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Original Article

Effect of Diode Laser on Proliferation and Differentiation of PC12 Cells

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Abstract

This study investigated the effects of diode (GaAlAs) laser irradiation at an effective energy density of 5 or 20 J/cm² on cell growth factor-induced differentiation and proliferation in pheochromocytoma cells (PC12 cells), and whether those effects were related to activation of the p38 pathway. Laser irradiation at 20 J/cm² significantly decreased the number of PC12 cells, while no difference was seen between the 5 J/cm² group and the control group ($p < 0.05$). Western blotting revealed marked expression of neurofilament and β -tubulin, indicating greater neurite differentiation in the irradiation groups than in the control group at 48 hr. Irradiation also enhanced expression of phospho-p38. The decrease in number of cells after laser irradiation was accelerated by p38 inhibitor, while neurite differentiation was up-regulated by laser irradiation, even when the p38 pathway was blocked. This suggests that laser irradiation up-regulated neurite differentiation in PC12 cells involving p38 and another pathway.

Key words: GaAlAs laser—PC12 cells—Proliferation—Neurite differentiation—p38 pathway

Introduction

The application of low level laser therapy is increasing in medicine and dentistry^{4,17}. Low

level laser therapy has been demonstrated to alleviate pain in various disease states and promote nerve regeneration, although the underlying mechanisms remain to be eluci-

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dated. There have been reports of guiding neurons with optical dipole forces²⁾. Rat pheochromocytoma cell line (PC12 cells) possesses neuronal characteristics, and is often utilized in neuronal *in vitro* models, as cell processes in undifferentiated PC12 cells can be extended by addition of nerve growth factor (NGF)⁵⁻⁷⁾. p38 mitogen-activated peptide kinase (MAPK) has been found to be essential for nerve differentiation, and is provoked by the combination of NGF with tropomyosin receptor kinase A receptors^{13,16)}. SB203580, an inhibitor of p38, blocks signal transmission of MAPK/Extracellular signal-regulated kinase (ERK) kinase, with subsequent inhibition of nerve process extension¹¹⁾.

On the other hand, exposure to nitric oxide (NO) causes a time-dependent increase in p38 activation and induction of apoptosis, which is inhibited by 203580 in PC12 cells. Therefore, it is possible that p38 plays a crucial role not only in differentiation, but also in survival of nerve cells¹⁵⁾. Nevertheless, p38 is activated by various stimuli³⁾, and it is still open to question as to whether it is activated by laser irradiation. Furthermore, changes in proliferation and differentiation in laser-irradiated PC12 cells with inhibition of the p38 pathway remain to be determined. In this study, we investigated the influence of laser irradiation on NGF-induced proliferation and differentiation in PC12 cells.

Materials and Methods

1. Cell culture and laser irradiation

Rat PC12 cells (Dainippon Sumitomo Pharma, Osaka, Japan) were cultured in RPMI 1640 medium (Invitrogen, Gaithersburg, MD, USA) containing 10% horse serum (Invitrogen), and 5% fetal bovine serum (Invitrogen). The cells were seeded in a collagen-coated 24-well microplate with collagen type I (IWAKI, Tokyo, Japan) at a density of 1×10^4 cells/well. In order to induce differentiation, PC12 cells were treated with NGF (50 ng/ml; Chemicon International, CA, USA). After 72 hr, the cultured cells were

irradiated with a GaAlAs diode laser (LD 15, wavelength; 810 nm; 10 watt, continuous wave; Dentek Laser Systems Production, Bremen, Austria) at a temperature of 37°C at 5 J/cm² or 20 J/cm². The relationships between laser irradiation distance and effective irradiation range and between source and effective irradiation energy have been described in an earlier report¹²⁾. To obtain a sufficiently wide area for irradiation, the distance from the bottom of the culture plate to the tip of the probe was set to 50 mm. Effective irradiation energy was equivalent to approximately 60% of the source energy. Thus, total energies of approximately 5 J/cm² and 20 J/cm² were achieved with 1.26 sec and 5.04 sec exposures, respectively. The PC12 cells were cultured for 24 hr or 48 hr after laser irradiation.

2. Cellular proliferation

After 24 hr and 48 hr, the cells were detached with a pipette and counted with a Coulter Counter (Beckman Coulter, Fullerton, CA, USA).

3. Cell morphology

After 48 hr, the cells were washed with phosphate-buffered saline, and morphological changes were observed with a phase-contrast microscope (Olympus, Tokyo, Japan).

