Comparison of Hsps Expression after Radio-frequency Field Exposure in Three Human Glioma Cell Lines

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Objective To investigate and compare the effect of radio-frequency (RF) field exposure on expression of heat shock proteins (Hsps) in three human glioma cell lines (MO54, A172, and T98). **Methods** Cells were exposed to sham or 1950 MHz continuous-wave for 1 h. Specific absorption rates (SARs) were 1 and 10 W/kg. Localization and expression of Hsp27 and phosphorylated Hsp27 ((78) Ser) (p-Hsp27) were examined by immunocytochemistry. Expression levels of Hsp27, p-Hs27, and Hsp70 were determined by Western blotting. **Results** The Hsp27 was primarily located within the cytoplasm, p-Hsp27 in both cytoplasm and nuclei of MO54, A172, and T98 cells. RF field exposure did not affect the distribution or expression of Hsp27. In addition, Western blotting showed no significant differences in protein expression of Hsp27 or Hsp70 between sham- and RF field-exposed cells at a SAR of 1 W/kg and 10 W/kg for 1 h in three cells lines. Exposure to RF field at a SAR of 10 W/kg for 1 h slightly decreased the protein level of phosphorylated Hsp27 in MO54 cells. **Conclusion** The 1950 MHz RF field has only little or no apparent effect on Hsp70 and Hsp27 expression in MO54, A172, and T98 cells.

Key words: Radio-frequency field; Heat shock protein 70; Heat shock protein 27; Protein expression; Phosphorylated Hsp27; Glioma cells

INTRODUCTION

In recent years, use of mobile phones has become popular in the world. Two common frequency bands are used in global system for mobile communication (GSM) - 850 MHz and 1900 MHz. Since mobile phones emit RF fields, considerable attention has been given to the issue whether exposure to RF generates adverse effects. Although a large number of epidemiological and experimental investigations have been conducted, the safety and potential biological effects of RF remain largely unknown. Some epidemiological studies have reported that cellular telephone exposure can result in a significantly increased risk of developing brain tumors[1], but their findings have not been confirmed by other epidemiological studies[2-3]. Results from experimental investigations are also controversial. It was reported that RF field can augment and suppress human cellular proliferation[4-5]. It has been shown that non-thermal RF-field exposure fails to elicit any detectable biological effect on three human-derived cell lines (TK6, HL60, and Mono-Mac-6)[6]. Conflicting results are also avaible regarding the genotoxic effects induced by exposure to RF fields[7-8]. Heart shock protein (Hsp) is recognized to act as a molecular chaperone. Hsp synthesis is induced not only by hyperthermia, but also by a wide variety of toxic conditions[9]. It has been shown that electromagnetic fields exert stress upon cells, inducing heat shock response as seen in human HL-60 cells[10] and human endothelial cells[11]. It was reported that 1950 MHz RF field exposure does not change the expression level of Hsp27 or Hsp70 in MO54 cells, but slightly inhibits the phosphorylation of Hsp27 in MO54 glioma cells[12]. As one of the commonly used mobile phone frequency bands, whether 1950 MHz RF field has the same effects on glioma cells as on other cell lines is unknown. In this study, we investigated and compared the effect of RF field exposure on expression of stress response genes (Hsps) in three human glioma cell lines (MO54,

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A172, and T98).

MATERIALS AND METHODS

Cell Culture

MO54, T98 cells, and A172 cells, derived from human malignant glioma in different patients, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (GIBCO, BRL) at 37 ℃ in an atmosphere containing 95% air and 5% CO_2.

RF Field Setup and Exposure

Exponentially growing cells were cultured in 35 mm dishes (3×10^5 cells/dish) and exposed to sham conditions or 1950 MHz (corresponding to the middle frequency allocated at the uplink band of IMT-2000 from mobile phone terminals) continuous-wave for 1 h. The specific absorption rate (SARs) was 1 W/kg and 10 W/kg, respectively. According to the records of exposure apparatus, none of the exposure conditions produced a rise in temperature higher than 0.1 ℃. Cells treated at 43 ℃ for 3 h served as a positive control for the induction of Hsp expression.

