Effect of Ga-Al-As Laser Irradiation at Wavelengths of 660 or 810 nm with Constant Output on the Ability of Human Dental Pulp to Form Hard Tissue

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Abstract

Purpose: In this study, to elucidate the ability of Ga-Al-As laser treatment to induce hard tissue formation, human dental pulp cells (hDPCs) stimulated with high concentration prostaglandin E2 (PGE2), which inhibits hard tissue formation, were irradiated with lasers at 660 or 810 nm. Differences in the molecular mechanisms underlying hard tissue formation using Ga-Al-As lasers at these wavelengths, including signaling via the bone morphogenetic protein (BMP)/SMAD pathway, were examined and compared.

Methods: hDPCs were harvested from third molars extracted under aseptic conditions from 20-year-old patients undergoing orthodontic treatment. hDPCs were cultured for up to 30 days. After adding PGE2, hDPCs were irradiated with a Ga-Al-As laser at an output of 300 mW and wavelengths of 660 or 810 nm, approximately 10 cm above the culture supernatant. The laser irradiation time period was set to 600 seconds. BMP2, phosphorylated-(p) SMAD1/5/8 and SMAD6 production were evaluated and calcified nodules stained.

Results: Ga-Al-As laser treatment resulted in decreased SMAD6 mRNA and increased protein expression of p-SMAD1/5/8 in groups irradiated at both wavelengths, compared with hDPCs stimulated with PGE2. Moreover, those irradiated at 810 nm exhibited lower BMP2 mRNA expression, but no definite difference in SMAD6 protein expression, compared with cells stimulated with PGE2.

Conclusion: Using Ga-Al-As lasers at the same output power, our results suggest that irradiation at 660 nm enhanced the ability of hDPCs to form hard tissue by suppressing SMAD6 expression; however, irradiation at 810 nm enhanced hard tissue generation via a different route that did not involve BMP2 and SMAD6.

Keywords:
- Low level laser treatment
- Ga-Al-As laser
- Calcification
- Prostaglandin E2
- Human dental pulp cells

Introduction

Preservation of dental pulp has a great influence on the prognosis of the tooth in conservation treatment. Ideally, the dental pulp is covered with hard tissue, consisting of dentin produced by the pulp itself. Calcium hydroxide preparations and mineral trioxide aggregate (MTA) are direct calcification methods in current clinical use (1, 2). The direct pulp capping method uses a calcium hydroxide preparation; however, due to its strong basic pH, this preparation can cause inflammation of the dental pulp and formation of a necrotic tissue layer (3). Hence, the success rate of this treatment is not high and the repaired dentin can be uneven. The use of MTA cement as part of a course of treatment by the direct braiding method using ProRoot MTA and Biodentine was reported by Linu et al (4). A follow-up survey was conducted, and the overall success rate was as high as 88.5%; however, diffuse mineralization was reported to persist for extended periods, leading to insufficient restoration of dentin, and relatively frequent progression to dental pulp excision treatment (4). Therefore, to protect the pulp tissue, methods to encourage more reliable hard tissue formation are required. When dental pulp is subjected to inflammation, the formation of hard tissue is promoted, and
reparative dentin is formed. Hence, hard tissue formation from dental pulp is closely related to inflammation.

Prostaglandin E₂ (PGE₂), a chemical mediator of inflammation, is believed to be involved in reparative dentin formation, and stimulation of human dental pulp cells (hDPCs) with low or high concentrations results in promotion or inhibition of hard tissue generation, respectively (5).

The activity and expression of bone morphogenetic proteins (BMPs) are regulated by inflammatory cytokines and chemical mediators. BMPs are among the most potent physiologically active inducers of bone formation; they regulate the differentiation of osteoblasts, and bone development, growth and regeneration. In particular, BMP2 has a strong osteogenesis promoting effect, and can induce MC3T3-E1 cells to form hard tissues through upregulation of alkaline phosphatase (ALP) (6). BMP2 is also involved in the differentiation of stem cells into dentinal blast cells in the pulp, and the acceleration of hard tissue generation (7).

