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Laser Effects on the Cell Growth and Migration of Fibroblasts Derived from Human Dental Pulp in Three-Dimensional Cell Culture

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The purposes of this study were to evaluate the effects on cell growth and migration of fibroblasts derived from human dental pulp by lasers using three-dimensional (3-D) cell culture system, and to compare the results with those from conventional methods using a two-dimensional (2-D) cell culture system. After extirpation from twenty freshly extracted human third molars showing no clinical sign of caries, pulp tissues were cultured in Minimum Essential Medium using a conventional 2-D cell culture. The cells between 5 and 18 passages were used for this experiment, and collagen gel (1 mg/ml) was used for the 3-D cell culture system. The energy densities used were 0.52 and 1.04 J/cm^2 for the gallium aluminum arsenide (GaAlAs) diode laser emitting at 830 nm, and 0.1 and 1.0 J/cm^2 for the argon (Ar)-dye laser emitting at 632 nm. After laser irradiation, cells were counted at 2, 4, 6, and 8 days, and observed at 3 days by stereomicroscopy. There was no significant difference in cell count between the control groups and the laser-irradiated groups (both lasers) on any day using the 2-D cell culture system ($p > 0.01$). However, there were significant differences in cell count between the control groups and the laser-irradiated groups (both lasers) at 8 days using the 3-D cell culture ($p < 0.01$). Cell migration appeared more accelerated by laser in 3-D cell culture than in the 2-D system. These results suggest that the 3-D cell culture system is useful for the evaluation of potential stimulative effects of lasers.

Keywords: Ar-dye laser; Biostimulation; Dental pulp fibroblasts; GaAlAs diode laser; Three-dimensional cell culture

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INTRODUCTION

The use of low-power lasers has been recommended by some authors for treating ulcers or wounds in order to accelerate healing processes. However, a considerable amount of conflicting literature exists concerning low-energy laser effects. Although a few negative results have been reported (Braekt *et al.*, 1991; Colver and Priestley, 1989; Hein *et al.*, 1992; Hunter *et al.*, 1984; Jongsma *et al.*, 1983), several studies have suggested the enhancement of wound healing, both in a human study (Mester *et al.*, 1985) and in experimental animal models (Abergel *et al.*, 1987; Kimura *et al*, 1991; Lyons *et al.*, 1987; Martin *et al.*, 1988; Mester *et al.*, 1971; Mester and Jaszsagi-Nagy, 1973). In *in vivo* studies, it is difficult to identify specific laser effects, because many factors are relevant to the process of wound healing and many variables modify laser effects in tissues. However, it is possible to distinguish a few growth factors related to specific laser effects in wound healing (Kimura *et al.*, 1993; 1997).

In vitro studies on cell cultures have also provided inconsistent results concerning the effects of lasers (Colver and Priestley, 1989; Hein *et al.*, 1992; Martin *et al.*, 1988), and relatively little is known concerning the effect of lasers at the biochemical level (Karu, 1988; 1989). In most *in vitro* studies, two-dimensional (2-D) cell culture systems have been used (Katada *et al.*, 1992; Nara *et al.*, 1992). This model provides a very simple and informative system for evaluating the factors related to laser effects, but excludes many relevant factors. It also differs in many significant aspects from the *in vivo* system. The three-dimensional (3-D) cell culture system has been used for several cell types and is gaining popularity (Benali *et al.*, 1993; Bouvier *et al.*, 1990; Hayashi *et al.*, 1991; Klein *et al.*, 1991). It is thought to provide a more accurate simulation of *in vivo* conditions than the 2-D system and is considered useful for evaluating biological effects *in vitro*. However, there currently exists almost no information on the usefulness of evaluating laser effects using a 3-D cell culture system.

Fibroblasts are the most widely tested cells used for studying the biological effects of low-power lasers *in vitro*. A possible biostimulative effect on these cells would imply relevant effects in several pathologies (Boulton and Marshall, 1986; Meyers *et al.*, 1987).

