Comparison of the reactions to stress produced by X-rays or electromagnetic fields (50Hz) and heat: induction of heat shock genes and cell cycle effects in human cells

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Summary
The effects of irradiation (200kV X-ray, 5 Gy), extremely low frequency electromagnetic fields (ELF-EMF, 50 Hz, 60 ± 0.2 μT) and/or heat shock (HS, 41°C for 30 min) on the expression of several heat shock genes (HSP27, HSP60, HSP70, HSP75, HSP78, HSP90) in human HL-60 leukaemia cells was studied by RT-PCR. In addition, the effects of these stressors on cell cycle progression in exponentially and asynchronously growing cell cultures were studied by flow cytometry. The dynamics of cell division during successive cell cycles was monitored by fluorescence-labelling of the cells with carboxyfluorescein succinimidyl ester (CFSE). Finally, the cell cycle distribution was studied by staining with propidium iodide (PI). With respect to HSP gene expression the three stressors produced similar effects. The combination of stressors (ELF-EMF and HS or Radiation and HS) strongly induced transcription of the HSP70 gene above the level induced by each stressor alone. The cell cycle analysis, however, revealed striking differences in the cellular response to each stressor. Of particular interest was the observed thermoprotective effect of ELF-EMF in heat shocked cells, an effect that was not seen in cells which were exposed to X-rays in the presence of thermal stress.

Keywords: electromagnetic fields – heat shock – radiation – thermoprotection – X-rays

INTRODUCTION

Cells are vulnerable to adverse environmental conditions and emergency programs to cope with unfavourable situations have a long evolutionary history and are strongly conserved in all organisms. Stress factors (henceforth referred to as stressors) that may upset the normal homeostatic mechanisms can be of a very varied nature. A stress reaction is elicited by extreme temperatures, various types of radiation, oxidative stress, heavy metals and a wide range of chemical substances. Despite the great diversity of stressors a rather uniform cellular stress response is elicited. A group of stress-inducible proteins, the heat shock proteins (HSP) were described first due to their characteristic appearance after heat shock (Ritossa 1962). A major function of some of these proteins, notably the HSP70

protein(s), is the proper refolding of the protein damaged as a result of the stress. The up-regulation of the "repair" proteins (chaperonin function) is accompanied by a down-regulation of other genes not involved in the stress response. As a result, normal cellular functions that are not essential for survival are temporarily suspended. Cell division is one of the most easily disrupted cellular functions and hence an early indicator of stress.

The down-regulation of housekeeping genes affects the homeostatic regulation of the cell in such a way that the cell will react differently to a second stressor. This situation is medically relevant. Several diseases are known to be accompanied by the expression of HSP thus indicating that cells encounter a stress situation. Particularly in cancer therapy (e.g. radiotherapy) the stress status of the cells is likely to determine the success of the

treatment. However, little research has been done to analyse the interactions of different stressors on the cellular level.

In this study we have analysed the cellular response to stressors which are known to act differently on the molecular level: while thermal stress is known to denature proteins, the energy of ionising radiation (like X-rays) is sufficient to damage proteins and DNA directly or indirectly by the production of reactive oxygen species (ROS). With respect to ELF-EMF the molecular targets are unknown, but the induction of the stress response with flux densities of only a few μT (Jin et al. 2000) indicates that despite the extremely low energy of ELF-EMF a clear cellular stress reaction can be induced. For energetic reasons, ELF-EMF can neither denature proteins nor damage cellular macromolecules directly. The mechanism of ELF-EMF induced biological reactions is likely to involve new molecular mechanisms which may change our current view on ELF-EMF as an environmental hazard. The observed differences in the cellular response to the three physical stressors analysed in this study are likely to reflect the inferred mechanistic differences.

MATERIALS AND METHODS

Cell Culture

The experiments were performed with the acute myeloid leukaemia cell line HL-60 (DSMZ, Germany). The cells were cultured at 37°C in RPMI 1640 medium with 10% heat-inactivated foetal calf serum (Gibco, France) in a humidified atmosphere containing 5% CO_2. The cultures were maintained at a density of 2×10^5 to 1×10^6 cells/ml^{-1} by re-suspending the cells in fresh culture medium every 2 days.

