcium production and alkaline phosphatase (ALP) activity were higher after laser irradiation. Expression of mRNAs for Smad1, Smad7, BMPs, ALP, and osteocalcin was greater after laser irradiation, whereas expression of Smad6 mRNA was inhibited. Production of BMP-2 and BMP-4 in conditioned medium was also higher after laser irradiation. These results suggest that Smads and BMPs play important roles in ALP activity and calcification upon laser irradiation of HDP cells. (J. Oral Sci. 50, 75-81, 2008)

Keywords: dental pulp; laser; Smads; calcification; BMPs; pulp capping.

Introduction

Dental pulp is a loose connective tissue consisting of odontoblasts, blood vessels, nerves, and collagen fibers

contained in a protective wall of hard tissue. When pulp tissue is irritated by mechanical, thermal, chemical, or bacterial irritants, inflammatory reactions occur and produce various bioactive molecules, particularly pro-inflammatory cytokines. Pulp repair characterized by dentin bridge formation is known to occur after direct pulp-capping of exposed pulp using calcium hydroxyl products (1). Although such direct pulp-capping is practiced widely, hard tissue formation often does not progress smoothly toward a cure, necessitating pulpectomy. As dental pulp conservation in the root canal is considered to yield the best long-term outcome, and pulpectomy has a major impact on the life of the affected tooth, various pulp-conservation treatments are being sought (2,3).

Recently, the clinical use of lasers has increased, and lasers are now employed frequently even for endodontic treatment (4). The short-wavelength Ga-Al-As (semiconductor) laser and He-Ne laser are used for coagulation of deep tissue in the body and for promoting hard tissue formation (5-7). Against this background, we have been studying the anti-inflammatory effect of Ga-Al-As laser irradiation when used as an adjuvant to accelerate hard tissue formation.

Utsunomiya (8) has reported that dentin bridge formation occurs earlier on exposed pulp surfaces in dogs one week after Ga-Al-As laser irradiation (0.3 W for 3 min) than in unirradiated controls. In contrast, it has been found that when cultured human dental pulp (HDP) cells are exposed to laser irradiation, calcified nodule formation increases upon long-term culture and alkaline phosphatase (ALP) activity is significantly accelerated (6). We have also shown that calcification of HDP cells is stimulated by laser irradiation, and that hydroxyl radicals generated by laser irradiation promote HDP cell mineralization (7). However, issues such as cell-propagated signaling immediately after laser irradiation and hard tissue-related

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mRNA expression have received little attention.

Bone morphogenetic proteins (BMPs) are growth factors belonging to the tumor growth factor (TGF)- β superfamily, whose members exert their effects by binding to two types of serine/threonine kinase receptor, both of which are essential for signal transduction (9,10). The type II receptors are constitutively activated kinases that transphosphorylate type I receptors upon ligand binding. The type I receptors activate intracellular substrates such as Smad proteins and determine the specificity of intracellular signals. Eight different Smad proteins have been identified in mammals, and these proteins are classified into three subgroups: receptor-regulated Smads (R-Smads), common partner Smads (Co-Smads), and inhibited Smads. R-Smads are directly activated by type I receptors, form complexes with Co-Smads, and translocate into the nucleus. Smad1, Smad5, and Smad8 are activated by BMPs, whereas Smad2 and Smad3 are activated by TGF- β and activin. Smad4 functions as a Co-Smad. Structurally, Smad6 and Smad7 are related only distantly to the other Smads, and act as inhibited Smads. There has been one previous report on the mechanism of suppression of TGF- β /Smad signaling by opposing stimuli mediated through the activation of inhibited Smad7 by NF- κ b (11).

BMP-2 has been shown to induce differentiation of cells of the osteoblastic lineage and to enhance their differentiated function (12). Furthermore, BMP-2 accelerates the differentiation of HDP cells into odontoblasts and increases ALP activity (13). In this way, inflammatory cytokines and mediators are thought to be associated with dental pulp calcification. However, the mechanism involved is not fully understood.