The purposes of this study were to evaluate the effects on cell growth and migration in fibroblasts derived from human dental pulp of the gallium aluminum arsenide (GaAlAs) and argon (Ar)-dye lasers using 3-D cell culture system, and to compare the results with those obtained using a conventional 2-D cell culture system.

MATERIALS AND METHODS

Cell Preparation

After approval by our University Human Subjects and Use Committee, the informed consent of all human subjects who participated in the experimental investigation was obtained once the nature of the procedure had been fully explained to them. Pulp tissues from twenty impacted extracted human third molar teeth with clinically no sign of caries were used in this study. They were placed in tissue culture flasks (Falcon, Franklinlakes, NJ, USA) and grown in Minimum Essential Medium (MEM, Gibco, Gaithersburg, MD, USA) containing 100 IU/ml penicillin, 100 μg/ml streptomycin, supplemented with 10% fetal bovine serum (FBS, Gibco) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The outgrowing cells were trypsinized 0.05% trypsin Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+}, and these were then subcultured at the rate of 1×10^6 cells/10 ml MEM in 75 cm^2 tissue culture flasks (Falcon) every 5 days (Nara *et al.*, 1992).

Between 5 and 18 passages of fibroblasts were obtained from the subcultures by trypsinization, and these were inoculated in 12-well plates (Falcon, diameter of well: 22 mm, thickness of base: 2.7 mm, Falcon) at 1×10^4 cells/well for 2-D cell culture.

For the 3-D cell culture system, collagen gel was used. Collagen was purified from rat tail tendon by the method of Fusenig (1992). The final concentration of collagen gel was 1 mg/ml, and 0.3 ml of collagen gel was used for each well in 12-well plate. For gelation, the plates were placed for 1 h at 37°C in a humidified incubator. After gelation, cells were inoculated on the collagen gel at the concentration of 2.5×10^4 cells/well for 3-D cell culture.

Laser Irradiation

Two kinds of lasers were used in this study. One was a GaAlAs diode laser (MicroLight 830, LASERMEDICS, TX, USA), which emitted at a wavelength of 830 nm, and an output of 45 mW. Irradiation times used were 1 min (0.52 J/cm^2) and 2 min (1.04 J/cm^2) (Nara *et al.*, 1992). Cells were irradiated through the bottoms of the plate wells. Laser output power was measured through the plastic well plate bottom and through collagen gel (3-D), and hence the laser energy densities were calculated. The second laser was an argon-dye laser (Coherent Laser Group, Santa Clara, CA, USA), which emitted at a wavelength of 632 nm, at an output of 60 mW. Two energy densities (0.1 and 1.0 J/cm^2) were used in this study (Nara *et al.*, 1992). The cells were irradiated through the bottoms of the plate wells. Laser output power was measured through the plastic well plate bottom and through collagen gel (3-D). Then the laser energy densities were calculated. Laser irradiation was performed on day 0 and day 1. Two kinds of controls were prepared in this study. One was a negative control, which was covered with aluminum foil and kept as dark as possible. The other one was a positive control, which was exposed to conventional light for 2 min to correspond with the exposure to conventional light experienced by the cells undergoing laser irradiation.

Cell Count

For the 2-D cell culture system, 0.05% trypsin in phosphate-buffered saline without Ca^{2+} or Mg^{2+} was used. The 0.1 μg/ml collagenase (Gibco) was used for the 3-D cell culture system to digest the collagen gel for the cell count at 2, 4, 6, and 8 days later. After floating the cells in liquid, a hemocytometer was used for the cell count.

Statistical analysis was performed using the Student's *t*-test (unpaired) between two groups, and a value of $p < 0.01$ was considered significant.

Cell Migration

A cover glass (thickness of 0.12 mm) was used to separate each well at the center for the 2- and 3-D cell culture system, and to keep the

cells on one side. After cells were fixed to the bottom, the cover glass was removed and cell migration was started. Ar-dye laser irradiation only was performed (0.1 J/cm^2) at day 0 and 1. At 3 days, cells were observed by inverted stereomicroscopy (Nikon, Tokyo, Japan) at a magnification of ×250.

