Varying responses of human cells with discrepant p53 activity to ionizing radiation and heat shock exposure

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Abstract. *Objectives:* Both heat shock (HS) and ionizing radiation have an impact on the cell cycle and may induce cell cycle arrest or apoptosis. Mutations of the *p53* gene are observed at a high frequency in human tumours and are recognized in about half of all human cancers. Sensitivity to radiation, heat and anticancer agents has been observed in *p53*^{+/+} cells, but not in mutated or *p53*-deficient cells. Moreover, enhancement of radiosensitivity by HS has been observed in wild-type *p53* cells but not in *p53*-deficient cells. The molecular mechanism of the differential cell response to HS or ionizing radiation is not yet understood. *Materials and Methods:* Differences in cellular response to radiation (200 kV X-ray, 1, 2, 5 Gy) and HS (39 °C, 41 °C and 43 °C for 30 min) on cell cycle progression of cultures of human *p53* mutant cells were investigated by flow cytometry. In addition, the effects of stressors used on the expression of several heat shock genes (*HSP27*, *HSP60*, *HSP70*, *HSC70*, *HSP75*, *HSP78*, *HSP90*) were studied by reverse transcriptase–polymerase chain reaction. *Results and Conclusions:* Yet, with respect to *HSP* gene expression, different stressors produced similar effects. Combination of HS and radiation treatment significantly induced the transcription of the *HSP70* gene above the level induced by each stressor alone. Cell cycle analysis, however, revealed striking differences in prolonged dynamics of cell division in response to each stressor. Thus, *p53* status could be a useful indicator in predictive assays for hyperthermia cancer treatment in combination with radiation and/or chemotherapy.

INTRODUCTION

The response of human cells to temperature elevation, exposure to ionizing radiation, toxic agents or other physiological and environmental stressors is universal and is mediated through the induction of highly conserved heat shock genes (Helmbrecht *et al.* 2000; Jolly & Morimoto 2000; Kuhl *et al.* 2000; Gutzzeit 2001). During stress conditions, heat shock proteins (HSPs) increase cell survival by protecting and disaggregating stress-labile proteins, as well as proteolyse damaged proteins (Morimoto 1998; Smith *et al.* 1998; Feder & Hofmann 1999; Zugel & Kaufmann 1999; Helmbrecht *et al.* 2000). In the absence of stressors, HSPs have multiple

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housekeeping functions, such as folding and translocation of newly synthesized proteins, activation of specific regulatory proteins (including transcription factors), protein degradation, protein signalling (including steroid hormone activation), tumour immunogenicity and antigen presentation (Helmbrecht *et al.* 2000; Jolly & Morimoto 2000; Takayama *et al.* 2003). The nomenclature of HSPs is assigned to their approximate molecular weight, for example, the 70 kDa HSP is known as the molecular chaperone HSP70. The most prominent members of the *HS* gene family included in this study are *HSP90*, *HSP78* (glucose-regulated HS protein 78), *HSP75* (mitochondrial HSP75), *HSP70*, *HSP60* and *HSP27* (Jolly & Morimoto 2000). The *HSP70* gene is exceptional among the chosen *HS* genes, as it is constitutively expressed and is only minimally induced by heat stress and radiation exposure (Turman *et al.* 1997; Tokalov *et al.* 2003).

Recently, considerable attention has been given to the fact that tumour suppressor protein p53 is involved in the cellular response to radiation and heat shock (HS) exposure (Zolzer & Streffer 2000; Ohnishi 2005). Mammalian cells respond to a number of stressors by activating many cellular processes, including the activation of the p53 protein and promotion of cell cycle arrest, mainly at G₁/S or G₂/M checkpoints. When stress is excessive, p53 can induce tumour-suppressive apoptosis (Helmbrecht *et al.* 2000; Jolly & Morimoto 2000). Disruption of p53 wild-type (p53^{wt}) function occurs frequently in many types of human cancer (Molinari 2000; Camplejohn & Rutherford 2001; Eicheler *et al.* 2002; Szymanska & Hainaut 2003) and this has been correlated with decreased sensitivity to chemotherapy, radiation and hyperthermia therapies (Takahashi *et al.* 2004). Potentiation of radiosensitivity was observed in wild-type p53^{wt} cells when they were additionally exposed to heat stress; p53-deficient (p53^{-/-}) cells did not exhibit this effect (Yasumoto *et al.* 2003). It was suggested that HS enhancement of irradiation-induced tumour growth alteration may result in p53-dependent apoptosis as a result of heat-induced inactivation of cell survival systems, through either regulation of the cell cycle or induction of DNA repair (Ohnishi 2005). Induction of p53-dependent apoptosis may at least partially explain the similarity of tumour response to radiation and HS exposure (Zolzer & Streffer 2000), but is still unclear why p53^{-/-} cells also show a variety of features in their response to HS and to radiation that are independent of wild-type p53 responses.

