Mitogen-activated protein kinase/extracellular signal-regulated protein kinase activation of cultured human dental pulp cells by low-power gallium-aluminium-arsenic laser irradiation

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Abstract

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Aim To examine whether low-power laser irradiation (LPLI) promotes cellular proliferation of human dental pulp-derived fibroblast-like cells (dental pulp cells).

Methodology Dental pulp cells were obtained by primary culture of human dental pulp tissues from extracted third molar teeth. The phosphorylation of the mitogen-activated protein kinase (MAPK) family after LPLI of these cells was investigated by Western blotting. By using a specific MAPK/ERK kinase (MEK) inhibitor (PD098059), the specific effect of LPLI on the MAPK pathway was also investigated by Western blotting as described above. The incorporation of [^{3}H]thymidine into the cells after LPLI was determined, and statistical analysis was performed by Wilcoxon signed-ranks test.

Results Extracellular signal-regulated protein kinase (ERK) 1/2 was phosphorylated between 5 and 30 min after LPLI. Moreover, PD098059 inhibited LPLI-mediated ERK1/2 activation. LPLI did not affect p38 MAPK or c-Jun N-terminal kinase (JNK) phosphorylation. But LPLI did not stimulate [^{3}H]thymidine incorporation into these cells.

Conclusions These results indicated that LPLI activated MAPK/ERK, a signal for proliferation, differentiation and survival, but did not activate the stress signals p38 MAPK and JNK in human dental pulp cells.

Keywords: dental pulp cells, low-power laser irradiation, MAPK/ERK activation.

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Introduction

Low-power laser irradiation (LPLI) therapy has been used for a variety of clinical applications. LPLI is thought to promote certain biochemical reactions without inducing any thermal effects, and is considered to reduce pain (Iijima *et al.* 1989), accelerate wound healing (Neiburger 1995) and exert a decreased effect on inflammatory processes (Shimizu *et al.* 1995, Sakurai *et al.* 2000). The effects of LPLI on pulpal analgesia (Whitters *et al.* 1995) and dentine hypersensitivity (Renton-Harper & Midda 1992) have been demonstrated in clinical studies, although the biological mechanisms that are involved remain unclear. LPLI has been reported to promote the growth of periodontal ligament fibroblasts and chicken embryo fibroblasts (Kreisler *et al.* 2003, Vinck *et al.* 2003). However, the possible effects on the proliferation of dental pulp-derived fibroblast-like cells (dental pulp cells) have not been demonstrated. To examine whether LPLI

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stimulates the growth of dental pulp cells, the phosphorylation of the mitogen-activated protein kinase (MAPK) family by LPLI, with focus on the activation of MAPK/extracellular signal-regulated protein kinase (ERK) was investigated.

Materials and methods

Culture of human dental pulp cells

Six dental pulp cells were obtained by primary culture of human dental pulp tissues from extracted third molar teeth. Permission from the Ethics Committee of Nihon University, School of Dentistry, was obtained for this experiment, and informed consent was obtained from each patient. The teeth were split open and the pulp tissue was then removed. Cultures were established by the migration of fibroblastic pulp cells from dental pulp explant cultures. All cells were cultured using α-minimum essential medium (α-MEM; Gibco, Invitrogen, Grand Island, NY, USA) containing 10% foetal bovine serum (FBS, Iwaki, Chiba, Japan) and antibiotics (50 U mL^{-1} penicillin, 50 μg mL^{-1} streptomycin, 100 μg mL^{-1} neomycin; Gibco) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. After primary culture, the cells were trypsinized and subcultured for between four and eight passages. The medium was changed every third day, and cell shape was observed using phase-contrast microscopy. The dental pulp-derived fibroblast-like cells were termed dental pulp cells.