4. Neurite assay

The percentage of cells showing neurite outgrowth was determined at 24 hr and 48 hr after irradiation. Cells revealing a neurite length greater than two times that of body length were considered positive.

5. Statistical analysis

Results of proliferation and neurite assay were compared with the Kruskal Wallis/Tukey test. Significance was set at 1% ($p < 0.01$) and 5% ($p < 0.05$).

6. Western blotting

At 1, 3, 6, 24 or 48 post-irradiation hr, the PC12 cells were washed twice with phosphate-buffered saline, lysed in RIPA buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris

[pH=7.4]) containing protease inhibitors, and then sonicated (Sonifier 250D; Branson, Dunbury, USA) for 1 min at a 0.1 pulse. The lysates were cleared by centrifugation at 15,000 rpm for 20 min (PF-2000; Wakenyaku, Kyoto, Japan) to obtain supernatant. Protein concentrations in the supernatant were determined by the Lowry method using Protein Assay Reagent (Bio-Rad Laboratories, Richmond, USA). The supernatants obtained from the cells at 1, 3, 6, 24 or 48 post-irradiation hr were used to determine neurite differentiation. Samples from after 1, 3, 6 and 24 hr were used to investigate activation of the p38 pathway. Equal amounts of protein (30 μ g) were separated by 7.5% sodium dodecyl

sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories). The membranes were then incubated with antibodies specific to neurofilament-L, β -tubulin isotype III (Chemicon International, CA, USA) and phospho-p38 (Cell Signaling, Beverly, MA, USA) in tris-buffer solution containing Tween 20 (TBS-T; 0.05% Tween 20, 150 mM NaCl, 20 mM Tris [pH 7.4]) overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase conjugated mouse or rabbit IgG (Amersham, Rochester, MI, USA). Immunoreactive bands were detected using an ECL™ Western Blotting Detection Reagent (Amersham).

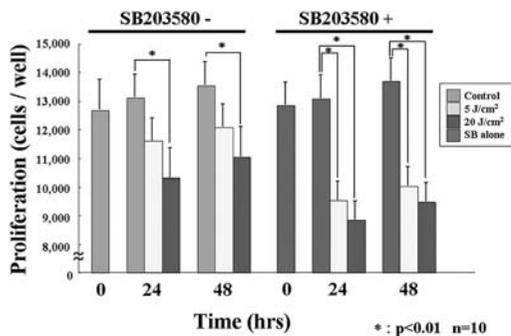


Fig. 1 Effects of laser irradiation and p38-inhibitor (SB203580) on proliferation of PC12 cells

Cell numbers were significantly decreased in irradiated cells without SB203580 after 24 and 48 hr. In SB203580-treated groups, cell numbers were significantly decreased at both irradiation levels (5 J/cm² and 20 J/cm²) after 24 and 48 hr. Data are expressed as mean \pm standard deviation (SD). *Significant p-value (p<0.01) versus control.

7. Inhibition of p38 pathway

In order to determine the role of p38 in the irradiated PC12 cells, an inhibitor (SB203580) was used to block the p38 pathway. SB203580 (10 μ M; Calbiochem, USA) was added 24 hr before laser irradiation, and its effect on proliferation and neurite outgrowth as a marker of differentiation was examined.

Results

1. Proliferation (Fig. 1, Table 1)

The number of cells (cells/well) showed a slight increase with time in the control group. A clear tendency toward a decrease was seen in cell number in the 5 J/cm² group in com-

Table 1 Values express mean (cells/well) and standard deviation under each experimental condition

SB203580 -			
	Control	5 J/cm ²	20 J/cm ²
24 hr	13,100 \pm 1,086.7	11,600 \pm 750.0	10,320 \pm 908.7
48 hr	13,540 \pm 836.3	12,060 \pm 842.1	11,040 \pm 916.8
SB203580 +			
	SB alone	5 J/cm ²	20 J/cm ²
24 hr	13,080 \pm 768.9	9,540 \pm 602.0	8,860 \pm 622.4
48 hr	13,680 \pm 795.8	10,400 \pm 619.6	9,480 \pm 656.6

parison with the control group, although the difference was not statistically significant. In the 20J/cm²-irradiated group, cell number showed a significant decrease after 24 hr and 48 hr ($p < 0.01$). No significant differences were observed between the control group and the groups treated with SB203580. The tendency toward a decrease in cell number after

laser irradiation at both 5J/cm² and 20J/cm² was more prominent in cells not treated with SB203580 ($p < 0.01$).

