Reactions of Keratinocytes to In Vitro Millimeter Wave Exposure

Imre Szabo,1 Mikhail A. Rojavin,2 Thomas J. Rogers,1 and Marvin C. Ziskin2*
1Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, Pennsylvania
2Center for Biomedical Physics, Temple University School of Medicine, Philadelphia, Pennsylvania

The effects of millimeter waves (MW) on human keratinocytes were studied in vitro using the HaCaT keratinocyte cell line. MW-induced modulation of keratinocyte function was studied in proliferation, adhesion, chemotaxis, and interleukin-1β (IL-1β) production assays. Spontaneous proliferation, adhesion to tissue culture plate, random migration, and IL-8- and RANTES-induced chemotaxis were not affected by exposure of cells to millimeter waves under the following conditions: frequency, 61.22 GHz; SAR, 770 W/kg; duration of exposure, 15–30 min. However, MW irradiation resulted in a modest but statistically significant increase in the intracellular level of IL-1β. These data suggest that exposure of human skin (with keratinocytes being the major component of epidermis) to MW can cause activation of basal keratinocytes resulting in an elevated level of IL-1β production. Bioelectromagnetics 22:358–364, 2001. © 2001 Wiley-Liss, Inc.

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INTRODUCTION

Millimeter waves (MW) of low power (<20 mW/cm²) have been used in several countries for medical purposes for more than 15 years [Pakhomov et al., 1998; Rojavin and Ziskin, 1998]. In most cases, MW as a treatment modality are applied to a certain skin area [Vinogradov et al., 1993; Korpan et al., 1994; Korpan and Saradeth, 1995; Lyan and Votoropin, 1996; Bakalyuk, 1997]. However, the mode of action of MW therapy is poorly understood, and little is known about either the efficacy of MW therapy or the underlying mechanisms responsible for the biological activity of MW [Korpan and Saradeth, 1995; Radzievsky et al., 1999]. Due to rapid absorption in human skin and other water containing tissues, the penetration depth of MW in the skin is, depending on the frequency of MW, between 0.2 and 0.5 mm [Gandhi et al., 1980; Gandhi, 1983]. Thus, any effects of MW must be initiated by interaction between the electromagnetic waves and skin within the cited penetration depth.

Among the possible cellular targets for MW in the upper layer of the skin, are keratinocytes, mast cells, melanocytes, Langerhans cells, and free nerve endings. Keratinocytes comprise up to 90% of the total skin cell population. While direct interaction between MW and neurons of various organisms has been shown to result in significant changes of neuronal activity [Akoev et al., 1995; Kolosova et al., 1996; Alekseev et al., 1997; Pakhomov et al., 1997; Alekseev and Ziskin, 1999], the reactions of keratinocytes to MW exposure remain undefined. However, MW have been reported to be effective in treating certain skin diseases affecting keratinocytes, such as atopic dermatitis and psoriasis [Adaskevich, 1995; Zaitseva and Donetskaia, 1997] as well as increasing the speed of wound healing, both in experimental animals [Detchav et al., 1996] and in humans [Cherkasov et al., 1978; Zemskov et al., 1988; Korpan and Saradeth, 1995].

In order to evaluate the possible role of keratinocytes in producing the biological effects of MW, we have conducted an in vitro study with human keratinocyte cell cultures, testing the hypothesis that exposure of these cells to MW may result in alterations of keratinocyte function. In these studies we analyzed the modulatory effect of MW exposure on proliferation,
migration, and adhesion of keratinocytes, processes that participate in wound healing and IL-1β production. IL-1β is a major pro-inflammatory cytokine, which can be produced and released by keratinocytes in response to various stimuli [Corsini and Galli, 1998; Wang et al., 1999; Ushio et al., 1999]. It is known to play an important role in the autocrine regulation of basic cellular properties of keratinocytes under resting and stimulated conditions [Bonifati et al., 1997; Zepter et al., 1997; Corsini and Galli, 1998; Takei et al., 1998; Maas-Szabowski et al., 1999]. We found that spontaneous proliferation, adhesion, random and chemotactic migration were not affected by exposure of cells to MW. However, MW irradiation resulted in a modest but statistically significant increase in the intracellular level of IL-1β.

