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Er:YAG laser irradiation increases prostaglandin E_2 production via the induction of cyclooxygenase-2 mRNA in human gingival fibroblasts

A. Pourzarandian, H. Watanabe, S. M. P. M. Ruwanpura, A. Aoki, K. Noguchi, I. Ishikawa
Periodontology, Department of Hard Tissue Engineering, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan

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Background and objectives: It has been reported that both prostaglandin E_2 (PGE_2) and Er:YAG laser irradiation accelerate wound healing. The stimulatory action of laser seems to occur during the proliferative stage of healing by stimulation of prostaglandin E_2 and cyclooxygenase-2 (COX-2), which are crucial early mediators in the natural healing process. We have then investigated the effect of Er:YAG laser irradiation on PGE_2 production and COX-2 gene expression in human gingival fibroblast *in vitro*.

Material and methods: Cultured fibroblasts were exposed to low-power Er:YAG laser irradiation with an energy density of 3.37 J/cm^2. The amount of PGE_2 production was measured by enzyme-linked immunosorbent assay (ELISA). COX-2 mRNA level, which is a critical enzyme for PGE_2 production, was analyzed by reverse transcriptase–polymerase chain reaction (RT–PCR).

Results: Er:YAG laser significantly increased PGE_2 production by human gingival fibroblasts. COX-2 mRNA, which was hardly detectable in control, increased dramatically after irradiation. COX-2 inhibitor, NS398, completely inhibited the PGE_2 synthesis stimulated by Er:YAG laser irradiation.

Conclusion: Our results showed that Er:YAG laser irradiation appears to exert its stimulative action on gingival fibroblasts proliferation through the production of PGE_2 via the expression of COX-2. This should be considered as one of the important regulatory pathways to accelerate wound healing after Er:YAG laser irradiation.

Dr Amir Pourzarandian, Section of Periodontology, Department of Hard Tissue Engineering, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113–8549, Japan
Tel; +81 3 5803 5488
Fax: +81 3 5803 0196
e-mail: amir.peri@tmd.ac.jp

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Low-power lasers have been reported to significantly enhance the healing rate in both soft and hard tissues. Early migration, attachment and proliferation of inflammatory cells and fibroblasts are necessary to promote the healing process. Many of these processes are regulated by bioactive substances, including interleukins, growth factors, extracellular matrix components, as well as arachidonic acid metabolites such as prostaglandins (PG). Several key enzymes, including phospholipase A_2 and cyclooxygenase (COX), regulate the

production of the prostaglandins (1). Phospholipase A_2 releases arachidonic acid from membrane phospholipids and COX converts arachidonic acid to PGs. Two isoforms of COX have been reported, a constitutively expressed COX-1 and an inducible COX-2. COX-2 is induced in gingival fibroblasts by inflammatory mediators such as interleukin-1β (2–4).

We have previously reported that low level Er:YAG laser stimulates cell proliferation in human gingival fibroblasts (5). However, little is known about the biological mechanism of Er:YAG laser irradiation. Gingival fibroblasts are capable of secreting impressive levels of prostaglandin E_2 (PGE_2) (2, 6), exhibiting the potential for tissue repair in periodontal treatment (7, 8). Gingival fibroblasts stimulated with interleukin-1β produce PGE_2 via *de novo* synthesis of COX-2 (4, 9, 10). Prostaglandin E_2 regulates cellular proliferation through interaction with specific receptors and modification of the levels of second messengers such as calcium and cAMP (11). To our knowledge, however, the effect of Er:YAG laser treatment on the production of PGE_2 and its regulation with special reference to COX mRNA expression has not been reported. Consequently, the aim of this study was to investigate the biological effects of Er:YAG laser irradiation on COX-2 and PGE_2 production in cultured human gingival fibroblasts.

Material and methods

Preparation and culture of human gingival fibroblasts

Human gingival fibroblasts were obtained from teeth extracted for orthodontic reasons. There were no clinical signs of periodontal disease. Informed consent was obtained from each patient before the cell culture was performed. The study protocol was approved by the Ethical Committee of the Tokyo Medical and Dental University. The teeth were rinsed three times in minimum essential medium-α (α-MEM) that contained 10% fetal bovine serum (Bioserum, Melbourne, Victoria, Australia), 100 U/ml penicillin (Sigma, St Louis, MO, USA), and 100 U/ml streptomycin (Sigma). Attached gingival tissue was dissected from the neck of the tooth. The samples were placed in 35-mm dishes (Falcon, Heidelburg, Germany) and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. When the human gingival fibroblasts that had migrated from the samples became confluent, the cultures were trypsinized with 0.25% trypsin/1 mM EDTA and transferred to 10-cm dishes. Human gingival fibroblasts that had reached confluence were defined as the first passage. Cells from passages 5–10 were used for the experiments. The cells were then plated to 35-mm dishes with approximately 5×10^4 cells/dish for the experiments.

