Microwaves From UMTS/GSM Mobile Phones Induce Long-Lasting Inhibition of 53BP1/γ-H2AX DNA Repair Foci in Human Lymphocytes

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We have recently described frequency-dependent effects of mobile phone microwaves (MWs) of global system for mobile communication (GSM) on human lymphocytes from persons reporting hypersensitivity to electromagnetic fields and healthy persons. Contrary to GSM, universal global telecommunications system (UMTS) mobile phones emit wide-band MW signals. Hypothetically, UMTS MWs may result in higher biological effects compared to GSM signal because of eventual "effective" frequencies within the wideband. Here, we report for the first time that UMTS MWs affect chromatin and inhibit formation of DNA double-strand breaks co-localizing 53BP1/γ-H2AX DNA repair foci in human lymphocytes from hypersensitive and healthy persons and confirm that effects of GSM MWs depend on carrier frequency. Remarkably, the effects of MWs on 53BP1/γ-H2AX foci persisted up to 72 h following exposure of cells, even longer than the stress response following heat shock. The data are in line with the hypothesis that the type of signal, UMTS MWs, may have higher biological efficiency and possibly larger health risk effects compared to GSM radiation emissions. No significant differences in effects between groups of healthy and hypersensitive subjects were observed, except for the effects of UMTS MWs and GSM-915 MHz MWs on the formation of the DNA repair foci, which were different for hypersensitive ($P < 0.02$[53BP1]//0.01[γ-H2AX]) but not for control subjects ($P > 0.05$). The non-parametric statistics used here did not indicate specificity of the differences revealed between the effects of GSM and UMTS MWs on cells from hypersensitive subjects and more data are needed to study the nature of these differences. Bioelectromagnetics
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INTRODUCTION

Microwave (MW) exposures vary in many parameters: power (specific absorption rate, incident power density), wavelength/frequency, near field–far field, polarization (linear, circular) continuous wave (CW) and pulsed fields (pulse repetition rate, pulse width or duty cycle, pulse shape, pulse to average power, etc.), modulation (amplitude, frequency, phase, complex), overall duration and intermittence of exposure (continuous, interrupted), acute and chronic exposures. With increased absorption of energy, thermal effects of microwaves are observed that deal with MW-induced heating. Specific absorption rate (SAR) or power flux density (PD) is a main determinant for the thermal MW effects. Many other physical parameters of exposure have been reported to be important for non-thermal biological effects, which are induced by MWs at intensities well below any heating. Reports of non-thermal effects started appearing in the 1970s and have previously been reviewed [Adey, 1981, 1999; Blackman, 1984, 1992; Gründler et al., 1988; Iskin, 1990; Devyatkov et al., 1994; Pakhomov et al., 1998; Belyaev et al., 2000; Betskii et al., 2000; Banik et al., 2003; Grigoriev et al., 2003; Grigoriev, 2004; Lai, 2005]. Some studies have reported stress response in exposed cultured cells [Kwee et al., 2001; Leszczynski et al., 2002; Blank and Goodman, 2004; Czyz et al., 2004]. In other studies, no effects of non-thermal microwaves were observed as it has recently been reviewed [Meltz, 2003]. Dependence of the MW effects on several physical parameters, including frequency, polarization, modulation and several biological variables could explain various outcomes of studies with non-thermal MWs [Adey, 1981, 1999; Blackman, 1984, 1992; Belyaev et al., 2000; Belyaev, 2005a]. Among other dependencies, the dependence of non-thermal effects of MWs on frequency has been reported [Pakhomov et al., 1998; Belyaev et al., 2000]. Frequency-dependent interactions of MWs with such targets as cellular membranes, chromosomal DNA, radicals, proteins and ions in protein cavities may be involved in effects of MWs [Ismailov, 1987; Belyaev et al., 1992b; Chiabrera et al., 2000; Binhi, 2002; de Pomerai et al., 2003; Ritz et al., 2004]. However, there is substantial lack of knowledge in biophysical modeling of MW-induced non-thermal biological effects.

It has been described that MWs under specific conditions of exposure either inhibited repair of radiation-induced DNA damage [Belyaev et al., 1992a,b,c,d, 1993] or induced single- and double-stranded DNA breaks (DSBs) [Lai and Singh, 1996; Lai and Singh, 1997]. The mechanisms of these effects are not understood but could be related to the induced changes in interaction of DNA with proteins [Belyaev et al., 1999].

Several proteins involved in DNA repair and DNA damage signaling such as phosphorylated H2AX (γ-H2AX) and the tumor suppressor TP53 binding protein 1 (53BP1) have been shown to produce discrete foci that co-localize to DSBs [Rogakou et al., 1999; Schultz et al., 2000; Rappold et al., 2001; Fernandez-Capetillo et al., 2002; Sedelnikova et al., 2002; Kao et al., 2003]. These foci are referred to as DNA repair foci and their identification is considered to be the most sensitive technique to study DSB. This technique allows measurement of a single DSB per cell.

The γ-H2AX and 53BP1 proteins are phosphorylated in response to DNA damage providing a scaffold structure for DSB repair [DiTullio et al., 2002]. According to the current model, this scaffold functions by recruiting proteins involved in the repair of DSB [Fernandez-Capetillo et al., 2002; Iwabuchi et al., 2003; Kao et al., 2003]. The scaffold is organized on a megabase-size chromatin domain containing a DSB regardless of the repair pathway that is involved in processing DSBs [Rogakou et al., 1999; Paull et al., 2000; Mochan et al., 2004]. Thus, identification of DNA repair foci provides ultimate sensitivity to detect DSBs regardless of the mechanism of their formation and repair.