To clarify the effects of Ga-Al-As laser irradiation on the calcification ability of HDP cells, we measured the expression of mRNAs for BMP-2 and BMP-4, Smad1, 6, and 7, ALP and osteocalcin (OCN), as well as production of BMP-2 and BMP-4 proteins, in HDP cells stimulated by laser irradiation.

Materials and Methods

Cell isolation and culture

This study was approved by the ethics committee of Nihon University School of Dentistry at Matsudo (Approval number No. EC 03-025). HDP cells were obtained from unerupted teeth extracted from young patients in the course of orthodontic treatment. Each patient gave informed consent before providing the sample. After the dental pulp had been extracted under sterile conditions, it was washed twice with Hanks' balanced salt solution (pH 7.4). Then the pulpal tissue was minced, placed on a 35-mm tissue culture dish, and covered with a sterilized glass coverslip

(14).

The culture medium used was α -minimal essential medium (Gibco, Grand Island, NY, USA) containing 10% fetal calf serum and antibiotics consisting of 100 μ g/ml penicillin G (Meiji Seika, Ltd., Tokyo, Japan), 100 μ g/ml gentamicin sulfate (Meiji Seika), and 100 μ g/ml fungisone (Gibco) supplemented with 50 μ g/ml ascorbic acid (Wako, Osaka, Japan) and 10 mM sodium β -glycerol-phosphate (Wako). Culture was maintained in an atmosphere of 5% CO₂, 90% N₂, and 5% O₂ at 37°C (14,15).

When cell growth from the explant had reached confluence, the cells were detached with 0.05% trypsin (580 BAEE units/mg; Gibco) in phosphate-buffered saline (PBS) and subcultured in culture flasks. Cells observed at confluence by phase-contrast microscopy had not formed the small mats typical of epithelial cells. For the experiment, HDP cells after 6 to 9 passages were plated at 1×10^5 cells (in 1.5 ml medium) per dish.

Cell numbers were recounted with a Coulter Counter ZM (Coulter Electronic Ltd., Luton, England).

Laser irradiation

A high-energy Ga-Al-As laser apparatus (Osada Light-surge 3000, Osada, Tokyo, Japan) with a wavelength of 810 nm (\pm 20 nm) and a maximum power output of 3.0 W was used. The laser beam was delivered by an optical fiber 0.6 mm in diameter, with a 10-cm distance from the tip of the fiber to the cell layer. The cells in the 35-mm dish were irradiated continuously at 1.0 W for 500 s (laser irradiation group), and unirradiated cells were used as a control group when they were 50% confluent. The conditions for laser irradiation were decided on the basis of ALP activity data obtained in a pilot study (data not shown) and in previous reports (6,7).

Alizarin Red S staining and measurement of calcium

Calcified nodules in the HDP cells were first washed for 30 days *in vitro* with PBS and then fixed in 10% buffered formalin for 1 h. They were then washed again and stained with Alizarin Red S (16). The calcium in the nodules was dissolved with 0.5 N HCl (1.0 ml) for 12 h, and the amount of calcium was then measured using Calcium C test Wako (Wako).

RNA extraction

HDP cells cultured in the medium were lysed with Trizol (Life Technologies, Inc., Rockville, MD, USA) in accordance with the manufacturer's protocol, and the total amount of RNA was extracted and stored at -80°C. RNA extraction times after laser irradiation were 3 h for Smad1,

Smad6 and Smad7, 6 h and 12 h for BMP-2, 12 and 24 h for BMP-4, 6 days for ALP, and 9 days for OCN.

