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The *in vitro* effects of semiconductor laser irradiation on inflammation: laser irradiation did not affect monocyte infiltration

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The effects of supernatants following irradiation of lymphocytes and monocytes with a semiconductor laser, on monocyte chemotaxis were investigated *in vitro*. Human peripheral lymphocytes and monocytes were obtained from healthy adult donors, and were suspended in medium, following laser irradiation. The laser irradiation (wavelength 900 nm, output power 1.5 mW) was carried out in the pulse wave mode at doses of 0.1 J cm^{-2} in total. After irradiation, the lymphocytes and monocytes were incubated with or without several kinds of stimulants for varying time periods. The supernatants of these cultures were then assayed for human monocyte chemotaxis. Through these experiments, we were unable to detect any significant levels of stimulative or depressive effects due to laser irradiation of the lymphocytes and monocytes, on the production of monocyte chemotactic activity.

1. Introduction

Quantitative studies have been performed to determine the action of low-intensity visible monochromatic light on various cells (*Escherichia coli*, yeasts, HeLa cells, Chinese hamster fibroblasts, human lymphocytes); in addition, irradiation conditions (wavelength, dose, intensity) necessary to stimulate vital activity have been also examined [1].

Recent studies have demonstrated that low-power lasers can modulate the subtle biological functions of cells *in vitro*, including cell proliferation and extracellular matrix production [2, 3]. To investigate the immune modulation by low-power laser irradiation, we earlier described the acceleration of the blastoid transformation of human lymphocytes by low-power laser irradiation *in vitro* [4]. Moreover, Ricevuti *et al.* [5] described the effect of helium–neon (He–Ne) laser irradiation on immunocompetent cells using the *in vivo* skin window method and the *in vitro* granulocyte function tests. They showed that cellular migration was markedly decreased by laser irradiation both *in vivo* and *in vitro* in a dose-independent manner, and that the laser irradiation produced no changes in random migration or in chemotaxis.

With inflammation, phagocytic monocytes next to granulocytes migrate to inflammatory lesions, and these cells are then responsible for removing materials and debris. Monocytes may also mediate both the immune system and the subsequent repair of injured tissue carrying out the cell-to-cell communication through the numerous cytokines. Thus, monocyte infiltration into an inflammatory lesion is essential for both the inflammatory and immune systems.

The present study was performed to determine whether photobiostimulation of the monocyte chemotactic response occurs, affecting the production of chemotactic lymphokines for human lymphocytes or affecting the chemotactic migration of human monocytes, when semiconductor laser irradiation is carried out on human lymphocytes and on human monocytes.

2. Materials and methods

2.1. The semiconductor laser

A semiconductor low-power laser (Yoshida Co., Ltd., Tokyo) was used for this experiment as follows: wavelength, 900 nm; pulse frequency, 1.2 k Hz; pulse width, 200 ns; pulse peak power, 10 W; average power intensity, 0.05 W cm^{-2}; and irradiation distance, 4 mm. The output power measured by the power meter (ANDO, AQ-2101 and AQ-2704, Tokyo, Japan) was 1.5 mW. The laser was perpendicularly irradiated to the cultures from the bottom of 24-well plates (Falcon, USA). The irradiation dose was 0.1 J cm^{-2}.

2.2. Chemotactic agent used as reference standard

N-formyl-methionyl-leucyl-phenylalanine (FMLP, Sigma, USA) was dissolved in ethanol at a concentration of 10^{-2} M [6], and was then stored at − 20 °C in

0.5-ml portions. Portions were thawed before use, diluted to the indicated concentrations with the medium described below, and then used as positive controls for chemotaxis assays [7].

2.3. Media for test specimens

Test materials and reference chemotactic agents were diluted with a mixture of seven parts of Gey's balanced salt solution containing 2% bovine serum albumin (Sigma, USA) and 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Sigma, USA) (Gey's BSA, pH 7.2), and five parts of Veronal buffer with 2 M $MgCl_2$ and 1 M $CaCl_2$ (VB^{2+}). This mixture will be referred to as Gey's BSA-VB^{2+} [8, 9].

