Nd:YAG Laser Irradiation Abolishes the Increase in Interleukin-6 Levels Caused by Peptidoglycan Through the p38 MAPK Pathway in Human Pulp Cells

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Abstract

The anti-inflammatory effects of low-power laser irradiation have previously been reported. However, how the laser irradiation regulates the expression of inflammatory cytokines remains unknown. In the present study, to elucidate the mechanism behind the anti-inflammatory effect, we examined the effects of low-power neodymium-doped yttrium aluminium garnet (Nd:YAG) laser irradiation on interleukin (IL)-6 expression in human pulp cells (HP cells) stimulated by peptidoglycan (PGN) and focused on intracellular signaling pathways. Low-power Nd:YAG laser irradiation obviated the PGN-induced increase in IL-6 levels in HP cells. A p38 MAP kinase inhibitor, SB203580, also inhibited the increase in IL-6 mRNA levels. PGN stimulated the activity of phosphorylated p38 in HP cells. Low-power laser irradiation inhibited the activity. Thus, suppression of the phosphorylated p38 activity by low-power laser irradiation in HP cells culminates in inhibition of the increase in IL-6 induced by PGN, suggesting that low-power laser irradiation regulates intracellular signaling molecule activities to exert its anti-inflammatory effect.

Introduction

Low-power laser irradiation causes anti-inflammatory effects *in vivo and in vitro* (1-3). However, the precise mechanism by which laser irradiation influences cell functioning remains unknown.

The neodymium-doped yttrium aluminium garnet (Nd:YAG) laser emits an infrared beam at a wavelength of 1064 nm and is widely used in dental treatment. With regard to its endodontic application, Nd:YAG laser has bactericidal effects and is suggested to be a candidate for root canal treatment (4). Furthermore, Nd:YAG laser irradiation can be of therapeutic benefit for direct pulp capping and pulpotomy in clinical practice (5-7). Inhibition of inflammation, elimination of bacteria, and promotion of pulp cell function are necessary for regeneration of pulp-dentin complexes in the treatment of pulpitis. We focus on the application of Nd:YAG laser in the regeneration of pulp-dentin complexes. In order to provide a new therapeutic approach using laser therapy, based on scientific evidence, we need to elucidate the mechanisms of the biological effects of laser irradiation at the cellular and molecular levels.

The mitogen-activated protein kinases (MAPKs), which mediate intracellular signal transduction, have been linked to the inflammatory cellular responses elicited by microorganisms and cytokines (8-10). There are three main MAPK subfamilies: the extracellular signal-regulated kinases (ERK1/2), the c-Jun NH2-terminal kinases (JNK), and p38 MAPK. Nd:YAG laser irradiation may influence the intracellular signaling molecules, since Ga-Al-As diode laser

irradiation inhibits the increased levels of IL-1β and PGE_2 induced by lipopolysaccharides in human gingival fibroblasts and the irradiation of He-Ne laser results in the reduction of the increase in the levels of IL-1β, TNF-α, and INF-γ induced by incisions on the surface of gingivae (3, 11, 12).

The cytokine, interleukin (IL)-6, is produced by several cell types, such as B- and T-lymphocytes, monocytes, macrophages, fibroblasts, and endothelial cells and is a major mediator of the host response to tissue injury and infection (13, 14). Therefore, high levels of IL-6 are connected with the pathogenesis of various inflammatory diseases. Inflamed human dental pulp tissues contain significantly higher levels of IL-6 than healthy pulps (15), suggesting that IL-6 derived from pulp cells plays a role in the mediation of the inflammatory and immune responses initiated by oral bacterial infection.

To clarify the mechanism of the anti-inflammatory actions by low-power laser irradiation, we investigated the effects of low-power Nd:YAG laser irradiation on the expression of IL-6 and activity of MAPKs in cultures of human pulp cells (HP cells) stimulated by peptidoglycan (PGN).

Materials and Methods

Cell Cultures

Human pulp (HP) cells were obtained from explant cultures of two pulps taken from two healthy premolars extracted in the course of orthodontic treatment from two donors with their informed consent according to a protocol approved by

the ethical authorities at Hiroshima University (16). HP cells were separately cultured to confluence in a 35 mm diameter plastic culture dish with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, Biological Industries Ltd., Israel), 100 units/mL penicillin, 100 μg/mL streptomycin, and 1 μg/mL amphotericin B (medium A). When the HP cells formed a confluent monolayer, the cells were harvested with 0.05% trypsin and 0.02% EDTA, and transferred to a 100 mm diameter plastic culture dish. HP cells at the 6th passage were used in the following experiments.