RESULTS

Stimulative Effect on Cell Growth

(1) GaAlAs laser: Figure 1 shows the result of GaAlAs diode laser irradiation on 2-D cell culture. At 8 days the cells had almost reached confluence, and no significant difference was observed between the 4 groups ($p > 0.01$). Figure 2 shows the result of GaAlAs diode laser irradiation on 3-D cell culture. The cell count at day 2 was almost same as that at day 0; after day 2, cells grew throughout the experimental period, but had not reached confluence at day 8. Cell growth in the positive control sample was somewhat faster than

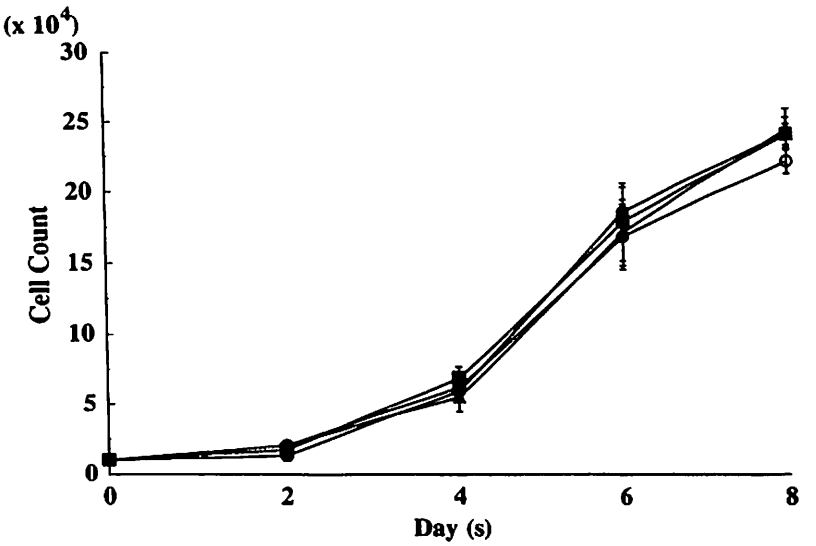

FIGURE 1 Effect of GaAlAs diode laser on 2-D cell culture. Data are expressed as the mean of nine determinations. Bars represent the standard error. (○), negative control; (●), positive control; (▲), 1 min (0.52 J/cm^2); (■), 2 min (1.04 J/cm^2).

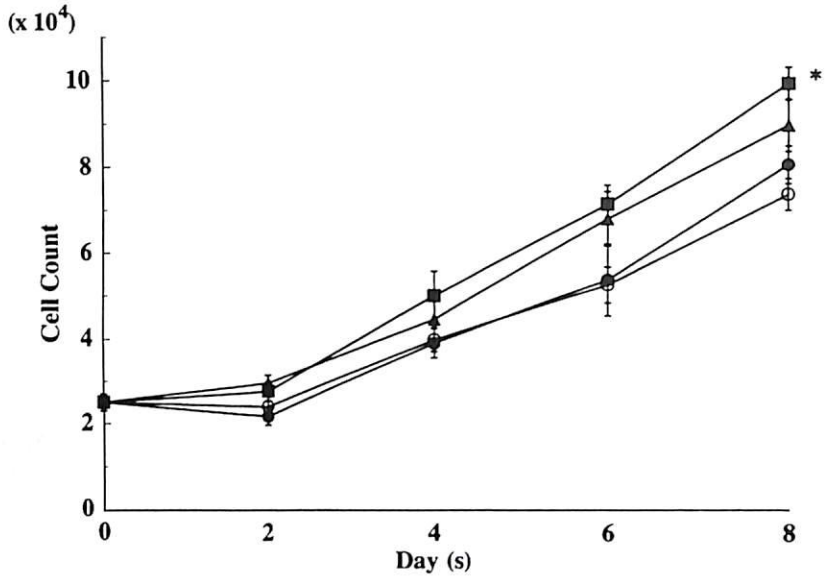

FIGURE 2 Effect of GaAlAs diode laser on 3-D cell culture. Data are expressed as the mean of nine determinations. Bars represent the standard error. (○), negative control; (●), positive control; (▲), 1 min (0.52 J/cm^2); (■), 2 min (1.04 J/cm^2). *Shows a significant difference compared with the positive control ($p < 0.01$).

