Low-Energy Helium-Neon Laser Irradiation Increases the Motility of Cultured Human Keratinocytes

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Helium-neon (HeNe) laser irradiation is known to stimulate wound healing. We investigated whether the biostimulatory effects of HeNe irradiation result from enhancement of keratinocyte proliferation or motility. HeNe effects on keratinocyte motility were evaluated by irradiating a "wounded" culture with 0.8 J/cm^2 3 times over a 20-h period. At 20 h post-irradiation, videocinemicroscopy and sequential quantitative measurements of the leading edge were taken over a 6-h period. There was a significant difference in migration of the leading edge in irradiated "wounds" compared to non-irradiated "wounded" controls (12.0 μm/h vs 4.0 μm/h, $p < 0.0001$). To determine if the increase in migration observed in irradiated cultures resulted from a proliferative effect of HeNe irradiation, subconfluent human keratinocyte cultures were irradiated with single or multiple doses of different fluences of HeNe irradiation (0.4 to 7.2 J/cm^2) and evaluated 72 h post-irradiation. Irradiated and non-irradiated keratinocyte cultures grown on a microporous membrane surface were co-cultured with irradiated and non-irradiated fibroblasts to determine if HeNe irradiation induced a paracrine effect on keratinocyte proliferation. No significant increase in keratinocyte proliferation was demonstrated in any of these treatments. The biostimulatory effects of HeNe irradiation may now be extended to include enhancement of keratinocyte motility in vitro; this may contribute to the efficacy of HeNe irradiation in wound healing. *J Invest Dermatol 94:822–826, 1990*

There is much evidence that low-energy lasers may stimulate biological processes and, as such, enhance wound healing. The definition of a "low-energy" laser is one that produces an energy density so low that temperature elevations are limited to less than 0.1–0.5°C [1] and any observed biologic effects are consequently ascribed to non-thermal events. A number of different laser light sources, including helium-neon (HeNe), ruby, and argon, as well as others, have been utilized to deliver low-energy irradiation in a multitude of different doses and treatment schedules. As a result, there are many conflicting reports as to the biostimulatory effects of low-energy laser irradiation [1,2]. In addition, the problems in study design and laser-parameter definition, particularly in some of the earlier studies, have made comparative analysis of biostimulatory effects difficult to interpret.

There is, however, significant evidence to indicate that HeNe irradiation of wounds in vivo may be beneficial at least in the early stages of wound healing in certain species [3–7]. On a cellular level, the effects of low-energy HeNe irradiation on fibroblasts has demonstrated increase in both collagen production [3,4,7–9] and procollagen types I and III mRNA in the irradiated dermis [10]. These laboratory findings correlate well with previously reported increased tensile strengths in HeNe-irradiated wounds [4,6,11].

Another important component of wound healing is re-epithelialization, which can be divided into two phases: migration of epithelial keratinocytes bordering the wound defect and proliferation. The known effectors capable of mediating either keratinocyte proliferation or migration is quite large and includes various growth factors, cytokines, and extracellular matriceal proteins, as well as many physical modalities [12,13]. Although some studies have noted increased epidermal thickness in low-energy-irradiated wounds [11,14], the possibility of a biostimulatory effect of HeNe irradiation on keratinocytes has not yet been addressed. For these reasons, the current studies were designed to assess the effect of low-energy HeNe irradiation on both keratinocyte proliferation and motility.

MATERIALS AND METHODS

Laser Specifications The optical system for this study provided 0.5 J/min distributed uniformly over a cylindrical volume having a diameter of 1.5 cm and a height of 1 cm. A HeNe laser (Spectraphysics Stabilite, Model 120, Mountain View, CA) with an output of 11.5 mW and a diverging lens (focal length approximately 65 cm) delivered 8.4 mW (as measured by a power meter, Metrologic, Model 45-450, Bellmawr, NJ) to a 1.3-cm aperture that was incorporated into a holding platform 57.5 cm above the lens, giving an energy fluence of 0.5 J/min. The laser power differed by no more than 10% between the edges and the middle of the aperture opening. Multiple measurements after warming showed a stable and accurate output with less than 2% fluctuation during a 2-h period.