Laser Irradiation

A Nd:YAG laser (Neocure Hyper, SOKKIA Co. Ltd., Kanagawa, Japan) was used in this study. The tip of the fiber was stabilized by a stand instrument at a distance of 60 mm from the bottom of the 6-well plate to irradiate one of the wells. The laser irradiation of HP cells was performed perpendicularly to the bottom of the 6-well plate with a Nd:YAG laser at 2W, 20 pps, and 100 mj for 60 s or 150 s. In this manner, 4 wells out of a 96-well plate were simultaneously and uniformly irradiated. The power density of irradiation was uniformly measured with a laser power meter.

Number of Viable Cells

HP cells in cultures at the 6th passage were harvested, seeded at a density of 5×10^3 cells/well in a 96-well plastic culture plate coated with type I collagen, and

maintained in medium A. After 10 days, confluent HP cells were washed 3 times with DMEM supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and 1 μg/mL amphotericin B (medium B) and then irradiated with the laser in the presence of medium B. After the laser irradiation, the HP cells were incubated for 4 h or 24h. The number of viable cells in each culture was calculated by a CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI).

RNA Preparation

HP cells in culture at the 6th passage were harvested, seeded at a density of 5 x 10^4 cells/well in 6-well plastic culture plates coated with type I collagen, and maintained in medium A. After 10 days, the HP cells were washed 3 times with medium B, exposed to 10 μg/mL PGN (derived from *Staphylococcus aureus,* Sigma, St. Louis, MO) in the presence of medium B, and then irradiated with the laser for 60 s or 150 s. After the laser irradiation, the HP cells were incubated for 4 h. To study the involvement of MAP kinase and nuclear factor (NF)-κB, SB203580 (a p38 MAP kinase inhibitor; Calbiochem, La Jolla, CA), PD98059 (an ERK inhibitor Calbiochem), SP600125 (a JNK inhibitor; Calbiochem) and PDTC (an NF-κB, inhibitor; Sigma) were used. Confluent HP cells were pretreated with PD98059 (50 μmol/L), SB203850 (10 μmol/L), SP600125 (10 μmol/L), or PDTC (10 μmol/L) for 1 h and then exposed to PGN (10 μg/mL) for 4 h. Total RNA was extracted using ISOGEN® (Wako Pure Chemical Industries, Osaka, Japan)

and quantified by spectrometry at 260 and 280 nm.

Real-time PCR

mRNA expression of IL-6 was quantified by real-time PCR. The PCR was carried out in two steps with a Lightcycler system using SYBR green (Roche Diagnostics, Mannheim, Germany). The following primers were used for real-time PCR: human GAPDH forward primer: 5'-AACGTGTCAGTGGTGGACCTG-3', human GAPDH reverse primer: 5'-AGTGGGTGTCGCTGTTGAAGT-3', human IL-6 forward primer: 5'-GGAGACTTGCCTGGTGAAAA-3', and human IL-6 reverse primer: 5'-GTCAGGGGTGGTTATTGCAT-3'.

ELISA for IL6

HP cells in cultures at the 6th passage were harvested, seeded at a density of 5 x 10^4 cells/well in 6-well plastic culture plates coated with type I collagen, and maintained in medium A. After 10 days, the HP cells were washed 3 times with medium B, exposed to 10 μg/mL PGN in the presence of medium B, and then irradiated with the laser. After the laser irradiation, the HP cells were incubated for 24 h. The concentration of IL-6 in the medium was determined using an IL-6 ELISA kit (Biosource International Inc., Camarillo, CA).

p38 Activity and Total p38 expression

HP cells were collected with 500 μL of cell lysis buffer (20 mmol/L Tris (pH

7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 μmol/L EGTA, 1 % Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 1 μg/mL leupeptin, and 1 mmol/L PMSF) and sonicated four times for 5 sec each time on ice. The samples were then microcentrifuged at 13,000 rpm for 10 min at 4 °C, and p38 activity in the supernatant was determined using a p38 MAP kinase assay kit (Cell Signaling, Beverly, MA). For total p38, the supernatant was mixed with 3 × SDS sample buffer and total p38 expression was detected by immunoblotting using rabbit polyclonal anti-p38 MAP kinase antibody (Cell Signaling; 1:1000).