Exposure to ELF-EMF, heat shock and radiation

Cell cultures (10^6 cells ml^{-1}, 15 ml per flask) were exposed to sinusoidal ELF-EMF (50 Hz, 60±0.2 μT) and / or thermal stress at 41°C for 30 min (for details see Tokalov and Gutzeit 2003a). Control cultures were maintained at 37°C. ELF-EMF was generated by a set of Merritt coils (Merritt et al. 1983, Kirschvink 1991) as described before (Junkersdorf et al. 2000). The correlation between coil current and the magnetic field was experimentally determined and was found to be linear in the range of 1–150 μT with a precision of ±2%. The harmonic distortion was determined to be smaller than 1%. During the exposure the magnetic

flux density was controlled by adjusting the coil current. with a precision of ±1.5% using a digital multimeter, (M-3860M, Conrad Electronic, Germany). For the experimental set-up, a location in the laboratory was chosen in which stray field sources could produce a magnetic field of less than 1 μT.

The temperature of the cell cultures was controlled using a specially designed plastic chamber with the dimensions 1800 × 1400 × 60 mm. Water of the desired temperature (±0.1°C; F15 waterbath, Julabo Labortechnik, Germany) circulated through cavities drilled in a serpentine way into the bottom plate of the chamber. One hour before the exposure of the cells to thermal stress the desired temperature in the chamber was reached and did not change until the end of the experiment. The temperature was controlled using a GTH 175/MO digital thermometer (Greisinger electronic, Germany) with a precision of ± 0.1°C. The plastic chamber was placed in the centre of the Merritt coils so that the two stressors (thermal stress and ELF-EMF) could easily be applied alone or simultaneously. After the exposure to the stressor(s) the cells were cultured under standard conditions at 37°C and analysed as described below.

Cell cultures were irradiated with a 200 kV X-ray tube (Isovolt 320/20, Seifert Roentgenwerke, Germany) using a 0.5 mm Cu filter. The tube was operated with current of 20 mA yielding a dose rate of 1.2 Gy per min.

Other cultures were exposed to two stressors to study their interaction (i.e. thermal stress in combination with ELF-EMF or radiation. HL-60 cultures were subjected to HS (41°C, 30 min) and simultaneously to ELF-EMF (60 μT, 30 min). Other cultures subjected to ELF-EMF or HS were, for technical reasons, cultivated for a further 60min before they were irradiated (5Gy). After exposure to the stressors the cells were cultivated for another 60min (for RT-PCR analysis) or for 2 days to study the dynamics of cell proliferation.

Analysis of HSP transcripts by RT-PCR

Total RNA was isolated from $4–5 \times 10^6$ cells using the Invisorb® RNA Kit II (Invitec GmbH, Germany) for total RNA extraction. After photometric quantification (Ultrospec 2000, Pharmacia Biotech, UK), 500 ng RNA was mixed with 10 μL reverse transcriptase (200 U of the enzyme, SuperScript, Qiagen, Germany), 40 units of RNase OUT (Promega, Germany), dNTPs (500 μmol/L), 50 pmol oligo-dT$_{15}$ primers (Life Technologies Inc., Germany), and buffer as recommended by the supplier. The samples were incubated at 37°C for 1 h followed by 20 min at 60°C. 1 μL of this cDNA