With new techniques in flow cytometry, it has become possible to investigate cell cycle progression in greater detail. Analysis of cell proliferation is commonly carried out by simultaneous analysis of DNA content and bromodeoxyuridine (BrdU) incorporation. Rice *et al.* (1986) were the first to quantify these parameters through the cell cycle in heat-treated cells. A similar protocol was employed to analyse radiation-induced G₂-phase block (McNally & Wilson 1986). Other researchers also have reported flow cytometric measurements of BrdU-labelled cells, further elucidating the cell cycle effects caused by heat or radiation (Higashikubo *et al.* 1993; Higashikubo *et al.* 1996; Pellegata *et al.* 1996; Zolzer & Streffer 2001). The technique of flow cytometry has been useful for cell cycle analysis, but quantification of DNA content did not allow for evaluation of different cell generations in a single experiment. However, when cells are labelled with the dye, carboxyfluorescein diacetate succinimidyl ester (CFSE), intensity predictably decreases during successive cell cycles (Lyons 1999; Tokalov & Gutzeit 2003), such that the cell generations can be distinguished. CFSE does not appear to be cytotoxic and, furthermore, it does not seem to affect lymphocyte differentiation (Hasbold *et al.* 1999). Results presented in this study illustrate the advantages of this technique in studying mixed populations of cycling and arrested cells. The CFSE labelling protocol reveals the prolonged arrest of the HL-60 (p53^{-/-}) cells in the G₂/M cell cycle checkpoint after HS (41 °C, 30 min) exposure (Tokalov & Gutzeit 2003). At the same time, it was found that a large fraction of the exposed cells permanently lost the ability to proliferate. These cells became apoptotic if the cell cycle was blocked by HS. Two cell populations, that is cycling and arrested cells, could be distinguished during the

entire experiment. The number of arrested cells decreased as a result of apoptosis and the cycling cells became more numerous. Their fraction increased continuously and the level of the control was reached within 10 days of culture (Tokalov & Gutzeit 2003). The purpose of the present study was to determine the kinetics of different effects of radiation and of HS on cell cycle progression of $p53^{-/-}$ human tumour cells. The dynamics of cell division during successive cell cycles were monitored by flow cytometry with time after radiation (different doses) and HS (different temperatures) exposure.

MATERIALS AND METHODS

Cell culture

Acute myeloid human leukaemia cells (HL-60, $p53^{-/-}$) were obtained from DSMZ (Braunschweig, Germany) and were maintained in Roswell Park Memorial Institute 1640 medium (Gibco, Cergy-Pontoise, France). Human cervical carcinoma cells (HeLa, $p53^{-/-}$) (DSMZ, Braunschweig, Germany) were grown in modified Eagle's medium (MEM; Bio Whittaker, Verviers, Belgium) with 1% of nonessential amino acids. Primary human diploid skin fibroblasts (AG01522, $p53^{wt}$) (Coriell Cell Repositories, Camden, NJ, USA) were grown in MEM with Earl's Balanced Salt Solution (Bio Whittaker, Verviers, Belgium) and 2 mM L-glutamine. All media were supplemented with antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin, Gibco, Cergy-Pontoise, France) and 10% (v/v) heat-inactivated foetal calf serum (FCS, Gibco, Cergy-Pontoise, France). All cells were grown at 37 °C in a humidified 5% CO₂ atmosphere and were maintained at a density of 2×10^5 – 1×10^6 cells/ml by suspending the cells in fresh culture medium every 2 days.