We have recently described the effects of mobile phone MWs of global system for mobile communication (GSM) on chromatin conformation and 53BP1/γ-H2AX DNA repair foci in human lymphocytes from hypersensitive and healthy persons [Sarimov et al., 2004; Belyaev et al., 2005; Markova et al., 2005]. These data have shown that stress response, DNA repair inhibition and/or DNA damage is induced by GSM MWs under specific conditions of exposure and dependent on carrier frequency. Contrary to GSM, universal global telecommunications system (UMTS) mobile phones emit wide-band, 5 MHz, signals. MWs representing wide-band signal may hypothetically result in higher biological effects since they may include "effective" frequency windows, presumably around 1–10 MHz, in the frequency range of mobile communication, 200–2000 MHz [Sarimov et al., 2004]. In our previous studies, we analyzed effects of GSM MWs immediately after exposure. In the present study we investigated effects of UMTS MWs in comparison to the effects of GSM MWs within 72 h post-exposure. Another aim of our project was to compare the response of cells from hypersensitive and healthy persons. In a Dutch study, the group of persons recruited based on their experience of being sensitive to MWs and the healthy control group reported reduced well-being during exposure to UMTS MWs [Zwambron et al., 2003]. The reported hypersensitivity in humans to electromagnetic field (EMF) is a fairly new phenomenon and the etiology of the phenomenon is not

yet known. There are several symptoms that hypersensitive people report when they are in the proximity of different sources of EMF such as video display terminals of personal computers, electrical appliances or mobile phones. The symptoms are not specific to this illness and there is no known pathophysiological marker or diagnostic test [Hillert et al., 1999]. In studies aimed at identifying possible health effects of MWs it is of interest to include groups that may have an increased sensitivity to this exposure. Therefore, cells were included in this study from persons who, based on their own experience and ill health, report to have such hypersensitivity.

MATERIALS AND METHODS

Donors and Blood Samples

Blood samples were obtained from five healthy donors and five patients reporting hypersensitivity to EMF. Patients referred to the Department of Occupational and Environmental Health, Stockholm County Council, who reported hypersensitivity to EMF including microwaves from mobile phones were asked to participate in the study. The first patients to give consent to participate were included in the study.

The group reporting hypersensitivity to EMF consisted of four women and one man, 28–49 years old (Table 1, Supporting Information). Control healthy subjects were matched by age (±6 years) and gender. In the hypersensitive group one person was working, one was unemployed and three persons were on sick leave or received sickness compensation. Aforementioned hypersensitive persons were sick because of hypersensitivity to EMF, that is, no other causes of their ill health were identified in the medical work-up. There were no smokers among the participants and no subject was on any regular medication. All hypersensitive subjects reported symptoms triggered by electrical equipment including mobile phones that were not sources of light (in all five cases) and were characterized with regard to the symptom profile, triggering factors, time relation and avoidance behavior [Hillert et al., 1999]. In all pairs, the hypersensitive person scored higher than the matched control in the questionnaire on symptoms; mean score 86 compared to 12 (29 symptoms scored 0–4 for frequency and severity, maximum score 232) [Hillert et al., 1998]. In four of the persons reporting hypersensitivity to EMF the neurovegetative symptoms headache, fatigue and difficulties concentrating were more pronounced than skin symptoms. The mean score per question and person for neurovegetative symptoms was 2.3 in the hypersensitive group and 0.4 in the control group (maximum 4). The corresponding score for skin symptoms in the face and upper chest were 1.7 and 0.1, respectively. In all cases of reported hypersensitivity the symptoms were experienced within 24 h after exposure to a reported triggering factor, in most cases within 1 h. All patients reported that they tried to avoid triggering factors.

Fresh blood samples from persons reporting hypersensitivity and matched controls were coded and all data were analyzed in blind. Ethical permission was obtained from the Ethic Committee of the Karolinska Institutet, Stockholm, Sweden.

Chemicals and Reagents

Reagent grade chemicals were obtained from Sigma–Aldrich (St. Louis, MI) and Merck (Darmstadt, Germany). Double cytoslides coated with polylysine and cytoslide chambers were purchased from Shandon (Pittsburg, PA). Anti-53BP1 mouse antibody was kindly provided by Dr. T. Halazonetis, The Wistar Institute, University of Pennsylvania, Philadelphia, PA, USA. The antibody recognizes the C-terminal domain of the protein that corresponds to the BRCT domains. Anti-Phosphorylated histone H2AX (γ-H2AX) rabbit antibody was purchased from Trevigen-BioSite (Täby, Sweden).

Cells

Lymphocytes were isolated 30 min after drawing the peripheral blood by density gradient centrifugation in Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) according to the manufacturer's instructions. The cells were transferred to basal medium (BM): RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco, BRL, Gaithersburg, MD) at 5% CO_2 and 37 °C in a humidified incubator. Adherent monocytes were removed by overnight incubation of the cell suspension in culture flasks (Falcon) at the cell density of 3×10^6 cells/ml in the volume of 10–40 ml. After this incubation, the cells in suspension were collected by centrifugation. The cell density was adjusted to approximately 2×10^6 cells/ml in fresh BM and the lymphocytes were pre-incubated for 2 h at 37 °C before exposure. The viability of cells was always above 98% as measured with trypan blue exclusion assay at the beginning of exposure and the fraction of blue cells did not exceed 10% at the end of cultivation. At different time points, samples were taken for assessment of apoptotic morphological changes. After staining with fluorescent dyes (acridine orange and propidium iodide), the cells with morphological changes characteristic for apoptosis, such as chromatin condensation, fragmentation of nuclei and

nuclei shrinkage, were scored by using fluorescence microscope as previously described [Belyaev et al., 2001].

Cell Exposure

Two samples with lymphocytes from matched hypersensitive and healthy persons, were simultaneously exposed to either GSM (905 MHz or 915 MHz) or UMTS (1947.4 MHz\, middle channel), output power being the same, 0.25 W. Exposure of cells in 14 ml round-bottom tubes (Falcon), to GSM and UMTS MWs were performed using two specially designed installations, each based on a transverse electromagnetic line cell (TEM-cell) and a test mobile phone. The construction of the TEM-cells allowed relatively homogeneous exposure of samples in these specific frequency ranges [Martens et al., 1993; Malmgren, 1998]. Cells from each person were exposed at each exposure condition once in one tube.