Immunocytochemistry

Hsp27 and p-Hsp27 were detected using immunofluorescent staining. Immediately after the sham or RF field exposure, cells were washed with cold phosphate-buffered saline (PBS), and fixed with 3.7% formaldehyde. Cell membranes were permeabilized with 0.25% Triton X-100 in PBS at room temperature. Non-specific binding sites were blocked with 1% bovine serum albumin (BSA) in PBS for 30 min. Cells were incubated with anti-Hsp27 goat polyclonal antibody (1:100, C-20 Santa Cruz Biotechnology) or anti-p-Hsp 27(^{78}Ser) rabbit polyclonal antibody (Stressgene) for 1 h, washed, incubated with FITC-conjugated anti-goat or anti rabbit IgG (1:100, Santa Cruz Biotechnology), and examined under an Olympus fluorescence microscope. Photos were taken using a digital camera. Immuno-positive Hsp27 or p-Hsp27 cells were counted in five different microscope areas, and percentage of positive cells was calculated by dividing the number of positive cells by the total number of cells in five areas.

Western Blotting

After treatment, cells were washed once with ice-cold PBS, scraped into 1 mL of PBS, and pelleted. The celLytic^{TM} mammalian cell lysis/Extraction reagent (Sigma) was used for extraction of cell proteins. Protein concentrations were determined using the SmartSpec 3000 system (BIORAD, Tokyo, Japan). Samples were boiled for 3 min before loading onto a 12% SDS-polyacrylamide gel. After electrophoresis, the gel was electroblotted onto a Hybond-ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Sweden). Non-specific binding sites on the membrane were blocked at 4 ℃ overnight with 5% milk in Tris buffered saline with Tween 20 (TBST). Following antibodies were used in this experiment, including anti-Hsp27 goat polyclonal antibody (C-20 Santa Cruz Biotechnology), anti-Hsp70 mouse monoclonal antibody (StressGen Biotechnologies Corp), anti-phospho-Hsp27 (^{78}Ser) rabbit polyclonal antibody (Stressgene), anti-β-actin mouse monoclonal antibody (Sigma), anti-goat IgG horseradish peroxidase (Santa Cruz Biotechnology), anti-mouse IgG horseradish peroxidase (Amersham-Pharmacia Biotech), and anti-rabbit IgG horseradish peroxidase (Amersham-Pharmacia Biotech). The blot was visualized with an ECL kit (Amersham-Pharmacia Biotech). Densitometric analysis was performed using the ATTO Image Analysis Software.

Data Analysis

Statistical analyses were performed using the Student's *t*-test. $P\leqslant0.05$ was considered statistically significant.

RESULTS

Effects of RF Field Exposure on Localization and Expression of Hsp27 and p-Hsp27

Hsp27 was primarily located within the cytoplasm of MO54, A172, and T98 cells. Of these cells, 25% exhibited a high Hsp27 expression level and strong immunofluorescence (green), 75% showed a weak immunofluorescence in MO54 cells. However, the Hsp27 expression level was high and uniform in A172 and T98 cells. RF field exposure (10 W/kg, 1 h) did not affect the distribution or expression of Hsp27 in three cell lines (Fig. 1), consistent with the results of Western blotting. Following treatment at 43 ℃ for 3 h, a large portion of Hsp27 was translocated into nuclei. Immunofluorescence within the cytoplasm became weak or disappeared completely (Fig. 1). As shown in Fig. 2, p-Hsp27 immunofluorescence was located both in cytoplasm and in nuclei. After RF field exposure (10 W/kg, 1 h), the p-Hsp27 immunofluorescence became slightly weaker in MO54 cells than in A172 and T98 cells compared

with the control cells. Following treatment at 43 ℃ for 3 h, the p-Hsp27 immunofluorescence became stronger in nuclei and weaker in cytoplasm of three cell lines, due to the phosphorylation and translocation of some Hsp27 into nuclei (Figs. 1 and 2).

