Original

He-Ne Laser Effect on Hard Tissue Formation
in Osteoblastic CellsKeiko YAMADA, Chika HAYASHI, Kano KOEDA
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骨芽細胞様細胞の硬組織形成に及ぼすヘリウムネオン
レーザーの影響について山田 恵子 林 千佳 小枝 佳乃
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要旨: ラット頭蓋冠より分離, 株化した骨芽細胞様細胞 (MC3T3-E1) に短時間 (3 分間, 5 分間) の He-Ne レーザーを照射した時の細胞の増殖に与える影響と, 細胞の無機質形成能の変化を調べるために実験を行い, 次の結果を得た。

- 1) レーザー照射 1 日後, 3 日後, 8 日後の細胞の形態を光顕下で観察したところ, 非照射細胞のそれとの違いは見られなかった。
- 2) 非照射群, 3 分間照射群, 5 分間照射群に細胞の増殖能の違いは認められなかった。
- 3) レーザーを照射した細胞内外に蓄積したカルシウム量は, 非照射細胞のそれよりも有意に大きな値を示した。

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Introduction

The effects of laser irradiation on hard tissue have been reported in medicine and dentistry. Several reports demonstrated that low power

laser accelerated bone regeneration in vivo experiments¹⁻³⁾.

Motomura⁴⁾ reported that He-Ne laser is the best to accelerate callus formation compared with dye or CO₂ laser. Kon³⁾ described that He-Ne laser ir-

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radiation on artificial bone defects of rat carvaria enhanced new bone formation and it could induce from acceleration of bone cell proliferation by laser irradiation. Recently, some papers published to demonstrate the biological effect of low power laser on cells. Yamada⁵⁾ reported that cell growth and DNA synthesis of clonal osteoblastic cells (MC3T3-E1) were enhanced by He-Ne laser irradiation. On the other hand, J. Rigau et al.⁶⁾ demonstrated that fibroblasts were not increased by He-Ne laser irradiation but the metabolism was activated. It is supposed that if the cell growth was accelerated by laser irradiation, bone tissue formation should be enhanced. However, it should be more important to investigate that whether the differentiation of bone tissue formation in an osteoblastic cell is activated or not by low power laser irradiation.

The purpose of the present study was to investigate the mineral formation effect of He-Ne laser on clonal osteoblastic cells (MC3T3-E1) when the cells were exposed to He-Ne laser with short duration time of 3 minutes or 5 minutes.

Materials and Methods

Cell Culture and Laser Irradiation

Clonal osteoblastic cells called MC3T3-E1 were used for this study. The cells were grown in alpha modification of Eagle's medium supplemented with 10% fetal bovine serum, 1% of penicillin-streptomycin-fungizone and 5mM β -glycerophosphate at 37°C in an atmosphere of 5% CO₂ in humidified air. They were subcultured every three days using 0.1% trypsin with 0.02% EDTA in Ca²⁺, Mg²⁺ free phosphate buffered saline (PBS-). Cells were seeded on 35mm plastic dishes at a density of 5×10^4 /dish for the present experiment. After culture of cells under the same condition for 2 - 3 hours, He-Ne laser (World Wide Laser Industry, 632.8nm of wavelength, 6mW of output energy) was irradiated for 3 or 5 minutes (114.3 mJ/cm² or 187.2mJ/cm²) from the bottom of dishes. Thereafter, they were placed in an incubator under the same condition to be followed by three kinds of experiments.

Experiment 1 : Microscopic Observation on Morphological Change

Cell cultures were observed under an inverted phase contrast microscope (DIAPHOTO-TMD, NIKON) at the time of 2-3 hours, a day, three days and eight days after laser irradiation.

Cells were seeded for the control at the same time without laser irradiation under same condition as irradiated cells.

Experiment 2 : Determination of cell numbers

At every 24 hours after laser irradiation, the medium was discarded and the cells were washed twice with PBC(-). Cells were dispersed with 0.1 % trypsin with 0.02% EDTA in PBS and centrifuged at 1000 r.p.m. for five minutes and cell numbers were counted by trypan blue exclusion test on a hemocytometer.

Experiment 3 : Accumulated calcium assay

On every seventh day during four weeks, the medium was discarded and the cells were washed with calcium and magnesium free PBC. They were then scraped into a porcelain crucible with a rubber-policeman and were left at room temperature to be dried naturally. The porcelain crucible were placed in a muffle furnace at 700°C for 24 hours to burn all kinds of organic matters and obtain only minerals. The ash was dissolved in 1ml of 15% HCl and then 0.5ml of the solution was diluted with 3.5ml of deionized water as well as 1ml of 0.1N HCl included 6250ppm Strontium. Thereafter, stable calcium in the solution was measured with an atomic absorption spectrophotometer (HITACHI 180).

Fig. 1 Photomicrograph of MC3T3-E1 cells ($\times 100$)

Cells are attached to the bottom of dishes and their processes just started to extend 2-3 hours after being seeded.

Fig. 2

Fig. 3

Fig. 4

Fig. 2-4 Photomicrographs of MC 3T3-E1 cells ($\times 100$) (24 hours after laser irradiation) 2 ; Control, 3 ; 3 min Lased, 4 ; 5 min Lased
Cells showed spindle-shaped morphology in any culture.

Results

1. Microscopic Observation

The most number of cells attached to bottom of

Fig. 5

Fig. 6

Fig. 7

Fig. 5-7 Photomicrographs of MC 3T3-E1 cells ($\times 1000$) (3 days after irradiation) 5 ; Control, 6 ; 3 min Lased, 7 ; 5 min Lased
The density of cells increased equally in each culture, and the processes showed longer compared with Fig. 2-4.

dishes and started to extending their processes in 2-3 hours after they had been seeded (Fig. 1), and then He-Ne laser irradiated to cells from bottom of dishes at the time.