Statistical Analysis

Differences between the two groups with or without PGN, between the two groups with or without laser irradiation in the presence of PGN, and between the two groups with or without the inhibitor in the presence of PGN were analyzed by the Student's *t* test.

Results

Nd:YAG laser irradiation for 60 s and 150 s did not influence the viability of HP cells 4 h and 24 h after the irradiation (Fig. 1), meaning that the laser irradiation can affect HP cell function without causing cell damage.

Nd:YAG laser irradiation alone did not affect the mRNA levels of IL-6 in HP cells (Fig. 2*A*). PGN at 10 μg/mL increased mRNA levels of IL-6 at 4 h (Fig.

2*A*). Nd:YAG laser irradiation suppressed the increased mRNA expression of IL-6 (Fig. 2*A*). This inhibitory effect was dependent on laser irradiation time (Fig. 2*A*). Furthermore, the laser irradiation diminished the PGN-induced increase in levels of IL-6 at 12h (Fig. 2*B*).

The increase in IL-6 mRNA levels caused by PGN was suppressed by pretreatment with SB203580 at 10 μmol/L (Fig. 2*C*). PD98059 at 50 μmol/L also inhibited the increase. Although there was a significant difference in IL-6 mRNA levels between no treatment and pretreatment with PD98059, the decrease in IL-6 levels caused by PD98059 was less pronounced than that induced by SB203580 (Fig. 2*C*). In contrast, pretreatment with SP600125 and PDTC did not influence the mRNA expression of IL-6 (Fig. 2*C*). All the inhibitors had little effects on the IL-6 mRNA levels in the absence of PGN (data not shown).

PGN at 10 μg/mL increased the activity of phosphorylated p38 and the maximal effect was seen at 20 min (data not shown). The laser irradiation of HP cells abrogated the PGN-induced increase in the activity of phosphorylated p38, dependent on the irradiation time (Fig. 3).

Discussion

Previous studies have shown that PGN induces the release of IL-1β, IL-6, and IL-8 through NF-κb and ERK in human eosinophils (17); PGN induces IL-6 production through p38 MAPK and NF-κb in the mouse macrophage cell line RAW 264.7 (18); and PGN stimulates multiple signaling pathways including

NF-κb, p38, and JNK to secrete IL-6, IL-8, and TNF-α in human corneal epithelial cells (19). In the present study, we have demonstrated that a p38 MAPK inhibitor most robustly blocked the PGN-induced increase in IL-6 level in HP cells. These findings suggest that PGN activates p38 MAPK to increase IL-6 levels in HP cells. Furthermore, the intracellular signaling molecules involved in the increase in IL-6 levels induced by PGN have been found to vary, depending on the cells examined.

Regarding the signal transduction during a cellular response induced by laser irradiation, the involvement of MARK cascades has been reported. Low-power Ga-Al-As diode laser irradiation and low-power He-Ne laser irradiation activate phosphorylation of ERK1/2, but not JNK or p38, to promote cellular proliferation of human dental pulp cells and mouse myoblasts, respectively (20, 21). Flashlamp pulsed-dye laser suppressed TGF-β1 expression and proliferation in cultures of human keloid fibroblasts through three subtypes of MAPK cascade, particularly phosporylation of ERK1/2 and p38 MAPK (22). In the present study, p38 MAPK mediated abolishment of the PGN-induced increase in IL-6 levels by Ne:YAG laser irradiation. Thus, we have, for the first time, demonstrated the involvement of MAPKs in the inhibition of inflammatory cytokine expression by low-power laser irradiation. However, it remains unknown whether Ne:YAG laser irradiation directly or indirectly inhibits the phosphorylation of p38 MAPK in HP cells.

Conclusion

Nd:YAG laser irradiation of HP cells inhibits the phosphorylation of p38 MAPK induced by PGN resulting in the suppression of IL-6 expression in HP cells. These observations provide critical new insights into the mechanism whereby low-power laser irradiation exerts an attenuation effect on inflammatory processes and suggest that low-power laser irradiation may be of therapeutic benefit for pulpitis.