Equipment for low-power semiconductor laser irradiation

A semiconductor laser machine (Osada Diotron 1000 V; Osada Electric Co. Ltd, Tokyo, Japan) was used for this experiment. This employs a semiconductor laser diode (Ga-Al-As) as a laser medium, with variation in output ranging from 0.1 to 1.0 W, and a wavelength of 660 or 810 nm. The equipment was modified to generate continuous laser light through a light guide with a diameter of 15.3 mm (the same diameter as each well of a 24-well plate). The experiments were carried out with an output of 0.5 W and wavelength of 810 nm (light guide tip output 0.425 W and power density 0.231 W cm^{-2}).

Western blot analysis

Western blotting was performed to investigate MAPK activation of human dental pulp cells by LPLI. Dental pulp cells were seeded into 24-well microplates (Sumitomo Bakelite, Tokyo, Japan) at 2.5×10^4 cells per well and incubated in 10% FBS-containing α-MEM for 3 days until confluent under the same conditions as those for the primary culture. After 48 h of serum starvation, a low-power 810-nm diode laser was used to irradiate the dental pulp cells for 90 s. At 0, 5, 15, 30, 60 or 120 min after laser irradiation, the cells were lysed in lysis buffer (50 mol L^{-1} Tris-HCl buffer pH 7.4, 1% Triton-X 100, 150 mmol L^{-1} NaCl, 0.2 mmol L^{-1} sodium orthovanadate, 0.5% NP-40) containing a protease inhibitor cocktail (P-2714; Sigma, St Louis, MO, USA). In a pilot study, the most appropriate period of serum starvation was investigated; it was found that phosphorylation of MAPK/ERK achieved a basal level after a further 48 h of serum starvation (data not shown).

Lysed proteins (20 μg per lane) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). Blocking was then performed using Block Ace (Yukijirushi, Tokyo, Japan). To detect MAPK activation, immunoblotting was conducted with anti-phospho MAPK/ERK (pT^{183}, pY^{185}), anti-phospho p38 MAPK (pT^{182}, pY^{184}) and anti-phospho c-Jun N-terminal kinase (JNK) (pT^{183}, pY^{185}) antibodies (rabbit polyclonal; Promega, Madison, WI, USA). Biotin-conjugated F (ab') goat anti-rabbit IgG (H + L) (Zymed, San Francisco, CA, USA) was used as a secondary antibody, followed by incubation with streptavidin horseradish peroxidase (HRP) (KPL, Gaithersburg, MD, USA). Immunoreactive proteins were detected by means of an enhanced chemiluminescence (ECL) reagent (Immunostar Reagent; Wako Pure Chemical Industries, Osaka, Japan), and exposed to X-ray film (Hyperfilm ECL; Amersham Biosciences, Little Chalfont, UK). Dr Western (Oriental Yeast Co., Tokyo, Japan) was used as a marker for molecular weight. This consists of six kinds of protein A repeats expressed by *Escherichia coli* (82, 68, 55, 42, 28, and 15 kDa). Total MAPK/ERK, p38 MAPK and JNK proteins were determined with antibodies for unphosphorylated proteins (Sigma) using reprobed membranes. A positive control consisted of PC12 cell extract (Promega) treated with nerve growth factor for MAPK/ERK, and treated with sorbitol for p38 MAPK and JNK. The negative control was untreated PC12 cell extract (Promega). Ten percent FBS containing α-MEM-treated cells served as a positive control.

The effect of LPLI on intracellular signal transduction pathways other than MAPK was performed by Western

blotting using six phospho-specific antibodies that are associated with cellular growth: janus kinase (JAK) 2 (pY^{1007}, pY^{1008}), Akt/protein kinase B (Akt/PKB) (pS^{473}), focal adhesion kinase (FAK) (pY^{397}), Src (pY^{418}), phospholipase C (PLC)-γ (pY^{783}) and paxillin (pY^{31}) (Biosource, Camarillo, CA, USA). The positive controls consisted of NIH3T3 cell extract treated with platelet-derived growth factor instead of PLC-γ and Akt/PKB, and FAK expression vector-transfected chick embryo fibroblasts for FAK. Negative controls were untreated cells (Biosource).