was used for PCR amplification. A 15μl PCR reaction mixture contained 25 ng of cDNA, 0.4 units Taq-DNA-polymerase (Peqlab Biotchnologie GmbH, Germany), 100 μM dNTPs and 1 μM of each primer pair. The analysed genes with the respective primer pairs are listed below: HSP27 (XM_004991): Homo sapiens heat shock 27kD protein 1 (HSPB1) gene, forward: 5´-ATGGCGTGGTGGAGATCACC-3´ GeneBank location: 451–470), reverse: 5´-CAAAAGAACACACAGGTGGC-3´ (location: 797–778); HSP60 (NM_002156): Homo sapiens heat shock 60kD protein 1 gene (chaperonin) (HSPD1), forward: 5´-ATTCCAGCAATGACCATTGC-3´ (1444–1463), reverse: 5´-GAGTTAGAACATGC CACCTC-3´ (1749–1730); HSP70a (M11717): Human heat shock protein (hsp 70) gene, forward: 5' TGTTCCGTTTCCAGCCCCCAA-3´ (435–455), reverse: 5' GGGCTTGTCTCCGTCGTTGAT-3' (993–974); HSC70 (NM_006997): Homo sapiens heat shock 70kD protein 8 (HSPA8), forward: 5'-TGTGGCTTCCTTCGTTATTGG-3' (39–59), reverse: 5' GCCAGCATCATTCACC ACCAT-3' (380–360); HSP75 (L15189): Homo sapiens mitochondrial HSP75, forward: 5´-TGGCAGTTA TGGAAGGTAAA-3´ (228–248), reverse: 5´-AGC AATGACTTTGTCTTCTG-3´ (752–732); HSP78 (XM_044201): Homo sapiens heat shock 70kD protein 5 (glucose-regulated protein, 78kD), forward: 5´-GATAATCAACCAACTGTTAC-3´ (1584–1603), reverse: 5´-GTATCCTCTTCACCA GTTGG-3´ (2162–2142); HSP90 (X15183): Human 90 kD heat shock protein (HSP90α), forward:5´-AAAAGTTGAAAAGGTGGTTG-3´ (1803–1822), reverse: 5´-TATCACAGCATCACTTAGTA-3´ (2426–2406); β-actin (M10277): Human cytoplasmic beta-actin gene (standard), forward: 5´-CAGCTCACCATGGATGATGAT-3´ (1084–1104), reverse: 5´-CTCGGCCGTGGTGGTGAAGCT-3´ (2280–2260).

Initial experiments were performed with a temperature-gradient thermocycler (Biometra, Germany) to determine the optimal temperature conditions of the PCR reaction and the range of PCR cycles over which the amplification efficiency remained constant. The amount of amplified PCR product was directly proportional to the amount of RNA used (data not shown). Amplification in the Biometra UNO-Thermoblock (Biometra, Germany) was set to 45 sec at 94°C, 30 sec at 58°C followed by 90sec at 72°C (30 cycles). Finally, primer extension was allowed for 10 min at 72°C. PCR products were analysed by agarose electrophoresis (run at 200 V) in 1.0% agarose gels in 1x TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) and visualized by staining with ethidium bromide (0.01% in 1xTAE buffer). The data were calculated with reference to the β-actin signal as internal standard. The size of the β-actin PCR

product from genomic DNA is 1198 bp compared to 625 bp for the RT-PCR product obtained from mRNA. Hence DNA contamination can easily be detected and excluded from the analysis. The specific bands were quantified by area morphometry analysis using a digital imaging system (Biometra, Germany) and appropriate software (Optimas Co, DC, USA).

Analysis of cell proliferation

Cells were stained with carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes, Eugene, OR) as described by Lyons (1999). Cells were incubated at 37°C in PBS containing 10 μM CFSE for 10 min, washed and placed in culture medium 1 hour before exposure to the stressor(s). Approximately 2×10^5 cells of the cultures were harvested after 2 days, fixed in 70% ethanol and stored overnight at –20°C. The cells were centrifuged for 5 min at 300 g, and the pellet was resuspended in PBS containing 50 μg/ml propidium iodide (PI) and 0.2 mg/ml RNase (Sigma, Germany) and incubated for at least 45 min. The fraction of cells present in different cell generations and their representation in the respective cell cycle phases was calculated using the FlowMax software (Partec, Germany).

Statistical analysis

Statistical analysis was performed using Student`s t-test. Significance levels were set at 2α= 0.05.