Exposure to radiation and HS

Cell cultures (10^6 cells/ml, 15 ml per flask) were exposed to thermal stress at 37 °C (sham-treated control cells) for 39, 41 or 43 °C for 30 min. The temperature of the cell cultures was controlled using a specially designed plastic chamber with the dimensions of $1800 \times 1400 \times 60$ mm³. Water of the desired temperature (± 0.1 °C, F15 Waterbath, Julabo Labortechnik, Germany) was pumped through cavities, drilled as serpentine in the bottom plate of the chamber. Desired temperature in the chamber was reached 1 h before the start of the experiment and was maintained to the end of exposure. Temperature was controlled using a GTH 175/MO digital thermometer (Greisinger Electronic, Regenstauf, Germany), precision ± 0.1 °C. After HS exposure cells were cultured under usual conditions at 37 °C and were analysed as described in succeeding discussions.

Cell cultures were irradiated in doses of 0 (sham-treated control cells), 1, 2 and 5 Gy 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.

One set of cell cultures was exposed to two stressors to study their inter-action (that is, thermal stress in combination with radiation). HL-60 cultures were subjected to HS (37 °C, 39 °C and 41 °C, 30 min), and were cultivated for a further 60 min before irradiation (5 Gy). At the end of exposure to the stressors, the cells were cultivated for an additional 30 min [for reverse transcriptase–polymerase chain reaction (RT-PCR) analysis] or for 6, 12, 18 h or 1, 2 and 4 days, to study the dynamics of cell proliferation.

Flow cytometry

Flow cytometry was carried out as previously described by Tokalov *et al.* (2003). Two parameters were quantified: the DNA content, by staining with propidium iodide (PI), and the fluorescence of

dye CFSE, which stains cell cytoplasm and can be used to identify proliferating cells by the decreasing fluorescent signal in successive cell generations (Lyons 1999). Briefly, 2×10^6 cells were stained with 10 μM CFSE (Molecular Probes, Eugene, OR) in phosphate-buffered saline (PBS) for 10 min at 25 °C. Cells were washed with PBS and culture medium was added. After appropriate culture times, cells were removed from the culture and were prepared for analysis by flow cytometry. Cells were washed once more with PBS and were centrifuged at 100 *g* for 10 min. The cell pellet was resuspended in 100 μl of PBS, fixed in 70% (v/v) ethanol by adding 1 ml of cold (−20 °C) ethanol and stored overnight at −20 °C. Cells were spun down once more and the pellet was resuspended in 1.5 ml of PBS at room temperature. After centrifugation it was re-suspended in 1 ml DNA staining solution containing 50 μg PI and 0.2 mg RNase (both Sigma, Disenhofen, Germany) and incubated for at least 45 min at room temperature in the dark. Between 1 and 5×10^5 cells per sample were analysed by flow cytometry (CyFlow, Partec, Muenster, Germany). The excitation wavelengths were 473 nm; green (520 nm for CFSE) and red fluorescence (> 590 nm for PI) were recorded. In addition, parameters for forward scatter (FSC) and side scatter (SSC) were determined. For each variable (exposure conditions, culture periods etc.) a minimum of six samples were quantified. The flow cytometer was calibrated with 2.5 μm polyfluorescent beads (AlignFlow, Molecular Probes, Eugene, OR) before each series of measurements. The fraction of cells present in different cell generations and their representation in respective cell cycle phases, was calculated using the mean level of measured fluorescence and phagocytic activity, with CYFLOW software (Partec, Muenster, Germany).

RT-PCR

Total RNA was isolated from 4 to 5×10^6 HL-60 cells using the Invisorb RNA Kit II (Invitac GmbH, Duisburg, Germany) for total RNA extraction. After photometric quantification (Ultraspex 2000, Pharmacia Biotech, Little Chalfont, UK), 500 ng of RNA was used in a 10-ml reverse-transcription reaction using 200 U of SuperScript reverse transcriptase (Qiagen, Hilgen, Germany), 40 units of RNase OUT (Promega, Mannheim, Germany), dNTPs (final concentration, 500 mmol/l), 50 pM oligo-dT15 primers (Life Technologies Inc., Karlsruhe, Germany), and buffer, as recommended by the supplier. Samples were incubated at 37 °C for 1 h followed by 20 min incubation at 60 °C. For PCR analysis, 1 ml of this cDNA preparation was used for each sample. Subsequent PCR amplification was carried out with cDNA derived from 25 ng of RNA in a 15 ml reaction mixture containing 0.4 unit *Taq* DNA polymerase (Peqlab Biotechnologie GmbH, Erlangen, Germany), 100 mM dNTPs, and 1 mM of each primer pair. Analysed genes and the respective primer pairs are listed in Table 1. Initial experiments were performed with a temperature-gradient thermocycler (Biometra, Goettingen, Germany) to determine the optimal temperature conditions of the PCR and the range of PCR cycles for 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, Goettingen, Germany) was set to 45 s at 94 °C, 30 s at 58 °C followed by 90 s 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% agarose in 1X TAE buffer (0.04 M Tris–acetate, 0.001 M ethylenediaminetetraacetic acid, pH 8.0) and were visualized by staining with ethidium bromide (0.01% in 1X TAE buffer; Sigma, Disenhofen, Germany). Results were calculated with reference to 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. Specific bands were quantified by area morphometric analysis using a digital imaging system (Biometra, Goettingen, Germany) and software (Optimas Co., Washington, DC, USA).

