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Effect of Ga-Al-As Laser Irradiation on *COX-2* and *cPLA$_2$*-α Expression in Compressed Human Periodontal Ligament Cells

Kotoe Mayahara, DDS, PhD,[1,2]* Akikuni Yamaguchi, DDS,[1] Masahito Sakaguchi, DDS,[1] Yu Igarashi, DDS,[1] and Noriyoshi Shimizu, DDS, PhD[1,2]

[1]*Department of Orthodontics, Nihon University School of Dentistry, 1-8-13 Kanda, Surugadai, Chiyoda-Ku, Tokyo 101-8310, Japan*

[2]*Division of Clinical Research, Dental Research Center, Nihon University School of Dentistry, 1-8-13 Kanda, Surugadai, Chiyoda-Ku, Tokyo 101-8310, Japan*

Background and Objective: Prostaglandin E_2 (PGE_2), which has bone-resorptive activity, is produced by human periodontal ligament (PDL) cells in response to mechanical stress. We previously reported that low level laser (LLL) irradiation inhibited PGE_2 production in PDL cells in response to mechanical stress; however, the mechanism underlying this inhibitory effect was unclear. Thus, we sought to determine the mechanism underlying the inhibitory effect of LLL on PGE_2 production in compressed PDL cells.

Study Design/Materials and Methods: A compressive force of 2.0 g/cm^2 was applied for 24 hours to human PDL cells obtained from premolars extracted for orthodontic treatment. LLL irradiation (Ga-Al-As laser, 830 nm, 3.82 J/cm^2) was applied 6 hours before, 1 hour before, and immediately after the application of compressive force. The mRNA expression of *COX-2* and *cPLA$_2$*-α was then examined by real-time PCR.

Results: LLL irradiation significantly inhibited *COX-2* and *cPLA$_2$*-α mRNA expression, which was increased in response to the application of a compressive force. Moreover, LLL irradiation immediately after compression had the strongest inhibitory effect on the expression of these genes.

Conclusions: The inhibition of PGE_2 production by LLL irradiation in compressed PDL cells may be due to the inhibition of *COX-2* and *cPLA$_2$*-α expression and is most pronounced immediately after the application of a compressive force. Lasers Surg. Med. 42:489–493, 2010.

Key words: COX-2; *cPLA$_2$*-α; low level laser; mechanical stress

INTRODUCTION

Orthodontic tooth movement enhances the loss of alveolar crestal bone and induces gingival regression, leading to esthetic problems, with a greater effect in adults than in younger patients [1–3]. Prostaglandin E_2 (PGE_2) is produced in periodontal ligament (PDL) cells in response to mechanical stress in vivo [4] and in vitro [5,6], and the PGE_2 produced by PDL cells in response to mechanical stress show bone-resorptive activity [6]. We previously showed that PGE_2 production in PDL cells subjected to a compressive force was increased in a donor age-dependent manner. PGE_2 production was dramatically increased in PDL cells collected from donors over the age of 35 [7]. These results are positively related to the acceleration of alveolar crestal bone loss during orthodontic treatment in adult patients. It is clinically important to prevent side effects, such as crestal bone resorption followed by gingival recession, during orthodontic treatment in adult patients.

PGE_2 is a pro-inflammatory agent and potent osteoclast-inducing factor synthesized from arachidonic acid (AA) by cyclo-oxygenase 2 (COX-2). The first step in eicosanoid production is the release of AA from membrane phospholipids by phospholipase A_2 (PLA2) [8].

Low level laser (LLL) irradiation is effective for fracture healing, tissue repair, and pain relief [9]. Several reports have suggested that a specific wavelength of laser irradiation may be used as an alternative anti-inflammatory tool for wound healing in the clinical setting [10–12]. Many physiological effects of LLL treatment, including pain reduction and anti-inflammation, have been reported [13,14]. It has also been reported that LLL irradiation inhibits PGE_2 production in stretched human PDL cells [15]. However, the mechanism underlying the inhibitory effect of LLL on PGE_2 is unknown.