in the negative control sample, but there was no significant difference between them ($p > 0.01$). Laser irradiation (both parameters) stimulated cell proliferation, and a significant difference was determined between the positive and the negative control on the one hand and the laser-treated group after 2 min irradiation (1.04 J/cm^2) only ($p < 0.01$). (2) argon-dye laser: Figure 3 shows the result of argon-dye laser irradiation on 2-D cell culture. The cells had grown throughout the experimental period and almost reached confluence at day 8, but no significant difference was observed between the 4 groups ($p > 0.01$). Figure 4 shows the result argon-dye laser irradiation on 3-D cell culture. The cell count at day 2 was almost the same as at day 0; after day 2 cells grew throughout the experimental period, but had not reached confluence at day 8. Cell growth in the positive control was at the same level as that in the negative control, and there was no significant difference between them ($p > 0.01$). Laser irradiation (both parameters) stimulated cell proliferation with a significant difference between the positive and negative control on

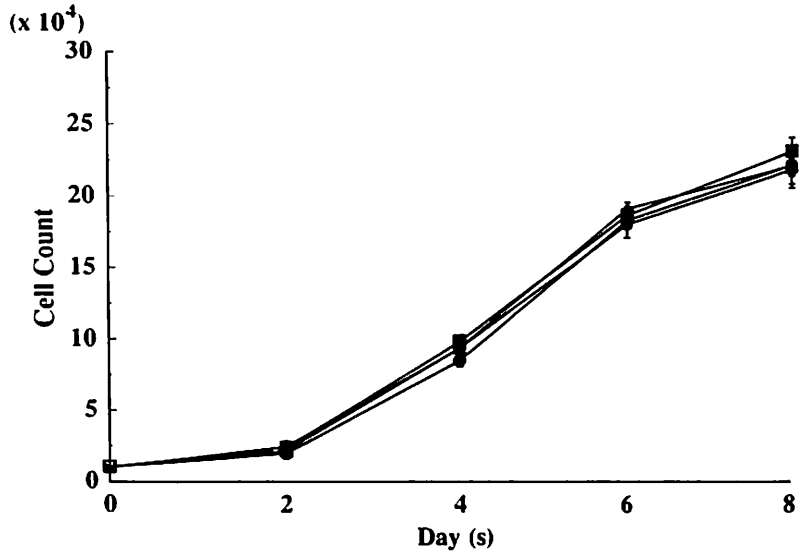

FIGURE 3 Effect of argon-dye laser on 2-D cell culture. Data are expressed as the mean of nine determinations. Bars represent the standard error. (○), negative control; (●), positive control; (▲), 0.1 J/cm^2; (■), 1.0 J/cm^2.

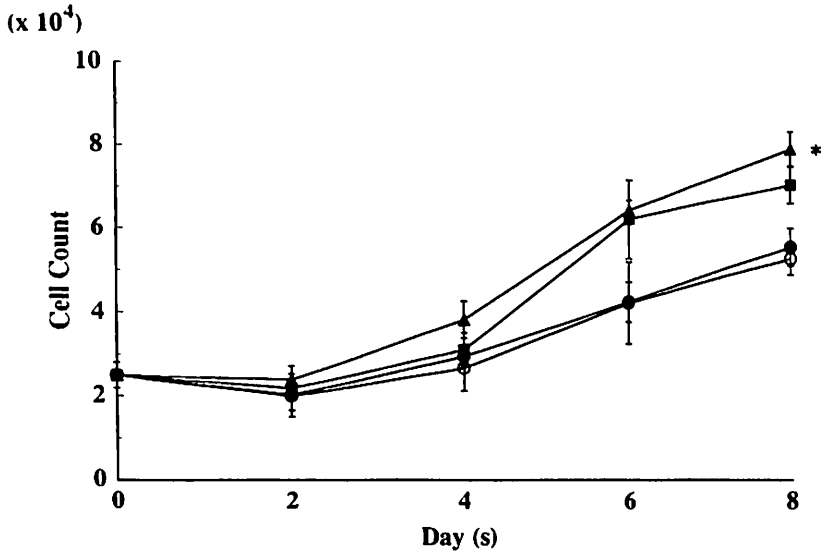

FIGURE 4 Effect of argon-dye laser on 3-D cell culture. Data are expressed as the mean of nine determinations. Bars represent the standard error. (○), negative control; (●), positive control; (▲), 0.1 J/cm^2; (■), 1.0 J/cm^2. *Shows a significant difference compared with the positive control ($p < 0.01$).