2.4. Lymphocyte culture

Heparinized peripheral venous blood was diluted with equal amounts of phosphate-buffered saline (PBS, pH 7.3), and then layered carefully onto Histopaque-1077. After density gradient centrifugation (400 × g, 35 min), the opaque interface was aspirated with a Pasteur pipette. To remove monocytes, centrifugation (850 × g, 10 min) with PBS was performed. Lymphocytes were cultured at a cell density of 2×10^6 cells ml^{-1} in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) containing 50 IU ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, and 2 mM L-glutamine with or without phytohemagglutinin-p (PHA-p, Difco, USA), concanavalin A (Con A, Sigma, USA) or pokeweed mitogen (PWM, Difco, USA). These mitogens were titrated in the optimal dilutions.The suspensions were incubated for 2–96 h in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. After incubation, the supernatants of these cultures were obtained by centrifugation at 850 × g for 10 min, and these were then stored at − 30 °C.

2.5. Monocyte culture

Heparinized peripheral blood drawn from healthy donors was subjected to fractionation by a modification of the Ficoll-Hypaque method [10]. The washed cells were routinely cultured at 3×10^6 cells ml^{-1} in Dulbecco's modified Eagle medium (DME, D-glucose 4500 mg ml^{-1}, Grand Island Biological Co., USA) containing 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 2 mM L-glutamine on 24-well plates in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. After 2 h of incubation, the non-adherent cells were removed by washing, leaving a population of > 95% phagocytic cells. These adherent monolayers were used for each experiment. After laser irradiation, the cells were incubated with or without 30 μg ml^{-1} of lipopolysaccharide (LPS, from *Escherichia coli* 055:B5, Difco Laboratories, Detroit, MI) and with or without 5 μg ml^{-1} of N-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP, Sigma, USA) for 24–72 h in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The supernatants of these cultures were obtained by centrifugation and these were then stored at − 30 °C.

2.6. Assay for migration of monocytes

Monocytes were obtained from a cell fraction in which they had been enriched by a modification of the Ficoll-Hypaque method. Cells were washed with PBS and suspended at a cell density of 3×10^6 cells ml^{-1} in Gey's BSA. Chemotaxis assays were performed by the use of a 96-well chemotaxis assembly (Neuro Probe, USA) [7, 8]. Each of the wells on a bottom plate was filled with 25 μl of the test or reference specimen, either dissolved or suspended in Gey's BSA-VB^{2+}. A polycarbonate filter sheet (Neuro Probe, USA) of 5 μm pore size was then placed on the bottom plate. A gasket and a top plate were fixed in place. 50 μl of leukocyte suspension were then added to each well on the top plate. The whole assembly was incubated at 37 °C for 90 min in a humidified incubator. After incubation, the filter was removed, fixed, and stained with Diff-Quik (International Reagents, Japan). Migration of leukocytes from the upper well to the lower well was determined by measuring the absorbance at 595 nm of each well by a microplate reader (BIO-RAD, Model 450, Japan). The assay for each specimen and each dose was carried out with triplicate filters. Data are expressed as the mean, plus or minus standard error (SE), of the absorbance obtained in at least three repeated experiments.

3. Results

Human lymphocytes were cultured with or without PHA-p for 72 h, and the supernatants from these cultures were tested for their ability to initiate monocyte migration. Significant numbers of monocytes migrated towards the PHA-p stimulated lymphocyte supernatants.

Monocyte migration assays for lymphocyte supernatants which had been cultured for 72 h were carried out on dilutions of 1/128 to 1/2 for monocytes. The chemotactic activities were then compared between the samples irradiated with the laser and the non-irradiated samples. Lymphocyte supernatants at a dilution of 1/4 had significant levels of chemotactic