MATERIALS AND METHODS

Culture Medium

RPMI-1640 medium with l-glutamine and HEPES buffer (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL) was used for cell culture (R10).

Chemokines

Human IL-8 and RANTES were used at a concentration of 100 ng/ml. All chemokines were purchased from PeproTech Inc. (Rocky Hill, NJ).

Cells

Spontaneously immortalized human keratinocyte HaCaT cells were a kind gift of Professor N. E. Fusenig (University of Heidelberg, Germany). Keratinocytes were continuously cultured in R10 at 37 °C in 5% CO₂. Pre-sheet cultures were used for all experiments. For the chemotaxis assay, the adherent keratinocytes were detached using 0.05% Tripsin–EDTA (Sigma, St. Louis, MO), and the cell number was adjusted to $2 \times 10^6$/ml.

Exposure Set Up

The basic exposure setup, as described in detail previously (Rojavin and Ziskin, 1997; Rojavin et al., 1998), consisted of a tuneable MW generator G4-142 (Russia), power meter ML 4803A (Anritsu, Japan), and a Hewlett Packard 8565B spectrum analyzer, located outside a shielded room. The shielded room was made of 12.5 mm thick low-carbon steel sheets. No electrical devices were employed inside the shielded room. The generator was connected through the waveguide to the exposure area inside the shielded room. Exposures were performed in a standard clean bench for tissue culture, and metal surfaces surrounding the horn antenna were covered with microwave-absorbing material to minimize reflection of MW. The MW field was guided to a rectangular 16 × 16 mm horn antenna, and cell cultures in the wells of a 24 well plate (diameter of each well, 15 mm) were placed for exposure resting on top of the antenna, one well at a time.

Exposure Conditions

Cells were exposed in the wells of a standard 24 well plate. Four corner wells of each plate were loaded with keratinocyte suspension. Two wells were exposed to MW and two were left as plate controls. Each corner well was individually exposed or sham exposed for 15 or 30 min. (The total exposure duration for each plate was 60 or 120 min.) The exposures were performed on a random basis by one experimenter, so that a second experimenter who did the subsequent analysis did not know which wells were exposed and which were sham-exposed. The Russian-made MW generator (G-141) produced a continuous wave 61.2 ± 2.1 GHz field with an output power of 20 mW that resulted in a specific absorption rate (SAR) of 770 ± 42 W/kg. The SAR was calculated using the following formula:

$$\text{SAR} = C \cdot (dT/dt)_0$$

where $C$ was set to 4186 J/kg·K, the heat capacity of water in SI units. $(dT/dt)_0$, the initial rate of temperature rise was 0.18 K/s, as measured using a calibrated 0.1 mm diameter thermocouple positioned in contact with the bottom of the well. Under these conditions, the steady-state temperature of the exposed samples never exceeded 1.6 °C. The incident power density ($I$) was calculated to be 29 ± 2 mW/cm² using the relationship described by Gandhi and Razzi [1986]:

$$I = \rho \cdot \delta \cdot \text{SAR}/[2 \cdot (1 - R)]$$

where $\rho$ is the density (1000 kg/m³), $\delta$ is the penetration depth (0.31 mm), and $R$ is the power reflection coefficient (0.59). The latter two quantities were measured at this frequency by the method described in our previous publication [Alekseev and Ziskin, 2000].

Keratinocyte Proliferation Assay

HaCaT keratinocytes ($2 \times 10^5$ in 500 µl R10) were placed into corner wells of 24 well tissue culture plates (Becton-Dickinson, Franklin Lakes, NJ) and
were exposed to MW for 30 min. Cells were then incubated for 24 h at 37°C, 5% CO₂ and the cell number was determined by the colorimetric method using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). Six determinations were performed in duplicate.

**Keratinocyte Migration Assay**

1 × 10⁶ HaCaT keratinocytes in 500 μl of migration medium (RPMI 1640 supplemented with 1% BSA and 25 mM HEPES, both from Gibco BRL) were placed into the corner wells of 24 well tissue culture plates and exposed to MW for 15 min. Cell suspensions were then diluted to 1 ml with migration medium and migration was immediately tested in a 48 well microchemotaxis chamber (Neuro Probe, Gaithersburg, MD) using a 12 μm pore size PVP-free membrane (Osmonics, Livermore, CA). Cell migration was terminated after 1 h incubation at 37°C, 5% CO₂. Spontaneous and chemokine induced migration was determined under light microscope by counting cells in a representative area at low magnification (100 × ). Ten experiments were performed in duplicate.

