Low-Energy Helium-Neon Laser Irradiation Stimulates Interleukin-1α and Interleukin-8 Release from Cultured Human Keratinocytes

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Clinical observations have suggested that low-energy lasers might promote wound healing. Evidence suggests that He-Ne laser irradiation induces an increase in the rate of keratinocyte migration and proliferation as compared with nonirradiated controls in vitro. This study sought to determine whether He-Ne laser could induce cytokine production in cultured keratinocytes. The results revealed (i) a significant increase in interleukin-1α and interleukin-8 production and their respective mRNA expression in He-Ne laser-treated groups as compared with nonirradiated controls, and (ii) under 1.5 joules/cm² irradiation, this stimulating effect of He-Ne laser treatment is concentration-dependent. Because interleukin-1α induces keratinocyte migration, this finding may partially explain the stimulatory effects on the motility of keratinocytes. As both interleukin-1α and interleukin-8 provoke proliferation of keratinocytes, it is not unreasonable to propose that these two cytokines play a profound role in the enhancement of keratinocyte proliferation as a result of He-Ne laser irradiation. Our findings provide further evidence of enhanced wound healing at the cellular and molecular level as a result of the He-Ne laser. Key words: He-Ne laser/cytokines/wound healing. J Invest Dermatol 107: 593–596, 1996

The beam of a low-energy laser produces a temperature elevation of less than 0.1 to 0.5°C in the irradiated tissues (Basford, 1989). Therefore, biologic effects are derived directly from the radiation itself rather than from thermal influences. The low-level laser has been studied as a possible therapeutic instrument for pain management (Kemmotsu et al., 1991), rheumatoid arthritis (Goldman et al., 1980), and modulation of wound healing (Lyons et al., 1987; Basford, 1989). The potential of low-energy lasers for enhancing wound healing noninvasively has been of particular interest, and the He-Ne laser has been employed most commonly for this purpose. A considerable amount of literature exists on the effects of low-energy lasers on wound-healing processes (Lyons et al., 1987; Basford, 1989; Basford, 1993; Wheeland, 1993; Neiburger, 1995). Several of these studies indicate improved wound healing in both animal studies and human trials (Kana et al., 1981; Kani et al., 1985; Abergel et al., 1987; Lyons et al., 1987; Bilhari and Mester, 1989). There is some disagreement in the literature concerning this issue, however, and negative findings have also been reported (Jongsma et al., 1983; McCaughan et al., 1985).

In the process of cutaneous wound healing, re-epithelialization occurs as a result of keratinocyte migration and proliferation into the wound site (Brown et al., 1986). Soluble regulatory factors derived from a variety of cell types in the skin may play an important role in keratinocyte motility and proliferation and cutaneous wound healing (Urtsinger et al., 1986; Mustoc et al., 1987; Nickoloff et al., 1988). He-Ne laser irradiation of cultured human keratinocytes demonstrated an increased rate of keratinocyte migration, as compared with nonirradiated controls (Haas et al., 1990). The operation did not appear, however, to lead to increased keratinocyte proliferation or an alteration in keratinocyte differentiation (Rood et al., 1992). On the other hand, low-level lasers revealed a greater stimulatory effect on keratinocyte proliferation in the unfavorable culture conditions of 1% fetal bovine serum than in the better culture conditions provided by 5% FBS (Steinlechner and Dyson, 1993). Therefore, elucidating the effect of He-Ne laser irradiation on cellular and molecular mechanisms underlying re-epithelialization is critical in furthering our understanding of wound healing. In this study, we investigated the effects of He-Ne laser on cytokine production from cultured human keratinocytes.