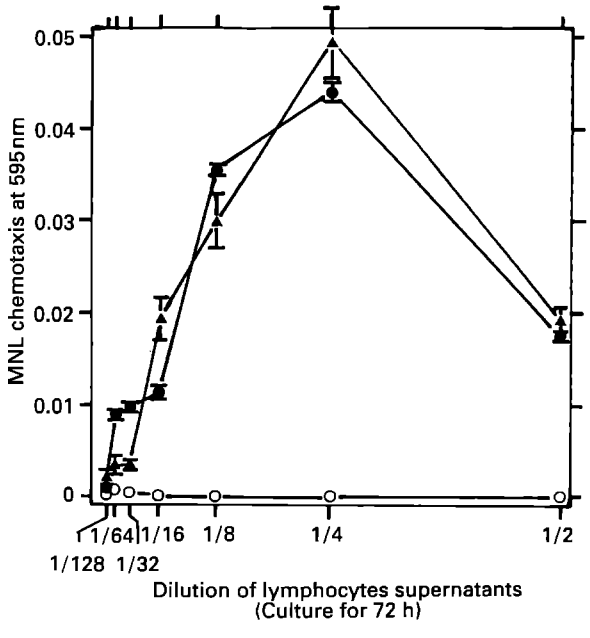

Figure 1 Dose-dependent monocyte chemotaxis to lymphocyte supernatants. Data represent the mean absorbance at 595 nm of triplicate filters ± 1SE: (▲) lased + PHA-p; (●) PHA-p; (○) control.

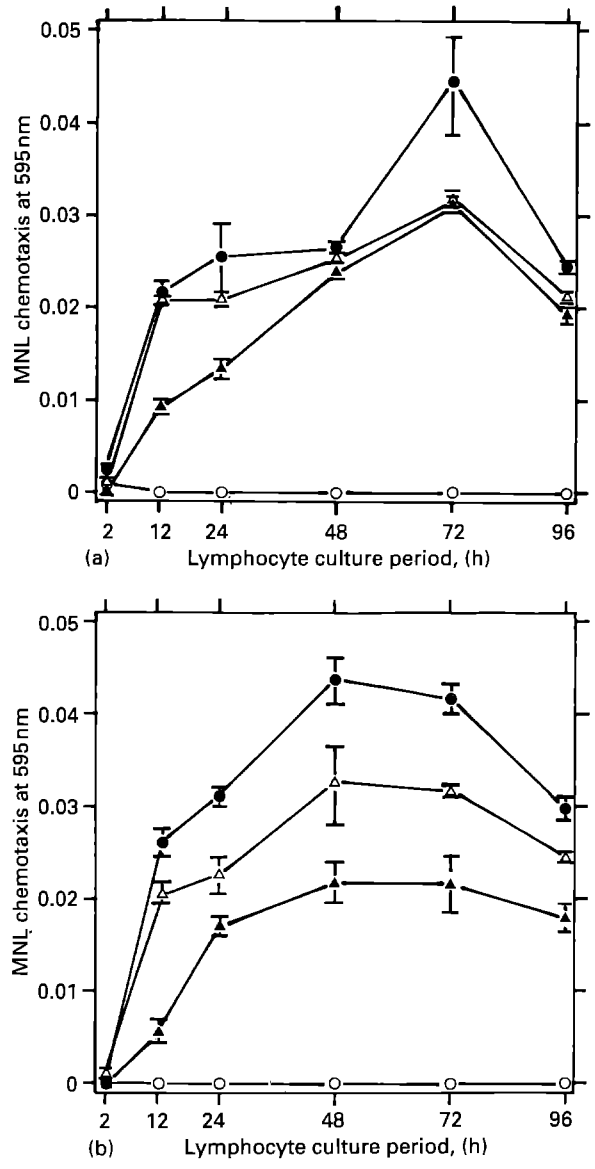

Figure 2 Kinetics of monocyte chemotactic factor production of lymphocytes. Human lymphocytes were cultured with PHA-p, Con A, PWM, or no stimulant for varying time period (2–96 h) and supernatants collected, diluted (1:4), and tested in the monocyte chemotaxis assay. Data represent the mean absorbance at 595 nm of triplicate filters ± 1 SE. (a) Non-irradiated control; (b) laser irradiated. (●) PHA-p; (△) ConA; (▲) PWM; (○) control.