In this study, we investigated the effect of the LLL irradiation of compressed PDL cells on the expression of COX-2 and PLA_2, which are important enzymes in PGE_2 production. Additionally, we investigated the time course of

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*Correspondence to: Kotoe Mayahara, DDS, PhD, Department of Orthodontics, Nihon University School of Dentistry, 1-8-13 Kanda, Surugadai, Chiyoda-Ku, Tokyo 101-8310, Japan.
E-mail: mayahara@dent.nihon-u.ac.jp
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the inhibition of PGE_2 production by LLL irradiation in response to mechanical stress. Based on our results, we hope to develop a method for the prevention of crestal bone resorption during orthodontic treatment in adult patients.

MATERIALS AND METHODS

Cell Culture

Human PDL cells were prepared in accordance with the methods of Somerman et al. [16] with minor modifications. Our experimental protocols were reviewed and approved by the Nihon University Department of Dentistry Ethics Committee. Premolars extracted from healthy patients during the course of orthodontic treatment were washed twice with 0.01 mol/L phosphate-buffered saline (PBS); tissue attached to the middle third of the root was then removed with a surgical scalpel. The coronal and apical portions of the root were not used to avoid contamination by cells from other tissues. The tissues were minced, placed in 35-mm tissue culture dishes (Falcon, Lincoln Park, NJ), and covered with sterilized glass coverslips to attach the tissue on the bottom of the dish. The medium used was α-MEM (Gibco, Grand Island, NY), supplemented with 100 U/ml penicillin-G sodium, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B (Gibco), and 10% fetal bovine serum (FBS; JRH Biosciences, Inc., Lenexa, KS). The cultures were maintained at 37°C in a humidified incubator containing 95% air/5% CO_2. Once the cells reached confluence, they were detached with 0.05% trypsin (Gibco) in PBS for 10 minutes and subcultured in culture flasks (Falcon). All cells still attached to the bottoms of the flasks were discarded during serial passage to avoid contamination by epithelial cells, which are less readily detached than fibroblasts [17]. Cells observed at confluence by phase-contrast microscopy had none of the small mats typical of epithelial cells. Cells that had been passaged five or six times were used in our experiments.

Application of a Compressive Force

PDL cells were plated onto 10-cm cell culture dishes at a density of 4.5×10^3 cells/cm^2. When the cells were subconfluent, the medium was changed to that described above, except that it contained 2% instead of 10% FBS. After incubation overnight, the cells were compressed continuously using the uniform compression method [6]. Briefly, the confluent cell layer in the dish was covered by a thin glass plate. The cells were then subjected to a compressive force of 2.0 g/cm^2 for 3, 6, 12, 24, and 48 hours according to the method of Kanzaki et al. [6] Control cells were covered with thin glass plates without lead weight. Compressive force of this condition was 0.035 g/cm^2.

Laser Irradiation

Ga-Al-As diode laser (model Panalas-1000 Matsushita, Inc., Osaka, Japan) with a wavelength of 830 nm (maximum power output of 500 mW, continuous mode) was used in this study. The laser beam was delivered by an optical fiber 0.6 mm in diameter that was defocused at the tip by a concave lens, allowing for uniform irradiation in a 100-mm diameter circular area at the level of the cell layer. LLL irradiation was performed at 280 mm from the bottom of dish, because lead weight may cut-off the laser beam irradiated from the top. The power density of the laser beam was monitored 6.5 mW at the center of the dish by a laser power meter (ORION PD; Ophir, Jerusalem, Israel). The total energy, corresponding to 10 minutes of exposure, was 3.82 J/cm^2. LLL irradiation was performed once 6 hours before, 1 hour before, or immediately after compression.