All exposures were performed at 37 °C in a CO_2-incubator, in Falcon tubes, 2.5 ml of cell suspension per tube, 2×10^6 cells/ml. Duration of all exposures was 1 h. Lymphocytes were exposed to MWs using either a GSM900 test-mobile phone (model GF337, Ericsson, Lund, Sweden) or a UMTS/GSM test-mobile phone (model 6650, Nokia, Helsinki, Finland) as previously described [Sarimov et al., 2004; Belyaev et al., 2005]. The output of each phone was connected by the coaxial cable to the correspondent TEM-cell. For GSM900 exposure we used the channels 74 and 124 with the frequencies of 905 and 915 MHz, respectively. The GSM signal included standard modulation, Gaussian Minimum Shift Keying (GMSK). Discontinuous transmission mode was off during all exposures. For UMTS exposure we used 1947.4 MHz middle channel, 5 MHz-wide band. The UMTS signal included standard modulation, Quadrature Phase Shift Keying (QPSK). Voice modulation was applied neither in GSM nor in UMTS exposures. The power was kept constant during exposures to GSM and UMTS as monitored on-line using either a power meter (Bird 43, Bird Electronic, Cleveland, OH) or a power meter (Hewlett-Packard 435A, Palo Alto, CA), respectively.

The specific absorption rate (SAR) was determined by measurements and calculations. Transmitted and reflected power was measured using a power meter (Hewlett-Packard 435A) and a coaxial directional coupler (Narda 3001-20, Hauppage, NY). A signal generator (Agilent 7648C, Santa Rosa, CA) connected to a power amplifier (Mini-circuit ZHL-2-8-N, Brooklyn, NY) was used. The SAR was calculated from the absorbed power and the mass of the sample to be 37 mW/kg for the frequency of 915 MHz and 40 mW/kg for the frequency of 1947 MHz. Good correlation between these measurements and calculations using the finite different time domain (FDTD) method has been observed [Sarimov et al., 2004]. The SAR value varied from 15 to 145 mW/kg at different locations of the exposed samples as calculated with FDTD using 0.75 mm $\times$ 0.75 mm $\times$ 0.75 mm size cells. More than 50% of cells had SAR values between 20 and 40 mW/kg. The measurement uncertainty budget for our setups has been accessed according to Nikoloski et al. [2005]. The uncertainty budget of the exposures did not exceed 48% with a confidence level of 95%. Taking into account all possible uncertainties, the SAR values in all cells were always well below thermal effects. Changes of frequency by 10 MHz change neither the SAR value nor the SAR variation in the exposed samples. In our TEM-cells, the measured power loss did not exceed 1.2% and that could not cause any temperature rise. Our TEM-cells were well ventilated through the special holes in the wooden cages of the TEM-cells. Temperature was measured in the MW-exposed samples before, during and after exposure with a precision of 0.1 °C. No changes in temperature were induced during exposures.

Sham exposures were performed in the same TEM-cells as MW exposures with MW power off. The order of MW- and sham-exposures was randomized among sessions. In each experiment and for each donor, the sham exposures were performed in duplicate, in the TEM-cell for GSM exposure and in the TEM-cell for UMTS exposure. No differences were observed between sham-exposed samples (sham–sham exposures) and the data from two sham exposures were pooled for comparison with exposed samples. The 1-h heat treatment in a water bath, 41 °C, was used as a positive control for stress response. As a positive control for genotoxic effect, the cells were irradiated with ^{137}Cs γrays, 3 Gy, using a Gammacell 1000 (Atomic Energy of Canada, Ottawa, Canada) source. The dose rate was 10.6 Gy/min.

AVTD Measurements

The conformation of chromatin was studied by the method of anomalous viscosity time dependencies (AVTD). This technique was shown to be a sensitive assay to measure genotoxic effects and stress response [Belyaev et al., 2001; Sarimov et al., 2004; Torudd et al., 2005]. Cell lysis was performed immediately after exposure as has been previously described [Belyaev et al., 1999]. Briefly, lymphocytes were lysed in polyallomer centrifuge tubes (14 mm, Beckman, Fullerton, CA) by addition of 3.1 ml lysis solution (0.25 M Na_2EDTA, 2% (w/v) sarcosyl, 10 mM Tris-base, pH 7.4) to 0.1 ml of cell suspension. The lysates were prepared in triplicate and kept at 23 °C for 4 h in

darkness before AVTD measurements. The AVTDs in lysates were measured using an AVTD-analyzer (Archer-Aquarius, Moscow, Russia) as described previously [Belyaev et al., 1999]. The AVTDs were measured at the shear rate of 5.6 s^{-1} and shear stress of 0.007 N/m^2. For each experimental condition, AVTD was measured in three replicates. AVTD parameters were described in detail previously [Belyaev et al., 1998, 1999]. Briefly, the AVTD is characterized by three main parameters: (1) maximum value of viscosity; (2) area under AVTD, and (3) time for maximum viscosity. All these parameters depend on conformation, rigidity and molecular weight of nucleoids [Belyaev et al., 1999]. Normalized relative viscosity (NRV) measured as normalized ratio of maximum viscosities in exposed and sham-exposed samples is the most sensitive parameter and was used here to characterize condensation of chromatin.

Immunostaining and Foci Analysis

Immediately after exposure, the cells were placed on ice for 1 h to prevent repair of eventual DSBs. Cytoslide samples were prepared by using cytospin centrifugation according to the manufacturer's instructions (Shandon). The immunostaining was performed according to Schultz et al. [2000] with some modifications. Cells were fixed in cold 3% paraformaldehyde in PBS, pH 7.4, permeabilized with cold 0.2% Triton X-100 in PBS (for 15 and 10 min, respectively), stained with primary mouse antibody 53BP1 (1:20) and primary rabbit antibody γ-H2AX (1:100) prepared in 2% FBS in PBS for 1 h, followed by 3 washes in cold PBS and incubated for 1 h with secondary goat anti-mouse IgG (H + L) antibody conjugated with Alexa fluor 488 (Molecular Probes, Eugene, OR) together with goat anti-rabbit IgG (H + L) antibody conjugated with Alexa fluor 555 (Molecular Probes), both in 2% FBS and in 1:200 dilution, followed by 3 washes in cold PBS. After 20 min DNA staining in ToPro (3-iodide, 1 mM stock solution in DMSO, Molecular Probes) and 5 min washing in PBS, cytoslides were mounted with equilibration solution and antifade reagent (Slow fade Light Antifade Kit, Molecular Probes) and sealed with cover slides. The images were recorded from 5 to 10 fields of vision that were randomly selected from two slides on the confocal laser scanning microscope Zeiss Axiovert 100 M using the planapochromat 63×/1.4-numerical-aperture oil immersion objective and the LSM 510 software. Optical magnification was 630. Through-focus maximum projection images were acquired from optical sections 1.00 μm apart and with a section thickness of 2.00 μm in the *Z*-axis. Resolutions in the *X*- and *Y*-axis were 0.20 μm. Five optical sections were usually obtained for each field of vision and the final image was obtained by projection of all sections onto one plane. For each independent exposure experiment and for each exposure condition (subject, type of exposure, duration after exposure), 300–600 cells were analyzed.