Device characteristics

A pulsed Er:YAG laser device (Delight™, HOYA ConBio, Fremont, CA, USA), with a wavelength of 2.94 μm, generating an output range of 30–350 mJ/pulse, with a maximum pulse repetition rate of 30 Hz and a pulse duration of 200 μs, was used.

Laser irradiation

Two days after the subculture, cells were serum starved in α-MEM with 0.5% fetal bovine serum for 24 h. The α-MEM was completely removed before laser treatment and replaced immediately afterwards. Er:YAG laser irradiation was performed with an energy density of 1.68 J/cm^2, 2.35 J/cm^2 and 3.37 J/cm^2 at a pulse repetition rate of 20 Hz. In a previous study, the energy density of 3.37 J/cm^2 was determined as the optimal stimulative with regards to its effect on fibroblasts proliferation as judged by cell counting and lactate dehydrogenase level measurement. The power density of irradiation was uniformly monitored with a power meter (Field Master, Coherent Co., Cincinnati, OH, USA) before and after treatment. The laser irradiation was performed perpendicularly to the bottom of a 35-mm culture dish. The laser beam was delivered with a hand piece without tip, which was stabilized by a stand instrument at the distance of 15 cm to irradiate a circular area of 35 mm at the cell layer level.

Enzyme-linked immunosorbent assay (ELISA) for PGE_2

PGE_2 levels in the condition media collected from control and laser-treated human gingival fibroblasts were measured using commercially available ELISA kits (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's instructions.

Reverse transcription–polymerase chain reaction (RT-PCR) for COX-2 mRNA

Human gingival fibroblasts were seeded in 35-mm plates and after 2 days they were serum-starved in α-MEM containing 0.5% fetal bovine serum to reduce the effect of serum on COX-2 production. After 24 h, the cells were irradiated (Sigma). After 24 h, cells were lysed and RNA was extracted by the guanidium thiocyanate/phenol/chloroform method, using ISOGEN (Nippon Gene, Toyama, Japan) cDNA primers synthesized from 2 μg of the total RNA with RAV2 reverse transcriptase and oligo(dT) primers (Takara Co., Shiga, Japan). The primers were COX-2: sense primer 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′, antisense primer 5′-AGATCATCTCTGCCTGAGTATCTTT-3′; β-actin: sense primer 5′-GTGGGCATGGGTCATCAGAAGGAT-3′, antisense primer 5′-CTCCTTAATGTCACGCACGATTTC-3′. PCR was performed in 25 μM of each primer, 2.5 mM of each dNTP and 2.5 U of Taq DNA polymerase (Takara Co.) in an automated DNA thermal cycler. The PCR amplification for COX-2 was comprised of 28 cycles of denaturation at 96°C for 30 s, annealing at 55°C for 1 min and extension at 72°C for 2 min. For β-actin, the PCR amplification was 28 cycles comprising denaturation at

96°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 3 min. the RT–PCR products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide. The intensities of obtained bands were determined using an UV-light box imaging system (Atto Corp., Tokyo, Japan).

COX inhibitors

To investigate whether the therapeutic range of Er:YAG laser-induced production of PGE_2 was mediated by changes in COX activity, indomethacin and NS-398 were used to block COX activity. Indomethacin blocks the activities of both COX-1 and COX-2, and NS-398 specifically inhibits COX-2 activity.

Experiment protocol

Thirty cultured human gingival fibroblasts dishes (35 mm) were divided into four groups:

- control, no laser application ($n = 3$);
- irradiated with energy density of 1.68 J/cm^2 Er:YAG laser ($n = 9$) (L1);
- irradiated with energy density of 2.35 J/cm^2 Er:YAG laser ($n = 9$) (L2);
- irradiated with energy density of 3.37 J/cm^2 Er:YAG laser ($n = 9$) (L3).

Three dishes of each group were used to measure the production of PGE_2 and COX-2. To investigate whether Er:YAG laser-induced production of PGE_2 was mediated by changes in COX-2 activity, the remaining six dishes from groups L1 to L3 were incubated with indomethacin (1 μM) and NS-398 (1 μM).

Statistical analysis

Statistical analysis was done using Sigma Stat software (version 2; Sigma). Values were calculated as mean ± SD. The ANOVA test was used for all group comparisons and Fisher's protected least significant differences was used to compare the differences between each group.