RNA Preparation and Reverse Transcriptase (RT)-PCR

In the time-course experiment, total RNA was extracted from the cells after application of the compressive force for 3, 6, 12, 24, and 48 hours using the TRIZOL reagent (Gibco) and stored at −80°C. In the laser irradiation experiment, total RNA was extracted from the cells after application of the compressive force for 24 hours using the TRIZOL reagent (Gibco) and stored at −80°C. cDNA synthesis and amplification by RT-PCR were carried out using a PrimeScript RT Reagent Kit (Takara, Tokyo, Japan), according to the manufacturer's instructions. A total of 2 μl of cDNA were subjected to real-time PCR using SYBR Green I dye. Real-time PCR was performed in a 25-μl volume containing 1× SYBR® Premix Ex Taq™ (Takara) and 20 μM specific primers. The sequences of the primers are shown in Table 1. The *COX-2* primers were designed using the Primer 3 software (Whitehead Institute for Biomedical Research, Cambridge, MA). All other primers were obtained from Takara. PCR was performed in a thermal cycler (Smart Cycler II System, Cepheid, Sunnyvale, CA) and the data were analyzed using Smart Cycler software (version 2.0). The real-time PCR conditions were 40 cycles of 95°C for 3 seconds and 60°C for 20 seconds. The specificity of the products was verified by melting curve analysis between 60 and 95°C. Each mRNA sample was tested three times. The levels of mRNA expression were calculated and normalized

TABLE 1. Primers used in this experiment

Target	Primer	Genbank acc. no.
COX-2	F 5′-AGCTTGGCCACTCGCTCGGTCTG-3′ R 5′-ATTCCATGGAGCCAGGCTTTC-3′	XM_001734
$CPLA_2$-α	F 5′-TGAGTGACTTTGCCACACAGGAC-3′ R 5′-AGGGATACGGCAGGTTAAATGTGA-3′	NM_024420
GAPDH	F 5′-AGGCAAGCAGCTAGACTGGTGAA-3′ R 5′-GCACCGTCAAGGCTGAGAAC-3′	NM_002046

to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA at each time point.

Statistical Analyses

All values represent the mean ± standard deviation (SD). The data were subjected to one-way analysis of variance (ANOVA), as indicated in the results. Student's *t*-test was used for analysis of the difference in some groups.

RESULTS

The time course of *COX-2* mRNA expression by human PDL cells in response to the compressive force is shown in Figure 1A. *COX-2* mRNA expression was increased in a time-dependent fashion (one-way ANOVA; $P<0.001$), and it was significantly increased, compared with control cells at 12, 24, and 48 hours after the application of the compressive force. The time course of cytosolic phospholipaseA$_2$-α (*cPLA$_2$-α*) mRNA expression in response to compression is shown in Figure 1B. *cPLA$_2$-α* mRNA expression was significantly increased, compared with control cells, at 24 and 48 hours after the application of the compressive force.

The inhibitory effects of LLL irradiation are shown in Figure 2B,C. *COX-2* mRNA expression was significantly increased in response to 24 hours compression (Fig. 2B). After 24 hours application of compressive force, *COX-2 mRNA* maximum increase was 23-fold, compared with that in control cells, whereas LLL irradiation 6 hours before, 1 hour before, and just after application of the compressive