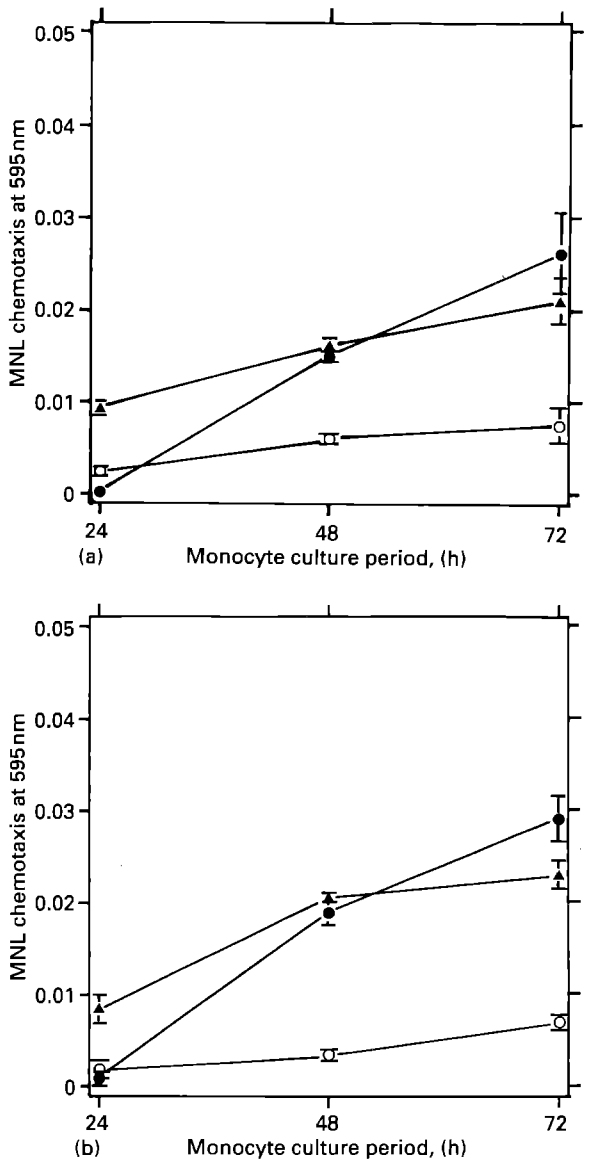

Figure 3 Kinetics of monocyte chemotactic factor production of monocytes. Human monocytes were cultured with LPS, MDP, or no stimulant for varying time period (24–72 h) and supernatants collected, diluted (1:4), and tested in the monocyte chemotaxis assay. Data represent the mean absorbance at 595 nm of triplicate filters ± 1 SE. (a) Non-irradiated control; (b) laser irradiated. (▲) MDP; (●) LPS; (○) control.

activity, however, there was no difference between the samples irradiated by the laser and the non-irradiated controls under these experimental conditions (Fig. 1).

After laser irradiation, the supernatants of lymphocyte cultures stimulated by PHA-p, Con A, or PWM were harvested at various time points (2–96 h) and assayed for monocyte chemotactic activity. The lymphocytes had elaborated the chemotactic mediator for monocytes within the first 72 h in the non-irradiated control, whereas the activity continued to increase until 48 h in the cultures irradiated by the laser. However, there was no difference between the samples irradiated with the laser and the non-irradiated control under these experimental conditions (Fig. 2a, 2b).

On the other hand, the supernatants of monocyte cultures to which LPS or MDP had been added following laser irradiation were harvested at various time points (24–72 h). The monocytes had elaborated the enhanced levels of the chemotactic mediator within 24–72 h in the supernatants. However, there was no difference between the laser-irradiated samples and the non-irradiated control under these experimental conditions (Fig 3a, 3b).

4. Discussion

Several studies which failed to find any acceleration of thymidine incorporation into DNA, succeeded in finding increased rates of protein and collagen synthesis following irradiation by He–Ne lasers [3, 11]. If these results were to be accepted, it would mean that the frequently [12–14] but not universally [15–18] reported accelerations of wound healing may possibly be due to changes in cellular migratory, metabolic, or secretory functions.

In the present study, irradiation by the semiconductor low-power laser did not show any modification of the production of chemotactic lymphokines for

human lymphocytes or of the chemotactic migration of human monocytes. Ricevuti *et al.* [5] described the effect of He–Ne laser irradiation on immunocompetent cells using the *in vivo* skin window method and the *in vitro* granulocyte function tests. It showed that cellular migration was markedly decreased by the laser irradiation both *in vivo* and *in vitro*, in a dose-independent manner. However, they used a high-energy dose (1–4 J cm^{-2}) for their laser irradiation.