Cell Culture Human keratinocyte cultures were initiated from neonatal foreskin epidermis using a previously described modifica-

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Abbreviations:
BrdU: bromodeoxyuridine
DMEM: Dulbecco's modification of Eagle's medium
FCS: Fetal calf serum
HeNe: helium-neon laser
PBS: phosphate-buffered saline

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tion [15] of the procedure of Rheinwald and Green [16]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories, McLean, VA) + 10% fetal calf serum (FCS, Gibco, Grand Island, NY), hydrocortisone (0.4 μg/ml, Sigma Chemical Co., St. Louis, MO), cholera toxin (10 ng/ml, Calbiochem-Behring Corp., LaJolla, CA) and epidermal growth factor (1 ng/ml, Collaborative Research, Bedford, MA). For studies of the effects of HeNe irradiation on proliferation, cells were plated at a density of $2.5 \times 10^4/cm^2$ in 24-well plates (Nunc, Inc., Denmark). To prevent absorption of HeNe laser energy by the colored culture medium, 24 h after plating, the subconfluent cultures were washed twice with phosphate-buffered saline (PBS) and the culture medium was replaced with 2 ml of (colorless) PBS. Designated wells were irradiated either with 0.4, 0.8, 3.0, or 7.2 J/cm². The maximal irradiation time was 20 min and all experimental wells, as well as control mock-irradiated wells, were kept at room temperature and atmosphere for the same period of time. After irradiation, the PBS was aspirated from the wells, and the dishes reincubated in fresh culture medium. When multiple exposures to HeNe irradiation were being investigated, this same procedure was followed at each interval, generally 6–8 h. Cell number was determined by Coulter counter (Coulter Electronics, Hialeah, FL) enumeration of trypsin-released cultures at 72 h after the first irradiation.

To evaluate possible paracrine effect of HeNe irradiation of fibroblasts on keratinocyte proliferation, dermal fibroblast cultures were established from human foreskin dermis. Trypsin-separated dermis was minced and allowed to attach to culture dishes, which were incubated in DMEM + 10% FCS at 37°C in 95% humidity 5% CO_2. After fibroblast explant growth was noted, cells were released by trypsinization and reseeded on fresh culture plates. Passage 3–4 fibroblasts were used for this study. Fibroblasts were plated in 24-well dishes at an initial density of $1.25 \times 10^4 cm^2$. Additionally, keratinocytes were plated on collagen-coated (Vitrogen, Collagen Corp, Palo Alto, CA) 12-mm microporous membranes culture plate inserts (Millicell-HA, Millipore Corp.), at an initial density of $1.5 \times 10^4/cm^2$. Both fibroblasts and keratinocytes were maintained in individual wells in DMEM + 10% FCS until laser exposure, at which time they were washed as previously described. Cells were irradiated 3 times over a 24 h period with 0.8 J/cm², and after each irradiation, the keratinocytes were co-cultivated with the fibroblasts by inserting the Millicell (keratinocyte) culture into fibroblast-coated culture wells. This allowed comparison of keratinocyte proliferation under four conditions: 1) irradiation of only the fibroblast layer, 2) irradiation of only the keratinocyte layer, 3) irradiation of both keratinocyte and co-cultured fibroblast layers, or 4) irradiation of neither. Keratinocytes were enumerated by Coulter counter as in the previous experiments.

Keratinocyte Motility Confluent keratinocyte cultures (passage 2) were "wounded" with a pulled, fire-polished glass rod of 0.1 mm in diameter. The "wound" was monitored microscopically to assure uniformity of width and minimize damage to the underlying extracellullar matrix laid down by the keratinocytes. The wounded cultures were re-incubated for 20 h, during which time they were irradiated 3 times with 0.8 J/cm² to the wounded area. Prior to each irradiation, culture medium was aspirated and substituted with (colorless) PBS, as outlined above. After each exposure, cultures were reincubated in fresh culture medium. Control cultures "wounded" but not irradiated were fluid changed in the same manner and removed from the incubator for the same length of times as the irradiated cultures. Twenty hours after wounding, gassed (95% humidity and 5% CO_2) culture plates were sealed by coating the edge of the cover with a thin layer of silicone and placing a strip of Parafilm (Parafilm "M", American Can Company, Greenwich, CT) around the culture plate. The temperature of the culture dish was maintained at 37°C with an air current incubator (Model 279 Sage Instruments-Orion Research, Inc., Cambridge, MA).