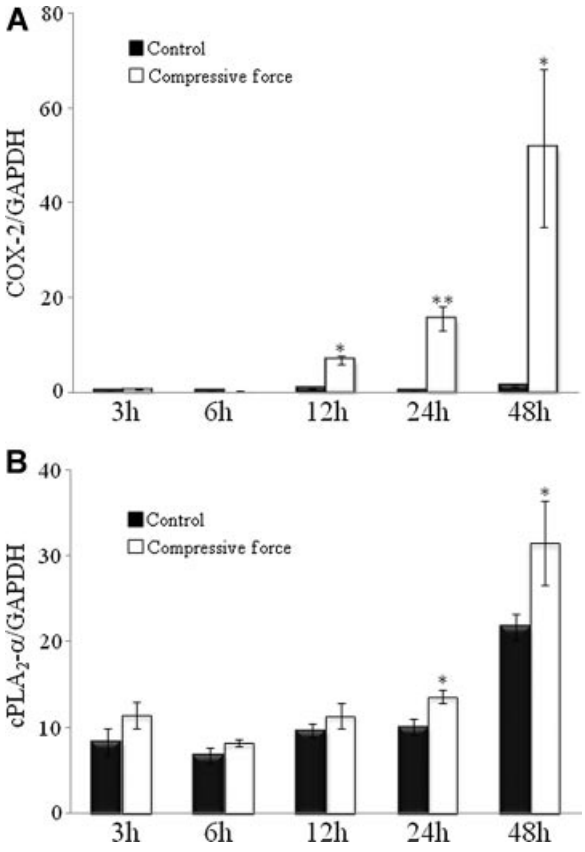

Fig. 1. Time course of *COX-2* (**A**) and *cPLA$_2$-α* (**B**) mRNA expression in human PDL cells after the application of a compressive force. The values shown are the means ± SD of three separate measurements for each time point. The increases were significantly different from those in the corresponding control cells (*$P<0.05$, **$P<0.01$). *COX-2* expression was increased in a time-dependent manner ($P<0.001$; one-way ANOVA).

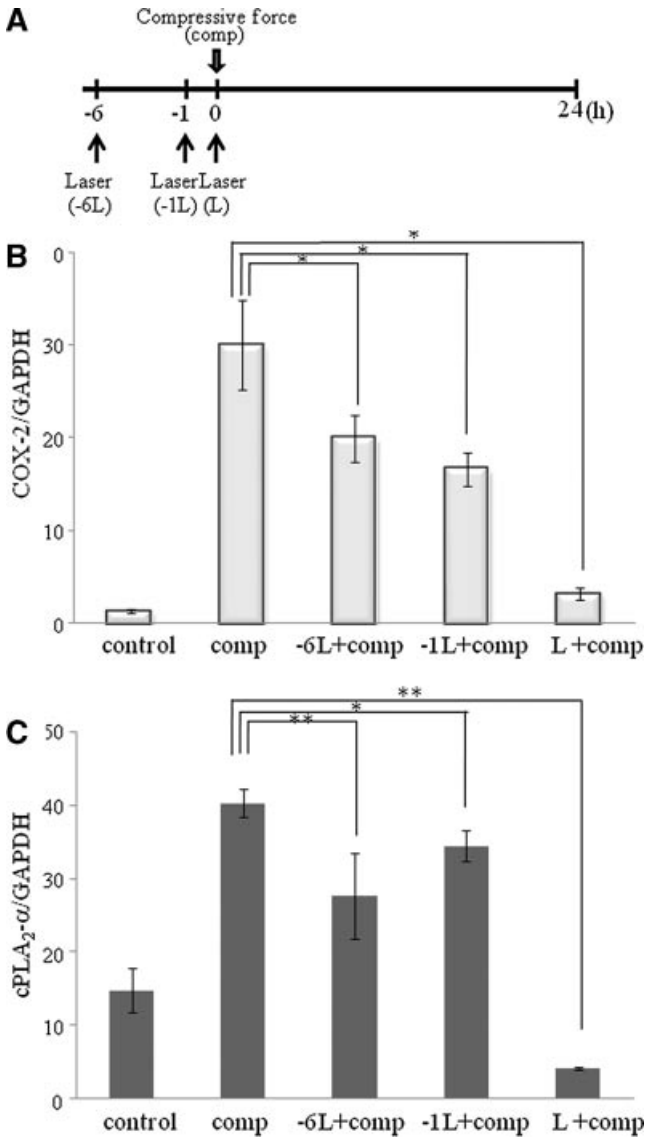

Fig. 2. The experimental protocol for LLL irradiation and application of compression. Irradiation was done 6 hours before, 1 hour before, and immediately after application of the compressive force (**A**). Effect of compression and the timing of LLL irradiation on *COX-2* (**B**) and *cPLA$_2$-α* (**C**) mRNA expression in human PDL cells. The values shown are the means ± SD of three separate measurements for each time point. The decreases were significantly different from those in the compression group (*$P<0.05$).

force significantly inhibited *COX-2* mRNA expression by 40%, 50%, and 85%, respectively, compared with unirradiated controls (Fig. 2B). *cPLA$_2$*-α mRNA expression was significantly increased in response to 24 hours compression (Fig. 2C). After 24 hours application of compressive force, *cPLA$_2$*-α mRNA maximum increase was 2.6-fold compared with that in the control cells, and LLL irradiation 6 hours before, 1 hour before, and just after application of the compressive force significantly inhibited *cPLA$_2$*-α expression by 31%, 15%, and 90%, respectively, compared with unirradiated controls (Fig. 2C).

