Effects of Semiconductor Laser Irradiation on Cultured Human Keratinocytes

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Summary We studied the influence of semiconductor laser irradiation on the cultured human epidermal keratinocytes and measured cell viability and the concentration of interleukin-1 α (IL-1 α) in the medium of irradiated cell cultures. We found that the number of viable cells in the cultures was significantly increased by giving semiconductor laser irradiation for 15 s ($115.0 \pm 0.5\%$), 20 s ($112.0 \pm 1.0\%$) compared with the number in the nonirradiated cultures ($p < 0.01$). The production of IL-1 α by cultured cells was increased by the laser irradiation for 20 s (35.8 ± 5.5 pg/ml) compared with the control (27.0 ± 5.6 pg/ml) after a 12-h incubation period ($p < 0.01$). These results imply that low-power laser irradiation has significant biological effects on human keratinocytes.

Key Words: semiconductor laser, cultured human keratinocytes, interleukin-1 α

Lasers are being applied in almost every aspect of our daily life. In the medical field, laser application is divided into high reactive level laser therapy (HLLT) and low reactive level laser therapy (LLLT) according to its energy power. Application of HLLT in the clinical field was started in 1961 for the treatment of retinodialysis [1]. This has been known as photo-coagulation therapy. From 1970, HLLT has been employed in general surgery as a surgical knife to maintain a bloodless operating field [2]. In these days, HLLT has been combined with endoscopy and applied to the treatment of gastric and colon

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cancer. For these HLLT, ruby, Nd-Yag, and carbon dioxide have been used as laser light-generating materials [3]. LLLT was applied to the clinical field in 1968 for the treatment of intractable ulcer of the skin, and successful results were reported [4]. LLLT has also been applied clinically to reduce pain, and the effectiveness of this use of the irradiation has been reported [5–7]. In these days, the application of LLLT has been recognized as an effective treatment for skin ulceration, painful arthritis and other various painful situations. For these LLLT, He-Ne, argon, and semiconductors have been used as laser light-generating materials. Whereas the mechanism of the effect of the HLLT irradiation has been easily recognized as cell destruction through heat generation, the mechanism underlying the effect of LLLT irradiation has remained unclear. Photo-chemical reaction to the cell and the improvement of the regional circulation, etc. have been suggested. To explain the clinical effectiveness of LLLT irradiation, we investigated the influence of semiconductor laser irradiation on the cultured human epidermal keratinocytes by measuring cell viability and the concentration of interleukin- 1α (IL- 1α) in the medium of irradiated cell cultures.

MATERIALS AND METHODS

Cell culture. With informed consent, normal human skin specimens were obtained from plastic surgery patients ranging from 20 to 56 years of age. The tissues were taken from the trunks or extremities of the patients. The skin specimens were washed in phosphate-buffered saline (PBS(–)) and connective tissue was trimmed away. The specimens were then treated with 1% dispase (Godo Shusei, Tokyo) in Dulbecco's modified Eagle's Medium (Gibco Laboratories, Grand Island, NY) with 10% fetal bovine serum (Cell Culture Laboratories, Cleveland, Ohio, USA) at 4°C overnight. Epidermal sheets were peeled from the dermis and stirred in 0.25% trypsin (Gibco Laboratories) and 0.2% ethylenediamine tetraacetic acid (Daiichi Kagaku, Tokyo) solution (trypsin-EDTA solution) for 30 min at room temperature. Cell suspensions were filtered through a nylon mesh filter (Falcon, Becton Dickinson, Franklin Lakes, NJ) and centrifuged at $180\times g$ for 5 min. Cell pellets were resuspended in serum-free, keratinocyte growth medium (K-GM, Kurabo, Osaka), which is composed of modified MCDB 153, bovine pituitary extract (0.4% v/v), epidermal growth factor (0.1 ng/ml), insulin (5 $\mu\text{g/ml}$), hydrocortisone (0.5 $\mu\text{g/ml}$), gentamicin (50 $\mu\text{g/ml}$), and amphotericin B (0.25 $\mu\text{g/ml}$), plated at 5×10^6 cells/50-ml flask, and cultured at 37°C in a humidified atmosphere with 10% CO₂. Cultures were fed every third day and were sub-cultured before reaching confluence by use of the trypsin-EDTA solution. Second-fourth passage cells were used for the experiments.

MTT assay. MTT assay [8, 9] has its biochemical basis in the reduction of tetrazolium salt 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial dehydrogenase [10] with resultant formazan in quantities proportional to the number of viable cells, which lends itself to colorimetric

determination because of its chromogenic decomposition by isopropanol. According to the method previously reported [8, 9], the cells were harvested by treatment with trypsin-EDTA, resuspended in fresh K-GM, and plated in a volume of 0.1 ml in the wells of 96-well flat-bottomed microtiter plates (Falcon, Becton Dickinson) at a concentration of 1.5×10^4 cells/well.