MATERIALS AND METHODS

Keratinocyte Culture Keratinocytes were isolated and maintained in culture as previously described (Jee et al., 1990). Adult foreskin specimens were washed throughly with ice-cold calcium-free minimal essential medium (Joklind’s modified Eagle’s medium) containing gentamicin sulfate (50 µg per ml) and penicillin-streptomycin (60 units, 60 µg per ml). They were cut into pieces about 5×5 mm² and immersed in 0.1% protease (type 14, Sigma Chemical Co., St. Louis, MO) and Joklind’s modified Eagle’s medium at 4°C for 24 h. After a brief exposure to Joklind’s modified Eagle’s medium containing 10% fetal bovine serum, the protease-treated tissue was transferred to F12 (GIBCO, Gaithersburg, MD). The epidermal sheet was separated from the dermis with a pair of forceps, and the epidermal cells were dissociated by teasing the sheets. While in the F12, the dermal surface was scraped gently with forceps to release the remaining epidermal cells. Next, F12 solution containing the dissociated cells and the cells scraped from the dermal surface were pooled and centrifuged at 100 × g for 5 min at 4°C. The cell pellet was then suspended in F12 supplemented with epidermal growth factor (20 ng per ml, Collaborative Research, Inc., Bedford, MA), insulin (5 µg per ml; Sigma), transferrin (5 µg per ml,
Sigma), hydrocortisone (1 μM; Collaborative Research, Inc.), cholela toxin (40 ng per ml; New England Biolabs, Inc., Beverly, MA), endothelial cell growth supplements (15 ng per ml, Collaborative Research, Inc.), and retinoic acid (1 × 10−7 M; Sigma). The cells were seeded on Vitrogen-coated tissue culture dishes (30–100 mg/cm2; Collagen Corp., Palo Alto, CA) at a density of 5 × 104 cells/ml. The cells were fed with fresh modified culture medium every day. When cells had reached approximately 60–80% confluency, the cells were trypsinized with 0.05% trypsin–ethylenediamine tetraacetic acid. The detached cells in the trypsin–ethylenediamine tetraacetic acid were mixed with 1 mg ice-cold trypsin inhibitor per ml (Sigma) and then centrifuged at 500 × g at 4°C for 5 min. The cell pellet was resuspended in the culture medium and seeded at a density of 5 × 107 cells/cm² on Vitrogen-coated tissue culture dishes. The second-passage cells were used for study.

He-Ne Laser Irradiation

The He-Ne laser used (Lasotronics Med-1000, Lasotronic Ag, Zugster, Switzerland) has an output of 10 mW with a diverging lens that delivered 7.0 mW (as measured by a power meter, Lasotronic Ag) to a platform 17 cm under the lens. Second-passage keratinocytes were plated on six-well plates in modified culture medium every 2 days. When cells had reached approximately 90% confluency, they were trypsinized, centrifuged, and the cell pellets were seeded in the culture medium at a density of 5 × 104 cells/well. After 24 h, the cultures (4.8 × 107 cells/well) were both rinsed with and irradiated in phosphate-buffered saline to minimize the loss of laser energy through absorption by colored culture medium. Wells were irradiated at 0, 0.5, 1.0, or 1.5 joules (J)/cm². Each experiment was performed in triplicate. During irradiation treatments, all wells within an experiment (including controls) were maintained in phosphate-buffered saline at room temperature and atmosphere for the length of the experiment. After irradiation, phosphate-buffered saline was aspirated and replaced with culture medium.

Measurement of Cytokines in Culture Supernatant

Cultured keratinocytes were irradiated with different dosages of He-Ne laser energy. Twenty-four hours after irradiation, cytokines in the culture supernatant were assessed by commercially available test kits. They were assayed using colorimetric immunoassays according to the manufacturers' specifications. The measurements included: interleukin-1α (IL-1α), interleukin-8 (IL-8), tumor necrosis factor-α, granulocyte macrophage-colony stimulating factor (Research and Diagnostic Systems, Minneapolis, MN) and interferon-γ (Holland biotechnology bv, Al Jieiden, The Netherlands).

Total RNA Extraction

Cultured human keratinocytes were collected 4 h after He-Ne laser irradiation. The time course for cell harvesting for keratinocytes mRNA expression was set as previously described (Kristensen et al, 1991). Total cellular RNA was isolated as previously described (Chomczynski and Sacchi, 1987). Briefly, about 10⁶ cells were lysed in solution D (4 M guanidium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol), after which 0.05 Molar of 2 M sodium acetate, 0.5 M water-saturated phenol, and 0.1 ml of chloroform/ isoamyl alcohol (24:1) was added to the lysate, shaken vigorously for 30 s, and cooled on ice for 15 min. The suspension was centrifuged at 12,000 rpm at 4°C for 15 min. The upper aqueous phase was then mixed with an equal volume of isopropanol and placed at −20°C for 1 h to precipitate RNA. After centrifugation at 4°C, 12,000 rpm for 15 min, the RNA pellet was mixed again with an equal volume of solution D and reprecipitated at −20°C for at least 1 h. This followed centrifugation again at 12,000 rpm for 20 min, and the remaining RNA pellets were washed in 75% ethanol, vacuum-dried, and dissolved in a diethylpyrocarbonate-treated ribonuclease-free solution. The RNA concentration was determined by OD₂₆₀.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Two micrograms of RNA were reverse transcribed into cDNA in the presence of 0.5 μg poly-deoxy(thymidines) (Pharmacia, Uppsala, Sweden), 8 μl of 1.25 mM deoxyinosine triphosphates (Pharmacia), Avian Myeloblastosis Virus reverse transcriptase (5 units per ml, Promega, Madison, WI) and 5× reaction buffer (250 mM Tris(hydroxymethyl)-aminomethane-HCl, 250 mM KCl, 50 mM MgCl₂, 50 mM dithiothreitol, 2.5 mM spermidine) at 42°C for 1 h. For the PCR reaction, we used the following primers: IL-1α 5′-CAGAGAAGACAGCATGGTGAGTAGCACAACCA CAG-3′ and 5′-TACGTGCGTTAGTTCAGGAG-3′; IL-8 5′-ACAGAGTCTTGCAGACAGCCAGGAGG-3′ and 5′-GGATATT CAGTGTTGGTCTACTCTATT-3′ (Paludan and Thorsrud-Pedersen, 1992) and β-actin 5′-GGGCGGCGGCCAGGACCA-3′ and 5′-CTCCT TAATGTCGACGAGTTC-3′ as described by Brenner et al (1987). Five-microliter aliquots of synthesized cDNAs were added to 95 μl of PCR mixture containing 10 μl of 10× PCR buffer (500 mM KCl, 100 mM Tris(hydroxymethyl)-aminomethane-HCl, 1% Triton X100, Promega), 1 μl of sense and anti-sense primers (0.2 μM), 8 μl of 1.25 mM deoxyinosine triphosphates, and 0.5 μl of Taq DNA polymerase (0.5 units per ml, Promega). The reaction mixture was overlaid with 50 μl of mineral oil. Amplification was initiated by 4 min of denaturation at 94°C for one cycle, followed by 25 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and 72°C for 7 min using an automatic thermal cycler. The PCR products were visualized by ultraviolet illumination following electrophoresis through 2.0% agarose gel (BRL, Gaithersburg, MD) at 50 volts for 40 min and staining in Tris(hydroxymethyl)-aminomethane-ethylenediamine tetraacetic acid buffer (BRL) containing 0.5 mg ethidium bromide per ml. The gels were photographed on Polaroid black and white film (type 667, Polaroid Corp., Cambridge, MA). PCR products were titrated to establish standard curves to document linearity and to permit semi-quantitative analysis of density. The films were scanned in a computerized laser densitometer, and the area under the curve was normalized for β-actin content.