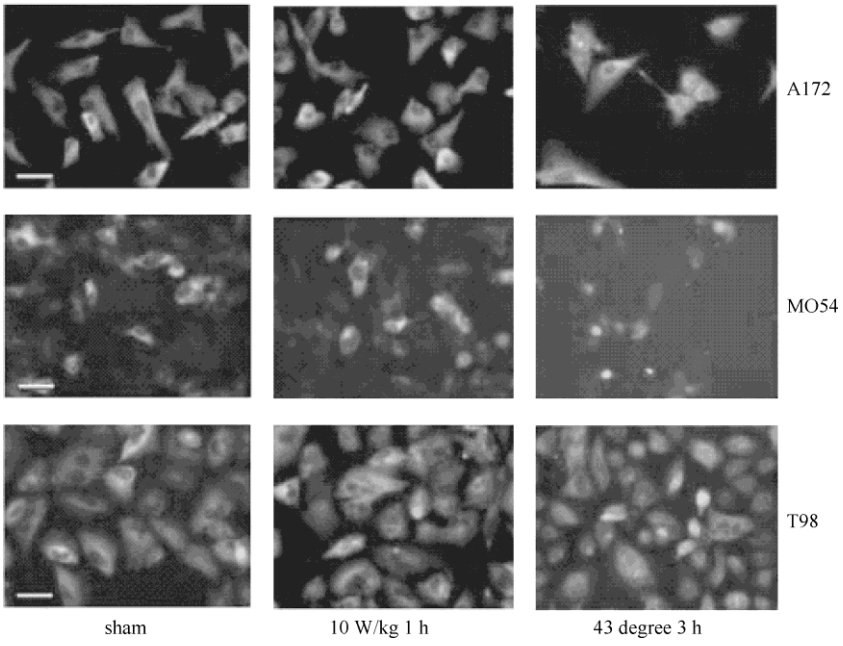

FIG. 1. Cellular localization of Hsp27 after RF field exposure. One hour after sham or RF field exposure (SAR: 10 W/kg), cells were fixed in 3.6% formaldehyde and immunostained with anti-Hsp27 antibody followed by FITC (green) conjugated secondary antibody. Bar=50 μm.

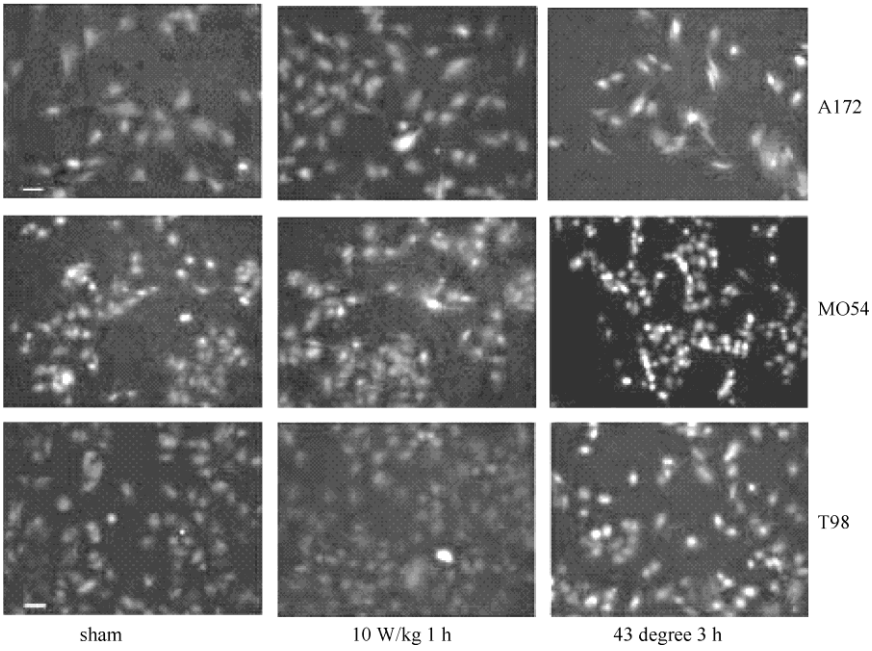

FIG. 2. Cellular location of p-Hsp27 after RF field exposure. One hour after sham or RF field exposure (SAR: 10 W/kg), cells were fixed in 3.6% formaldehyde and immunostained with anti-p-Hsp27 antibody followed by FITC (green) conjugated secondary antibody. Bar=50 μm.