SMAD family proteins contribute to BMP signaling. SMAD1, 5 and 8 (SMAD1/5/8) are phosphorylated by BMP receptors and promote downstream transcription, while SMAD6 and 7 suppress BMP signaling (8-14). According to Adachi et al. (15) treatment of hDPCs with 1 μM PGE₂ promotes SMAD6 expression and inhibits phosphorylation of SMAD1/5/8, thereby inhibiting BMP signaling and hard tissue formation. Where dental pulp treatment is required, inflammation has already occurred in the dental pulp, particularly on the surface of the exposed medulla; therefore, establishment of successful clinical treatment necessitates control of dental pulp inflammation.

Numerous studies have been conducted into the promotion of hard tissue formation using Ga-Al-As laser treatment of hDPCs for the purpose of developing measures auxiliary to direct pulp capping. Kunimatsu et al. (16) showed that irradiation of hDPCs with a Ga-Al-As laser (810 nm) at 1.0 W for 10 seconds resulted in enhanced expression of ALP, type I collagen (Coll-1) and dentin sialoprotein (DSP), and the effectiveness of this treatment in promoting calcification. In addition, Theocharidou et al. (17) reported that Ga-Al-As laser (660 nm) irradiation of human dental pulp stem cells at 140 mW is effective in promoting calcification, through enhancement of the expression of BMP2, DSP and Osterix. Moreover, Komine et al. (18) reported that the Ga-Al-As laser irradiation of hDPCs at 660 nm (20 mW) and 810 nm (1.0 W) promoted ALP activity and the formation of hard tissue through increasing BMP2 and BMP4 expression levels. These studies were conducted to investigate the induction of hard tissue formation by the dental pulp with lasers at single wavelengths, or lasers at different wavelengths; however, it is difficult to compare the effects reported because of the differing output conditions and/or irradiation times used to promote hard tissue formation. Few studies have analyzed whether factors related to hard tissue formation are affected by laser wavelength. In our previous research we focused on wavelength to elucidate the mechanism underlying the ability of Ga-Al-As lasers to induce hard-tissue formation, and examined the difference in hard tissue formation of cells exposed to Ga-Al-As lasers at 660 or 810 nm under the same output conditions, by comparing ALP activity, calcified nodule staining, BMP2 mRNA expression, and hard tissue formation. Treatment at these two wavelengths did not result in any clear difference in hard tissue formation; however, differences were noted between the two wavelengths regarding the mRNA expression level of BMP2. Therefore, we hypothesized that there are differences in the intracellular pathways induced at the two wavelengths and conducted further investigation of BMP signaling in response to laser treatment to test this hypothesis.

In this study, we investigated the influence of Ga-Al-As laser treatment at different wavelengths (660 and 810 nm) on hard tissue formation by hDPCs treated with high concentrations of PGE₂.

**Materials and Methods**

**Cell Culture**

This study was approved by the ethics committee of Nihon University School of Dentistry at Matsudo (No. EC15-009). Informed consent was obtained from all individual participants included in the study. hDPCs were obtained from the extracted third molars of young patients. All molars were extracted during the course of orthodontic treatment, and all patients gave informed consent before providing the sample. After the dental pulp had been extracted under sterile conditions, it was minced, placed on a 35-mm tissue-culture dish and then covered with a sterilized glass coverslip. The culture medium used was α-minimum essential medium (α-MEM; Gibco, USA) supplemented with 100 μg/ml penicillin G (Gibco, USA), and 10% fetal bovine serum (FBS; Gibco, USA), and cells were grown under 5% CO₂ in air at 37°C. When the cell growth from explants reached confluence, the cells were detached with
0.05% trypsin (580 BAEE units/mg, Gibco, USA) in phosphate-buffered saline and subcultured in culture flasks. For experiments, hDCPs were plated at 2 x 10^5 cells (1.0 ml medium) in 35-mm dishes after six to nine passages. hDCPs subcultures were incubated for 72 h in medium supplemented with 50 μg/ml ascorbic acid (Wako, Japan) and 10 mM sodium β-glycerol-phosphate (Sigma Chemical Co., USA) and were then stimulated with PGE2 (Cayman Chemical, USA).