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Figure Legends

FIGURE 1 The effects of Nd:YAG laser irradiation on the viability of HP cells. Values are means ± S.D. of three cultures.

FIGURE 2 IL-6 expression in HP cells stimulated by PGN. (A and B), The effect of the laser irradiation. (A) IL-6 mRNA levels. The ratio of IL-6 mRNA to GAPDH mRNA in the HP cells without both PGN and laser irradiation was arbitrarily assigned a value of 1, and the ratio of IL-6 mRNA to GAPDH mRNA in the HP cells stimulated with PGN and (or) laser irradiation was normalized to this. (B) IL-6 levels in the medium. (C) The effects of MAP kinase inhibitors or a NF-κB inhibitor. The ratio of IL-6 mRNA to GAPDH mRNA in the HP cells without both PGN and the inhibitor was arbitrarily assigned a value of 1, and the ratio of IL-6 mRNA to GAPDH mRNA in the HP cells stimulated by PGN in the absence or presence of each inhibitor was normalized to this. Values are means ± S.D. of three cultures. $*p < 0.01$

FIGURE 3 The effect of Nd:YAG laser irradiation on the PGN-induced increase in the activity of phosphorylated p38 in HP cells. HP cells were exposed to PGN at 10 μg/mL and irradiated with Nd:YAG laser for 60 and 150 s. After the laser irradiation, HP cells were incubated for 20 min. HP cells were collected, resuspended in 500 μL of cell lysis buffer, and sonicated as described in the Materials and Methods section. The bands were detected with horseradish peroxidase-linked secondary antibody and ECL.

Figure 1.

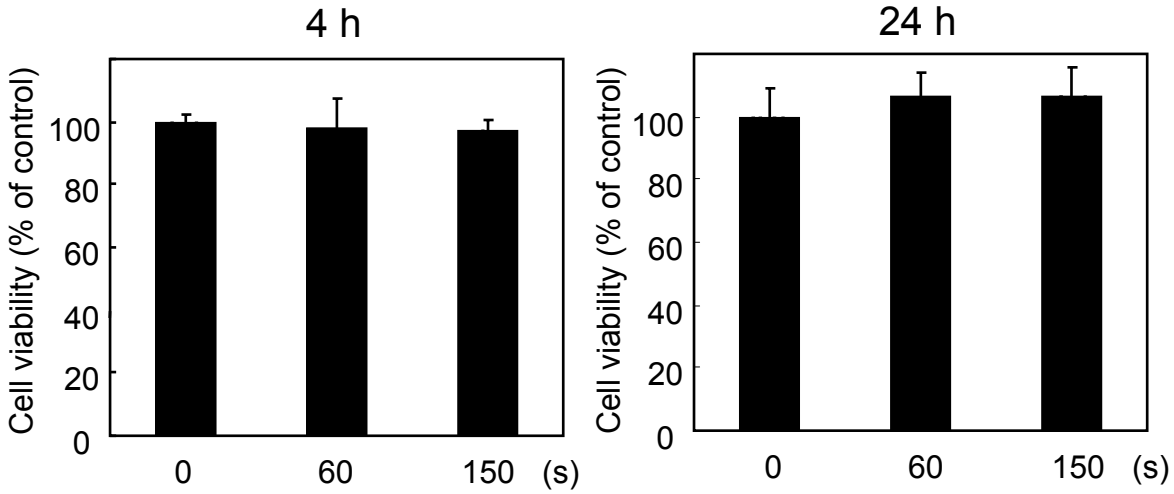


Figure 2.

