Investigation of the non-thermal effects of exposing cells to 70–300 GHz irradiation using a widely tunable source

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ABSTRACT

This study investigated the effects of millimeter wave (MMW) irradiation with a wide range of frequencies on the proliferation and activity of normal human skin fibroblast (NB1RBG) and human glioblastoma (A172) cells. Very few studies have focused on low-power, long-term irradiation of cells with a widely tunable source. Our research examined non-thermal effects on cells exposed to radiation at low power with tunable frequencies from 70 GHz to 300 GHz. A widely tunable MMW source was set within a cell culture incubator. To avoid the effect of heat generation due to irradiation, the intensity was maintained below 10 μW and the device was arranged such that the irradiation came from underneath the cells. Irradiation was performed by sweeping from 70 GHz to 300 GHz in 1.0 GHz steps. The MMW source was positioned 100 mm away from the container in which the cells were cultured. Cells were exposed to MMWs for either 3, 70 or 94 h. Measurements of cell proliferation were made using the alternating current measurement method. We found no difference in proliferation between cells exposed to MMWs and unexposed cells. A colorimetric method using novel tetrazolium compound: MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] was used for cell activity and cytotoxicity assays. We found no difference in cellular activity or toxicity between MMW-exposed cells and sham cells. Our study thus found no non-thermal effect as a result of exposure of cells to 70 GHz to 300 GHz of radiation.

Keywords: non-thermal effect; widely tunable MMW source; exposure of cultured cells to electromagnetic fields; human fibroblast cell line

INTRODUCTION

Many studies have been carried out on the biological effects of millimeter wave (MMW) irradiation [1–9]. However, results have been mixed, with some studies reporting effects due to irradiation and others finding no effects. This discrepancy may be due to the varying irradiation frequencies, irradiation intensities, and target organisms used in previous studies. Thus, although practical applications of MMWs were expected, safety evaluations have not been concluded, especially regarding frequencies of ≤300 GHz. The European Commission’s Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) reported recent research trends and found that few studies have examined the biological effects of the several hundred GHz region [10]. More relevant research was deemed necessary, and SCENIHR encouraged new studies into the effects of exposure of skin and eyes to radiation (low-level, long-term exposure and high-level, short-term exposure, respectively).

In addition, Fröhlich proposed resonance vibrations in the cellular phospholipid bilayer membrane at frequencies ranging from 0.1 THz to 1 THz [11]. This hypothesis was based on the expectation that irradiation at weak intensities might cause some kind of non-thermal effect. This hypothesis has never been verified due to the lack of source of waves that vary over a wide range of frequencies. With the advancement of wave-source technologies in recent years, however, NTT Electronics Co. has developed the uni-traveling-carrier photodiode (UTC-PD), a wave-source with wide tunability, ideal for verifying Fröhlich’s hypothesis. In the past, the thickness of the cell...
membrane had been estimated to be 10 nm, although exact measurement was not then possible [12]. Molecular biology research in recent years has found that the cell membrane is 4 nm thick, and that sound velocity within the cell membrane is ~3 km s\(^{-1}\) [13]. From these findings, it is surmised that the specific frequencies that might evoke non-thermal effects would be within the range of several hundred GHz. Most of the hypothesis-verification research hitherto has used frequencies of <100 GHz [14–16], because there were no MMW sources with frequencies exceeding 100 GHz at that time. However, it is possible that frequencies that have not yet been tested could bring about a non-thermal effect.

The goal of our study was to determine whether irradiation at 70–300 GHz frequencies had effects on cells, and/or whether these frequencies have non-thermal effects on cell activities. To avoid the thermal effects of irradiation, we decided to use an MMW power source of 10 μW or less. Moreover, the resonance frequency in cells was investigated at 70–300 GHz by sweeping in 1 GHz increments. We examined cell proliferation and activity for cultured human skin fibroblast (NB1RGB) and glioblastoma (A172) tumor cell cultures. Since the MMWs do not penetrate the human body deeply, normal skin cell culture is used in our study. The proliferation rate of cancer cells differs from that of normal cells. Additionally, neuronal activity was changed by MMW exposure, as reported earlier [8, 9]. Thus, we selected glioblastoma cells. Cell proliferation was measured using an electrical impedance system capable of monitoring in real-time during exposure of cells to MMWs. Furthermore, cell activity upon MMW exposure was measured by a colorimetric method using bioreduction.