**Laser Irradiation**

A Ga-Al-As laser supplied by Osada Electric Co Ltd (Japan) was used as the irradiation source. hDCPs were irradiated with a Ga-Al-As laser at wavelengths of 660 or 810 nm and an output of 300 mW, approximately 10 cm above the culture supernatant. The laser irradiation time period was set to 600 seconds.

**RNA Extraction and Real-time PCR**

hDCPs were cultured to confluence in 35-mm tissue culture dishes containing α-MEM supplemented with 10% FBS. Cells were then incubated for 24 h in α-MEM containing 2% FBS, and washed twice with PBS. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Germany), following the manufacturer’s protocol and stored at -80°C. RNA concentrations were determined by measuring absorbance at 260 and 280 nm. Real-time PCR was performed in 25 μL reactions using a One-Step SYBR Prime Script RT-PCR Kit 2 (TaKaRa, Japan) and a Thermal Cycler Dice Real-Time System (TaKaRa, Japan). Reactions contained 1 μL RNA (100 ng total), 12.5 μL 2 x One-Step SYBR RT-PCR buffer 4, 1 μL Prime Script One-step Enzyme Mix 2, 400 nM each of Forward and Reverse primers, and 8.5 μL RNase-free dH2O. RNA was reverse transcribed for 5 min at 42°C, denatured at 95°C for 10 s, and then amplified using 50 cycles of denaturation at 95°C for 5 s, and annealing/extension at 60°C for 30 s. Primers for BMP2, SMAD6 and GAPDH are listed in Table 1 (15, 19, 20). Gene expression levels were calculated using the ΔΔCt method, with normalization against GAPDH levels.

**Western Blotting**

hDCPs (1 x 10^6) were cultured to confluence in 10-cm tissue culture dishes with α-MEM and 10% FBS and then incubated for 24 h in α-MEM and 1% FBS. Cells were lysed with CelLytic M (Sigma-Aldrich, UK) supplemented with Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche, Germany), 100 mM PMSF, 0.2 mM EGTA and 2 mM EDTA. Protein concentrations were measured using the Bradford method. Samples were boiled for 5 min at 95°C in buffer containing sodium dodecyl sulfate, separated on 7.5% polyacrylamide gels, and transferred to nitrocellulose membranes at 12.5 V overnight. Membranes were blocked at room temperature for 50 min with skim milk (Wako, Japan), probed for 120 min with mouse antibodies against SMAD6 (1:1,000, abcam, GB) and rabbit antibodies against phosphorylated-(p) SMAD1/5/8 (1: 2000, Cell Signaling Technology, USA) and rabbit antibodies against Beta-actin (1:1000; Cell Signaling Technology, USA), washed three times with 4% skim milk containing 0.05% Tween 20, and labeled for 90 min with HRP-conjugated anti-mouse IgG (1: 3,000; Cell Signaling Technology, USA) and HRP-conjugated anti-rabbit IgG (1: 2,000; Cell Signaling Technology, USA). Immunoreactivity was detected using an ECL Prime Western Blotting Detection System (GE, NJ) and quantified in Adobe Photoshop.

**Examination of Calcified Nodule Formation**

For examination of calcified nodule formation, hDCPs were cultured for 30 days in vitro and stained using the von Kossa technique.

**Statistical Analysis**

Results are reported as means ± SEM of the indicated
number of experiments. Data were analyzed using Tukey tests. Differences with P values < 0.05 were considered significant.

Results

Effects of Ga-Al-As Laser and PGE$_2$ on BMP2 and SMAD6 mRNA Expression in hDPCs

Levels of BMP2 and SMAD6 mRNA were evaluated by real-time PCR to compare the effects of treatment of hDPCs with Ga-Al-As lasers at equal power (300 mW), but different wavelengths (660 and 810 nm) on molecules involved in regulation of hard tissue formation. After 1 h BMP2 mRNA expression was significantly higher in cells treated with PGE$_2$ alone and those treated with PGE$_2$ at 660 nm Ga-Al-As laser than in those treated with PGE$_2$ at 810 nm laser or controls (Fig. 1A). In contrast, SMAD6 mRNA expression was significantly higher in the PGE$_2$ group than in cells treated with PGE$_2$ alone with Ga-Al-As laser at either wavelength or controls (Fig. 1B).