Both laser irradiated and control cultured cells were washed twice with PBS(—) and cultured further in 0.1 ml of fresh K-GM for 1 day, and $10 \mu\text{l}$ of MTT (Sigma Chemical Co., St. Louis, MO) solution (5 mg/ml of PBS(—)) was added to each well. After incubation with MTT at 37°C for 4 h, 0.1 ml of 0.04 M HCl with isopropanol was added to each well and the formazan crystals were dissolved. The absorbance at 570 nm was measured with a micro plate reader (MPR-A4i; Tosoh, Tokyo). Wells containing the medium and MTT solution, omitting the cells, were used as the blank. The results were expressed as the ratio, as a percentage, of the absorbance for the treated cells divided by that for the control cells.

IL-1 α enzyme-linked immunosorbent assay (ELISA). Harvested cells were plated in the wells of 48-well plates at a concentration of 3×10^4 cells/well. After a 4-day incubation, the cells were irradiated. The concentration of IL-1 α in the culture supernatants was measured by use of a commercially available IL-1 α ELISA kit (Otsuka Pharm., Tokyo). The absorbance at 490 nm was measured with a microplate reader (MPR-A4i; Tosoh).

Laser irradiation. Keratinocytes (1.5×10^4 cells/well) were plated in K-GM in the wells of 48- or 96-well flat bottomed plates and then irradiated with a semiconductor laser (Matsushita, Tokyo) for 15 s, 20 s, 30 s or 60 s with a wavelength of 830 nm and an energy density of $0.78 \text{ mW}/\text{cm}^2$.

Temperature changes in the well were measured with an AM-3000 thermometer (Anritsu, Tokyo) after irradiation (Fig. 1).

Statistics. The numerical values of the data were given as means $\pm$ SEM ($n = 4$). Student's t test was used for statistical evaluation. A p value of 0.05 or less was regarded as significant.

Fig. 1. Experimental setup of semiconductor laser irradiation system.

RESULTS AND DISCUSSION

The MTT assay showed that the keratinocyte survival rate after semiconductor laser irradiation was $115.0 \pm 0.5\%$ for 15 s and $112.0 \pm 1.0\%$ for 20 s of irradiation time at 24 h after the irradiation. These data exhibited a significant difference with the control ($p < 0.01$). However, the survival rate after 60 s irradiation time was $83.0 \pm 12.0\%$, reflecting cell damage (Fig. 2).

Fig. 2. Survival rate of keratinocytes laser-irradiated for 20 s, 30 s or 60 s. △, measured 6 h post irradiation; ○, measured 12 h post irradiation; ●, measured 24 h post irradiation. Data are shown as number of viable cells (% of control). Mean $\pm$ SE is given. $n=3$. Significantly difference vs. control: * $p < 0.01$.

Fig. 3. IL-1 α level in the medium of keratinocytes laser-irradiated for 20 s, 30 s or 60 s. ○, measured 12 h post irradiation; ●, measured 24 h post irradiation. Mean $\pm$ SD is given. $n=4$. Significant difference vs. value 24 h post irradiation: * $p < 0.05$, the difference vs. control: ** $p < 0.01$.

The temperature of the well in the nonirradiated condition was 25.4°C, and the change in the well temperature was within 1°C in the various irradiation conditions (data not shown). There was no change in cell proliferation in nonirradiated keratinocytes at 37°C (control), 30°C, or 25°C.

The keratinocytes laser-irradiated for 20 s showed an increase (35.8 ± 5.5 pg/ml) in the IL-1 α level in the medium compared with the control (27.0 ± 5.6 pg/ml), $p < 0.01$ by measuring 12 h post irradiation. There was a significant difference in the IL-1 α level in the 20 s irradiation group between 12 h after (35.8 ± 5.5 pg/ml) and 24 h after (25.8 ± 10.4 pg/ml) irradiation, $p < 0.05$ (Fig. 3).

Semiconductor laser irradiation is widely used clinically for pain alleviation [11, 12] and for wound healing [13, 14]. However, the mechanism by which a low-power laser acts on the human body or cells remains largely unelucidated to date. Thus, we investigated this mechanism with regard to direct epidermal impact. It is suggested that the IL-1 α formed by these epidermal cells interacts with a receptor on the plasma membrane of the IL-1-producing cell, thus causing cell proliferation in an autocrine manner [15, 16]. Also, even in the normal condition, a small amount of IL-1 is released from keratinocytes; and it has been reported that under various types of stimulation (lipopolysaccharide, UV irradiation), it shows a marked increase [17]. It was reported earlier that semiconductor laser irradiation of K-562 cells for 15 s at a 500 mW output resulted in $31.4 \pm 1.63\%$ of the cells in the G₂/M phase vs. $18.5 \pm 1.26\%$ for the control cells, which difference indicates cell proliferation [18]. Thus, in the present experiment, enhanced production of IL-1 α by cultured keratinocytes 12 h after the semiconductor laser irradiation for 20 s may suggest that the external photic impact on the cells' own self-producing mechanism resulted in cell proliferation and that the increase in IL-1 α release reflects an increase in cell number. This allows the consideration for the idea that the 15 s semiconductor laser irradiation in the present clinical setting is an optimum treatment period, and that it provides a treatment-enhancing effect in the cases of ulcers refractory to treatment. Henceforth, in order to clarify the mechanism by which semiconductor laser irradiation activates the keratinocytes, we shall investigate signal transduction via the IL-1 α receptor or cells themselves so as to elucidate the reason for the clinical effectiveness.

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