DNA and Protein Synthesis Assay

Immediately after He-Ne irradiation, the medium for all control and experimental groups was replaced with 2 ml of serum-free medium containing 5 μCi [methyl-¹H]Hymidine per ml (specific activity 6.7 Ci/mmol, Amersham International, Little Chalfont, U.K.) for DNA synthesis assay, or with 2 ml of serum-free medium containing 5 μCi [³H]Leucine per ml (specific activity, 69 Ci/mmol, Amersham International) for protein synthesis assay. After incubation for 24 h, cells were washed with phosphate-buffered saline and resuspended in trypsin-ethylenediamine tetraacetic acid solution. After centrifugation, the cell pellets were lysed in 1 N NaOH and incorporated [³H]Hymidine and [³H]Leucine were counted after the addition of hydrofluor (National Diagnostics, Manville, NJ). The radioactivity was counted in a scintillation counter (Minax-B Tri-Carb 4000 series, Packard, Gowers Grove, IL). The amount of incorporation was expressed as counts per minute.

RESULTS

He-Ne Laser Irradiation Showed No Significant Thermal Effect on Cultured Keratinocytes

The temperature effect of He-Ne laser irradiation on cultured keratinocytes was assessed according to the same irradiation protocol. Thermistors (Takara, Tokyo, Japan) were used for this measurement. The mean ± SD of the temperature change on cultured wells before and after irradiation were 21.2 ± 0.2°C, 21.0 ± 0.1°C, and 21.0 ± 0.2°C, respectively. He-Ne laser irradiation resulted in no significant temperature change on cultured keratinocytes.

A Significant Increase in IL-1α and IL-8 Release from He-Ne Laser-Treated Keratinocytes

IL-1α A significant increase in IL-1α production was noticed in He-Ne laser-treated groups as compared with nonirradiated controls (Table 1). The stimulatory effect of He-Ne laser treatment was concentration-dependent; irradiation rates of 0.5, 1.0, and 1.5 J/cm² brought about progressively greater stimulation of IL-1α release.

IL-8 A significant increase in IL-8 production was observed in He-Ne laser-treated groups compared with untreated controls (Table 1). The stimulation effect of He-Ne laser treatment was dose-dependent; irradiation rates of 0.5, 1.0, and 1.5 J/cm² resulted in progressively greater stimulation of IL-8 release.

He-Ne laser treatment showed no stimulatory effect on granu-
locate macrophage-colony stimulating factor, tumor necrosis factor-α, or interferon-γ release by keratinocytes.

RT-PCR Confirmed IL-1α and IL-8 Production from Cultured Keratinocytes After He-Ne Laser Irradiation Cytokine expressions as detected by the RT-PCR method were evaluated for IL-1α and IL-8 in keratinocytes after low-energy laser irradiation. The cDNA was normalized to yield equivalent amounts of β-actin to standardize the amount of total cellular mRNA in each PCR reaction. PCR products of IL-1α and IL-8 cytokines were found to be proportional to laser dosage (Fig 1a,b). Calibration of β-actin was displayed in Fig 1c. Our findings were compatible with those measured by enzyme-linked immunosorbent assay and revealed that low-energy laser can stimulate mRNA expression of IL-1α and IL-8 for keratinocytes.