Table 1. Primer sequences used for reverse transcriptase and PCR

Genes	Primers (sense/antisense)	Location	mRNA ^a	GenBank
Hsp27	5'-ATGGCGTGGTGGAGATCACC-3' 5'-CAAAAGAACACACAGGTGGC-3'	451–470 797–778	347 bp	XM_004991
Hsp60	5'-ATTCCAGCAATGACCATTGC-3' 5'-GAGTTAGAACATGCCACCTC-3'	1444–1463 1749–1730	306 bp	NM_002156
Hsp70	5'-TTCCGTTTCCAGCCCCCAATC-3' 5'-CGTTGAGCCCCGCGATGACA-3'	435–455 993–974	559 bp	M11717
Hsc70	5'-TGTGGCTTCCTTCGTTATTGG-3' 5'-GCCAGCATCATTACCACCAT-3'	39–59 380–360	342 bp	NM006997
Mthsp75	5'-TGGCAGTTATGGAAGGTAAA-3' 5'-AGCAATGACTTGTCTTCTG-3'	228–248 752–732	525 bp	L15189
Grp78	5'-GATAATCAACCAACTGTAC-3' 5'-GTATCCTCTTCACCAGTTGG-3'	1584–1603 2162–2142	579 bp	XM_044201
Hsp90	5'-AAAAGTTGAAAAGGTGGTTG-3' 5'-TATCACAGCATCACTAGTA-3'	1803–1822 2426–2406	624 bp	X15183
β-actin	5'CAGCTCACCATGGATGATGAT3' 5'CTCGCCGTGGTGGTGAAGCT3'	1084–1104 2280–2260	626 bp	M10277

^aExpected size of PCR products if mRNA is amplified.

Statistics

Experimental results data are expressed as the mean $\pm$ standard deviation of several independent experiments. Statistical significance of the recorded effects was assessed using Student's *t*-test assuming two-tailed distributions and unequal variances.

RESULTS

Expression of the most prominent members of the *HS* gene family was analysed 30 min after exposing HL-60 and HeLa cells to X-rays (5 Gy) and HS (41 °C) exposure alone or in combination (Fig. 1). The data were compared to unexposed controls (set to 1). It was found that the observed reaction of HL-60 and HeLa cells to the both stressors was similar. The *HSP70* genes were inducible by both X-rays and HS alone ($P < 0.05$) and for HL-60 cells, the reaction was strongest when both stressors acted jointly ($P < 0.05$). Maximal induction was observed for *HSP70* transcripts as their level was approximately 5-fold higher in HL-60 cells than in unstressed controls ($P < 0.01$). Apart from *HSP70*, a significant response to the HS ($P < 0.05$) was only noted for *HSP75* and *HSP78* genes in HL60 cells. There were no changes in *HSC70* expression in response to the experimental stressors ($P > 0.05$).