**Keratinocyte Adhesion**

5 × 10⁵ HaCaT keratinocytes in 500 μl R10 were placed into corner wells of a 24 well tissue culture plate and were immediately exposed to MW for 30 min. Cells were then incubated at 37°C, 5% CO₂ for 1 h. Nonadherent cells were removed by gentle washing with a warm medium, and the number of adherent cells was determined by the MTT colorimetric method. Four experiments were performed in duplicate.

**MTT Colorimetric Method**

To determine the cell number of HaCaT keratinocytes, supernatants from exposed and control samples were replaced with 300 μl of phenol red free RPMI 1640 (GibcoBRL, Life Technologies), and 30 μl of 5 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma) was then added to each well. After 90 min of incubation at 37°C, supernatants were removed and the cells were subjected to two cycles of freeze/thaw for 30 min. Formazan was solubilized in ethanol/acetone (60/40 w/v) for 30 min at 4°C, and the optical density of the resulting solution was determined at 535 nm using a micro-ELISA reader [Mosmann, 1983].

**IL-1β Measurement**

Keratinocytes (5 × 10⁵ in 500 μl R10) were placed into corner wells of 24-well tissue culture plates. Following overnight incubation at 37°C the cell confluence was typically complete. Supernatants were then replaced with 500 μl of a fresh medium, and the monolayers were exposed to MW for 15 or 30 min at room temperature. After 24 h of additional incubation, supernatants were harvested for determination of IL-1β release, and an equivalent volume of fresh medium was added to the monolayers. The cells were lysed by freeze/thaw three times. Supernatants and lysates were centrifuged to remove cells or cellular debris and stored at -70°C until the time of assay. IL-1β was determined using the Quantikine Colorimetric ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Each set of experiments contained six determinations in duplicate.

**Statistical Analysis**

All measurements were carried out in a blind manner. Each determination consisted of the analysis of a single plate, in which two wells were exposed to mm waves and two wells were sham exposed. The individual making the measurements did not know which of the four wells were exposed and which were the sham controls. After all measurements were performed, the code was revealed.

The subsequent statistical analysis was performed using the two-factor analysis of variance with replication [Zar, 1974]. The IL-1β concentration, or other measurements, were the variables under consideration, and the two factors being simultaneously tested were the effects of MW exposure and different sample plates. There were two levels in the first factor (MW and sham exposures) and six in the second factor (plate number). There were two replications of each measurement yielding a total of e · p · r data values in each analysis, where e is the number of exposure levels (= 2), p is the number of plates, and r is the number of replications (= 2). F tests were used to determine statistical significance. All tests were performed at the 0.05 level of significance. The results are presented as the mean ± standard deviation of the mean (SD).

**RESULTS**

The spontaneously immortalized human keratinocyte cell line HaCaT was grown without addition of growth factors and was used to analyze MW exposure-induced alterations in function of cells representing the most active cell type of the epidermis. In the preliminary experiments we found that cell proliferation, adhesion, and IL-1β production were not affected by a 30-minute incubation at room temperature (without MW exposure); however, the same incubation period strongly altered the migration of HaCaT cells. This is why we chose the shorter, 15 minute exposure in this latter assay (data not shown). Our first studies showed
that the proliferation rate of HaCaT keratinocytes, as determined by MTT assay, was not significantly altered by MW exposure for 30 min (Fig. 1.), although the absorbance which represents the number of cells transforming the soluble tetrazolium salt to formazan crystals was slightly higher in the exposed group. However, statistical analysis showed no significant difference between the two groups.

We also assessed spontaneous and chemokine-induced cell migration after 15 min of MW exposure in a 48 well microchemotaxis chamber using a 12 μm pore size uncoated membrane (Fig. 2). Keratinocytes were allowed to migrate at 37°C for 60 min, and the number of migrated cells was determined by light microscopy. Under certain growing conditions (cell density <2×10⁵/cm²) keratinocytes were found to migrate toward IL-8 and RANTES (data not shown), chemokines involved in the recruitment of inflammatory cells into the skin. Under our experimental conditions these chemokines at a concentration of 100 ng/ml induced a moderate enhancement of keratinocyte migration compared to the untreated control (56 and 44% increase, respectively), which was not significantly altered by MW exposure. However, MW-exposed cells exhibited less spontaneous migration and relatively higher chemotactic migration toward IL-8 (75%) and RANTES (49%), but these changes were not statistically significant.