RT-PCR technique

cDNA synthesis and amplification by RT-PCR were carried out with a GeneAmp RNA PCR kit (Perkin-Elmer, Norwalk, CT, USA). The PCR mixture was subjected to amplification with a GeneAmp PCR system 9600 (Perkin-Elmer) set at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. PCR was carried out for 24 cycles for Smad1 (17), 36 cycles for Smad6 (18), 27 cycles for Smad7 (17), 24 cycles for BMP-2 (14), 24 cycles for BMP-4 (7), 33 cycles for ALP (7), 27 cycles for OCN (15), and 27 cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (17) using primers designed and synthesized with reference to the reported cDNA sequences. These primers were as follows: GAPDH (forward) 5'- ATC ACC ATC TTC CAG GAG - 3', (reverse) 5'- ATG GAC TGT GGT CAT GAG - 3'; Smad1 (forward) 5'- GAG GGG GCC GTG CAG ATG TAT - 3', (reverse) 5'- GCC CTC TCT GGA TAT CTT CT - 3'; Smad6 (forward) 5'- TGA ATT CTC GGA TAT CTT CT - 3', (reverse) 5'- GCT CGA AGT CGA ACA CCT T - 3'; Smad7 (forward) 5'- GCT GCA TAA ACT CGT GGT CA - 3', (reverse) 5'- ATC ACC ATC TTC CAG GAG - 3'; BMP-2 (forward) 5'- GCT GTA CTA GCG ACA CCC AC - 3', (reverse) 5'- TCA TAA AAC CTG CAA CAG CCA ACT - 3'; BMP-4 (forward) 5'- GCT GAA GTC CAC ATA GAG CGA GTG - 3', (reverse) 5'- ACT GGT CCA CCA CAA TGT GAC ACG - 3'; ALP (forward) 5'- GAA AGA GAA AGA CCC CAG - 3', (reverse) 5'- ACC ACC CAT GAT CAC ATC - 3'; and OCN (forward) 5'- GAT ACC TAT CAA TGG CTG GGA GCC - 3', (reverse) 5'- GTC GAC ATA GGC CTC CTG AAA GCC - 3'.

PCR fragments were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide.

Effects on BMP-2 and BMP-4 production

BMP-2 and BMP-4 were released into the culture medium over 24 h to 72 h of incubation, and quantified with a Quantkine BMP-2 ELISA kit[®] (R&D Systems, Minneapolis, MN, USA) and a Quantkine BMP-4 ELISA kit[®] (R&D Systems), respectively, at 450 nm (7).

Assay of ALP activity

HDP cells cultured in 35-mm dishes were rinsed three times with PBS. Glycine (100 mM, pH 10.5) buffer containing 2 mM MgCl₂ and 8 μM p-nitrophenylphosphate (p-NPP) was then added and the amount of p-NP was measured at 405 nm. One unit of enzyme was defined as the activity that liberated 1 mM product/min at 37°C, and ALP activity was expressed as mUnits/dish (14).

Effects on OCN production

OCN released into the culture medium over 12 to 24 days of incubation was quantified with an OCN instant ELISA kit[®] (Bender MedSystems, USA) at 570 nm (6).

Statistical analysis

All values are presented as means ± SD, and the significance of differences was determined by Student's *t*-test.

Results

Alizarin Red S staining

After 30 days of culture, calcified nodules present in the 35-mm dishes were stained with Alizarin Red to a markedly greater extent in the laser irradiation group than in the control group. The amount of calcium in the irradiated group was significantly greater than in the control group (Fig. 1) ($P < 0.01$) ($n = 7$).

RT-PCR

To determine the effects of laser irradiation on expression of mRNAs for Smad1, Smad6, Smad7, BMP-2, BMP-4, and ALP, we used RC-PCR analysis. An increase in mRNA expression was recognized in the laser irradiation group at 3 h (Smad1 and Smad7), 6 h (BMP-2 and BMP-4), 12 h (BMP-2 and BMP-4), 6 days (ALP), and 9 days (OCN) in comparison with the control group (Figs.2-4). However, laser irradiation decreased the quantity of Smad6 mRNA at 3 h (Fig. 2).