Changes in cell cycle distribution of 10^5 cells were studied first by DNA flow cytometry 24 h after exposure to radiation with 0 (control), 1, 2 and 5 Gy (dose dependence). Results are presented in Table 2 and in Fig. 2, and indicate that radiation induced a G_2 block, which was dose dependent in all three cell lines. HL-60 cells showed the maximal response. Percentages of cells in the $G_2 + M$ phases increased up to $25 \pm 3\%$ (1 Gy, $P < 0.05$), $52 \pm 5\%$ (2 Gy, $P < 0.005$) and $81 \pm 6\%$ (5 Gy, $P < 0.001$) in comparison to the control cell population ($10 \pm 2\%$). For HeLa and AG01522 cells, the effect was much less prominent and significant changes were only registered after 5 Gy radiation exposure. In these cases the percentage of cells in the $G_2 + M$ phases

Figure 1. Expression of genes encoding several HSPs 1 h after radiation: X-rays (200 kV source, 20 mA, 0.5 mm Cu filter, 5 Gy at a dose rate of 1.2 Gy/min; labelled 'Rad'), HS (41 °C for 30 min; labelled 'HS'), and combined treatment (labelled 'HS + Rad') in HL-60 and HeLa cells analysed by RT-PCR and software-aided quantification. Quantification of respective mRNA levels calculated with reference to unstressed control cells (= 1). Mean of 11 experiments. Statistically significant differences ($P < 0.05$) are indicated by asterisks.

increased up to $39 \pm 6\%$ (compared to 20 ± 2 in controls, $P < 0.05$) and $31 \pm 5\%$ (compared to $9 \pm 2\%$ in controls, $P < 0.05$).

Differences in the behaviour of p53 wild type and p53 mutant cell lines were evident. Wild-type cells (AG01522) showed a G_1 block, that is, small changes in the population of cells in G_1 phase with a strong concomitant decrease of cells in S phase of the cell cycle. Amount of DNA synthesized in the cells reduced up to $7 \pm 2\%$ (in comparison to $16 \pm 3\%$ in controls, $P < 0.05$) after radiation exposure with a 5-Gy dose. With the p53 mutant cells (HL-60 and HeLa), a G_1 block was not apparent. Percentage of cells in the G_1 phase was dose-dependent reduced for HL60 cells up to $33 \pm 4\%$ (2 Gy, $P < 0.01$) and $7 \pm 2\%$ (5 Gy, $P < 0.001$), in comparison to $54 \pm 2\%$ in the control cell population, which was accompanied by the reduction of proportion of cells in S phase (Table 2). For HeLa cells, similar changes were found, but the effect was much weaker and significant reduction in percentage of G_1 phase cells was registered only after radiation exposure with 5 Gy ($27 \pm 6\%$ in comparison to $56 \pm 8\%$ in controls, $P < 0.05$). Thus, for the following comparative investigation of dynamics of the cellular response to the radiation and HS exposure, only HL60 cells were used.

Table 2. Changes of cell cycle distribution in 24 h after X-ray exposure

Cell type	Cell cycle distribution ^a			
	Dose (Gy)	G ₀₊₁ (%)	S (%)	G ₂ + M (%)
AGO1522	0	75 ± 5	16 ± 2	9 ± 2
	1	77 ± 8	11 ± 5	12 ± 3
	2	68 ± 5	12 ± 4	20 ± 5
	5	62 ± 7	7 ± 2*	31 ± 5*
HeLa	0	56 ± 8	24 ± 5	20 ± 2
	1	56 ± 5	26 ± 4	24 ± 3
	2	47 ± 6	20 ± 5	33 ± 5
	5	27 ± 6*	34 ± 2*	39 ± 6*
HL60	0	54 ± 2	36 ± 5	10 ± 2
	1	52 ± 5	23 ± 5	25 ± 3*
	2	33 ± 4**	15 ± 5*	52 ± 5***
	5	7 ± 2****	12 ± 6*	81 ± 6****

^aPercentage of cells in different phases of cell cycle (%) were calculated as average ± SD of four independent experiments. The level of statistically significant differences (bold) to control culture (0 Gy) is indicated by asterisks (**P* < 0.05, ***P* < 0.01, ****P* < 0.005, *****P* < 0.001).

Figure 2. Dose dependences. Dose dependence of AGO1522 (*p53*^{wt}), HeLa (*p53*^{-/-}) and HL60 (*p53*^{-/-}) cell cycle distribution 1 day after exposure to radiation with of 0 Gy (control), 1 Gy, 2 Gy and 5 Gy.

Figure 3. Time dependences. Time dependence of HL60 (*p53*^{-/-}) cell cycle distribution 1 day after exposure to radiation with a dose of 5 Gy or HS (41 °C, 30 min).