No difference was found in Hsp27 expression level between A172, MO54, and T98 cells exposed to RF fields (SAR: 1 W/kg, 10 W/kg) for 1 h and sham-exposed cells (Fig. 3). Since phosphorylation of Hsp27 is important for its function[13-14], the level of phosphorylated Hsp27 was measured using an antibody that recognizes phosphorylated Hsp27, specific for phosphorylation on the serine 78 site of Hsp27. As shown in Fig. 3, there was no difference in phosphorylated Hsp27 level between A172, MO54, and T98 cells exposed to RF fields (SAR: 1 W/kg) for 1 h and sham-exposed cells. However, exposure to RF field at 10 W/kg for 1 h slightly decreased the level of phosphorylated Hsp27 in MO54 cells other than in A172 and T98 cells (Fig. 3). Treatment at 43 ℃ for 3 h obviously increased the level of phosphorylated Hsp27 in three cell lines (Fig. 3).

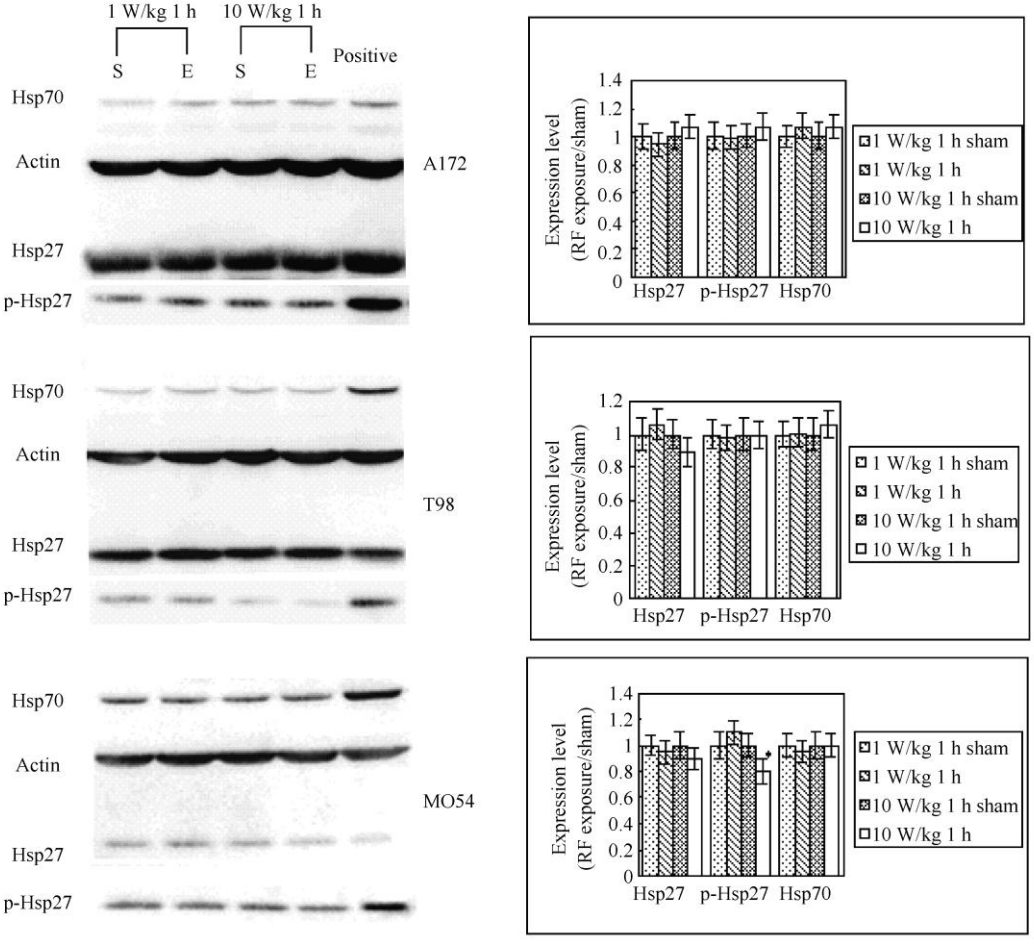

FIG. 3. Representative Western blots and expression ratio (RF exposure/sham) of Hsp27, p-Hsp27, and Hsp70 in A172, MO54 and T98 cells after exposure to RF field. Exponentially growing cells were exposed to RF field for 1 h at SARs of 1 W/kg and 10 W/kg, and lysates were prepared immediately after exposure. Western blot analysis was performed as described in the section "Materials and Methods". The experiment was performed at least twice. $^{*}P<0.05$ *vs* sham-exposed cells.

Effects of RF Field Exposure on Hsp70 Expression