To confirm whether interleukin-1β (IL-1β) activates p38 MAPK and tumour necrosis factor-α (TNF-α) activates JNK of these cells, Western blotting was performed after stimulation with 1 ng mL^{-1} IL-1β and 10 ng mL^{-1} TNF-α as described above.

To know the upstream of the MAPK/ERK, serum-starved dental pulp cells were pretreated with α-MEM containing either 5 μmol L^{-1} PD098059, a specific noncompetitive inhibitor of MAPK/ERK kinase (MEK) (Biomol, Plymouth Meeting, PA, USA) (Alessi *et al.* 1995) dissolved in dimethyl sulfoxide (DMSO) or DMSO only, for 60 min, whilst controls remained untreated. The cells were then irradiated and harvested at 0, 5, 15 and 30 min after irradiation and assayed for phospho-specific MAPK/ERK phosphorylation by Western blotting as described above. Ten percent FBS-containing α-MEM-treated cells served as a positive control.

[^{3}H]thymidine incorporation

The effect of LPLI on cellular DNA synthesis was monitored by the uptake of [^{3}H]thymidine. Six dental pulp cells were cultured by the method described above. After 48 h of serum starvation, a low-power diode laser was used to irradiate the dental pulp cells for 10, 30 and 90 s, and nonirradiated cells served as a control. Twenty hours after laser irradiation, pulse labelling of the dental pulp cells was carried out by exposure to [^{3}H]thymidine (37 kBq $well^{-1}$) for 2 h. Labelled cells were fixed with 5% trichloroacetic acid (TCA) at 4 °C for 10 min. The radioactivity of the TCA-insoluble fraction was determined in a Packard 2000 CA Liquid Scintillation Counter (Packard USA, Meriden, CT, USA). Ten percent FBS-containing α-MEM-treated cells served as a positive control. All experiments were performed in quadruplicate. All values were represented as mean ± SD. Wilcoxon signed-ranks test was used for statistical analysis at a significance level of $P < 0.05$.

Results

LPLI specifically activates ERK phosphorylation

Phosphorylation of ERK1/2 was induced in low-power laser-irradiated cells after 5, 15, and 30 min (Fig. 1a).