Spatial co-localization of 53BP1 and γ-H2AX foci was analyzed in all cells and samples as previously described [Markova et al., 2007]. Briefly, at least partially overlapping foci were considered to co-localize while co-localization was not counted in the cases of a distinct gap between 53BP1 and γ-H2AX foci.

Statistical Analysis

We set the statistical power to 0.80 based on previously obtained data on effects of GSM MWs on human lymphocytes [Sarimov et al., 2004; Belyaev et al., 2005]. The data were analyzed with the Mann–Whitney *U*-test, Kruskal–Wallis test or by the Wilcoxon matched pairs signed rank test. A correlation analysis was performed using Spearman rank order correlation test. Results were considered as significantly different at $P < 0.05$.

RESULTS

Chromatin Conformation

Irradiation with 3 Gy resulted in a statistically significant 3-fold increase in AVTD dealing with radiation-induced relaxation of chromatin (data not shown). In Table 2 (Supporting Information), primary data for all subjects obtained immediately after exposure to MWs are provided to document the variability in responses between different exposures/subjects. These data indicate possible individual variability in the effects. However, this conclusion seems to be premature. Repeated experiments with cells from the same donors are needed to prove individual variability; this was not the aim of our study. In those cases where heat shock significantly affected cells (donors 314, 315, and 906) a decrease in NRV that corresponds to chromatin condensation was observed immediately following 1 h treatment. MWs at the frequency of 915 MHz induced significant chromatin condensation in cells of four subjects (314, 315, 809, and 906) ($P < 0.05$, Mann–Whitney *U*-test). Significant decrease in NRV was also observed after exposure to MWs at 905 MHz in cells from three donors (314, 315, and 413). UMTS MWs at 1947.4 MHz resulted in significant condensation only in cells from donor 314. These data suggested that effects of MWs might be

frequency-dependent and various responses might be observed in cells from different individuals.

Although statistically significant chromatin condensation was observed in cells from some donors at all treatment conditions immediately after exposure (Table 2), no such consistent response was seen 24 h following exposure to MWs when chromatin could be either condensed or decondensed (not shown). We tested the hypothesis that effects of different treatments would differ between groups of hypersensitive and normal persons using Wilcoxon matched pairs signed rank test. Only four matched pairs were included in this analysis because no cells from donor 801 were available to study changes in chromatin conformation by the AVTD technique. No statistically significant differences in the effects on chromatin conformation were seen, $P > 0.05$, between cells from control and hypersensitive groups as measured after all treatment conditions either immediately or 24 h following exposure. Therefore, the data pooled from all subjects, normal and hypersensitive, were analyzed for each treatment condition (Fig. 1). Based on the pooled data, statistically significant chromatin condensation was found in lymphocytes immediately after MW exposure at 915 MHz and heat shock at 41 °C ($P < 0.05$, Mann–Whitney U-test). From all treatments, only heat shock resulted in significant effects ($P < 0.02$, Mann–Whitney U-test) in the pooled data obtained 24 h following treatments (Fig. 1).

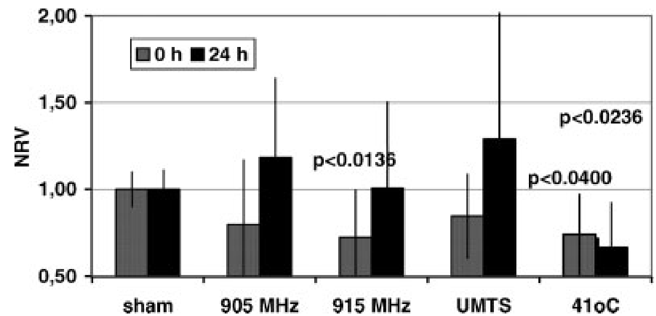

Fig. 1. The conformation of chromatin was studied by the method of anomalous viscosity time dependencies (AVTD) in five experiments with lymphocytes from nine subjects, four hypersensitive and five healthy subjects. Normalized relative viscosity (NRV) was used to characterize condensation of chromatin. For each subject and treatment condition the AVTD measurements of exposed and sham-exposed samples were performed in triplicate. Data are shown as mean and standard deviation (SD). In this figure and other histograms, P-values belong to bars that are situated below.

53BP1/γ-H2AX Foci

Irradiation of lymphocytes with 3 Gy resulted in a statistically significant increase in 53BP1/γ-H2AX foci dealing with radiation-induced DSBs, around 10 foci/Gy/cell as analyzed 1 h post-irradiation (data not shown). For three control donors, the data were obtained up to 72 h after irradiation of lymphocytes with doses of 0.5, 1, and 2 Gy providing clear dose response and time kinetics for radiation-induced 53BP1/γ-H2AX (data not shown). These data will be analyzed elsewhere. Typical images of lymphocytes with DNA repair foci under various treatment conditions are shown in Figure 2. The primary data obtained from cells of each subject immediately after 1 h exposure are shown in Tables 3A and 4A (Supporting Information). We observed a distinct MW-induced reduction in the level of 53BP1 and γ-H2AX foci both in cells from control and hypersensitive subjects in response to 915 MHz. UMTS MWs also consistently reduced 53BP1 foci in cells from all subjects and reduced γ-H2AX foci in most subjects. Very similar reductions in 53BP1/γ-H2AX foci were