Observations were made by inverted phase microscopy and recorded for 6 h by time-lapse videocinematography. To analyze migration, the images of the wounded area at time zero and at 6 h were projected onto a video monitor and traced onto acrylic transparencies. Migration was quantitated by measuring the distances migrated by cells at the wound margin into the wound at 1-cm intervals along a 10-cm area of the projected wound border. Measured distances were then converted to actual distance (1.3 μm real distance = 10 mm on the projection). Significant differences between control and experimental culture were determined using a paired Student t test. For still photographs, the videotapes were played back through a time-base corrector which allows for freeze-frame image, and the projected image was photographed using a 35-mm camera. There was some loss of resolution and contrast using this system.

BrdU Labelling of Proliferative Cells Confluent keratinocyte monolayers were prepared, wounded, and irradiated as described earlier. After irradiation, cells were labeled with bromodeoxyuridine (BrdU), a thymidine analogue which is incorporated into the DNA of proliferating cells, following the method of and using the reagents supplied in the Cell Proliferation Kit (Amersham, Arlington Heights, IL). Cells were then fixed, reacted with mouse anti-BrdU antibody, and stained with a peroxidase-conjugated anti-mouse antibody and a diaminobenzidine indicator system.

RESULTS

The effect of HeNe irradiation on keratinocyte motility was analyzed using a wounded monolayer model and measuring the rate of advance of the leading edge of the wound margin in irradiated and control non-irradiated wounds. Initial retraction of the wound margin was noted during the first 6 h after wounding. From 20–26 h after wounding, keratinocytes migrated into the wounded area. HeNe-irradiated wounds demonstrated a threefold (12 μm/h) increase in the rate of migration of the wound edge as compared to non-irradiated controls (4 μm/h, $p < 0.0001$) (Fig. 1). Examination of the edge of the wound margin at the end of the 6-h recording period demonstrates that both cell spreading of the wound-edge cells as well as migration of adjacent cells into the wound margin (Fig 2) contribute to closure of the wounded area. These results suggest that HeNe irradiation results in stimulation of keratinocyte migration.

Because keratinocyte proliferation may account for some of the enhanced wound healing noted in studies of laser-irradiated wounds [11,14] as well as the increased migration we observed in our test

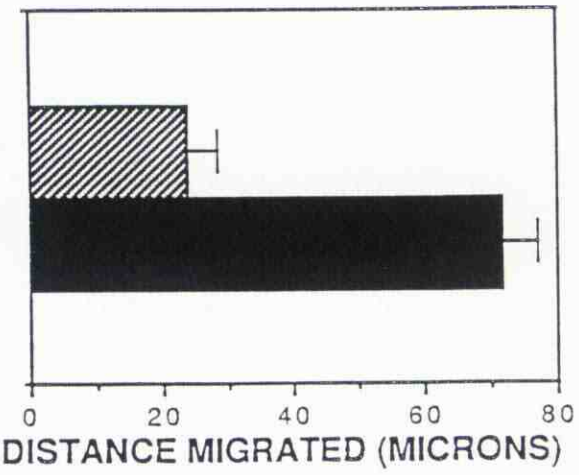

Figure 1. Effect of HeNe-irradiation on keratinocyte motility. "Wounded" monolayers received 3×0.8 J/cm² irradiation over a 20-h period, after which time the rate of migration of the wound margin was measured over a 6-h period. *Hatched bar:* control, mock-irradiated culture ($n = 20$); *solid bar:* irradiated cultures ($n = 40$). Data is expressed as the means of 10 different measurements in two (control) and four (irradiated) separate experiments. Experimental values differed from controls significantly ($p < 0.0001$) using the Student t test.