RESULTS AND DISCUSSION

We have studied the stress reaction of human cells (acute leukaemia cells, HL-60 cell line) to heat shock, X-rays, and to an energetically very different kind of stressor, namely weak extremely low frequency electromagnetic fields (ELF-EMF) of 50 Hz. Since the heat shock genes are typically induced when cells are exposed to stress we first analysed the expression of the most prominent members of the heat shock gene family and compared their induction after exposing HL-60 cells to the respective stressors. The pattern of induction was similar after ELF-EMF, X-Ray or heat shock (HS) exposure and HSP70 was consistently the most strongly induced gene (Fig. 1). The induction of HSP27, HSP75 and HSP78 genes was much weaker and a significant induction above control levels was only observed in HS samples but not for the other stressors. For the genes HSP60 and HSP90 no

significant change was recorded under any treatment. This stress response may vary qualitatively and quantitatively in a wide range

between different cell types *in vivo* and this also holds true with respect to various commonly used cell lines.
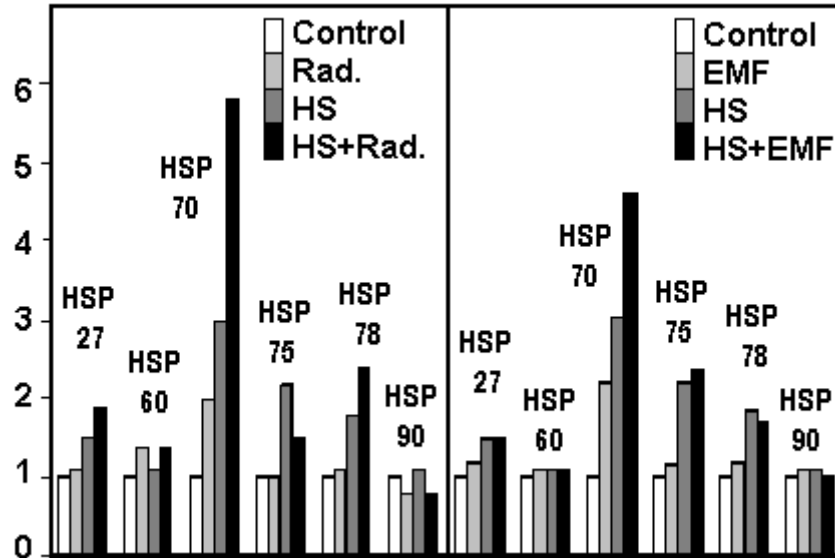

Fig. 1. Expression of human HSP genes in HL-60 cells analysed by RT-PCR and software-aided quantification. Exposure conditions: X-rays (200kV source, 20 mA, 0.5 mm Cu filter, 5Gy at a dose rate of 1.2 Gy/min; labelled "Rad."), heat shock (41°C for 30 min; labelled "HS") , and ELF-EMF (60µT, 50 Hz for 30 min; labelled "EMF"). The quantification of the respective mRNA levels is calculated with reference to unstressed control cells (=1). Mean of 11 experiments.

The observed reaction of HL-60 cells to the three stressors was remarkably similar in view of the different physical qualities of the stressors. With respect to ELF-EMF our findings are in keeping with the results of Goodman et al. (1994) who reported increased HSP70 transcript concentrations in HL-60 cells after exposure to weak ELF-EMF (60Hz). The combination of stressors (X-rays or ELF-EMF in combination with thermal stress) increases the HSP70 mRNA concentration further above the level of each stressor alone. Since the molecular mechanisms of stress induction by the three treatments are different it would be of interest to find molecular explanations for the observed effects. Is the expression of the HSP70 gene at the end of the stress-induced signal transduction pathways which converge at this point, or must alternative mechanisms be considered? Since the molecular targets of ELF-EMF are not known this question cannot be answered at present. A new and interesting suggestion has been made recently by Lin et al. (2001) who provide evidence that ELF-EMF may act directly on the DNA. Specific short DNA sequences have been identified that appear to be associated with ELF-EMF inducible genes.

There are, however, numerous open questions that need to be addressed. For example, we have shown before that there is no linear dose-response relationship in the range of the flux densities studied (10 to 140 µT). The maximal induction of heat shock gene expression, in particular of the HSP genes, was observed at about 60–80µT while higher flux densities showed a weaker effect (Tokalov and Gutzeit, 2003b). Such "window" effects have also been observed with respect to different frequencies of ELF-EMF (Berg 1999).