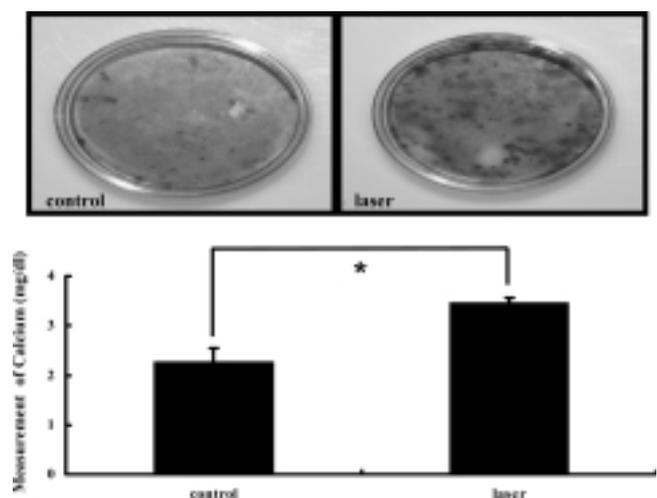


Fig. 1 Effects of 30-day laser irradiation on calcified nodule formation and calcium levels in HDP cells. HDP cells were stained to a significantly greater extent in the laser-irradiated group than in the controls ($*P < 0.01$).

Effects on BMP-2 and BMP-4 production

BMP-2 production in the laser irradiation and control groups increased in a time-dependent manner and peaked at 48 h; the production level was significantly greater in the laser-irradiated group than in the control group (Fig. 5) ($P < 0.01$) ($n = 8$). BMP-4 production in the laser irradiation and control groups increased in a time-dependent manner and peaked at 72 h; the production level was significantly greater in the laser-irradiated group than in the control group (Figs. 3 and 4) ($P < 0.01$) ($n = 8$).

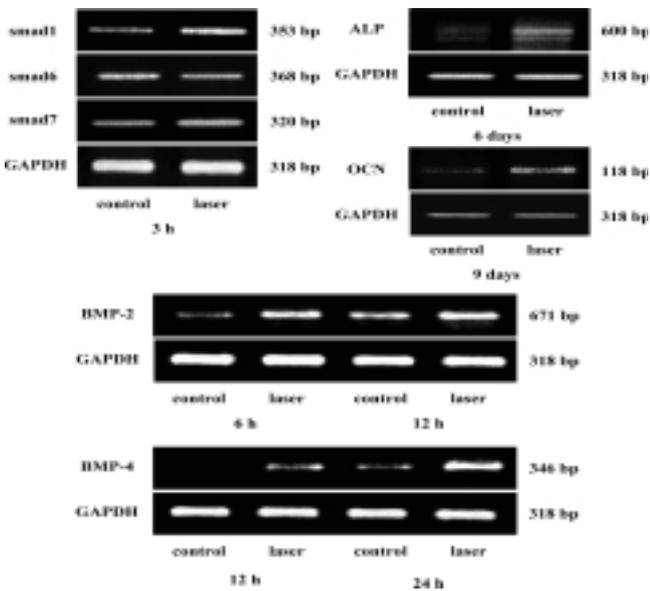


Fig. 2 Effects of laser irradiation on mRNA in HDP cells. mRNA expression was markedly greater at 3 h (Smad1, 6, 7), 6 h (BMP-2), 12 h (BMP-2, 4), 24 h (BMP-4), 6 days (ALP), and 9 days (OCN) in the laser-irradiated group than in the control group.

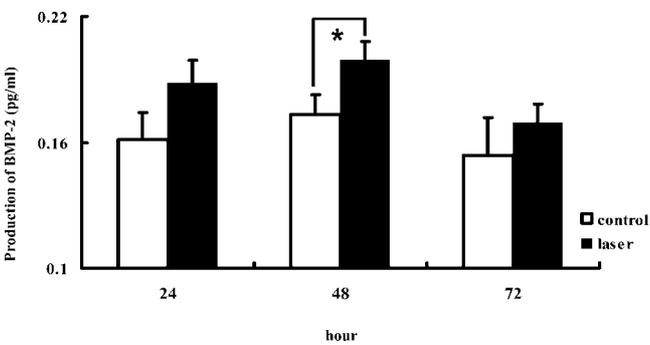


Fig. 3 Effect of laser irradiation on BMP-2 production in HDP cells. BMP-2 production was significantly greater in the laser-irradiated group than in the control group at 48 h ($*P < 0.01$).