The expression of Hsp70 in A172, MO54 and T98 cells after exposure to RF fields (SAR: 1 W/kg and 10 W/kg) for 1 h and the ratio of exposure to sham conditions are shown in Fig. 3. There was no obvious change in Hsp70 expression between RF field-exposed cells and sham-exposed cells. Treatment at 43 ℃ for 3 h obviously increased the level of Hsp70 in three cell lines.

As shown in Fig. 4, the morphologies of RF field-exposed (10 W/kg, 1 h) cells and sham-exposed cells

were nearly identical under phase-contrast microscope. Meanwhile, round shape MO54 and T98 cells and fusiform shape A172 cells were observed following treatment at 43 ℃ for 3 h.

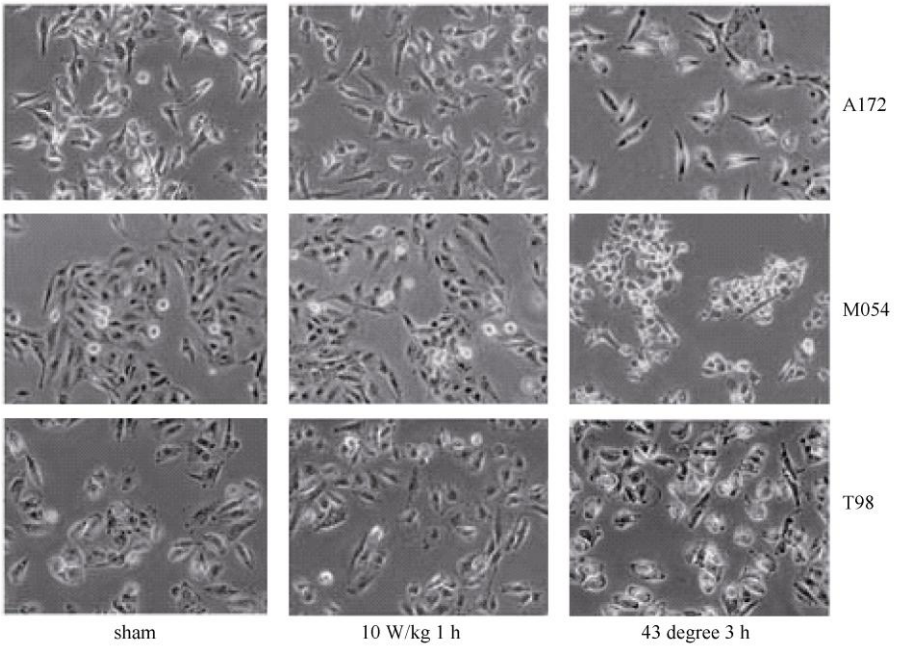

FIG. 4. Morphology of A172, MO54, and T98 cells after exposure to RF field for 1 h at a SAR of 10 W/kg (×100).

DISCUSSION

In this study, the effect of RF field exposure on expression of stress response genes (Hsps) in three human glioma cell lines (MO54, A172, and T98) were investigated and compared. The results show that 1950 MHz RF field had little or no apparent effect on Hsp70 and Hsp27 expression in MO54, A172, and T98 cells, which is consistent with the previous findings[15]. The result of RF-induced slight inhibition of Hsp27 phosphorylation in MO54 cells was successfully replicated in this study, but this result could not be found in two other cell lines (A172 and T98), suggesting that slight inhibition of Hsp27 phosphorylation is only a specific effect of RF field exposure on MO54 cells. In addition, our data show that RF field exposure did not affect the morphology of MO54, A172, or T98 cells, indicating that RF field couldoes not contribute to some adverse effects on cultured human glioma cells.