Effects of Ga-Al-As Laser and PGE$_2$ on SMAD Protein Expression in hDPCs

Next, to further explore the molecular effects of treatment of PGE$_2$-treated hDPCs with Ga-Al-As lasers at 660 and 810 nm, expression of SMAD6 and p-SMAD1/5/8 was evaluated by western blotting. No p-SMAD1/5/8 expression was detected in cells treated with PGE$_2$ alone 3 h after irradiation; however, irradiation of PGE$_2$-treated hDPCs at either wavelength resulted in clear p-SMAD1/5/8 expression. SMAD6 protein expression was observed in all groups. Treatment with PGE$_2$ increased SMAD6 levels, while decreased levels were observed after semiconductor laser irradiation of PGE$_2$-treated hDPCs at 660 nm. In contrast, cells treated by semiconductor laser irradiation at 810 nm exhibited no clear decrease in SMAD6 expression compared with those treated with lasers at 660 nm (Fig. 2).

Calcified Nodule Formation by hDPCs Treated Using Ga-Al-As Laser Irradiation and PGE$_2$

The effects of Ga-Al-As laser treatment on calcified nodule formation by hDPCs stimulated with PGE$_2$ were then determined using the von Kossa method. 30 days after treatment, less intense staining (indicating calcified nodule formation) was observed in hDPCs treated with PGE$_2$ alone compared with controls. In addition, cells treated with PGE$_2$ and irradiated at 660 and 810 nm exhibited an increase in calcified nodule formation compared with cells treated with PGE$_2$ alone (Fig. 3).

Discussion

Current clinical practice for induction of direct calcification of dental pulp the use of methods involving calcium hydroxide preparations or MTA (1, 2); however, the treatment is not effective in all cases because of insufficient formation of repaired dentin, hence clinical treatment often progresses to pulp excision.

Dental pulp is affected by inflammation. Therefore, to preserve the dental pulp tissue, there is a requirement for more reliable promotion of hard tissue formation. In addition, inflammation and hard tissue formation are closely related in the dental pulp, since dental pulp generates repair dentin when it receives mild stimulation. Sakamoto et al. (5) reported that the capacity of dental pulp cell cultures to
form hard tissue is promoted and inhibited by stimulation with low and high concentrations of PGE₂, respectively. BMPs, which are regulated by inflammatory chemical mediators and cytokines, are among the physiologically active substances with the strongest influence on bone formation and are important osteoblast differentiation, and bone development, growth, and regeneration (7). TGF/β superfamily signal transduction, which includes BMP signaling, is mainly mediated through SMAD family proteins. Eight SMADs have been reported in mammals, and share SMAD-specific functional domains. SMAD1/5/8 are receptor-regulated SMADs phosphorylated by the BMP receptor, that interact with either SMAD4, or the repressive SMADs, SMAD6 and 7, which compete for phosphorylation by SMAD receptors, or for binding to specific SMADs, generating different complexes. Adachi et al. (15) reported that treatment with 1 μM PGE₂ promotes the expression of SMAD6 and inhibits the phosphorylation of SMAD1/5/8, thereby inhibiting BMP suppression of signal transduction, and leading to hard tissue formation.

The application of Ga-Al-As lasers for promotion of anti-inflammatory activity and hard tissue formation has also been investigated (21–25). Honmura and colleagues generated a rat experimental inflammation model and applied semiconductor laser irradiation to the inflamed region, leading to 20%–30% inhibition of inflammation via increased vascular permeability, edema of the acute phase, and granuloma formation (26). In addition, Shimizu et al. (27) reported that inflammation caused by mechanical stress was reduced low energy laser irradiation. These anti-

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![Western blotting (3 hour)](image1)

**Fig. 2.** Expressions of p-SMAD1/5/8 and SMAD6 signal proteins in hDPCs exposed to PGE₂ and laser irradiation at the 3 hour.