Fig. 8

Fig. 9

Fig. 10

Fig. 8-10 Photomicrographs of MC 3T3-E1 cells ($\times 100$) (8 days after irradiation)
8; Control, 9: 3 min Lased, 10; 5 min Lased
Cells are seemed to be in confluent and showed round-shaped morphology.

Irradiated cells showed spindle-shaped morphology on one day after irradiation and it was not different from the morphology of unirradiated cells on one day after they had been seeded (Fig. 2, 3, 4). On 3 days after laser irradiation, the density

Fig. 11 Cell Proliferation (Logarithm No of cells/ dish)

of cells increased equally in 3 minutes, 5 minutes irradiated or unirradiated cultures. The morphology in each cells were not different (Fig. 5, 6, 7). Cells were seemed to be in confluent on the 8 day culture and round-shaped in irradiated cells as well as control cells (Fig. 8, 9, 10).

2. Cell Proliferation

Mean numbers and S. D. of cells according to culture time are shown in Table 1 and the logarithms of the cell numbers are shown in Fig. 11. The numbers of irradiated cells (3 or 5 minutes of duration) were not different from unirradiated cells at any day. The number of cells increased rapidly from 1 through 4 days after irradiation, but the speed of proliferation was slow down at 4th day through 6th day.

3. Accumulated Calcium Assay

The mean amounts of calcium which was accumulated in irradiated or unirradiated cultures were shown in Table 2 and Fig. 12. The amounts of calcium accumulated inside and outside of cells during 1, 3, 4, weeks after 5 minute irradiation were higher than the calcium accumulation in 3 minute irradiated or unirradiated cell cultures at the respective periods. The amounts of calcium in 3 minutes irradiated cell cultures were not significantly different with unirradiated cell cultures at any periods.

Table 1 The mean number of cells according to culture time ($\times 10^4/\text{dish}$)

Days	Control	3min. Lased	5min. Lased
1	8.5 (0.7)	8.6 (0.4)	7.8 (0.8)
2	21.3 (2.1)	18.0 (2.2)	17.7 (1.9)
3	27.7 (3.6)	31.3 (2.7)	26.9 (4.4)
4	59.0 (5.7)	64.7 (1.3)	63.3 (9.8)
5	76.7 (9.9)	70.0 (6.5)	70.7 (4.2)
6	113.3 (26.2)	103.0 (21.3)	106.3 (12.0)

(S.D.)

The changes of cell numbers in each cultures are not different significantly.

Table 2 The amount of stable calcium in cultues ($\mu\text{g}/\text{dish}$)

	1 week	2 week	3 week	4 week
Control	7.6 (0.93)	11.6 (0.22)	12.2 (0.21)	15.6 (0.85)
3 min. Lased	8.2 (1.22)	11.6 (0.36)	13.1 (2.08)	18.2 (1.31)
5 min. Lased	14.5 (0.14)	14.6 (1.11)	15.2 (0.90)	21.9 (0.46)

Mean Value (S.D.)

*: $p < 0.05$ **: $p < 0.01$

The mean amount of stable calcium accumulated in cultures which were exposed to laser for 5 minutes were different from calcium amount of control culture at the time of 1, 3 and 4 weeks after laser irradiation.

Fig. 12 Calcium Accumulation ($\mu\text{g}/\text{dish}$)

Discussion

It is known that wound healing and bone regeneration are accelerated by low power laser irradiation in vivo. Especially, He-Ne laser is most popular in clinical application. Trellas and Mayayo¹⁾ observed an important increase in vascularization and faster formation of osseous tissue with a dense trabecular net in the tibia of mice which was exposed by He-Ne laser radiation compared to the control group. On the other hand, several authors have described that cell proliferat-

ion and differentiation were activated by low power laser irradiation to fibroblast or osteoblastic cells. In respect of laser effect on cell proliferation, however, the findings are different among papers and it seems to depend on the setting condition of cells, output energy and the kind of laser apparatus.

On the present study, low output energy ($114.3 \text{ mJ}/\text{cm}^2$ or $187.2 \text{ mJ}/\text{cm}^2$) was applied to osteoblastic cells to focus on the laser effect on the cell function of bone tissue formation, not on the proliferation of cells. As a result, cell growth was not accelerated, but calcium accumulation was increased by the laser irradiation under the setting condition compared to unirradiated cells.

From this point, enhancement of calcium accumulation inside and outside of MC3T3-E1 cells seemed to be induced from functional differentiation which was accelerated by He-Ne laser irradiation. With the microscopic observation on cultured cells, the effect of He-Ne laser irradiation on cell morphology was not observed as Yamada⁷⁾ reported no effect of He-Ne laser irradiation on RA cell morphology as well as osteo-

blastic cells⁵⁾.

Schreider et al.⁸⁾ demonstrated that enhancement of Golgi apparatus in fibroblast which was exposed to He-Ne laser beam had recognized under TEM observation. It is known that the Golgi body includes microfibers and microgranules and it participates the formation of collagen as well as polysaccharides⁹⁾.

On the other hand, it has been reported that low power laser accelerated collagen synthesis in fibroblast^{10,11)}.

It seems to be the possibility of the mechanism of laser effects on hard tissue formation in osteoblastic cells that the low power laser stimulates procollagen synthesis in osteoblastic cells and collagen synthesis as well as calcification in and outside of cells.

Conclusion

He-Ne laser stimulates differentiation of osteoblastic cells (MC3T3-E1) for hard tissue formation even if the duration of the laser is too short to accelerate cell proliferation.

The outline of this paper was reported on 3rd International Congress on Laser in Dentistry (Salt Lake City, August, 1992).

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