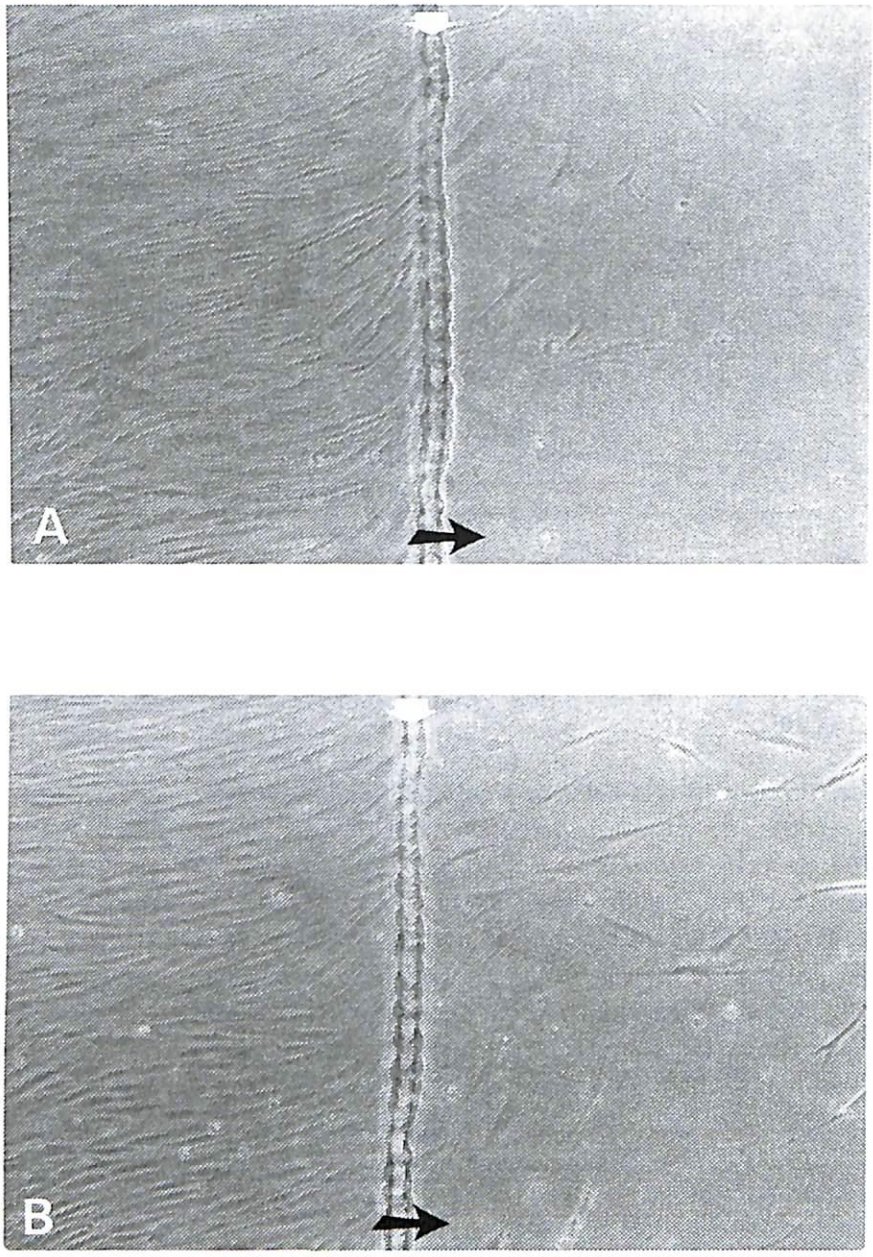

FIGURE 5 Representative photographs by stereomicroscopy at 3 days in 2-D cell culture. (A) is control, and (B) is irradiated by argon-dye laser (0.1 J/cm^2). Black arrow shows the migration direction, and white arrow shows the center line (start line of migration). (Original magnification ×250).

the one hand and the laser-treated groups at the parameter of 0.1 J/cm^2 only ($p < 0.01$).