Results

The induction of PGE_2 production by Er:YAG laser irradiation

The average production of PGE_2 by human gingival fibroblast cells in control and in response to Er:YAG laser irradiation with different energy densities are shown in Fig. 1. Control cultures synthesized a low amount of PGE_2, whereas Er:YAG laser significantly increased PGE_2 production. There was no significant cell death by Er:YAG laser irradiation, as measured by LDH levels in the medium or cell counting using trypan blue exclusion method (data not shown). Cultures irradiated with an energy density of 3.37 J/cm^2 showed maximum PGE_2 release. These results showed that the Er:YAG laser with the energy densities used in this experiment induces PGE_2 production in a laser energy-dependent manner without damage to the cells.

Effect of Er:YAG laser irradiation on COX-2 mRNA

To determine if the increased production of PGE_2 by Er:YAG laser is due to increased synthesis of its cognate enzyme, COX-2 mRNA levels in all groups were examined by RT–PCR analysis. As shown in Fig. 2, the COX-2 mRNA was markedly induced in human gingival fibroblast cells irradiated by Er:YAG laser. The bands of the laser-treated human gingival fibroblast cells were clearly more intense than those for the corresponding control. Cultures irradiated with an energy density of 3.37 J/cm^2 showed maximum COX-2 mRNA expression.

Effect of COX-2 inhibitor on PGE_2 release

In order to substantiate the involvement of COX-2 in laser-induced PGE_2 production in human gingival fibroblast cells, we investigated the effects of NS-398 and indomethacin, inhibitors of COX, on laser-induced PGE_2 production (Fig. 3). In the fibroblasts pre-treated with indomethacin and NS-398, Er:YAG laser-induced PGE_2 release was significantly reduced. Indomethacin and NS-398 inhibited laser-induced PGE_2 synthesis in human gingival fibroblast cells with similar efficiency. These results suggest that COX-2 is involved in PGE_2 release induced by Er:YAG laser.

Discussion

Although Er:YAG laser is clinically used as a treatment modality, the molecular pathway that mediates this action is virtually unknown. We investigated the effect of low-level Er:YAG laser irradiation on the biosynthesis of PGE_2 and COX-2 in human gingival fibroblasts. We demonstrated that low-level Er:YAG

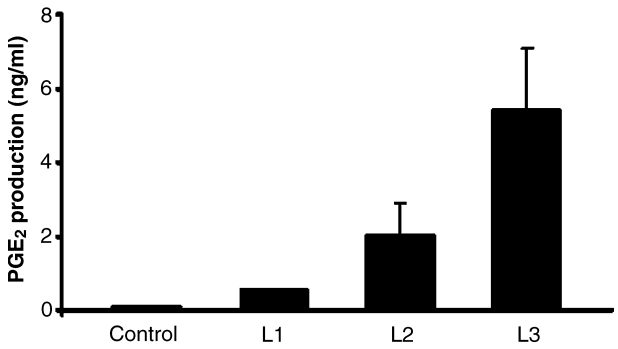

Fig. 1. Prostaglandin E_2 (PGE_2) release from human gingival fibroblasts irradiated with low-level Er:YAG laser. Each histogram bar represents the mean prostaglandin recovered ± SD. Laser irradiation stimulated PGE_2 production in a laser-energy dependent manner. One-way ANOVA revealed significant differences between treatment categories ($p < 0.001$). Control vs. L2 and L3; L3 vs. L1 and L2 were significantly different by Fisher's least significant difference test (significant at 95%).

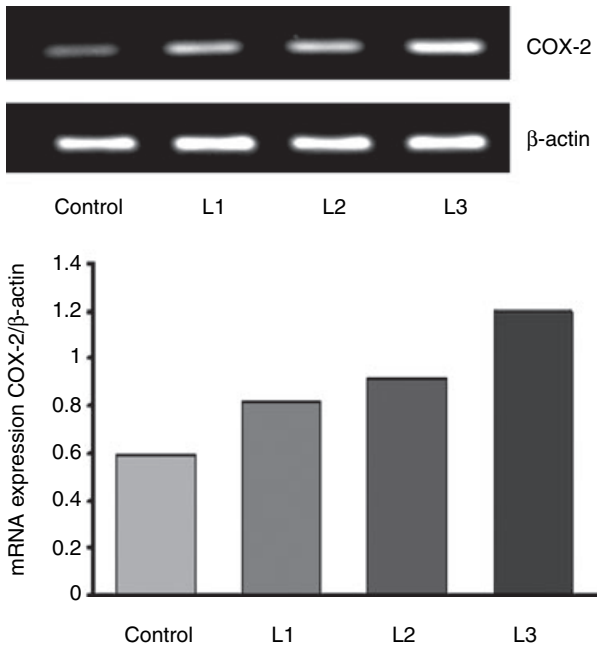

Fig. 2. Cyclooxygenase-2 (COX-2) mRNA expression in human gingival fibroblasts. Human gingival fibroblast cells were irradiated with laser, and total RNA was extracted and COX-2 mRNA expression was analyzed by reverse transcription–polymerase chain reaction. The COX-2 mRNA was highly induced in human gingival fibroblast cells irradiated by Er:YAG laser in a laser-energy dependent manner.