2. Morphological change (Fig. 2)

NGF-treated PC12 cells showed a clear increase in inter-connected neurites after 48 hr (Fig. 2a). No difference was observed

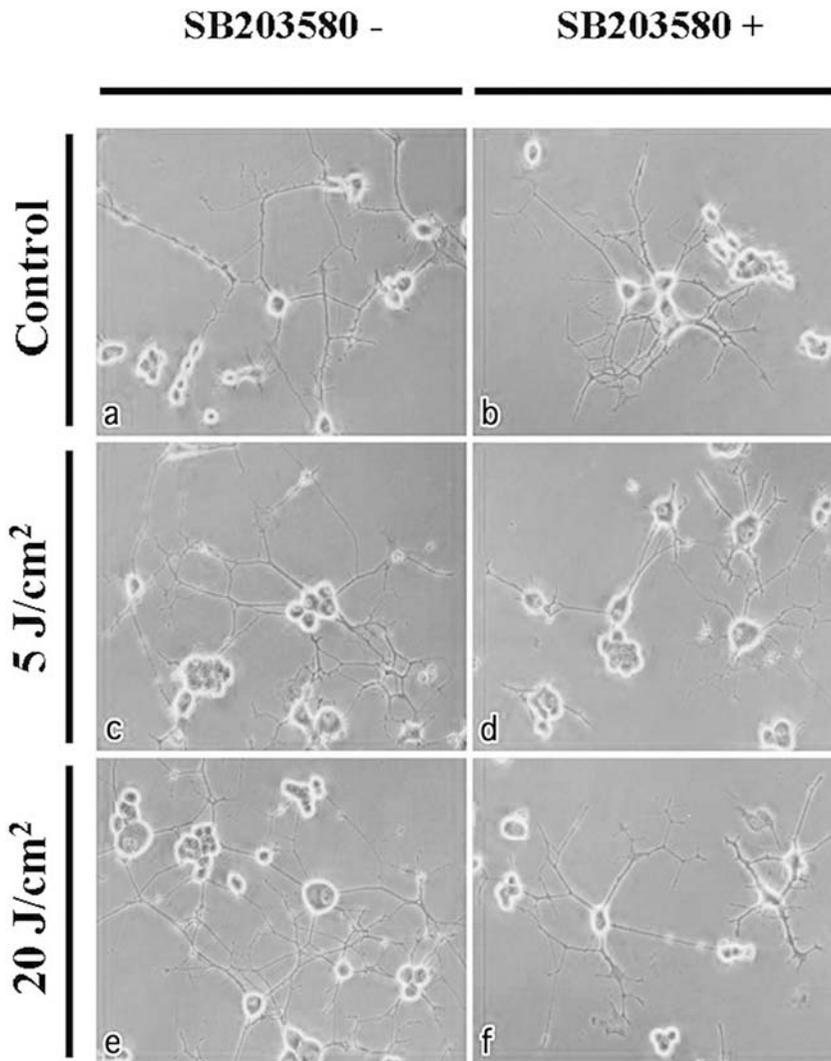


Fig. 2 Phase-contrast microscopic pictures of PC12 cells

Cultured PC12 cells treated with NGF (a, c, e) and with both NGF and SB203580 (b, d, f). Cells shown in Figs. a and b were non-irradiated, c and d were irradiated at 5J/cm², e and f were irradiated at 20J/cm². Well-developed interconnecting cell processes (neurite outgrowth) were detected in non-irradiated (control) (a), at 5J/cm² (c), and at 20J/cm² (e) in irradiated PC12 cells. Neurites in SB203580-treated cells show poor outgrowth (b, d, f).

in terms of morphological change between irradiated and non-irradiated PC12 cells (Figs. 2c, e). Cell processes were shorter in the SB203580 group than in the control

group (Fig. 2b). No remarkable change was observed in the SB203580 group, even after laser irradiation. No difference was noted between the 5J/cm² and 20J/cm² groups (Figs. 2d, f).

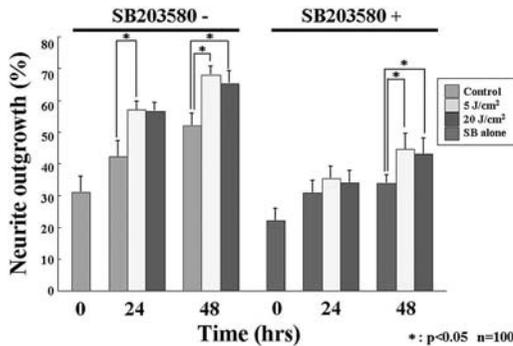


Fig. 3 Effects of laser irradiation and p38-inhibitor (SB203580) on neurite outgrowth in PC12 cells. Neurite outgrowth increased with incubation time. SB203580 totally inhibited neurite outgrowth. In SB203580 non-treated experiments, percentage in irradiation at 5 J/cm² was greater than that in control group after 24 and 48 hr. Percentage at 20 J/cm² was significantly higher after 48 hr than that of control group. In SB203580-treated group, neurite outgrowth was enhanced significantly at both irradiation levels after 48 hr. Data are expressed as mean ± SD. *Significant p-value (p<0.05) versus control.