Figure 3 illustrates the time dependence of cell cycle distribution in 10^5 cells in 0 (control), 6, 12 and 18 h after the radiation (5 Gy) and HS (41 °C, 30 min) exposure as average of six independent experiments. Six hours after radiation or HS exposure, the percentage of the cell population in S phase increased up to $50 \pm 3\%$ (HS, $P < 0.05$) and $66 \pm 8\%$ (radiation, $P < 0.05$) in comparison to controls ($36 \pm 5\%$). This effect was probably the result of a delay in progression through the S phase, which is the predominant cell cycle effect after HS exposure (Zolzer & Streffer 2000). However, after 12 and 18 h of culture, differences between the effects of radiation and HS became apparent. The percentage of cells in $G_2 + M$ phases increased in time after radiation exposure up to $53 \pm 8\%$ (12 h, $P < 0.001$) and $65 \pm 6\%$ (18 h, $P < 0.001$) in comparison to controls ($10 \pm 2\%$). In contrast, HS-treated cells remained unchanged compared to controls ($P > 0.05$) after 12 and 18 h of culture.

In order to determine the long-term kinetics of different effects of radiation and HS on cell cycle progression of HL60 cells, the dynamics of cell division during successive cell cycles was monitored for 2, 4 and 6 days after radiation (with doses 0 (control), 1, 2 and 5 Gy) and HS (37 (control), 39 °C, 41 °C and 43 °C) exposure in six independent experiments. Cells were stained with CFSE at the beginning of the experiment. Labelled cells lose approximately half of their stain to daughter cells after completion of cell division. Thus, successive generations of cells can be identified on the basis of the CFSE fluorescence intensity. Finally, CFSE labelled cells were stained with PI so that cell distribution in different cell cycle phases could be analysed simultaneously for two or more cell generations.

Reduction of CFSE fluorescence after 2 days of culture is illustrated in Fig. 4. In sham-treated HL-60 cells (controls) almost ($75 \pm 3\%$) of the analysed cells were in the second cell division after 2 days of culture. As cells go through cell cycle at different rates some ($12 \pm 3\%$) had entered the G_1 phase of the third cycle, whereas another fraction of cells ($8 \pm 2\%$) cycled more slowly had not yet completed the first cycle. A small fraction of the cells ($5 \pm 3\%$) did not cycle and retained the original CFSE content (marked as 'delay'). These cells became hypodiploid

Figure 4. Proliferation of HL-60 cells analysed by quantifying CFSE content and PI fluorescence (DNA content). Because of the reduction of CFSE fluorescence with each cell division, the first, second and third divisions can be distinguished (labelled 1, 2 and 3; 0 indicates cell cycle at the time of CFSE labelling). The two-dimensional plots indicate CFSE and PI determination of cells cultured for 2 days after exposure to radiation (with dose 0 (sham treated control), 1, 2 and 5 Gy) of HS exposure (37 °C (sham treated control), 39 °C, 41 °C and 43 °C for 30 min). A population of arrested cells (in G₁/S phase, named 'delay') after HS, can clearly be separated from cycling cells. Two different populations of arrested cells ('delay' and in G₂/M phases) can be distinguished from cycling cells after radiation.

during incubation and presumably underwent apoptosis. For the purposes of this study, delayed and apoptotic cells were not distinguished from each other, and were together considered as 'delayed'. The numbers of these cells were compared to the population of proliferating cells.

Heat shock (39 °C, 41 °C, and 43 °C) increased the percentage of arrested cells. A rise in temperature by 2 °C (39 °C) above control (37 °C) had only a minor effect with respect to cell division, as most cells (80 ± 8% on the second day) continued their cell cycle. However, when cells were exposed to 41 °C, the percentage of proliferating cells decreased to 32 ± 4% ($P < 0.001$). At 43 °C only a small fraction of cells (4 ± 3%, $P < 0.001$) underwent division.

Two days after radiation exposure, two different populations of arrested cells could be identified easily. One population of cells remained arrested, closely resembling the HS-induced delay described previously. The percentage of such cells increased significantly in a dose-dependent manner by up to 17 ± 3% (1 Gy, $P < 0.05$), 24 ± 4% (2 Gy, $P < 0.005$) and 56 ± 8% (5 Gy, $P < 0.001$) compared to controls. However, in radiated cells, another prominent cell population could be distinguished. These cells arrested in the G₂/M stage and were characterized by DNA content typical for G₂/M phase, yet a CFSE content typical for G₁ phase cells of the same generation, but not for the larger G₂ cells. The percentage of such cells also increased with increasing radiation dose by up to 8 ± 2% (1 Gy, $P > 0.05$), 15 ± 2% (2 Gy, $P < 0.05$) and 35 ± 5% (5 Gy, $P < 0.001$) compared to controls (8 ± 2%), which did not complete the first division.