In the case of quantitative studies concerning the action of low-power lasers on various cells, the experimental conditions (dose of irradiation, thermal effect of laser, origin of cells) ought to be discussed. Nara *et al.* [19, 20] described how the semiconductor laser accelerated the cell proliferation of human dental pulp fibroblasts *in vitro* when it was irradiated at between 0.04 and 0.40 J cm^{-2}. Moreover, the He–Ne laser has shown the ability to accelerate the proliferation of pulp fibroblasts at a low dose (0.1 J cm^{-2}) of irradiation, and the maximum acceleration for the cell growth appeared at a dose of 0.1 J cm^{-2}. Berki *et al.* [21] described how when the irradiation energy of the He–Ne laser was between 0.14 and 14.0 J cm^{-2}, significant levels of cell activation (increased immunoglobulin secretion and phagocytosis) appeared on the *in vitro* cultured lymphatic cell lines and macrophages. However, higher energy irradiation caused the rapid destruction of these cells. In this study, therefore, we mainly used the dose of 0.1 J cm^{-2} for irradiation with the semiconductor laser. However, laser irradiation using other low-power doses and waves was also examined (data not shown), and the results which were obtained showed that there was no difference between the laser-irradiated samples and the non-irradiated control.

Clinical observations have suggested that low-power lasers may stimulate wound healing. Several studies have indicated the enhancement of wound healing both in animal models and in patients. On the other hand, some of the studies were unable to document the beneficial effects of low-power lasers on wound healing [17, 22]. Ikeuchi *et al.* [23] studied the effects of He–Ne laser irradiation on the wound healing ability of guinea pig skin, and it was suggested that low-power laser irradiation accelerated the compositon of type III collagen for wound healing following incisions. Furthermore, preliminary trials have suggested that He–Ne lasers may enhance the healing of chronic skin ulcers in humans [24]. In the dental field, it has been reported that pain relief in hypersensitive dentine has been achieved by He–Ne lasers [25–27]. Iwase *et al.* [28] examined the possible mechanism of the He–Ne laser on the nervous system. They stated that the membrane potential was hyperpolarized and the neuronal membrane was stabilized by the He–Ne laser irradiation, and that the excitability of nerve cells was depressed. Moreover, they demonstrated that the He–Ne laser irradiation was very effective where there was moderate pain with slight damage or slight inflammation [29]. However, further controlled clinical trials are needed to attest to the clinical efficacy of low-power lasers in enhancing wound healing in human diseases.

In the inflammatory lesion, lymphocytes and phagocytic monocytes are responsible for removing materials and debris, and these cells also mediate the immune system and subsequent repair of the injured tissue by carrying out cell-to-cell communication through the numerous cytokines. Thus, the monocyte infiltration into an inflammatory lesion is essential for the inflammatory and immune systems. Enhancement of the immune function *in vitro* has been investigated in several studies using low-power lasers, especially the He–Ne laser. It was noted that the effects of intensive irradiation by a visible region cause not only quantitative changes (increase in numbers of T-active rosette-forming cells and B cells determined by immunofluorescence) but also qualitative changes, since the number of erythrocytes around individual lymphocytes increases and since the percentage of determinants giving off positive fluorescence is also raised [30]. Prospective application of non-damaging laser irradiation is due to its positive effects, such as increasing interferon production on certain links of the immune system [31]. Moreover, the enhancement of the adherence of *Salmonella* to lymphocytes has been described following irradiation with the He–Ne laser [32].

The cell activation processes are preceded by early biochemical events such as increases in internal calcium, increased inositol phospholipid metabolism, activation of protein kinase C, and membrane depolarization. According to a recent paper [33], the effect of He–Ne laser irradiation is thermally correlated to an alteration in the fluxes of transmembrane sodium. Exposure of neutrophils to laser irradiation resulted in a less partial depolarization of the resting cell membrane and a blunted depolarization response to stimuli than did control cells in physiologic conditions. Karu [34] referred to the short-term responses in which the laser beams are absorbed by the respiratory chain. The primary photochemical and photophysical events seemed to occur in the mitochondria in the case of eucaryocytes, and the absorption of light quanta produces changes in the redox state of the respiratory chain. From these reports, it would seem that the light quantum acted only as a trigger for the regulation of cellular metabolism.

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