While on the level of HSP70 gene expression ELF-EMF exposure produces effects similar to X-rays or heat shock the effects on the cell cycle revealed interesting differences. Cell proliferation was analysed by labelling with CFSE at the beginning of the experiment. The intensity of CFSE decreases predictably during successive cell cycles (Lyons, 1999) such that different cell generations can be distinguished. CFSE does not seem to be toxic for cells (Hasbold et al. 1999) so that the analysis can be carried out over several cell generations and cell cycle arrested and apoptotic cells can be distinguished from cycling cells (Tokalov and Gutzeit 2003a).
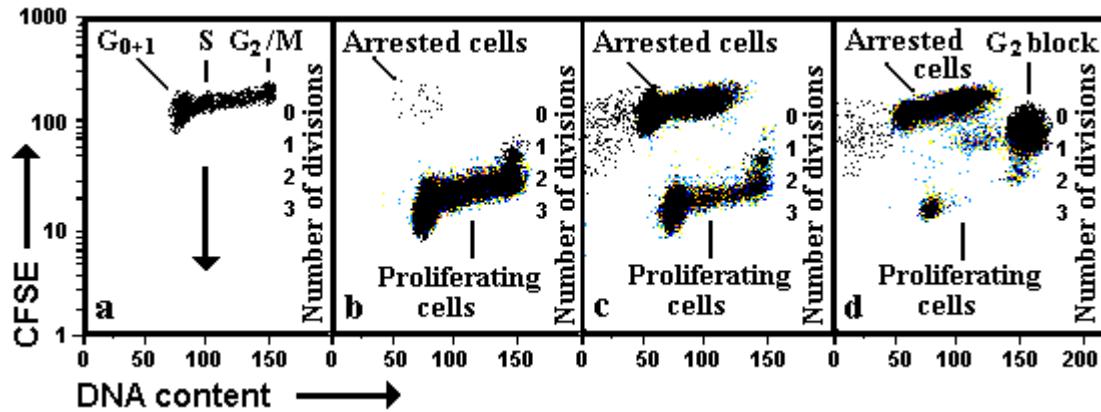
Fig. 2. **Proliferation of HL-60 cells analysed by quantifying CFSE content and PI fluorescence (DNA content)**. Due to the reduction of CFSE fluorescence with each division, cells in the first, second, and third cell division can be distinguished (labelled 1, 2 and 3; 0 indicates the cell cycle at the time of CFSE labelling). The two-dimensional plots show CFSE and PI determinations of cells at the beginning of the experiment (a) after 2 days of culture (b) and 2 days after exposure to thermal stress at 41°C for 30 min (c) and radiation (5 Gy, shown in d). Cell cycle arrested cells after HS can clearly be separated from cycling cells (c). After radiation the population of cycling cells is small but two different populations of arrested cells (in G_1 and S and in G_2) can be distinguished (d).

The reduction of CFSE fluorescence after 2 days of culture is illustrated in Fig. 2. During the course of the experiment it became apparent that cells cycle with different speed. In normally cycling HL-60 cells (controls) most cells were in the second round of cell division after 2 days of culture. Some cells had entered the G_1 phase of the third cycle while a few cells cycled more slowly and had not completed the first cycle (G_2/M). A small fraction of the cells ($5\pm3\%$, not visible in Fig 2b) did not cycle and retained the original CFSE content. These cells became hypodiploid during the incubation and presumably underwent apoptosis.

Compared to control cultures ELF-EMF exposure alone (50 Hz, 60 µT) did not change the rate of cell division, nor the total number of cells nor the percentage of dividing cells (data not shown). From these data it can be concluded that ELF-EMF did not affect the proliferation of asynchronously dividing HL-60 cells under the chosen experimental conditions and this holds also true for HL-60 cell cultured for up to 10 days following ELF-EMF exposure (Tokalov and Gutzeit 2003a).