Assay of ALP activity

ALP activity in the laser-irradiated and control groups increased in a time-dependent manner and peaked on day 12. ALP activity was significantly greater in the laser-irradiated group than in the control group (Fig. 5) ($P < 0.01$) ($n = 8$).

Effects on OCN production

OCN production in the laser-irradiated and control groups increased in a time-dependent manner and peaked at 18 days; the production level was significantly greater in the laser-irradiated group than in the control group (Fig. 6) ($P < 0.01$) ($n = 8$).

Discussion

Various bio-stimulatory effects of Ga-Al-As laser irradiation have been reported, including wound healing (19), fibroblast proliferation, and collagen synthesis (5,6). Cytological studies have reported that calcified nodule

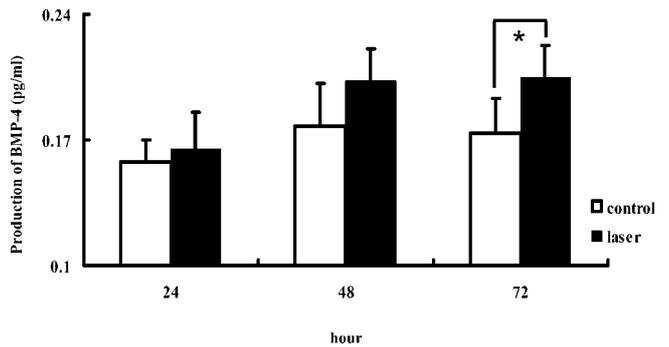


Fig. 4 Effect of laser irradiation on BMP-4 production in HDP cells. BMP-4 production was significantly greater in the laser-irradiated group than in the control group at 72 h ($*P < 0.01$).

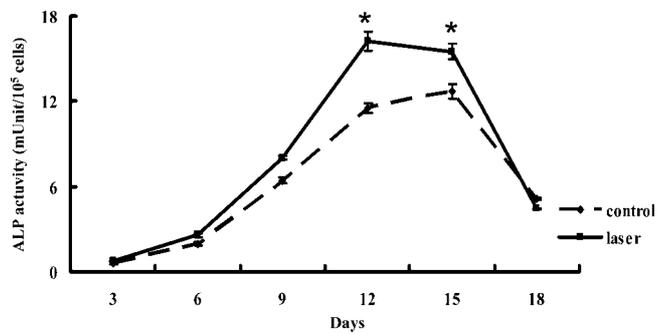


Fig. 5 Effect of laser irradiation on ALP activity in HDP cells. ALP activity was significantly greater in the laser-irradiated group than in the control group at 12 days ($*P < 0.01$).

formation is increased and that ALP activity enhanced when HDP cells are exposed to laser irradiation (16). However, few studies have investigated the cellular responses and gene expression after laser irradiation (7).

To elucidate the mechanism of increased calcification in response to Ga-Al-As laser irradiation, we focused on Smads and BMPs and investigated the effects of irradiation on calcification by HDP cells.

BMPs are known to induce the differentiation of osteoblastic cell lineages and to enhance their differentiated functions (20). ALP is known to be associated with bone metabolism and differentiation of osteoblasts (21). ALP activity and BMP production are among the most frequently used indicators of osteoblast differentiation and osteogenic properties (21). In addition, because ALP activity and BMP production are highest in the subodontoblastic layer of dental pulp tissue and are closely related to dentin formation, they have been suggested as suitable markers of differentiation during dental pulp dentinogenesis (6,7,13,14). It is thought that ALP and BMPs are produced in the early stages of calcification through a series of processes, and it has been reported that high ALP activity and BMP production are evident in HDP cells. Thus it may also be possible to use ALP and BMPs as markers of cellular differentiation during the formation of hard tissue in dental pulp, as they appear to play important roles in a series of processes that lead to calcification. Saito et al. (13) reported that BMP-2 increases ALP activity in HDP cells, and Nakase et al. (21) also reported that ALP activity is promoted in mouse preosteoblastic cell lines by treatment with BMP-2 and BMP-4. In addition, expression of BMP-2 mRNA is upregulated, and ALP activity promoted, in HDP cells cultured at alkaline pH (14). These findings suggest that BMPs and ALP are also involved in the calcification of HDP cells. We investigated the expression