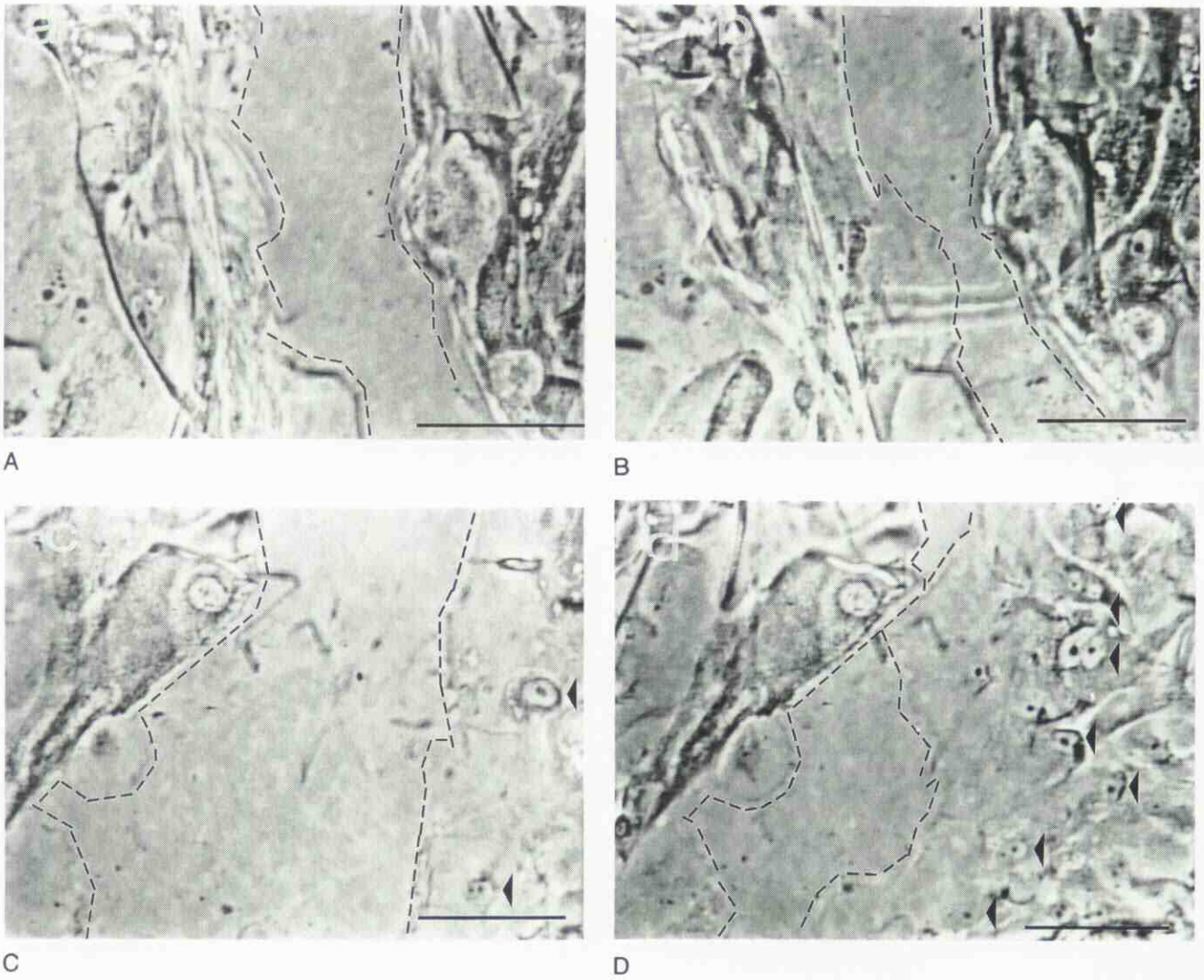

Figure 2. Photomicrograph of representative "wounded" monolayer experiments quantitated in Fig 1. Methods for cultivation and irradiation are as for Fig 1. Measured fields were projected onto video monitor using freeze-frame technique, and the image on monitor was photographed. *A,B*: control, non-irradiated cultures. *C,D*: HeNe-irradiated cultures. *A,C*: time 0 h. *B,D*: time 6 h post-irradiation. *Scale bar:* 0.1 mm; *dotted line* outlines edge of cells which have migrated into wound area; *arrowheads* identify nuclei at the wound margin. Note the increased numbers of nuclei at the right wound margin of the irradiated wound at 6 h.