DISCUSSION

It is known that mechanical stress such as tensile force and compressive force induces PGE_2 production in human PDL cells [5–7]. Furthermore, we showed that LLL irradiation inhibited PGE_2 production in stretched human PDL cells [15]; however, the mechanism was not elucidated. In the present study, we found that the inhibitory effect of LLL on PGE_2 production involved the inhibition of *COX-2* and *cPLA$_2$*-α mRNA expression.

PGE_2 is induced in response to growth factors, proinflammatory agents, such as cytokines, mitogens, and lipopolysaccharides [18,19], and mechanical stress [5]. COX-2 catalyzes the oxygenation of AA to PGE_2. The first step in eicosanoid production is the release of AA from membrane phospholipids by PLA_2. $cPLA_2$ is a member of the PLA_2 enzyme superfamily, which includes secretory PLA_2, cytosolic PLA_2 ($cPLA_2$), and other members. $cPLA_2$, which activates AA hydrolysis, exists in three isoforms: α, β, and γ. Of them, $cPLA_2$-α is known to be a major component of the arachidonate-releasing signal transduction pathway [20–22].

The early phase of orthodontic tooth movement involves an acute inflammatory response related to PGE_2 produced by PDL cells in response to mechanical stress [5]. PGE_2 has also been shown to be a potent bone-resorptive factor during tooth movement [6]. Several studies have shown that alveolar bone loss in adult orthodontic patients is greater than in younger patients [1–3]. Many orthodontists have observed gingival recession, followed by alveolar crestal bone resorption, during orthodontic treatment in adult patients. We also found that PGE_2 production and *COX-2* mRNA expression in PDL cells in response to mechanical stress was greater when the cells came from adult donors than from younger donors [7]. Thus, excessive PGE_2 production from PDL cells in response to mechanical stress may be associated with alveolar crestal bone resorption.

LLL has been used in the treatment of specific diseases [23], including reducing the duration of acute inflammation, stimulating tissue repair, and relieving pain [9]. In the present study, LLL irradiation immediately after the application of a compressive force significantly decreased *COX-2* mRNA expression in PDL cells. This suggests that LLL irradiation may be used to reduce inflammation, similar to non-steroidal anti-inflammatory drugs, such as aspirin or indomethacin, which inhibit COX activity. When these drugs are used to reduce pain and inflammation, they can also produce side effects, such as urticaria, angioedema, and peptic ulcers, whereas LLL irradiation has not been shown to produce any side effect. PGE_2 is an important local factor for bone resorption during orthodontic tooth movement; thus, excessive inhibition of PGE_2 may lead to decreased tooth movement during orthodontic treatment.

In this study, we used multiple courses of irradiation before the application of a compressive force. The most effective course of LLL irradiation for the inhibition of *COX-2* and *cPLA$_2$*-α mRNA expression was immediately after the application of the compressive force. This indicates that LLL treatment immediately after the activation of tooth movement may have the largest inhibitory effect on PGE_2 production. LLL irradiation 6 hours and 1 hour before the application of a compressive force inhibited *COX-2* and *cPLA2*-α expression, but was less effective than irradiation immediately after the application of a compressive force. COX-2 expression has been linked to the activation of protein kinase C (PKC) and mitogen-activated protein kinase (MAPKs) signaling [24]. Additional studies are needed to investigate the effects of LLL irradiation on signal transduction cascades, leading to the activation of PKC and MAPKs.

In conclusion, we found that LLL irradiation inhibited *COX-2* and *cPLA$_2$*-α mRNA expression in response to a compressive force in PDL cells, and that the strongest inhibitory effect was produced immediately after the application of a compressive force. Because these factors are known to possess potent bone-resorptive activity, it is possible that LLL treatment upon the activation of tooth movement may effectively reduce alveolar crestal bone loss.

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