Because asynchronously proliferating cell cultures were used in all experiments, a difference of one cell cycle is to be expected when analysing proliferation during several rounds of division. This may easily be seen when comparing cells cultured for 2, 4 and 6 days (Fig. 5). For example, after 4 days of culture, cells passed through a minimum of three and a maximum

Figure 5. The effect of HS and radiation on cell division. Analysis of cell proliferation was carried out after 2, 4 and 6 days of incubation following exposure to the stressors: radiation (5 Gy) and HS (41 °C for 30 min). The different cell generations are labelled '1–7' and '0' for cell populations that did not divide.

of six cycles of cell division. At this time only a small fraction of cells did not divide or was apoptotic ($5 \pm 3\%$), whereas the remaining cells ($95 \pm 3\%$) proliferated.

After HS (41 °C) exposure, the populations of arrested cells showed high levels of CFSE under experimental conditions, indicating that they did not re-enter the cell cycle. According to their DNA content (PI staining), the arrested cells were in the G₁ and S stages, but not in the G₂/M. None of the cells delayed in S phase for 2 days were present later in the first or second division, no new cell generations arose after 4 or 6 days. The number of arrested cells decreased as a result of apoptosis and proliferation of cycling cells. Therefore, the fraction of proliferating cells increase continuously as shown for cultured cells 4 and 6 days after the HS treatment.

After radiation exposure, cell populations that were delayed in S phase did not re-enter the cell cycle as did HS-treated cells. None of the cells with high CFSE, which were delayed in the S phase for 2 days, reached the G₂ phase in day 4 or 6 of culture after radiation exposure with dose 5 Gy (Fig. 5). As was the case for HS treatment, the number of these cells decreased with time as a result of apoptosis and proliferation of cycling cells. Cells that were blocked after 2 days in G₂/M, and which could be registered even 4 days after irradiation with a dose of 5 Gy ($6 \pm 2\%$, $P < 0.001$), showed quite different long-term dynamics. Some of them became apoptotic without division, had a reduced DNA amount, a high CFSE content and in 4 days after radiation exposure occupied the area near the cells delayed in S phase, as may be found in the dot plot histogram (Fig. 5). Other cells underwent mitosis associated with the expected reduction in

the amount of DNA. They then re-entered the cell cycle or became apoptotic after one or several divisions. Some cell populations occupied an area of the dot plot histogram with smaller CFSE content and sub-diploid DNA content and appeared in cell cultures 4 and 6 days after irradiation. As was found in the case for after HS treatment, the percentage of arrested and apoptotic cells was reduced because of further proliferation of cycling cells. For example, in the following 2 days of culture (from second to fourth) after radiation exposure with a dose of 5 Gy, the fraction of proliferating cells almost doubled and reached $20 \pm 5\%$ ($P < 0.05$) of total cell population analysed.

DISCUSSION

This study has investigated the cellular response to ionizing radiation and to HS, two stressors that are known to act differently at the molecular level. It is well established that thermal stress denatures proteins, whereas the energy of ionizing radiation (such as X-rays) is sufficient to damage proteins and DNA directly (or indirectly) by the production of reactive oxygen species (Bryant 1997; Kang *et al.* 2006). In this manuscript, we have paid particular attention to the varied cellular responses to these stressors.