3. Neurite assay (Fig. 3)

The percentage of cells revealing neurite outgrowth increased with time (Fig. 3), and was significantly greater in the 5J/cm²-irradiated group than in the control group after 24 hr and 48 hr (p<0.05). The percentage of neurite outgrowth in the 20J/cm² group, however, showed a significantly higher value after 48 hr (p<0.05). No significant difference was seen between the two irradiated groups at 24 hr and 48 hr. Treatment with SB203580 resulted in a marked decrease in NGF-induced neurite outgrowth with 48 hr laser irradiation. The percentage of neurite outgrowth with 48 hr laser irradiation was significantly higher than that in non-irradiated cells treated with SB203580 alone (p<0.05). No significant differences were observed between the two irradiated groups at 24 hr or 48 hr.

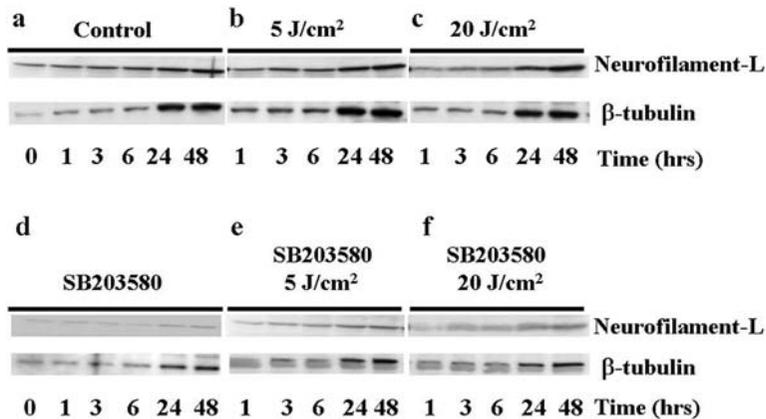


Fig. 4 Effects of laser irradiation and p38-inhibitor (SB203580) on expression of neurofilament and β-tubulin by Western blotting. Expression of neurofilament and β-tubulin was up-regulated by both levels of laser irradiation. SB203580 clearly inhibited expression of both proteins, but laser irradiation slightly enhanced expression in cells treated with inhibitor. a, non-irradiated cells without SB203580 (control); b, 5 J/cm²-irradiated cells without SB203580; c, 20 J/cm²-irradiated cells without SB203580; d, non-irradiated cells with SB203580; e, 5 J/cm²-irradiated cells with SB203580; f, 20 J/cm²-irradiated cells with SB203580.

4. Expression of neurofilament and β -tubulin (Fig. 4)

Expression of neurofilament and β -tubulin showed an increase with time (Fig. 4a). Expression of neurofilament and β -tubulin after laser irradiation was analogous to that in the control group until 24 hr. On the other hand, expression of these proteins after 48 hr was up-regulated in both the $5\text{J}/\text{cm}^2$ and $20\text{J}/\text{cm}^2$ groups in comparison with in the control group. No differences were observed in the expression of either protein between $5\text{J}/\text{cm}^2$ and $20\text{J}/\text{cm}^2$ laser irradiation (Figs. 4b, c). Expression of neurofilament and β -tubulin showed a clear reduction in comparison with that in the control group (Fig. 4d). Expression of neurofilament and β -tubulin was more prominent in the $5\text{J}/\text{cm}^2$ irradiation in comparison with that in cells treated with SB203580 alone (Fig. 4e). A small change in the expression of these proteins was detected with $20\text{J}/\text{cm}^2$ irradiation in comparison with that in cells treated with SB203580 alone (Fig. 4f).

5. Activation of p38 (Fig. 5)

Western blot revealed a small change in anti phospho-p38 from 1 to 24 hr in the control group (Fig. 5a). Expression of phospho-p38 was gradually enhanced from 1 to 3 hr after irradiation at $5\text{J}/\text{cm}^2$ in comparison with in the control group (Fig. 5b). Although phospho-p38 was up-regulated by laser irra-

diation at $20\text{J}/\text{cm}^2$, no remarkable change was observed from 1 to 24 hr post-treatment (Fig. 5c). Treatment with SB203580, on the other hand, totally blocked p38 activation after laser irradiation (Fig. 5d, e, f).