**MATERIALS AND METHODS**

**MMW source**

As an MMW source variable over wide frequencies, we used the UTC-PD (IOD-PMAN-13001; NTT Electronics Co., Yokohama, Japan), a technology that has undergone rapid development in recent years. The only one widely tunable MMW source is the UTC-PD. Using photomixing of two wavelength laser lights on the photodiode, the UTC-PD can generate MMWs in the range of 10 GHz to \(\geq 1\) THz in \(-1\) GHz steps [17]. To change the frequency, the loss time was 10 s per 1 GHz. MMWs irradiated through a silicon lens have a divergence angle of \(-15\)° [18]. We used an irradiation frequency of 70–300 GHz at a maximum power output of 10 μW. Irradiation was performed from the bottom of wells in which the cells were seeded, and the light source was set at a distance of 100 nm from the well bottom. Cells in wells of a 96-well plate were equally irradiated with MMWs owing to the silicon lens of the source. Although the incubator had been saturated with vapor, the absorption by vapor in the range of 70–300 GHz was at a negligible level because the distance travelled by the MMWs was short [19].

Exposure evaluations were performed by measurements using a Physikalisch-Technische Bundesanstalt (PTB)-calibrated pyroelectric sensor-type detector (THz20; Sensor und Lasertechnik, Neuenhagen, Germany) and an international output standard red-laser (633 nm) traceable pyroelectric-type sensor (THZ251-MT-BNC; Gentec-EQ, Quebec, Canada). The optical system required for measurements was set up outside the incubator. We confirmed an approximate output of 10 μW at 100 GHz, and 3 μW at 300 GHz.

Temperatures in the incubator chamber containing the MMW source were measured with a thermocouple (+channel digital thermometer, MT-309, MOTHERTOOL Co., LTD, Japan) in order to detect a temperature increase of 0.2°C and above due to MMW irradiation.

**Exposure apparatus used for cell proliferation measurements**

As the exposure apparatus for cell proliferation measurements, the head of the wave source UTC-PD was inserted into an aluminum pipe and set under the cells to be exposed. To avoid exposure of other cells, the bottoms of wells containing unexposed cells were covered in aluminum foil to block the MMWs from reaching the cells. This exposure apparatus was set in the incubator. Cell culture wells were fixed in place with a 2-mm thick polycarbonate plate. To expose the bottom of each well, a 0.785-cm² hole was opened in the middle of each polycarbonate plate. Well bottoms were made with a 0.7-mm thick quartz glass, and the MMW transmittance of each well bottom surfaces was \(-10\%\) (Fig. 1A). As the cells adhered to the bottom of the well, 10% of the incident MMW power reached them, i.e. 1.27 μW/cm² at 100 GHz and 0.38 μW/cm² at 300 GHz.

**Exposure apparatus for measuring cell activity**

The exposure apparatus for cell activity measurements was set up inside the incubator using a polystyrene board as an irradiation table. A 700 mm × 550 mm hole was made at the center of the cell culture placement site on the polystyrene board and MMWs were radiated from underneath the hole. A 96-well plate (Corning, 3596, USA) was used to measure cell activity and cytotoxicity. The MMW transmittance of the wells was 90%. As the cells adhered to the bottom of the well, 90% of the incident MMW power reached them, i.e. 0.23 μW/cm² at 100 GHz and 0.07 μW/cm² at 300 GHz. To compare the effects on exposed and sham cells, these two sets of cells were kept in individual, identical incubators (Fig. 1B).

**Cells used for exposure tests**

Two types of cell cultures were used in our tests: normal human skin fibroblast: NB1RGB (RCB0222; RIKEN BioResource Center, Tsukuba, Japan) and human glioblastoma cells: A172 (RCB2530, RIKEN BioResource Center). MEM 1xALPHA plus GlutaMax (32561-037; Thermo Fisher Scientific K.K., Yokohama, Japan) was used as the medium for the NB1RGB cells, and RPMI1640 (R8758; Sigma-Aldrich, Tokyo, Japan) was used as the medium for the A172 cells. Ten percent fetal bovine serum (HyClone; Thermo Fisher Scientific K.K., Yokohama, Japan) was added to each broth. Cells were maintained in an incubator set at 37°C with 5% CO₂ in a saturated vapor state.