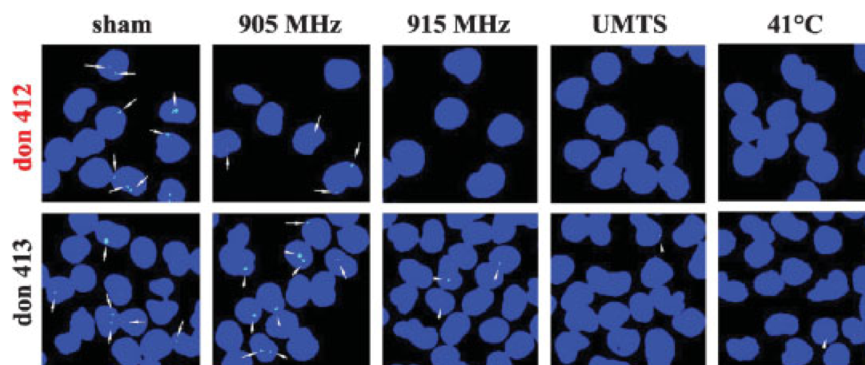

Fig. 2. Panels show typical images of fixed human lymphocytes (counterstained in blue with ToPro-3-iodide) from hypersensitive (subject 412) and matched healthy subject (subject 413) with 53BP1 foci (stained in green with Alexa fluor 488 and designated by arrows) as revealed by immunostaining and confocal laser microscopy. Foci were seen in sham-exposed cells. Significantly fewer foci were observed after 1-h exposure to GSM MWs at 915 MHz, UMTS MWs at 1947.4 MHz and heat shock, 41 °C (Table 3). [The color figure for this article is available online at www.interscience.wiley.com.]

observed in lymphocytes from control and hypersensitive subjects in response to heat shock at 41 °C (Tables 3A and 4A, Supporting Information).

The response to 905 MHz was not consistent among subjects and either increase or decrease in amount of 53BP1 and γ-H2AX foci or no effect was observed dependent on subject (Tables 3 and 4, Supporting Information). In particular, a statistically significant induction of γ-H2AX was seen in cells from donor 907, suggesting that 905 MHz may induce DSBs in cells from this donor (Table 4, Supporting Information).

There was no statistically significant difference in effects between groups of hypersensitive and healthy persons ($P > 0.05$, Wilcoxon matched pairs signed rank test) under all conditions of exposure. All data were pooled and highly significant inhibitory effects on formation of DNA repair foci were found as analyzed immediately after 1 h exposure to UMTS, 915 MHz and heat shock (Tables 3A and 4A, Supporting Information; Fig. 3).

The most striking observation was that these MW-induced inhibitory effects continued up to 3 days following 1 h exposure to MWs (Fig. 3). This long-lasting inhibition of the 53BP1/γ-H2AX foci was consistently observed in lymphocytes from both control and hypersensitive subjects in response to GSM MWs at 915 MHz and UMTS MWs (Tables 3B and C and 4B and C; Supporting Information). Responses to these MW exposures were stronger than response to heat shock at 41 °C that tended to disappear and was not statistically significant as analyzed 3 days following exposure (Tables 3C and 4C, Supporting Information; Fig. 3). The viability of cells was always above 98% as measured with trypan blue exclusion assay at the beginning of exposure and the fraction of blue cells did not exceed 10% at the end of cultivation. Apoptosis varied from 5% at the beginning to 20% at 72 h after beginning cultivation. These data are in line with previously published results [Torudd et al., 2005]. Despite increasing level of apoptosis, the inhibition of DNA repair foci was observed in a majority of cells at all time points showing that the observed effects did not correlate with onset of apoptosis. No activation of lymphocytes was observed by analysis of cell morphology and DNA content using obtained images of sham-exposed and MW-exposed lymphocytes. Therefore, inhibitory effects were unlikely to be caused by activation of lymphocytes or by alternation of cell cycle distribution from G0 to G1-S-phases.

Similar to the data obtained immediately after exposure, the response to 905 MHz was not consistent among subjects, and either an increase or decrease in the amount of foci was observed 24 and 72 h after exposure