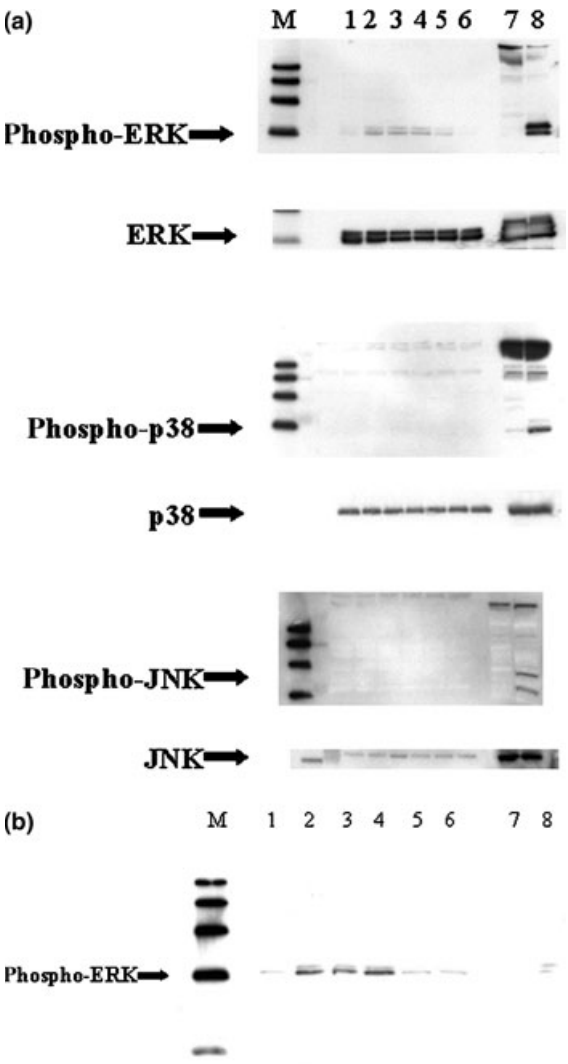

Figure 1 Phosphorylation of the MAPK family after LPLI in dental pulp-derived fibroblast-like cells. Phosphorylation of ERK1/2 was induced 5, 15 and 30 min after application of LPLI, and LPLI did not affect p38 MAPK or JNK phosphorylation (a). Phosphorylation of ERK1/2 was detected from 5 to 30 min in 10% FBS-treated positive control cells (b). Results presented are one of these cell populations. (M, molecular weight marker; lane 1, non-irradiated; lane 2, 5 min after LPLI; lane 3, 15 min after LPLI; lane 4, 30 min after LPLI; lane 5, 60 min after LPLI; lane 6, 120 min after LPLI; lane 7, negative control; lane 8, positive control).

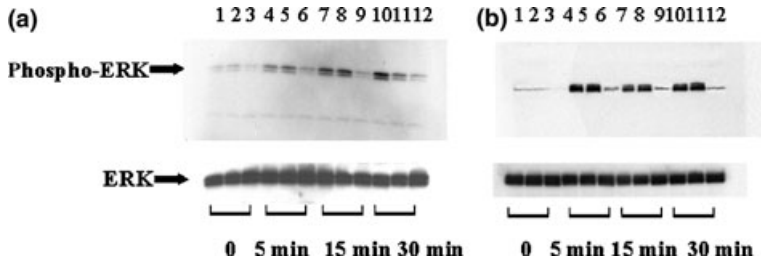

Figure 2 Effect of the MEK inhibitor, PD098059, on MAPK/ERK activation. A MEK inhibitor, PD098059, inhibited LPLI-mediated ERK1/2 activation, whereas in controls and DMSO-treated cells, ERK1/2 was activated by LPLI (a). Similar data were obtained by treatment with 10% FBS (b). PD098059 inhibited both LPLI and FBS-mediated ERK1/2 activation. Results presented are one of these cell populations. (lanes 1, 4, 7 and 10, control; lanes 2, 5, 8 and 11, DMSO; lanes 3, 6, 9 and 12, PD098059).

These levels declined gradually, and were barely detectable after 120 min. Total ERK1/2 protein levels were similar in the control and test groups at all time points. Phosphorylation of ERK1/2 was detected from 5 to 30 min in 10% FBS-treated positive control cells (Fig. 1b). In contrast to MAPK/ERK, LPLI did not affect p38 MAPK or JNK phosphorylation, because total p38 MAPK and JNK protein levels were similar. Phosphorylation of MAPK/ERK, or the total amount of MAPK/ERK, did not change within 120 min without LPLI (data not shown).

A MEK inhibitor, PD098059, inhibited LPLI-mediated ERK1/2 activation, whereas in controls and DMSO-treated cells, ERK1/2 was activated by LPLI (Fig. 2a). Similar results were obtained using FBS as a stimulant instead of LPLI (Fig. 2b).

These results showed that ERK1/2 was phosphorylated by LPLI, whereas p38 MAPK and JNK1/2 were not.

[^{3}H]thymidine incorporation

[^{3}H]Thymidine incorporation showed a tendency to increase with prolonged irradiation time, and was about 1.3 times higher than the control level that was irradiated for 90 s (Fig. 3). However, Wilcoxon signed-ranks test failed to show any significant differences.