Stimulative Effect on Cell Migration

A stimulative effect on cell migration was observed at 3 days in both 2-D and 3-D cell culture systems (Figs. 5 and 6), but was more clearly discernible in the 3-D (Fig. 6A and B) than in the 2-D system (Fig. 5A and B).

DISCUSSION

In this study, effects by lasers on cell growth and migration were investigated using a 3-D cell culture system, and these results were compared to those observed in a conventional 2-D model. No significant difference was observed between the control groups and the laser-irradiated groups using either laser and the 2-D cell culture system. Several factors may be relevant to this observation. The difference between the controls and laser-treated groups may have been too small to discern with the techniques used in this study. In

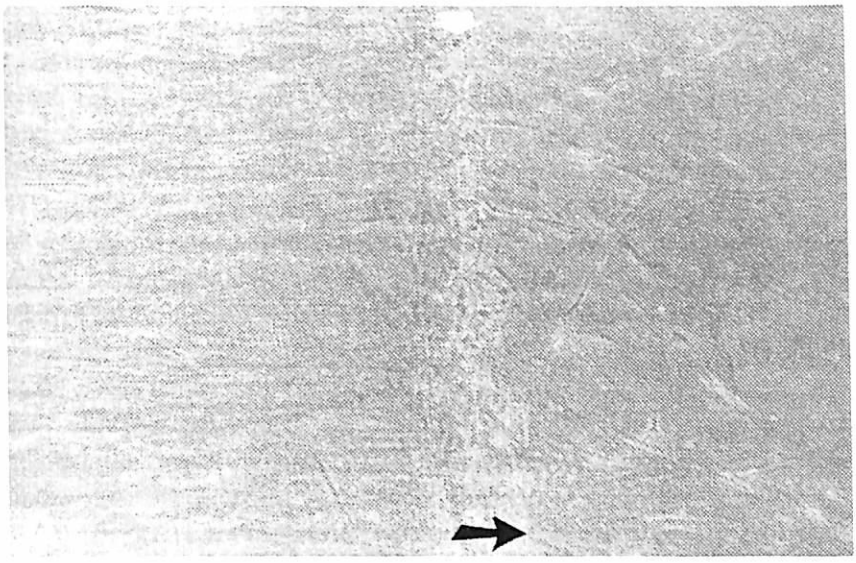

FIGURE 6(A)

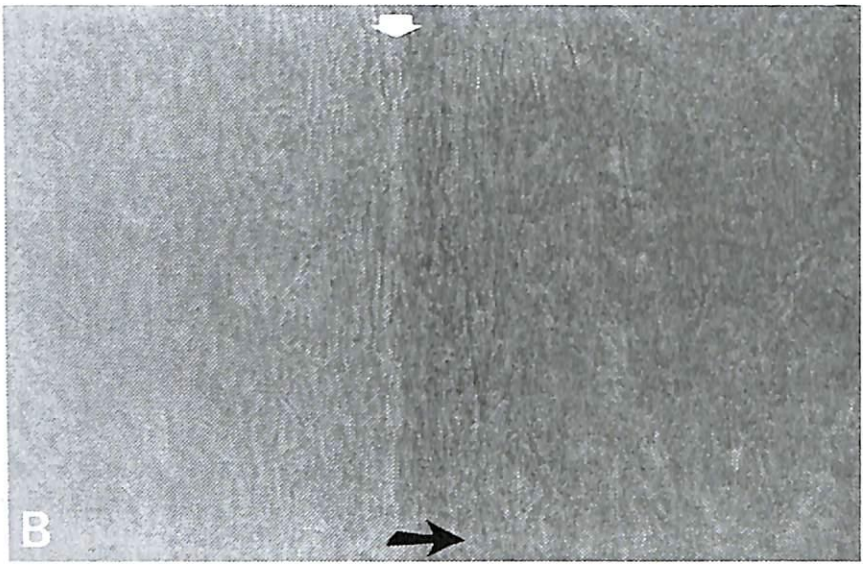

FIGURE 6(B)