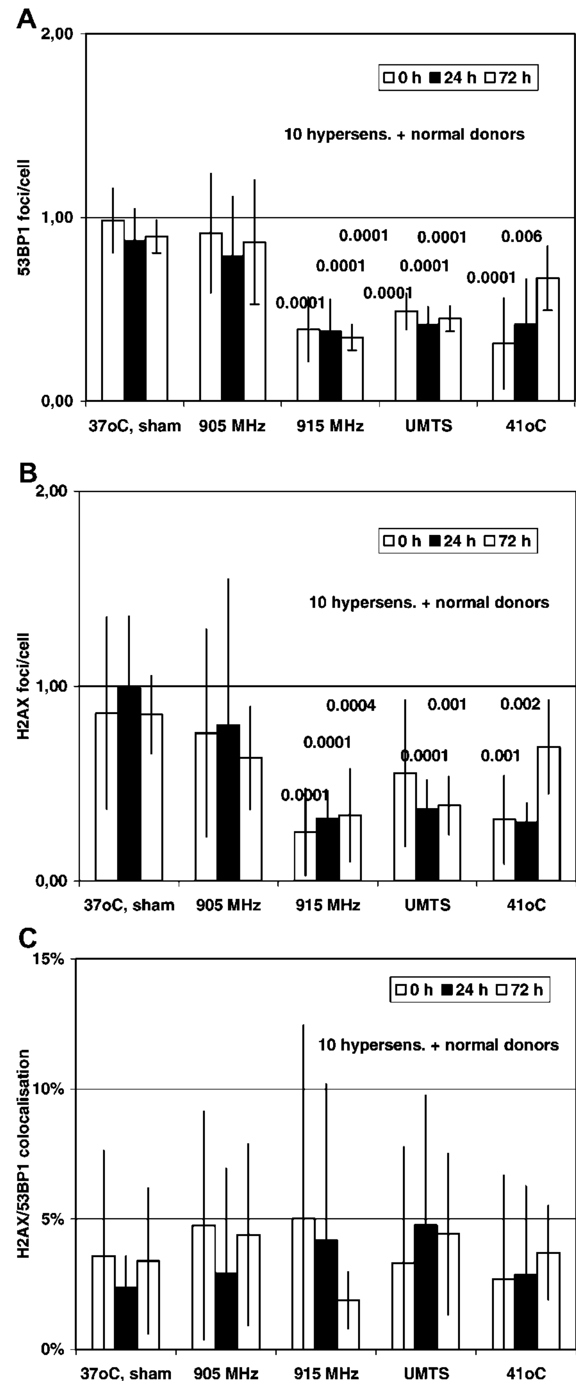

Fig. 3. 53BP1 foci (**A**), γ-H2AX foci (**B**), and co-localization of 53BP1/γ-H2AX foci as normalized to amount of γ-H2AX foci (**C**) in human lymphocytes immediately (0 h), 24 and 72 h following exposure to GSM MWs at 905 and 915 MHz, UMTS MWs at 1947.4 MHz, and heat shock at 41 °C, as measured by immunostaining and confocal laser microscopy. Mean values for cells from 10 subjects (five hypersensitive and five matched healthy subjects) and standard deviations are shown. Three hundred to 600 cells from 5 to 10 images were analyzed per treatment condition for each subject. *P*-values are shown for those treatments that were statistically significantly different from sham-exposure as analyzed by the Wilcoxon matched pairs signed rank test.

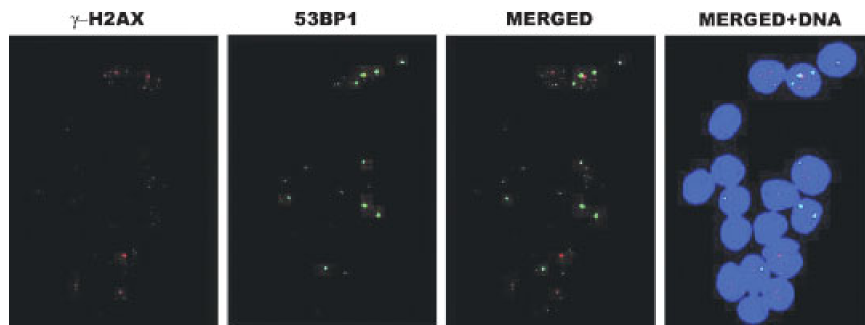

Fig. 4. Typical image showing the lack of co-localization between majorities of 53BP1 and γ-H2AX foci is shown in sham-exposed lymphocytes from one control donor. [The color figure for this article is available online at www.interscience.wiley.com.]

(Tables 3B and C and 4B and C, Supporting Information). Interestingly, γ-H2AX was statistically significantly induced by exposure to 905 MHz in cells from donor 907 as analyzed 24 h following exposure. This observation was in line with the induced level of foci as observed in cells of this donor immediately after exposure (Table 4, Supporting Information). Similar increases in 53BP1 foci, although statistically insignificant, were seen in cells from this donor (Table 3, Supporting Information).

For each group of subjects, we verified the hypothesis that MW exposure affects formation of 53BP1 and γ-H2AX foci. For this purpose, we compared effects of microwave exposures with sham (multiple comparisons of sham, 905, 915, and 1947.4 MHz) using the Kruskal–Wallis ANOVA by ranks. This multiple comparison showed that MWs affected both 53BP1 and γ-H2AX foci in cells from both hypersensitive and matched control persons at very high significance levels (Table 5, Supporting Information). Even stronger significance levels were obtained if the data from two groups were pooled and analyzed together with the Kruskal–Wallis ANOVA by ranks. These data show that the MW exposures as used in this study significantly affect the formation of DNA repair foci in human lymphocytes.

We next verified the hypothesis that the effects of GSM MWs are frequency-dependent. This was done by comparison of MW effects at 905 MHz and 915 MHz in cells from both hypersensitive and matched control persons by the Mann–Whitney *U*-test or Wilcoxon matched pairs signed rank test when applicable. This comparison showed that GSM MWs inhibit formation of the 53BP1/γ-H2AX foci dependent on frequency in cells from both normal and hypersensitive subjects (Table 6, Supporting Information).

We also tested whether effects of GSM MWs at the effective frequency of 915 MHz were the same as effects of UMTS MWs (Table 7, Supporting Information). Comparison of the MW effects on cells from hypersensitive subjects and matched control healthy persons was performed by the Wilcoxon matched pairs signed rank test. This comparison demonstrated that the effects of UMTS MWs and GSM MWs at 915 MHz on the formation of the 53BP1/γ-H2AX DNA repair foci were different for hypersensitive ($P < 0.02$ for 53BP1 and $P < 0.01$ for γ-H2AX, respectively) but not for control subjects ($P > 0.05$). The non-parametric statistics used here do not indicate the specificity of the differences between the effects of GSM and UMTS MWs on cells from hypersensitive subjects.

For all treatment conditions, a correlation between 53BP1 and γ-H2AX foci was observed ($R > 0.5$, $P < 0.000001$, Spearman rank order correlation test) both in cells from control and hypersensitive subjects. However, the majority of 53BP1 and γ-H2AX foci did not co-localize as the co-localization did not exceed 5% (Fig. 3C). These data are in line with results of our previous publications showing very low co-localization of γ-H2AX and 53BP1 foci in normal human lymphocytes and primary human fibroblasts [Markova et al., 2005, 2007]. Figure 4 shows that the majority of 53BP1 and γ-H2AX foci do not co-localize.

DISCUSSION

It is widely accepted that γ-H2AX and 53BP1 foci mark the locations of DSBs. Quantitative analysis of these foci show that these and other markers of DSB repair co-localize in the majority of DNA repair foci induced by radiations and genotoxic chemicals [Sengupta et al., 2004; Lee et al., 2005; Bocker and Iliakis, 2006; Markova et al., 2007]. However, this co-localization is usually only partial and, to our knowledge, 100% co-localization has never been established by quantitative analysis. We confirm here our previously reported finding that the majority

of 53BP1 and γ-H2AX foci do not co-localize in either untreated or MW/heat-shock treated lymphocytes [Markova et al., 2005]. Similarly, a low level of 53BP1 and γ-H2AX foci co-localization was observed in untreated primary human VH-10 fibroblasts [Markova et al., 2007]. However, radiation-induced foci showed significant co-localization that was dependent both on dose and post-irradiation time [Markova et al., 2007]. Perhaps different marker proteins have different kinetics of binding to and remaining at the locations of DSBs that may be dependent on treatment dose, post-treatment time, origin of DSB and cell type.