Keratinocyte adhesion to plastic in uncoated 24-well tissue culture plates was also studied after 30 min of MW exposure. Cell adhesion was determined by MTT assay after a 60-minute incubation at 37°C followed by removal of nonadherent cells. Measurement of the absorbance, which represents the number of adherent cells, showed no significant difference between exposed and nonexposed groups (Fig. 3).

From previous experimental data it is known that a wide range of in vitro stimuli might induce an increase in IL-1 production by keratinocytes. We studied whether MW exposure induces IL-1β production in HaCaT cells and whether exposed cells exhibit altered release of this cytokine. Extracellular release of IL-1β and intracellular cytokine production in cell
lysates were determined after 15 and 30 min of MW exposure, followed by cultivation for 24 h at 37°C. Extracellular release of IL-1β was not significantly altered by MW exposure for either time period tested; however, the cytoplasmic level of IL-1β increased at both 15 and 30 min of exposure. Although the increase at 15 min was not sufficient to reach statistical significance (P = 0.057), the intracellular IL-1β levels in the exposed cells exceeded those of the controls in every plate tested. At 30 min, the increase in cytokine production became statistically significant (Fig. 4). We also found that an equal volume of cell lysate always contained at least 100-fold higher IL-1β levels that found in the supernatant.

DISCUSSION

Our efforts were aimed at identifying functional activities of keratinocytes that might explain the mechanisms of experimental and clinical effects of MW on skin. These effects include acceleration of wound healing and improvement of atopic dermatitis. In order to reduce the wide range of functional variabilities in primary keratinocytes due to differences in age and gender of patients, or the site of skin biopsies, we used the human HaCaT keratinocyte cell line. Under certain conditions these cells are able to build up a three-dimensional epidermis showing several morphological similarities to normal skin [Boelsma et al., 1999]. Under our experimental conditions of MW exposure, HaCaT cells were found to retain function as highly activated keratinocytes, suggesting little alteration in keratinocyte functions. However, in resting human keratinocytes MW exposure might induce greater enhancement of these cellular functions.

According to our data, exposure of keratinocytes to MW in vitro within the power, frequency, and duration limits characteristic of the therapeutic application of MW generators results in modestly elevated levels of intracellular IL-1β. We found a relatively high variability in the intracellular IL-1β concentration in HaCaT keratinocytes in repeated experiments; however, in every case the MW exposure resulted in a 7–30% increase compared to its corresponding sham control.

Interleukin-1 is a pleiotropic proinflammatory cytokine produced by many cell populations upon stimulation. A wide variety of stimuli has been found to induce IL-1 production in humans including the elevation of body temperature. However, in vitro experimental data suggest that only high temperatures (above 40°C) can induce IL-1 production in epidermal keratinocytes [Gatto et al., 1992; Bowers et al., 1999]. Nevertheless, we conducted additional experiments measuring IL-1β production of keratinocytes at various temperatures. In these experiments, cells in 24-well plates were incubated at 22, 30, and 37°C for 1 h followed by additional 24 h culture at 37°C. Even a 15°C rise in temperature did not significantly alter the spontaneous IL-1β production (data not shown). Although our experimental data strongly suggest the non-thermal effect of MW irradiation on IL-1β production in keratinocytes, we cannot completely rule out that the local temperature elevation might contribute in some way, either in whole or in part to the IL-1β production observed.

The experimental finding that MW exposure causes an increase in intracellular content of IL-1β is important because it may help explain the mechanism of MW therapy. That is, the MW stimulate keratinocytes to produce IL-1β. IL-1β is released into the blood stream acts as a molecular messenger carrying the “translated” MW signal to other cells, and ultimately
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induces biochemical and possibly other changes in distant parts of the body. Further studies regarding the role of IL-1β and possibly other cytokines produced by keratinocytes in response to electromagnetic MW irradiation should provide a fruitful direction of research aimed at identifying biochemical mechanisms of MW therapy.

REFERENCES