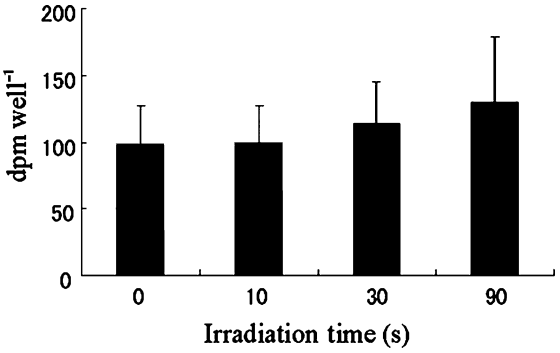

Figure 3 Effect of LPLI on [^{3}H]thymidine incorporation into cells. [^{3}H]thymidine incorporation showed a tendency to increase as the duration of LPLI was prolonged; however, the difference was not significant (Wilcoxon signed-ranks test).

Effect of LPLI on intracellular signal transduction pathways other than MAPK

The findings provided evidence that LPLI resulted in MAPK/ERK activation downstream of MEK; however, its upstream pathways and pathways other than MAPK are unknown. Therefore, Western blotting was performed. JAK2, FAK, Akt/PKB and paxillin were phosphorylated slightly at all times regardless of LPLI (Fig. 4). PLC-γ and Src were not phosphorylated at all (Fig. 4).

Discussion

Mitogen-activated protein kinase was identified as a serine/threonine-specific protein kinase in the second half of the 1980s (Ray & Sturgill 1987, Hoshi *et al.* 1988). Present in all eukaryotes, it is a mainstay of cellular signal transduction. Extracellular signals activate MAPK cascades to execute complex cellular programmes such as proliferation, differentiation and apoptosis (Nishida & Gotoh 1993).

The MAPK cascade includes MAPK/ERK, JNK and p38 MAPK. MAPK/ERK is known to be activated by growth and differentiation factors, peptide hormones, or cytokines and neurotransmitters. JNK and p38

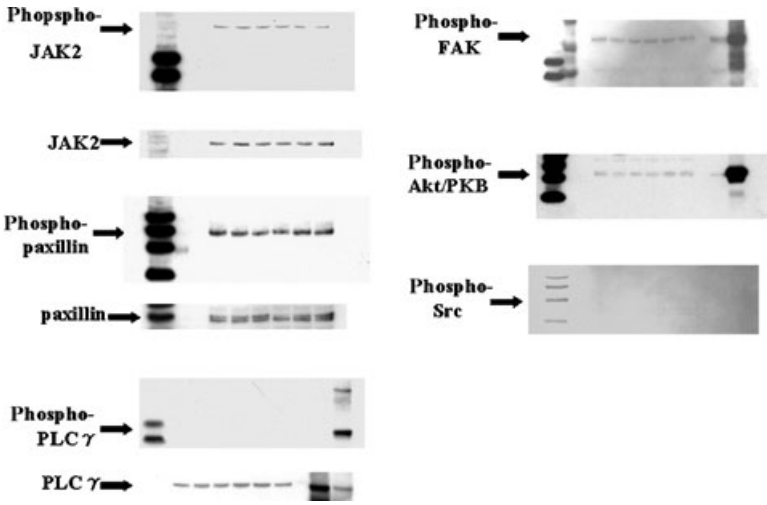

Figure 4 Effect of LPLI on intracellular signal transduction pathways other than MAPK. JAK2, FAK, Akt/PKB and paxillin were phosphorylated slightly at all time points, regardless of LPLI. PLC-γ and Src were not phosphorylated at all. Results presented are one of these cell populations.

MAPK are also activated by physical and chemical stimuli such as radiation, heat and pressure (Zanke *et al.* 1996, Li *et al.* 2001). Although various signal transfer routes operate in the organizational and immune mechanisms of a cell, the MAPK cascade may play a central role (Seger & Krebs 1995).