It had been noticed before by several authors (Nitta et al. 1997, Kuhl et al. 2000) that heat shock halts the cell cycle and, depending on the experimental conditions, cells may become arrested for several hours at the G_1 and/or G_2/M cell cycle checkpoints. For example, Hang and Fox (1996) observed that heat shocked CHO cells prolong the S-phase and finally arrest at the G_2/M phase. The CFSE labelling technique reveals that two distinct cell populations can be distinguished (Fig. 2c). Under our experimental conditions the majority of

cells ($68\pm5\%$) became arrested and did not re-enter the cell cycle within the 2-day culture period and even when the incubation was prolonged for up to 6 days the cells remained arrested and finally became apoptotic (data not shown). According to their DNA content (PI staining) the arrested cells were in the G_1 and S stage but not in the G_2/M stage (Fig. 2c). A similar finding was reported for heat shocked human melanoma cells which had the DNA content of S phase cells but did not synthesise DNA as shown by two-parameter (BrdU/PI) flow cytometry (Zolzer and Streffer 2000). The absence of cells in the G_2/M phase amongst the arrested cells could be explained either by phase specific cell death or by resistance to the HS so that the cells contributed to the actively cycling cell populations. Evidence for the latter explanation is provided by the finding that G_2 cells are more stress resistant than S phase cells (Read et al. 1983, Rice et al. 1984).

Following X-ray exposure (200kV, 5 Gy) the majority of cells were strongly affected by the stressor. One population of cells remained arrested in the G_1 and S phase and this situation closely resembled the HS induced block described before (compare Figs 2c and 2d). However, in radiated cells another prominent cell population can be distinguished: cells arrest at the G_2/M stage and they are characterised by the DNA content typical for G_2/M phase and by a CFSE content typical for G_1 phase cells of the same generation but not for the larger G_2 cells. Further experiments with different incubation times following the radiation, clearly showed that the cells blocked in the G_2 phase indeed derive from the G_1 (and S) cells of the same generation and, finally, become arrested. A similar arrest in G_1 and delay in the S phase followed by a

final block in G₂/M was shown in mice Ehrlich Ascites carcinoma cells *in vivo* after exposure to X-rays (Tokalov 1990). What is the fate of the cells that were in the G₂/M phase at the beginning of the experiment and which possessed a higher CFSE content than the G1 cells of the same generation (Fig. 1a)? Apparently these cells became, after a short radiation-induced delay, the "founder" population for the few actively cycling cells (third cycle, Fig. 2d). This interpretation is supported by published data which show that CHO cells are more resistant to radiation at the late S and G₂ phases of the cell cycle (Sinclair 1968). Further studies using different human cell lines showed that the cell cycle specific response to radiation is qualitatively similar but quantitative differences exist between different cell types (Tang et al. 1994, Biade et al. 1997, Zolzer and Streffer 2000).

Clear biological effects of ELF-EMF are notoriously difficult to demonstrate. Despite the induction of HSP70 no effect on cell proliferation was detected (see above and Fig. 3). However, we have shown before, that clear effects of ELF-EMF can be uncovered in the presence of thermal stress (Michel and Gutzeit 2000, Junkersdorf et al. 1999). This "costress" situation may challenge the homeostatic mechanisms of the cell such that ELF-EMF significantly alters the response to the second stressor (HS) in a reproducible way (Gutzeit 2001). Furthermore, these studies showed that the combination of stressors may result in qualitatively different reactions compared to the response to each stressor alone. For this reason we also tested the effects of a combination of stressors on cell proliferation.
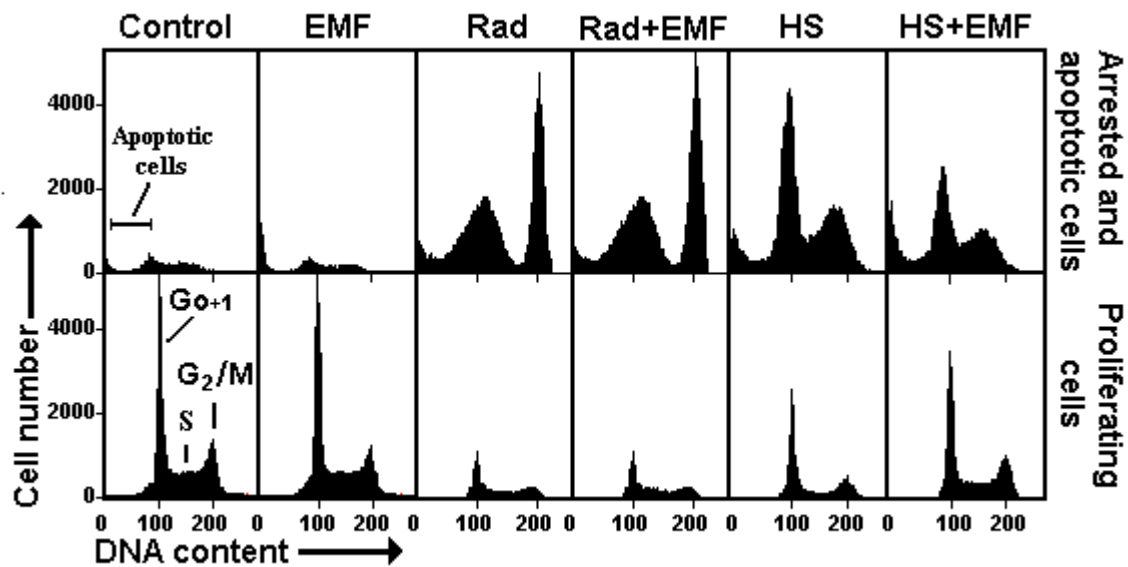