system, we evaluated the effect of low-power HeNe irradiation on cell proliferation. Cultured keratinocytes received either one or multiple (3 in 24 h) HeNe exposures of varying energy fluences (0.4, 0.8, 3.0, or 7.2 J/cm^2). Cell number was determined 72 h after irradiation. No significant difference in the rate of cell proliferation between irradiated and non-irradiated controls was seen (data not shown). However, when skin is irradiated in vivo, both epidermal keratinocytes and dermal fibroblasts receive irradiation, and it is therefore possible that HeNe irradiation of fibroblasts may induce keratinocyte proliferation by a paracrine mechanism. To test for this possibility, cultures of either keratinocytes or fibroblasts were irradiated (3×0.8 J/cm^2/24 h) and subsequently co-cultivated in various combinations of irradiated and non-irradiated populations. Although there was an increased number of keratinocytes at 24 h in the experimental combination wherein neither keratinocyte nor fibroblast was irradiated, this was not statistically significant (Fig 3). At all later time points, no combination yielded any significant difference in the rate of keratinocyte proliferation.

Nevertheless, there was the further possibility that HeNe irradiation may induce a localized proliferative effect at the wound margin in our wounded monolayer model, which, because localized to such a relatively small area of the culture dish, might not be readily apparent when cell number of the entire culture plate is determined. To examine this possibility, wounded cultures were BrdU labeled and immunocytochemically stained. A small number of proliferative cells (BrdU labeled) were noted along the wound margins of both irradiated and non-irradiated wounds, with no significant change in either pattern or quantity of BrdU-labeled cells (data not shown).

DISCUSSION

This study demonstrates that irradiation of "wounded" keratinocyte cultures with low-energy HeNe laser irradiation at an energy fluence of 0.8 J/cm^2 significantly enhances keratinocyte motility from the leading edge of the wound (12.0 μm/h vs 4.0 μm/h in control non-irradiated cultures). Goslen [12] suggests that the rate of epidermal migration is optimally 12–21 μm/h, and is subject to interference by many factors, such as the type of provisional matrix, local oxygen tension, and the presence or absence of infection. Hence, the rate of migration observed in this in vitro system, although slower than that reported by Goslen, is a reasonable approximation of migration rates achieved in vivo.

HeNe irradiation did not induce keratinocyte proliferation, either generalized or localized at the wound margin. The induction of migration and not proliferation is consistent with other studies that

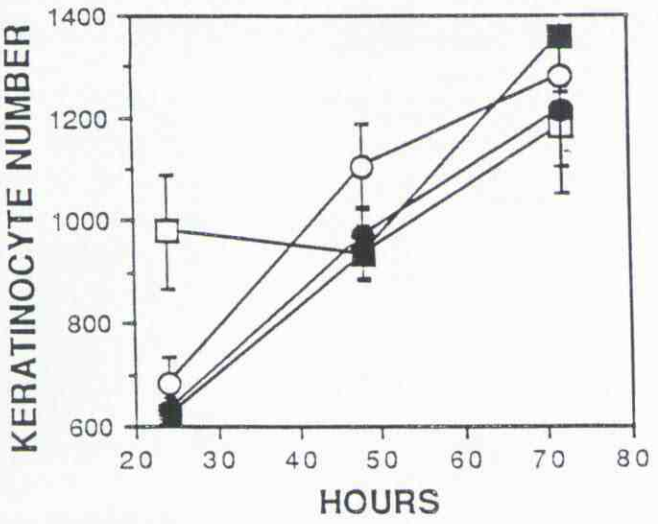

Figure 3. Effect of HeNe irradiation of different cell populations on keratinocyte proliferation. Keratinocytes were enumerated at 24, 48, and 72 h following HeNe irradiation (3 × 0.8 J/cm²/24 h) of either cultured keratinocytes (●), fibroblasts (■), both cell populations (○), or neither population (□). After irradiation, cell populations were co-cultivated as outlined in *Materials and Methods.*

have found that keratinocyte migration can occur in the absence of proliferation [17–19]. The lack of HeNe-stimulated keratinocyte proliferation mirrors the results obtained in recent fibroblast studies where increases in collagen production by HeNe-stimulated fibroblasts have been well documented [7–9] but not increases in fibroblast growth rate [9,20,21]. A recent study by Colver and Preistley [21] has demonstrated, similar to the current work, an absence of a proliferative effect of HeNe irradiation on epithelial cells. In contrast to the current report, however, that study failed to demonstrate an inductive effect of HeNe irradiation on keratinocyte migration. The disparity in the observations may stem from the considerable differences in the experimental protocols of the two studies: with the former study utilizing an assay system that measures outgrowth from full-thickness skin explants rather than the "wounded" monolayer system we used, and the use of a narrow (1 mm) beam laser with approximately half the laser power and lower total doses of irradiation than those used in the current studies.