In this study, LPLI specifically activated MAPK/ERK. Irradiation of serum-starved dental pulp cells resulted in phosphorylation of ERK1/2, and this was blocked by a MEK inhibitor, PD098059. These data imply that LPLI activates the MAPK pathway through MEK, as PD098059 has been shown to block the upstream protein kinase, MEK1/2. Laser irradiation can be considered a stress signal for cells (Leitz *et al.* 2002). However, the semiconductor laser used in the present study did not appear to promote any stress response in dental pulp cells. The results suggest that LPLI does not act via a stress signal but via a mitogen-activated pathway that leads to cellular proliferation.

Promotion of cell proliferation by LPLI occurred through signalling of MAPK/ERK from MEK; however, the possibility that LPLI activates other signal-transduction pathways cannot be ruled out, because its upstream pathways are unknown. Therefore, it could be considered that there may be specific pathways other than MAPK/ERK involved in the activation of cultured dental pulp cells by LPLI, and employed six phospho-specific antibodies, JAK2 ($pY^{1007}pY^{1008}$), Akt/PKB (pS^{473}), FAK (pY^{397}), Src (pY^{418}), PLC-γ (pY^{783}) and paxillin (pY^{31}), that are associated with cellular growth (Chan *et al.* 1999, Sekiya *et al.* 1999, Carter-Su *et al.* 2000, Nakamura *et al.* 2000, Sieg *et al.* 2000, Tatosyan & Mizenina 2000) to examine other possible pathways. None of the examined proteins were further phosphorylated by LPLI.

In this study, ERK1/2 was activated immediately after LPLI. However, [^{3}H]thymidine incorporation following LPLI was not as effective as expected. It has been shown that hypoxia-induced vascular endothelial growth factor (VEGF) production enhances tumour survivability via suppression of serum deprivation-induced apoptosis (Baek *et al.* 2000). Induction of ERK phosphorylation and a decrease of the Bax/Bcl-2 ratio by recombinant human VEGF imply that hypoxia-induced VEGF prevents apoptosis by activating the MAPK/ERK pathway. Furthermore, MAPK/ERK activation induces not only cell survival (Xia *et al.* 1995, Allan *et al.* 2003) but also stimulates collagen production (Atamas *et al.* 2003, Papakrivopolou *et al.* 2004), chemotaxis (Brahmbhatt & Klemke 2003) and cell motility (Fincham *et al.* 2000).

Several studies have reported the promoting effect of LPLI on cellular proliferation (Shefer *et al.* 2001, Vinck *et al.* 2003). Because LPLI was performed after the cells had reached confluency, activation of MAPK/ERK did not induce cellular proliferation but induced other unknown activities such as growth factor secretion. However, as it was not possible to clarify any effects downstream of activated MAPK/ERK, further study will be required to clarify the role of MAPK/ERK activation induced by LPLI in human dental pulp cells.

In addition, it has been reported that in skeletal muscle cells LPLI is able to activate the phosphorylation of c-Met as well as hepatocyte growth factor, suggesting a possible mechanism by which LPLI affects the MAPK/ERK pathway (Shefer *et al.* 2001). There is

speculation that LPLI creates ligand-free dimerization of specific receptors that are in the appropriate energetic state to accept the laser energy, leading to their autophosphorylation, and affecting the downstream pathways. As human dental pulp cells do not express c-Met, and the six kinds of pro-growth signals occurring upstream of the MAPK cascade were not phosphorylated by LPLI, the pathways upstream of MEK still remain unknown.

The clinical relevance of the present *in vitro* findings remains unclear. Further studies are being performed to examine the effects of frequent LPLI on these cell populations. Taken together, the results indicate that LPLI activates MAPK/ERK phosphorylation specifically, and not JNK or p38 MAPK, in dental pulp cells.

Conclusions

LPLI activated MAPK/ERK, a signal for proliferation, differentiation and survival, and did not activate stress signals, p38 MAPK and JNK, in human dental pulp cells.

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