**Cell proliferation measurement**

The alternating current (AC) impedance method (BM2401; HIOKI E.E.Co., Nagano, Japan) was used to measure cell proliferation. For this method, we used a dedicated measurement well (bottom area of 0.785 cm²), to the bottom of which was fixed an ultra-thin indium tin oxide (ITO) membrane electrode on quartz glass, which has a
transmittance of ~10% for MMWs. As cells that adhere to and grow on the ITO electrode impede current flow, changing the impedance value and creating a curve in the graph of cell growth. A reactance ratio was calculated from this measured impedance value, and thus cell growth was indirectly measured. Each well was seeded with 12,000 cells, then the cells were cultured for 24 h. An electrode pin was then set in the well and the cells were cultured for an additional 2 h. Impedance values were measured at 5-min intervals, with 100 kHz measurement frequency continuously over 94 h. Simultaneously, we irradiated the cells while sweeping from 70 GHz to 300 GHz in 1 GHz steps every 24.17 min. There were a total of 231 sweeping steps and the exposure time was 94 h. Absorbance at 490 nm was then measured. This assay was designed to ascertain whether cells showed evidence of toxicity after a 3-h exposure at 0 GHz, during which no difference in frequencies would have been generated.

Cytotoxicity assay
MTS was used in the cytotoxicity assay in a similar way to how it was used in cell activity measurements. The same number of cells were seeded as in the activity measurement method, and these cells were cultured for 70 h with no irradiation. After cells had reached 90–100% confluence, the MTS reagent was added. As cells were cultured for 3 h for colorimetric reactions, we irradiated the cells while sweeping from 70 GHz to 300 GHz in 1 GHz steps every 36 s. Absorbance at 490 nm was then measured. This assay was designed to ascertain whether the power of the pump lasers (2-wavelength total: 17 mW) as UTC-PD inputs had a thermal effect on cell activity, cells were irradiated with 0 GHz. The UTC-PD generated MMWs due to the difference in the frequency generation between the two lasers. The sham exposure refers to when the two lasers were switched off. On the other hand, the 0 GHz exposure means when the wavelengths of the two lasers were exactly the same, i.e. no MMW generation. Under these conditions, cell activity at 0 GHz irradiation was measured with the MTS method.

Data analysis
Measurements were independently performed three times for each respective test, and the mean value and standard deviation (SD) were calculated. In the MTS method used for cell activity measurements and toxicity assays, cell activity rate (%) was calculated as follows: \[ \left( \frac{\text{sample absorbance}}{\text{sham absorbance}} \right) \times 100 \]. We compared each unit of data between exposed cells and unexposed cells or sham cells, and significance tests (significance level 95%) were performed using the Tukey–Kramer multiple comparison test and the Welch test.

RESULTS
Cell proliferation measurement
Following the AC impedance method to measure cell proliferation, NB1RGB and A172 cells were irradiated for 94 h with frequencies ranging from 70 GHz to 300 GHz in 1 GHz steps every 24.17 min. As shown in Fig. 2, the temperature during incubation did not increase during the MMW irradiation. We found no difference in reactance ratios between exposed and unexposed NB1RGB cells (Fig. 3A). Similarly, we detected no change in the reactance ratio of the exposed A172 cells compared with the control cells (Fig. 3B). Microscopic observations of cells were made after measurement of proliferation; however, there was no finding of morphological changes due to cell damage (data not shown).
Cell activity measurement
The results of the activity measurements of cells irradiated for 70 h with frequencies ranging from 70 GHz to 300 GHz are shown in Fig. 3A. Seventy hours of irradiation did not significantly change the activity rates of either NB1RGB cells or A172 cells. Positive control cells were cultured for 70 h in an incubator chamber heated to 42°C. Compared with sham cells, the activity of positive control NB1RGB cells declined by 55% ($P < 0.05$), and the activity of the positive control A172 cells declined by 73% ($P < 0.05$). As confirmation that the power of the 2-wavelength lasers did not produce a thermal effect, we found that 70-h exposure to 0 GHz did not affect cell activity rates (Fig. 4A).

Cytotoxicity assays
We used toxicity assays to test whether exposure of cells to MMWs caused cytotoxicity. After culturing for 70 h, MTS reagent was added, and these cells underwent a colorimetric reaction assay for 3 h. During this reaction time, cells were irradiated with frequencies ranging from 70 GHz to 300 GHz. We found no significant decline in absorbance for exposed cells or for sham cells (Fig. 4B). As a positive control, cells were treated with toxic DMSO. We found that cell activity declined in correlation with increased concentrations of DMSO in more than 0.35 M (Fig. 5). At the highest concentration of 1.4 M DMSO, cell activity rates had significantly ($P < 0.05$) declined for the NB1RGB cells by 87%, and for the A172 cells by 95%, compared with sham cells (Fig. 4B).