BMP-2 and BMP-4 mRNAs at 6 h and 12 h, and that of ALP mRNA at 6 days, and also studied the production of BMP-2 and BMP-4 proteins from 24 h to 72 h, and ALP activity from 3 to 21 days. The highest values of, and increases in, BMP mRNA expression and protein production were observed in the laser-irradiated group. Furthermore, the greatest increase of ALP mRNA expression was observed in the laser-irradiated group. ALP activity in the laser-irradiated and control groups increased in a time-dependent manner and peaked on day 12, the increase in the laser-irradiated group being significantly greater than in the control group. These results suggest that laser irradiation increased BMP production and ALP activity in HDP cells.

Our RT-PCR data showed that expression of mRNAs for Smad1 and Smad7 was increased by laser irradiation of HDP cells, whereas expression of Smad6 mRNA was inhibited. Imamura et al. (22) reported that Smad6 inhibits the phosphorylation of Smad1 induced by the bone morphogenetic protein type IB receptor in mouse. Okabe et al. (17) reported that expression of mRNAs for Smad1 and Smad7 was upregulated, and expression of BMP-2 mRNA promoted, in HDP cells, indicating that Smad1 and Smad7 play an important role as mediators in a series of processes that lead to calcification in HDP cells. Their data were in accord with ours, and appear to support the hypothesis that expression of Smad1 mRNA (as a BMP-activated Smad) increases calcification ability, whereas expression of Smad7 mRNA (as a BMP-inhibited Smad) inhibits excessive calcification in HDP cells upon laser irradiation.

Furthermore, the observation that OCN is present in odontoblastic cells and in the odontoblastic process, as well as in the dentin matrix, but not in predentin, supports the view that it is synthesized within odontoblasts, transported through the odontoblastic processes, and deposited in the mineralizing dentin (23). A more likely explanation is that OCN is secreted by young odontoblasts but is not contained within the matrix because of the absence of mineral. Such a concept would imply that the presence or absence of OCN is not the limiting factor in dentin mineralization, but that the deposition of OCN in dentin follows calcification, as observed in bone (23). During laser-irradiation, the expression of OCN mRNA was significantly greater than in the unirradiated controls. It is generally believed that OCN production occurs after cell differentiation (23). Moreover, Yao et al. (24) have reported that the mRNAs of collagen, ALP, and OCN are expressed before the formation of mineralized nodules, but that OCN is not produced during the initial formation of bony tissue. Their results support ours.

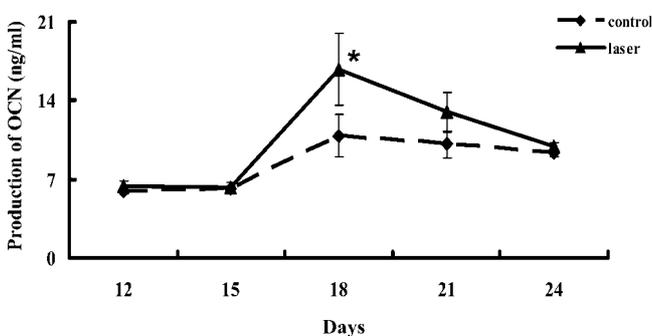


Fig. 6 Effect of laser irradiation on OCN production in HDP cells. OCN production was significantly greater in the laser-irradiated group than in the control group at 18 days (* $P < 0.01$).

Furthermore, we used Alizarin Red S staining to observe the effect of laser irradiation on calcified nodule formation in HDP cells. It has been established in vitro that the HDP cell is capable of cell-associated matrix calcification. The amount of calcium was higher in the laser-irradiated group than in the control group, suggesting that laser irradiation promotes calcified nodule formation in HDP cells.

In conclusion, our findings indicate that increased expression of mRNA for Smads in response to laser irradiation promotes BMP production and calcification in HDP cells. This suggests that the increased BMP production induced by Ga-Al-As laser irradiation activates cell-signaling molecules such as Smads, thereby promoting calcification of HDP cells.

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