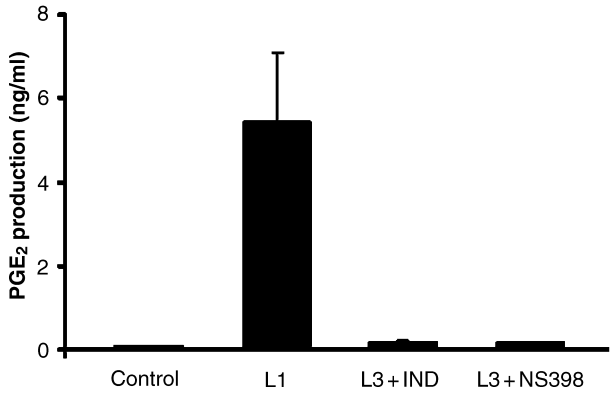

Fig. 3. Effects of indomethacin (IND) and NS-398 on Er:YAG laser-induced prostaglandin E_2 (PGE_2) synthesis in human gingival fibroblast cells. Values are mean ± SD. Both indomethacin and NS-398 inhibited PGE_2 production in laser-irradiated cultures ($p < 0.001$).

laser irradiation significantly stimulated the PGE_2 production in human gingival fibroblast cells. We then demonstrated by RT–PCR that the enhanced production of PGE_2 in culture of gingival fibroblasts was accompanied by an increase of COX-2 mRNA expression, which is reported as the rate-limiting enzyme for the production of PGE_2. In addition, PGE_2 production by Er:YAG laser was suppressed to a lower level by a selective inhibitor of COX-2. These results indicate that Er:YAG laser induced PGE_2 production in human gingival fibroblasts via the induction of COX-2 mRNA expression. Notably, by irradiation with different energy densities, the optimal stimulative energy density was found to be 3.37 J/cm^2. These findings showed the stimulatory effect of Er:YAG laser on COX-2 mRNA expression and PGE_2 production in a laser-energy dependent manner.

There is another study (12), based on biochemical observations, investigating the stimulatory effects of low-level diode laser irradiation on healing in human gingival fibroblasts. The authors concluded that the low-level diode laser irradiation inhibits lipopolysaccharide-induced PGE_2 in human gingival fibroblast cells through a reduction of COX-2 mRNA level.

COX is the rate-limiting enzyme that is involved in PGE_2 production. RT–PCR analysis showed that Er:YAG laser markedly induced COX-2 mRNA in human gingival fibroblasts in contrast to unexposed cells. In our RT–PCR analysis, the control cells, which were not exposed to Er:YAG laser, also expressed COX-2 mRNA very weakly. This weak expression of COX-2 could be due to fresh serum, since previous studies have shown that fresh serum by itself induces COX-2 mRNA levels (13).

To determine if there is any direct link between PGE_2 production and COX-2 induction by Er:YAG laser, activity of COX-2 was specifically inhibited using NS-398 and indomethacin. Indomethacin blocks the activities of both COX-1 and COX-2, and NS-398 specifically inhibits COX-2 activity. Induction of PGE_2 synthesis was completely abolished by COX inhibitors, establishing a direct association between the production of PGE_2 by Er:YAG laser and the induction of COX-2.

In a previous study, an enhanced proliferation of fibroblasts was observed after treatment with Er:YAG laser (14). To understand the biological mechanism of stimulative effect of Er:YAG laser on human gingival fibroblast, PGE_2 production was measured. PGE_2 is one of the important early mediators in the induction of cell proliferation (15–17). Up-regulation of PGE_2 is associated with an induction of COX-2 mRNA, which may subsequently provide a means for amplifying the cellular response to laser (13, 18) and contribute in certain stages of cell proliferation (19). We confirmed a marked induction of PGE_2

and COX-2 protein within 24 h after irradiation with Er:YAG laser. This period is characterized by a significant migration and proliferation of fibroblasts at the wound edge (20). Our data show that Er:YAG laser irradiation appears to exert its stimulative action on gingival fibroblasts proliferation through the production of PGE_2 via the expression of COX-2. This should be considered as an important regulatory aspect for human gingival fibroblasts proliferation in healing and may be one of the cellular pathways to elicit a biological effect after Er:YAG laser irradiation.

In conclusion, Er:YAG laser irradiation strongly stimulated PGE_2 production partly due to enhanced COX-2 mRNA expression in human gingival fibroblast cells. Production of PGE_2 via the induction of COX-2 by Er:YAG laser irradiation may play an important role in acceleration of gingival fibroblasts proliferation. These findings confirm that Er:YAG laser irradiation may facilitate wound healing, particularly in the early acute phase.

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