It has previously been shown that non-thermal MWs affected conformation of chromatin in *E. coli* cells, rat thymocytes and human lymphocytes under specific conditions of exposure [Belyaev et al., 2000, 2005; Markova et al., 2005]. Usually, in human lymphocytes, non-thermal MWs transiently condensed chromatin in contrast to decondensation, which has been observed immediately after genotoxic impacts such as ionizing radiation [Belyaev et al., 1999, 2001; Torudd et al., 2005]. The AVTD data obtained in this study are in line with the data published previously. GSM MWs at 915 MHz resulted in statistically significant and transient condensation of chromatin similar to condensation induced by heating (Table 2, Supporting Information). No heating was induced in samples exposed to MWs. The SAR values at different locations of the exposed samples were always well below thermal effects. Therefore, the MW effects could not be attributed to the heating, although a similar response was observed both after MW exposure and heat shock. This similarity indicates that MW exposure at 915 MHz is a stress factor for human peripheral blood lymphocytes. Stress response proteins and particularly hsp70 was activated by MWs of mobile phones in some previous studies as measured by Western blot [Kwee et al., 2001; Weisbrot et al., 2003] and even candidate gene sequences in molecular mechanism of this stress response were identified [Blank and Goodman, 2004]. Notably, both responses to heating and non-thermal MWs varied among donors, suggesting individual variability in chromatin condensation induced by these factors. In general, the effects of heating and MWs on chromatin condensation were less pronounced compared to the effects on DNA repair foci (Tables 2–4, Supporting Information).

We have recently described the effects of MWs from GSM mobile phones on 53BP1/γ-H2AX DNA repair foci in human lymphocytes [Belyaev et al., 2005; Markova et al., 2005]. GSM MWs at 915 MHz inhibited the formation of the DNA repair foci in lymphocytes from hypersensitive and control subjects [Belyaev et al., 2005; Markova et al., 2005]. Here, we extend the previously published data and report that exposure to GSM MWs at 915 MHz consistently inhibits formation of the 53BP1/γ-H2AX DNA repair foci in cells from 26 tested hypersensitive and normal persons. The prevalence of women among persons reporting hypersensitivity to electromagnetic fields, around 70%, is typical for Sweden. The ratio of women:men in this study, 8:2, represents the prevalence of women. Effects of UMTS MWs were observed in all eight samples obtained from women. Therefore, the UMTS findings seem to be general for women. There are reports that non-thermal effects of MWs may be gender-dependent [Belyaev, 2005a]. Thus, more data are needed to complement our finding on the UMTS effects on lymphocytes from men. Our findings regarding effects of GSM MWs at 915 MHz on chromatin conformation and DNA repair foci seem to be general with respect to gender because these effects were observed in cells from both men and women as has been shown in this paper and previously [Belyaev et al., 2005; Markova et al., 2005].

Our previous data have shown that MWs at specific frequencies inhibit repair of radiation-induced DNA damage in *E. coli* cells [Belyaev et al., 1992b, 1993]. Thus, our working hypothesis was that a decrease in 53BP1/γ-H2AX foci could be a manifestation of the inhibitory effects of MW on repair of spontaneous DSBs. Notably, both 53BP1 and γ-H2AX foci are similarly inhibited by heat shock and MWs from mobile phones. This inhibition may be caused by a decrease in accessibility of DSBs to proteins because of stress-induced chromatin condensation. The molecular mechanisms of the inhibitory effects observed here may also include downregulation of 53BP1 or delocalization of this protein from DNA repair foci. Analysis of these mechanisms was not within the scope of this study. While molecular mechanisms are unknown, the obtained data have clearly shown that MW from mobile phones, similar to moderate hyperthermia, can significantly inhibit DNA repair in human lymphocytes. Disruption of the balance between cellular repair systems and DNA damage may be of significant importance in the genotoxic effects of GSM/UMTS mobile communication including genomic instability and cancer [Galeev, 2000].

In contrast to 915 MHz exposures, MWs at 905 MHz did not affect cells in most cases. However, 905 MHz could either decrease or increase the amount of foci in cells from some subjects. Similar interindividual variability for the 905 MHz effects was observed in a previous study, where 905 MHz MWs induced DNA repair foci in cells of 4 donors out of 10 [Markova et al., 2005]. Does this mean that 905 MHz exposures

induced DSBs in those cases where foci increased? The data obtained here neither exclude nor directly support such a possibility. Replicated experiments with cells from the same donors may provide information regarding the nature of this variability in response to 905 MHz. Stronger variability of MW effects at 905 MHz compared to effects at 915 MHz provides additional evidence of the importance of carrier frequency in studies of MW effects.

It should be noted that the formation of DNA repair foci involves phosphorylation of 53BP1/γ-H2AX proteins [DiTullio et al., 2002; Fernandez-Capetillo et al., 2002]. It is thus possible that the observed effects of MWs and heat shock at the level of 53BP1/γ-H2AX foci formation were due to a change in phosphorylation.

Some recent publications show that heat shock can induce formation of γ-H2AX foci in cultured cells [Takahashi et al., 2004; Kaneko et al., 2005]. Mechanisms of this induction are not known. The strongest effects were observed in cancerous S-phase cells that usually have very high background levels of γ-H2AX foci, up to 20 foci/cell. It is therefore supposed that heat affects replication resulting in formation of DSBs at the replication forks [Takahashi et al., 2004]. However, this mechanism cannot account for our findings because we used primary human lymphocytes in G0 with relatively low background levels of foci, up to 2 foci/cell. Differences in cell type and stage of cell cycle may provide an alternative explanation for the discrepancies in our findings and the aforementioned literature data. This suggestion is supported by a recent publication where the authors did not observe induction of γ-H2AX foci by heat shock in cultured human amnion FL cells [Zhou et al., 2006]. It is interesting to note here, that effects of heat shock on chromatin as measured with AVTD technique in primary G0 human lymphocytes, had a nonlinear dependence on temperature within 40–45 °C [Sarimov et al., 2004]. Contrary to chromatin condensation that was induced in human lymphocytes at 40–42 °C and 0.5–2 h treatment, chromatin decondensation was observed at heat shock with higher temperature and longer duration of treatment. It may be another reason for discrepancies between data. Indeed, inhibitory effects of heat shock on γ-H2AX foci were observed at 41 °C and one-two h treatment in our studies. The temperature-dependent induction of H2AX phosphorylation was observed at temperatures of more than 41.5 °C and at longer durations of treatment [Takahashi et al., 2004].