The energy fluences used in this study are in the same range as those previously reported which demonstrated biologic responses. These fluences are generally in the 1–2 J/cm² range. Increased tensile strength was reported by Lyons et al [4] using 1.22 J/cm², and by Braverman et al [11] with a dose of 1.65 J/cm². Surinchak et al [6] used a dose of 2.2 J/cm² which yielded a significant increase in tensile strength of irradiated rabbit wounds, but higher doses of 4.5 J/cm² did not produce a significant effect on tensile strength. Higher doses were also noted to inhibit wound closure as in the study of Kana et al [3], where an energy fluence of 4 J/cm² yielded the best wound healing results (increased collagen production) and an energy fluence of 20 J/cm² inhibited healing.

Previous reports have also indicated that multiple exposures might stimulate wound healing. Kubasova et al [22] reported that a single exposure of fibroblasts to 1 and 5 J/cm² produced no change in fibroblasts but multiple daily exposures for 4 d resulted in specific surface changes of cell membranes. The multiple-dosing schedule used by Mester et al [14] of 1 J/cm² at days 3, 7, and 12 also induced significant stimulation of wound healing in rats. For these reasons, our studies on possible effects of HeNe irradiation on cell proliferation utilized energy fluences ranging from 0.3–7 J/cm², delivered in either single- or multiple-dosage protocols. An energy fluence of 0.8 J/cm² was selected for both the combined keratinocyte/fibroblast proliferation experiments and for the motility experiments because it is approximately the energy fluence used in many of the recent fibroblast studies and in many of the current in vivo wound-healing studies which showed significant results [4,5,7–11].

Low-energy HeNe irradiation is presumed to effect biologic processes by a non-thermal mechanism [1,2,7,9,11,20]. In these experiments, the maximal increase in temperature is negligible: 0.12°C as calculated using a previously derived formula.* However, the mechanism by which HeNe irradiation enhances keratinocyte motility is unknown. Reports suggest that perhaps an intracellular target or cytochrome is stimulated by HeNe irradiation [24], or that HeNe irradiation may have an effect on cell membranes [22] by changing the ability of irradiated human fibroblasts to bind ^{3}H-concanavalin A. Membrane properties such as receptors for matrix molecules and cytokines [18,25] are involved in both adhesion and migration. Hence, HeNe-induced membrane abnormalities may account for the enhanced motility observed in irradiated keratinocytes.

Another possible mechanism to explain HeNe-irradiation enhanced cell motility may be the induction of proteases in irradiated cells. Local increases in the protease plasminogen activator have been shown by Morioka et al [19] and Nakajima et al [26] to be associated with keratinocyte migration. Plasminogen activator has been induced in some cell types by other forms of radiant energy. Short-wave ultraviolet irradiation (UV) has been shown to induce plasminogen activator in fibroblasts [27] and longer-wave UVB induces plasminogen activator in a mouse keratinocyte line [28]. It is, therefore, reasonable to suggest that HeNe irradiation may similarly induce this enzyme in irradiated keratinocytes, thus facilitating migration. Our current efforts are directed at examining this possibility.

Although the exact mechanism of this enhancement of keratinocyte migratory activity by HeNe irradiation remains to be defined, it appears that the biostimulatory effects of HeNe irradiation extend to keratinocyte motility in vitro. This finding may help explain the previously noted efficacy of HeNe irradiation in clinical studies of wound healing. Additional basic research and clinical studies should help improve our understanding of the mechanism and clinical indications for this laser system.

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*$T_o - T_e = [(dE/dx)I/4\pi wk][1 + 2\ln(r_e/r_o)]$, where T_o = final temperature of sample, T_e = temperature at periphery of sample, dE/dx = energy loss rate averaged over w, w = width or thickness of sample, r_o = radius of beam, r_e = radius of sample, I = beam current, k = thermal conductivity of sample (for H_2O = 0.581 W/m/°C) [23].

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