![von Kossa staining (30 days)](image2)

**Fig. 3.** Dental pulp cells treated with different doses of laser irradiation at day 30 with von Kossa staining.
inflammatory effects occurred through significant inhibition of PGE2 and IL-1β production by periodontal ligament cells; however, despite these various investigations, the detailed mechanism underlying the effects of semiconductor lasers on inflammation processes remains unknown.

To further study the influence of Ga-Al-As laser irradiation on BMP2 signaling, we focused on their anti-inflammatory effects. We evaluated the ability of hDCPs stimulated with the same concentration of PGE2 to generate hard tissue following irradiation with a Ga-Al-As laser at 660 or 810 nm under the same output conditions. The results indicated that cells stimulated with PGE2 alone, or treated with PGE2 + 660 nm irradiation exhibited significantly increased BMP2 mRNA expression levels compared with those of the other two groups (controls and cells treated with PGE2+810 nm irradiation). In addition, the PGE2 group showed a significant increase in the amount of SMAD6 mRNA and protein expression compared with the other three groups, while p-SMAD1/5/8 expression of was suppressed. Moreover, analysis by von Kossa staining indicated that hard tissue formation was suppressed in the PGE2 group. BMP2 levels increased in response to PGE2 stimulation; however, those of SMAD6 increased. Therefore, the role of this inhibitory SMAD in hard tissue formation via inhibition of phosphorylation of SMAD1/5/8 was also suppressed; however, SMAD6 levels decreased in the PGE2 + 660 nm laser stimulation group relative to PGE2 stimulation alone, with a consequent increase in the expression of p-SMAD1/5/8 and promotion of hard tissue formation.

These data indicate that p-SMAD1/5/8 was expressed, signal transduction activated, and hard tissue generated as a result of suppression of SMAD6 expression by irradiation with a Ga-Al-As laser at 660 nm. In contrast, western blotting indicated that, unlike the PGE2+810 nm irradiation group, SMAD6 protein expression was suppressed in the PGE2 + 660 nm irradiation group, compared with cells stimulated with PGE2 alone, while expression of p-SMAD1/5/8 was induced. Regarding BMP2 mRNA expression, the PGE2+810 nm irradiation group exhibited predominantly decreased mRNA expression; however, von Kossa staining indicated that hard tissue formation was promoted relative to the PGE2 stimulation group. These data may indicate that the pathway by which hard tissue formation is induced in the PGE2+660 nm irradiation group differs from that induced by cells irradiated at 810 nm, in that the phosphorylation of SMAD1/5/8 is induced by the increase in BMP2.

Regarding the difference in cellular response according to wavelength, Nicolau et al. (28, 29) carried out laser irradiation of the mouse nerve junction and observed no effect was observed using a visible light laser at 655 nm; however, using a near infrared laser (830 nm), efficient suppression of synaptic transmission was detected. Furthermore, laser irradiation of rat liver mitochondria at various wavelengths led to increased ATP content at 415, 633, 650 and 725 nm; however, no such increase was observed using wavelengths of 477, 511 and 554 nm (30–32). In the field of photobiology, photoreceptor substances with different wavelength absorption spectra have been postulated to exist in cells. Based on these reports and our results, we speculate that photoreceptor substances with different wavelength are also present in hDCPs. This would infer that absorption spectra that differ depending on the wavelength hDCPs are involved in the mechanism of hard tissue formation from dental pulp. In future, further consideration of the receptor involved in this process in hDCPs will be necessary.

These findings suggest that irradiation with a semiconductor laser at 660 nm enhances the ability of hDCPs to form hard tissue by suppressing SMAD6. Moreover, the effects of the Ga-Al-As laser at 810 nm with the same output are not mediated via BMP2 and SMAD6; we speculate that it irradiation at this wavelength induces hard tissue formation via another mechanism.

**Conclusion**

Treatment of hDCPs with semiconductor lasers with the same output indicated that irradiation at 660 nm enhanced their ability to form hard tissue through suppression of SMAD6; however, irradiation at 810 nm enhanced hard tissue forming ability via a route that did not involve BMP2 and SMAD6.

**Conflicts of interest**

The authors have no conflict of interest to declare.

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