FIGURE 6 Representative photographs by stereomicroscopy at 3 days in 3-D cell culture. (A) is control, and (B) is irradiated by an argon-dye laser (0.1 J/cm^2). Black arrow shows the migration direction, and white arrow shows the center line (start line of migration). (Original magnification ×250).

this study, cell growth and migration were observed over a period of 8 days; potentially, evidence of differences in cell behavior might have become more obvious with a longer observation period. Significant differences were observed between the control groups and some of the laser-irradiated groups using the 3-D cell culture system. The main advantage of the 3-D cell culture system is related to the fact that the cells can easily migrate through the flexible matrix (Benali *et al.*, 1993; Emerman *et al.*, 1979). Our results regarding cell migration supported this fact. Thus, it appears that the 3-D cell culture system may provide a more sensitive means of evaluating laser effects at a cellular level. Therefore, this system may also provide more relevant and useful information on potential *in vivo* laser effects than the conventional 2-D system. Disadvantages of the 3-D system include the cost and time involved in this procedure.

In this study, we used collagen gel at the concentration of 1.0 mg/ml. This concentration was used to keep the gel intact throughout

the 8 day observation period. This concentration provided relatively high structural integrity which may have impeded fibroblast proliferation. In further studies, we have observed that cell growth is faster at lower concentrations of gel than that at the concentration used in this preliminary study (unpublished data). Another factor which may be relevant is the type of collagen used: Type I collagen exists in dentin, and type III collagen exists in pulp *in vivo* (Selter and Bender, 1984). The collagen used in this study consisted mainly of type I collagen (Fusenig, 1992), so that the fibroblasts derived from dental pulp in this study were not in an environment exactly paralleling their natural surroundings. This factor may have affected cell growth and may also be relevant to the phenomenon that cell count at day 2 was almost same as at day 0. However, excepting these points, there were no problems in performing the experiments using the 3-D cell culture system in this study.

Two kinds of parameters were used for each laser, but significant differences in cell behavior were obtained using only one parameter for each laser. Our result (0.1 J/cm^2) obtained the significant difference of argon-dye laser emitted at 632 nm in 3-D cell culture system was coincident with best condition of He–Ne laser emitted at 632.8 nm in 2-D one, and that (1.04 J/cm^2) of GaAlAs laser in 3-D cell culture system was very near with best condition (1.0 J/cm^2) of semiconductor laser emitted at 790 and 830 nm in 2-D one using the same cells (Nara *et al.*, 1992). Perhaps an energy density of 0.52 J/cm^2 for a GaAlAs laser was too low an energy to have observable effects.

It is well known that the proliferation of cells depends on numerous environmental factors such as the nutrient medium ionic composition, presence of hormones and growth factors, temperature, etc (Karu, 1988). In this study, all environmental factors were standardized. According to Karu (1988, 1989), light need not be coherent to have an effect, but cell growth is dependent on the energy density and wavelength of the light used. The cells in the positive control group were stimulated by exposure to non-coherent (room) light, but not sufficiently to achieve statistical significance. However, significant differences were observed in 2 laser-treated groups in the 3-D cell culture system. Therefore, these parameters may also affect cell growth *in vivo*.

A possible mechanism for these effects on cellular level may be that photo-energy is converted by the mitochondria into chemical and electrical signs and transmitted to the nucleus via several steps (Karu, 1988). In this process, the activity of ATP synthetase is enhanced. However, further work is required to investigate the many factors relevant to a process of this type.

CONCLUSION

These results show that a 3-D cell culture system is useful for evaluating the effects on cell growth and migration of fibroblasts derived from human dental pulp by lasers. Moreover, this system may provide a better indication of potential clinical effects than conventional 2-D systems.

Acknowledgments

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