DISCUSSION
The aim of this study was to address the need for research regarding biological effects on skin of low-level, long-term exposure to THz fields, as specifically stated by the SCENIHR 2015 report. We irradiated cultured cells long-term at a low power, which evokes few thermal effects, in order to investigate non-thermal effects.

In investigating a possible biological effect, exposure at a specific frequency has often been described, but the use of a widely tunable MMW source has seldom been reported. In this study, we irradiated different cells with MMWs during sweeping at increments of 1 GHz. Since the MMWs do not penetrate deep into the human body, we considered their effect on skin. We selected normal skin cells and investigated the effect on cells during MMW exposure for 3–94 h. We found no difference between reactance values of cells irradiated during their growth phase for 94 h and those of unexposed cells (Fig. 3A). As a positive control, we added 0.07 M DMSO, then the reactance values declined and the cells did not grow, which reflected its cytotoxicity (Fig. 3A and B). After measuring the proliferation in culture wells to which DMSO had been added, microscopic observation revealed that the cells had died (data not shown), thereby confirming that our method was able to successfully measure proliferation of cells in their growth process.

Williams et al. (2013) exposed human epithelial cells and human embryonic stem cells to high-intensity THz waves for 3–6 h and reported that there were no effects on cell growth [21]. The positive effect of neuronal activity has been reported during short-term exposure.
exposure of 60 GHz with low intensity [8]. We exposed cultured cells to long-term, low-power and continuous waves. From our results for cell proliferation, we concluded that continuous MMW exposure for 94 h does not affect cell growth, and has no additional non-thermal effects.

The same results were also obtained in tests performed using the MTS method, and activity rates of NB1RGB and A172 cells irradiated for 3 h. Sham cells, exposed cells and positive control cells treated with 1.4 M dimethyl sulfoxide (DMSO) added, and cells exposed to 0 GHz are shown. The results represent the mean values ± SD of three independent replicates.

Fig. 4. Activity of cells exposed to frequencies ranging from 70 GHz to 300 GHz. (A) Activity of NB1RGB and A172 cells irradiated for 70 h measured using the MTS method. Sham cells, exposed cells and positive control cells cultured in a 42°C incubator, and cells exposed to 0 GHz are shown. (B) Cytotoxicity assay results obtained using the MTS method, and activity rates of NB1RGB and A172 cells irradiated for 3 h. Sham cells, exposed cells and positive control cells treated with 1.4 M dimethyl sulfoxide (DMSO) added, and cells exposed to 0 GHz are shown. The results represent the mean values ± SD of three independent replicates.

0.43 mW was reported to have induced spindle abnormalities. Although our study included frequencies at levels similar to those in their report, we found no evidence of damage to the exposed cells. Nevertheless, it is possible that cells exposed to high-power MMWs were damaged due to thermal effects. As shown in Fig. 3, we found no decline in cell proliferation. It has been reported that cells irradiated for 2, 8 and 24 h at 0.106 THz and intensities of 0.04–2 mW/cm² presented no evidence of genetic damage [5]. Exposure to 0.12 THz radiation of 5 mW/cm² for 24 h had no significant effect on morphological changes in human eye cells that were not indicative of genotoxicity [23]. We also irradiated cells at a frequency of 0.106 THz or 0.12 THz. Although the respective irradiation intensities differed, we obtained similar results.

As a positive control to measure cellular activity, DMSO was added over a range of concentrations. We found that high concentrations of DMSO caused reduced cell activity and cytotoxicity (Fig. 5). Cells cultured for 70 h at 42°C, a temperature that causes heat shock, exhibited a decrease in cell activity and cell damage (Fig. 4A). We thus confirmed that cytotoxicity can induce a decline in cellular activity. However, MMW irradiation did not result in a decrease in cell activity, indicating that MMWs did not cause any cytotoxicity. We also irradiated cells at 0 GHz, which means no difference frequencies generation, but inputted the 2-wavelength laser lights into the UTC-PD were not affecting cell activity in a heat-related manner (Fig. 4A and B). Our results showed that there was no change in cell activity and no cytotoxicity as a result of irradiation for 70 h with frequencies sweeping from 70 GHz to 300 GHz. With regard to the Fröhlich hypothesis concerning resonance vibration and non-thermal effects within the cell membrane [12], our present study was unable to confirm the phenomena affecting cell proliferation and cell activity by sweeping from 70 GHz to 300 GHz in 1.0 GHz steps.

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CONFLICT OF INTEREST
The authors have declared that there are no conflicts of interest.
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