Discussion

The bio-stimulatory effect of laser irradiation on culture cells is closely related to energy density¹⁰. Previous studies have suggested that an energy density of $1\text{--}5\text{J}/\text{cm}^2$ is most effective for cell proliferation and differentiation^{8,14}, whereas higher densities induce inhibition⁹. One study investigating the effect of different power densities (142.85 and $428.57\text{mW}/\text{cm}^2$) of laser irradiation on proliferation of human fibroblasts found that a lower density yielded a larger increase than a higher density¹¹. It is possible that the different effects found in studies on laser irradiation, even when the same energy density was employed, resulted from differences in equipment and conditions.

Apart from energy density, the conditions of irradiation used in this study were the same as those used in an earlier study¹⁰, demonstrating that they did not induce an increase in the temperature of the culture medium. In the present study, we selected energy densities of $5\text{J}/\text{cm}^2$ and $20\text{J}/\text{cm}^2$ to clarify the stimula-

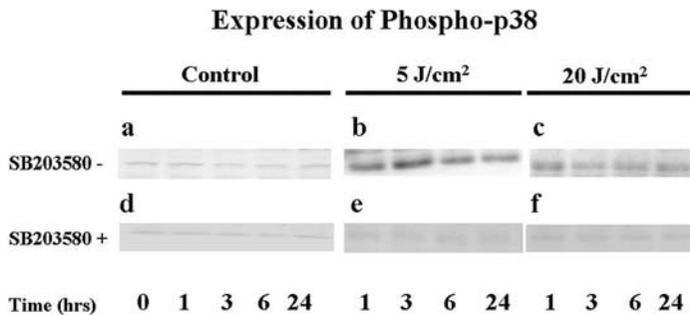


Fig. 5 Effects of laser irradiation and p38-inhibitor (SB203580) on activation of p38 in PC12 cells

There was little change from 1 to 24 hr in control group (a). Activation of p38 was apparently enhanced after 1 and 3 hr by irradiation at $5\text{J}/\text{cm}^2$ (b) and $20\text{J}/\text{cm}^2$ -irradiation (c). SB203580 clearly blocked p38 activation (d) even by irradiation (e, f).

tory and inhibitory effects of irradiation on proliferation and differentiation, respectively, in PC12 cells. Laser irradiation at 20J/cm² significantly decreased the number of PC12 cells, while there was no difference between the 5J/cm² and control groups ($p < 0.05$). This implies that a power density of 20J/cm² damaged the PC12 cells, resulting in those cells detaching from the culture plate or induction of cell death. However, it was not possible to confirm whether laser irradiation at 5J/cm² was effective in proliferation of PC12 cells.

Laser irradiation at 5J/cm² enhanced the percentage of neurite outgrowth after 24 and 48 hr ($p < 0.05$). Significant up-regulation by 20J/cm² irradiation was evident after 48 hr ($p < 0.05$). Western blotting revealed clear enhancement of expression of neurofilament and β -tubulin compared with that in the control group at 48 hr at both irradiation levels. These results suggest that laser irradiation exerts a positive effect on neurite differentiation in PC12 cells, although there was no difference in neurite differentiation between 5J/cm² and 20J/cm².

It has been reported that the bio-stimulatory effect of GaAlAs laser irradiation is influenced by the cellular cycle¹⁰⁾. In this study, we first induced differentiation in PC12 cells with NGF. We found that subsequent laser irradiation accelerated this differentiation, similar to its effect in osteoblasts¹⁾.

There was a tendency toward a decrease in cell number with laser irradiation, which was accelerated by treatment with SB203580. The p38 pathway has been associated with apoptosis following inflammation, and SB203580 plays an important role in avoidance of apoptosis. Furthermore, it has been demonstrated that NO and H₂O₂ induced p38 activation and apoptosis-like cell death, which were blocked by a p38 specific inhibitor¹⁵⁾. On the other hand, an investigation demonstrating similar results to the present study has indicated that SB20190 significantly enhances apoptosis induced by TNF- α in PC12 cells¹⁾. Our data suggest that activation of p38 signaling reduces cell death by laser irradiation.

In conclusion, GaAlAs diode laser irradiation up-regulated neurite differentiation *via* p38 or another signaling pathway, but not proliferation in NGF-treated PC12 cells.

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