It was reported that RF field energy primarily acts as a stress inducer, causing changes in protein conformation, which in turn symulates synthesis of Hsp in exposed cells and tissue. These changes may disturb the normal regulation of cells, finally leading to an increased risk of developing cancer[16]. Whether RF field exposure from mobile phones constitutes to environmental stress has aroused many researchers' interest. Hsp27 and Hsp70 have been reported as the most inducible molecules of Hsp family members under stressful conditions[17]. Hsp70 protects cells against a variety of stresses, including heat shock, anoxia, and heavy metals[18]. It was reported that exposure to 2.45 GHz electromagnetic fields for 2-16 h induced Hsp70 at a high SAR of more than 20 W/kg but not at 5 W/kg in human glioma MO54 cells[19], which is consistent with the present finding that exposure to a 1950 MHz RF field for 1 h had no effect on Hsp70 expression in A172, T98, and MO54 cells when the SAR was less than 10 W/kg (Fig. 3). However, Weisbrot *et al.*[20] found that non-thermal radiation from GSM mobile phones elicites a rapidly elevated Hsp70 level with *Drosophila melanogaster*. Kwee *et al.*[21] reported that the expression of Hsp70, instead of Hsp27, is induced when transformed human epithelial amnion cells are exposed to a GSM signal of 960 MHz at a SAR of 0.0021 W/kg for 20 min. Fritze *et al.*[22] measured the level of Hsp70 messenger RNA in rat brain after exposure to RF

fields from mobile phones, and found that a continuous wave exposure of 7.5 W/kg (but not 1.5 W/kg) slightly increases the hsp70 messenger RNA level in cerebellum and hippocampus. RF/microwave radiation (27/2450 MHz, CW signal) at a much higher SAR (25 and 100 W/kg) for 2 h fails to induce heat shock response in HeLa and CHO cells[23]. No significant difference in Hsp70 and Hsp27 expression can be detected after exposure to a 900 MHz RF field in human leukocytes[24]. Hsp27, a cytoplasmic protein, phosphorylates and translocates to nuclei in response to stress[25]. Phosphorylation of Hsp27 is an early sign of cell response to stress factor. Cellular Hsp27 is in an unphosphorylated state of unstressed cultured cells. Exposure to thermal stress for minutes, the unphosphorylated Hsp27 level decreases while the phosphorylated Hsp27 level increases[26-27]. In the present study, Hsp27 was located mainly within the cytoplasm of three glioma cell lines under normal conditions (Fig. 1), and a large portion of Hsp27 was translocated into the nuclei following treatment at 43 ℃ for 3 h. Exposure to RF field(s) failed to affect the distribution and expression of Hsp27 in MO54, A172, and TG98 cells (Figs. 1 and 3). It has been reported that Hsp27 phosphorylation but not synthesis of Hsp can be induced[28]. In order to study whether RF field affects the phosphorylation of Hsp27, the phosphorylated Hsp27 level was examined using anti-Hsp27 antibody specific for phosphorylated Hsp27. The phosphorylated Hsp27 level was slightly inhibited only in MO54 cells exposed to RF field (SAR: 10 W/kg), suggesting that RF field exposure (SAR: 10 W/kg) can inhibit the constitutive phosphorylation of Hsp27 in MO54 cells.

It has been shown that exposure to mobile phone signals can affect cell morphology[29]. However, the morphology of three cell lines remained unchanged after RF field exposure in our study, suggesting that RF field at a SAR of up to 10 W/kg has little effect on morphology of human glioma cells. In addition, Stagg's[30] reported that 836.55 MHz, packet-modulated RF field at three power densities: 0.09, 0.9, and 9 mW/cm^2 (SARs ranging from 0.15 to 59 mW/g) does not increase cell proliferation in normal or transformed cultures of glial origin, suggesting that RF field exposure at 1950 MHz is not associated with cancer.

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