Fig. 3. **Cell cycle analysis by flow cytometry**. HL-60 cells were stressed (for conditions see Fig. 1) and after 2 days of culture cells were stained with propidium iodide and the DNA content of 100000 cells was analysed. In addition, the cells were labelled with CFSE to distinguish cell cycle arrested and apoptotic cells from cycling cells (upper and lower row of figures, respectively). The populations were separated and quantified by gating (FlowMax software, Partec, Germany).

The experimental method (CFSE labelling at the beginning of the experiment and finally PI labelling) was identical to the experiments described above and illustrated in Fig. 2. In order to illustrate the effects under costress situations the population of proliferating cells and of arrested and apoptotic cells were analysed separately by gating (Fig. 3). The DNA profiles of both populations are illustrated and for each experimental condition exactly 100 000 were analysed. In this way changes between the populations of arrested and proliferating cells are readily apparent and, furthermore, the stress-induced

abnormal DNA distributions after X-ray and HS can be quantified.

The results show that ELF-EMF alone has no effect on cell proliferation while HS and radiation lead to characteristic deviations from the normal pattern of cell cycle distribution. The radiation induced G₂ block is clearly visible when the DNA profiles are plotted (Fig. 3). The few cells that escaped the cell cycle arrest (HS and radiation) continued proliferating and showed a normal DNA distribution. The effect of ELF-EMF in the presence of radiation or thermal stress is of particular interest.

ELF-EMF had no significant effect in combination with radiation stress but in combination with HS the number of proliferating cells strongly increased while at the same time the number of arrested cells decreased (Fig. 3). This effect was reproducible and observed in 11 independent experiments (p<0.05; see also Gutzeit and Tokalov 2003a). Cells are typically more resistant to a stressor if they were previously exposed to stress. The phenomenon is usually referred to as thermoprotection. Interestingly, thermoprotection can be achieved by overexpressing HSP70 and the property is lost when the HSP gene is deleted (Gabai et al. 2000). The ELF-EMF induced induction of HSP70 is consistent with this notion.

In view of the possible beneficial effects of thermoprotection it has been suggested that ELF-EMF treatment be used as an alternative to current hyperthermia protocols in medical therapies (Carmody et al. 2000). The data presented here which show that ELF-EMF induces HSP70 but has no inhibitory effect on cell proliferation and can hence be considered a weak stressor is encouraging in this respect.

We do not know why ELF-EMF only protects cells in combination with HS but not in combination with X-ray treatment (Fig. 3). Apparently the three stressors analysed in this study act differently and because of the different target molecules different signal transduction systems will be activated which will lead to different cellular reactions despite the fact that with respect to HSP gene expression there are striking similarities. Further studies concerning the molecular action of the stressors are required and may be rewarding since the medical treatments both depend on and affect the stress status of cells.

Acknowledgements

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