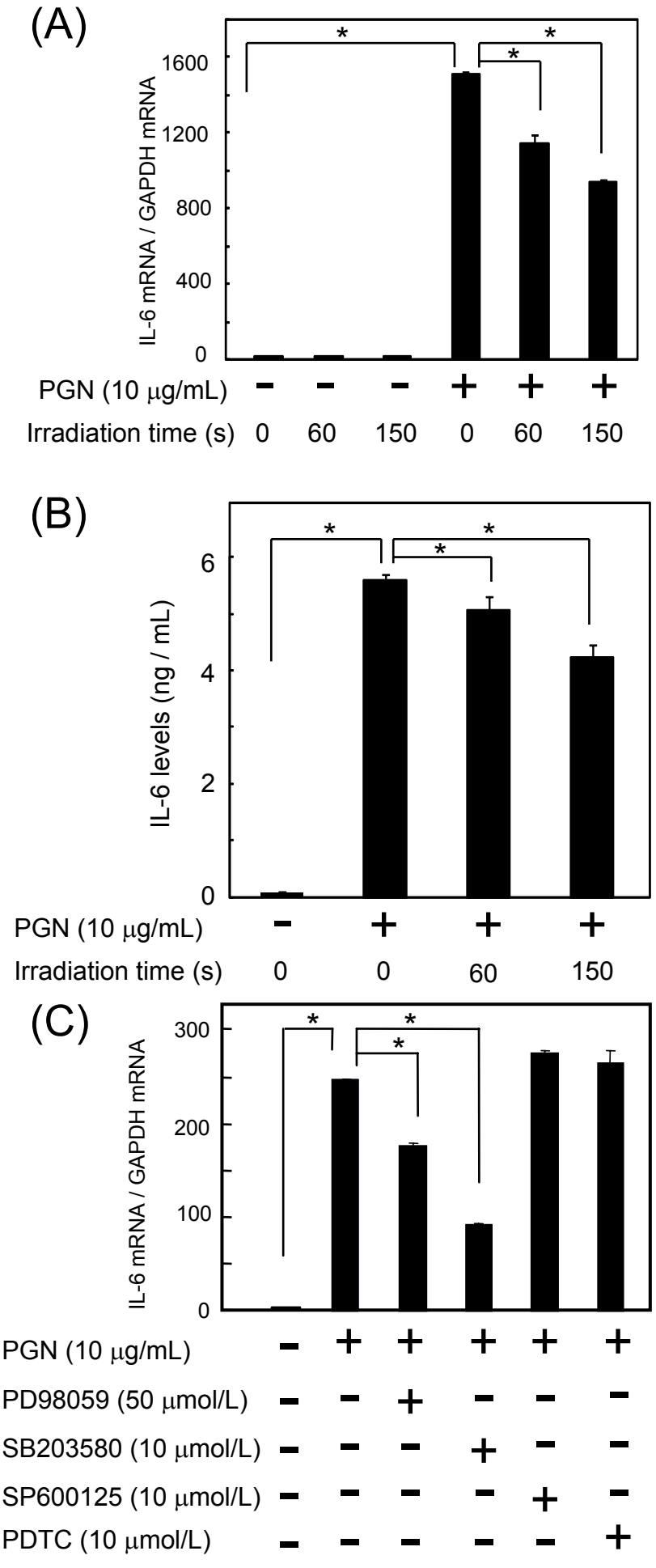
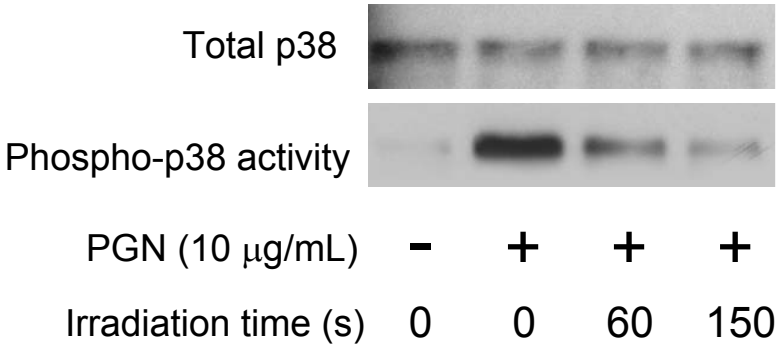

(A)
IL-6 mRNA / GAPDH mRNA
1600
1200
800
400
0
*
*
*
PGN (10 μg/mL) - - - + + +
Irradiation time (s) 0 60 150 0 60 150
(B)
IL-6 levels (ng / mL)
6
4
2
0
*
*
*
PGN (10 μg/mL) - + + +
Irradiation time (s) 0 0 60 150
(C)
IL-6 mRNA / GAPDH mRNA
300
200
100
0
*
*
*
PGN (10 μg/mL) - + + + + +
PD98059 (50 μmol/L) - - + - - -
SB203580 (10 μmol/L) - - - + - -
SP600125 (10 μmol/L) - - - - + -
PDTC (10 μmol/L) - - - - - +

Figure 3.


Total p38
Phospho-p38 activity
PGN (10 μg/mL) - + + +
Irradiation time (s) 0 0 60 150