Significant variations in the response of cells were observed in both hypersensitive and control groups of subjects. This investigation and previous studies [Belyaev et al., 2005; Markova et al., 2005] provide unequivocal evidence that MWs from mobile phones induce adverse effects in lymphocytes from hypersensitive and healthy subjects. However, the only difference between the groups was found here by comparing the effects of UMTS MWs and GSM MWS at 915 MHz on the formation of the 53BP1/γ-H2AX DNA repair foci (Table 7, Supporting Information). These effects were different for hypersensitive but not for control subjects. The non-parametric statistics that were used do not reveal the specificity of the differences between the effects of GSM and UMTS MWS on cells from hypersensitive subjects. More data are needed to study the nature of these differences.

In general, the comparison of pooled data obtained with all treatments did not show significant differences between the groups of controls and hypersensitive subjects. This result might be explained by the heterogeneity in groups of hypersensitive and control persons. Even if there is such a difference, it would be masked by the large individual variation between subjects, which was observed in both control and hypersensitive groups. An additional problem may be the lack of any objective criteria for selection of a study group consisting of persons that are either truly hypersensitive or insensitive to EMF (although this has yet to be proven). One cannot exclude that compensatory reactions are less efficient in the hypersensitive persons providing stronger connection of reactions to MWs at the cellular level with symptoms of hypersensitivity.

The data obtained in a previous study [Markova et al., 2005] and here clearly show that MWs from GSM mobile phones affect the formation of 53BP1/γ-H2AX DNA repair foci in human lymphocytes dependent on carrier frequency. This result, obtained in lymphocytes from 10 healthy and 10 hypersensitive persons, is of great importance. First, such frequency dependence suggests a mechanism for microwave effects that does not deal with heating. Investigation of this mechanism and the molecular targets of the frequency-dependent effects of MWs is a fundamental problem. Second, the data indicate that a possibility may exist to choose those carrier frequencies for GSM mobile communications that do not adversely affect human cells.

Inhibition of DNA repair foci in human lymphocytes was induced by specific GSM/UMTS signals from mobile phones at intensities well below the safety standards of the International Commission on Non-Ionizing Radiation Protection (ICNIRP) [ICNIRP, 1998]. The SAR values at different locations of the exposed samples were always well below thermal effects. In addition, the effects of GSM 915 MHz and UMTS MWs on DNA repair foci were observed in a majority of cells excluding any explanation based on micro-thermal points due to SAR variation. Therefore,

the MW effects were non-thermal. Changes of frequency by 10 MHz changed neither the SAR value nor the SAR variation between cells in the exposed samples. Dependence of the MW effect on frequency, 905 MHz versus 915 MHz, provided further evidence for the non-thermal nature of the MW effect described here. Thus, an important aspect of our findings is that criteria other than ''thermal'' criteria need to be established and utilized in the development of safety standards. In particular, our data indicate that different frequencies should be considered separately in setting the limits for safety standards.

In our pilot study on the effects of GSM MWs on the DNA repair foci we used 2 h exposure [Belyaev et al., 2005]. In the current study and in the previous one [Markova et al., 2005], the effects of 1 h exposure were investigated. Regardless of the time of exposure, formation of DNA repair foci was almost completely blocked by GSM MWs. These data suggest that the inhibitory effects of MWs reach saturation at least at 1 h exposure. Our yet unpublished results show that GSM-induced condensation of chromatin has approximately linear dependence on duration of exposure within 30 min and levels off at longer exposures. Similarly, inhibitory effects of MW exposure regarding DNA repair may be dependent on exposure time at short periods and leveling off for longer exposures.

Based on the established dependencies of non-thermal effects of MWs on frequency we hypothesized that MW representing wide-band signals such as UMTS (5 MHz) may result in higher biological effects compared to relatively narrower GSM signal (200 kHz) because of the higher probability of ''effective'' frequencies within the UMTS bands [Belyaev, 2005b]. The data obtained here are consistent with our hypothesis and show, for the first time, that UMTS MWs (1947.4 MHz, middle channel) inhibit formation of the 53BP1/γ-H2AX DNA repair foci in human lymphocytes both from hypersensitive and healthy subjects. In addition, UMTS signals significantly differ from GSM signals due to different modulation techniques. Modulation might be of great biological significance, thereby providing a possible alternative explanation for more pronounced effects in response to UMTS signals. Remarkably, inhibitory effects induced by MWs from UMTS mobile phones were rather stable and persisted for at least 72 h, even longer than the stress response following heat shock. Contrary to GSM communication, where all providers use the same fixed frequency channels, different UMTS frequency bands are usually assigned to different providers. Technically, it would be easy to adopt specific UMTS bands for mobile communication if it were proven in replicated studies that some of these frequency bands do not produce adverse effects compared to other bands. Identification of those signals and frequency channels/bands for mobile communication, which do not affect human primary cells, is a high priority task in the development of safe mobile communication.

CONCLUSIONS

Microwaves from UMTS/GSM mobile phones at non-thermal levels lower than the ICNIRP safety standards affect formation of 53BP1/γ-H2AX DNA repair foci and chromatin conformation in human lymphocytes from subjects reporting hypersensitivity to electromagnetic fields and healthy subjects. The MW effects on DNA repair foci were more pronounced. These effects depended on carrier frequency and type of signal and suggested misbalance between DNA damage and DNA repair. The results also show that inhibition of DNA repair foci is rather stable and observed up to 3 days following 1 h exposure to non-thermal microwaves from GSM/UMTS mobile phones. No significant differences in effects between groups of healthy and hypersensitive subjects were observed, except for the effects of UMTS MWs and GSM-915 MHz MWs on the formation of the DNA repair foci, which were different for hypersensitive but not for control subjects.

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