He-Ne Laser Treatment Showed No Significant Effect on Protein and DNA Synthesis in Keratinocytes There were no significant differences of [3H]thymidine and [3H]leucine uptake of keratinocytes between He-Ne laser-treated and control groups. He-Ne laser irradiation (less than 1.5 J/cm²) did not affect protein or DNA synthesis in cultured keratinocytes.

DISCUSSION

In animal experiments, He-Ne laser irradiation has been reported to bring about systemic effects, in addition to local effects, on the healing of cutaneous wounds. Irradiation of only one side of a bilaterally infected cutaneous wound enhanced recovery in both sides, compared to the nonirradiated control group (Braverman et al., 1989; Rokchind et al., 1989). Production of growth or tissue factor (Braverman et al., 1989) and/or increase of phagocytic capacity of leukocytes (Mester et al., 1985) following laser irradiation may be involved in the systemic effect. On the other hand, low-level lasers encouraged macrophages in vivo to release factors that stimulated fibroblast proliferation above control levels (Young et al., 1989). These findings indicated that low-level laser therapy stimulated release of growth factors in vivo or in vitro.

One aim of this study was to investigate whether He-Ne laser could induce cytokine production in cultured keratinocytes. We noticed a significant increase in IL-α and IL-8 production and their respective mRNA expressions in He-Ne laser-treated groups compared with nonirradiated controls. He-Ne laser irradiation less than 1.5 J/cm² is dose-dependent, resulting in progressively greater stimulation of IL-1α and IL-8 release. Their respective mRNA expressions were confirmed by RT-PCR. Although both IL-1α and IL-8 induce proliferation of keratinocytes (Ristow, 1987; Tuschil et al., 1992), in this study He-Ne laser irradiation showed no stimulatory effect on DNA synthesis (as an indicator of cell proliferation) in spite of an increase in IL-α and IL-8 production. Two possible explanations for this paradox are (i) low-level laser therapy has a greater effect on cultures grown in the unfavorable conditions of 1% fetal bovine serum than in the better conditions of higher fetal bovine serum concentrations (Steinlechner and Dyson, 1993), and (ii) following cell migration, there is a lag phase before the onset of keratinocyte proliferation (Sullivan and Epstein, 1963).

Effects produced by low-energy laser may be due to the effects of light in general rather than to the unique qualities of lasers. Wavelength-dependent photo-biochemical reactions occur throughout nature (Basford, 1989). The primary photo-acceptors for light are thought to be the components of the terminal oxidation redox chain; cytochrome aa₃ absorbs red light (Brunner and Wilson, 1982), and it may be the photoactive molecule upon which the He-Ne light exerts its effect. It is thought that an effect of low-level laser therapy is to oxidize the primary photoreceptor (Steinlechner and Dyson, 1993). Low-level laser therapy has been demonstrated to stimulate cellular respiration; the He-Ne laser has been shown to increase the proton gradient across the inner mitochondrial membrane and to stimulate ATP production (Passarella et al., 1984), bringing about cell migration and proliferation. Low-level laser therapy might also alter the rate of cell proliferation by changing intracellular H⁺, K⁺, and cAMP levels (Karu, 1988). In this study, He-Ne laser irradiation has been shown to stimulate IL-1α and IL-8 release from keratinocytes. Because IL-1α causes keratinocyte migration (Ristow, 1987), it may partially explain the stimulatory effect on the motility of keratinocytes (Haas et al., 1990). Both IL-1α and IL-8 induce keratinocyte proliferation. It is reasonable to propose that these two cytokines play a profound role in the way He-Ne laser enhances keratinocyte proliferation. Our findings provide further evidence of enhanced wound healing at the cellular and molecular level brought about by the He-Ne laser. The mechanism of this stimulation of cytokine release from keratinocytes by He-Ne irradiation, however, remains to be defined.

REFERENCES


Figure 1. The effects of He-Ne laser treatment on mRNA expression in IL-1α and IL-8 are dependent upon the dose of radiation as shown by RT-PCR. (a) IL-1α, (b) IL-8; (c) β-actin. M, pGEM DNA marker. Lane 1, control cells without laser irradiation; lane 2, 0.5 J/cm²; lane 3, 1.0 J/cm²; lane 4, 1.5 J/cm². The results of densitometric analysis for cytokines/β-actin ratios were as follows. IL-1α: lane 1, 0.76; lane 2, 1.02; lane 3, 1.30; lane 4, 1.76. IL-8: lane 1, 0.28; lane 2, 0.44; lane 3, 0.69; lane 4, 1.39.


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