We chose the HL-60 and HeLa cell lines for gene expression experiments as these cells are known to respond to various stressors with quite different induction of the *HSP70* gene (Wang *et al.* 1999; Tokalov *et al.* 2003). To demonstrate and quantify HS gene induction, the RT-PCR method was used. Under experimental conditions, the RT-PCR method is highly reproducible and provides information concerning the concentration of respective transcripts in the exposed cells. Six human *HSP* genes were included in the analysis and their mRNA levels were determined at the end of HS or radiation exposure. Kinetics of the induction and down-regulation of the *HSP70A* gene following short-term HS exposure (for example, 30 min) have been studied previously (Tokalov & Gutzeit 2004). Thus, an appropriate response under the chosen experimental conditions could be expected. The induction of the HS genes by chemical and physical stressors is a universal property of all cells (Morimoto 1998; Helmbrecht *et al.* 2000). However, the response may vary widely, both qualitatively and quantitatively between different cell types *in vivo* and in various commonly used cell lines. The strongest induction is typically observed for the *HSP70* gene (Wang *et al.* 1999; Tokalov *et al.* 2003; Tokalov & Gutzeit 2004), and is in accordance with the results of this study. However, the response may differ quantitatively in different human cell lines. For example, following an HS at 42 °C for 2 h, about a 6-fold induction of HSP70 transcripts was found in human fibroblasts, HeLa, and Jurkat cells, whereas the increase was 23-fold in MCF-7 cells (Wang *et al.* 1999).

Recent data indicate that the cellular response to HS involves activation of various signalling pathways, although the molecular mechanism triggering these responses is still unclear (Gabai *et al.* 2002). Although HS exposure produces similar effects, in common with X-rays, on the level of *HSP70* gene expression, analysis of the cell cycle using CFSE allows demonstration of the effects with respect to the dynamics of cell proliferation. This study confirmed earlier findings that radiation and HS can induce a delay in proliferation of the *p53* mutant cells (Kuhl *et al.* 2000; Zolzer & Streffer 2000; Tokalov *et al.* 2003).

Experiments following radiation with differing incubation times clearly showed that cells blocked in the G₂ phase indeed were derived from the G₁ (and S) cells of the same generation, and, finally, became arrested (Zolzer & Streffer 2000). A similar delay in the S phase followed by a final block in G₂/M was shown in dynamics of mouse Ehrlich ascites carcinoma cells, after

exposure to a range of doses (2, 4, 6, 8, 10, 15 and 20 Gy) of X-rays *in vivo* using single-parameter DNA flow cytometry (Tokalov 1990). The phenomenon of HS induction of the S-phase delay was first reported for MeWo human melanoma cells and its time and dose dependence was quantified by BrdU incorporation (Zolzer & Streffer 1993). A similar effect has been demonstrated in 4451 human squamous carcinoma cells, both with the mutated *p53* gene (Zolzer & Streffer 2000). This may be explained by, perhaps, the cells' increasing difficulty of DNA replication with *p53* absence. The cells are not subjected to G₁/S block and therefore have no temporal opportunity for DNA repair before entering into the S phase (Pellegata *et al.* 1996). As a result, DNA synthesis in such cells is completely blocked. However, the method of BrdU incorporation does not allow a long-term analysis of cell cycle dynamics. Kuhl *et al.* (2000) have described HS blocking the cell cycle and, under certain conditions, an increased fraction of cells accumulating in G₁ or G₂/M cell cycle checkpoints. It is possible that a G₂/M block existed for a short period after HS exposure, but was not registered here because of the experimental design. Meanwhile, the CFSE labelling technique revealed that a large fraction of the exposed cells lost the ability to proliferate and never regained it. Cells became apoptotic if the cell cycle was blocked by HS. The two cell populations, that is, cycling cells and arrested cells, could be distinguished during the entire experiment, and a widening gap between these populations in the two-dimensional dot plot histogram was noticeable (Fig. 5). Cells in the G₂/M phase possessed a higher CFSE content than G₁ cells of the same generation. It appeared that these cells composed a 'founder' population, after a short radiation-induced delay, for a few actively cycling cells (third cycle) noted in this study. This is supported by research showing that cells are more resistant to radiation in the late S and G₂ phases (Sinclair 1968). Further studies using a variety of human cell lines showed that the cell cycles' specific response to radiation is qualitatively similar, although quantitative differences exist between different cell types (Zolzer & Streffer 2000).

For a substantial amount of time, ionizing radiation or elevated temperatures have been used to treat cancer in a variety of forms. Mutations of the *p53* gene are observed very frequently in human tumours (Wang *et al.* 2001; Eicheler *et al.* 2002; Szymanska & Hainaut 2003) and loss of *p53* function has been correlated with decreased sensitivity to chemotherapy, radiation and hyperthermia therapies in a variety of human tumours (Takahashi *et al.* 2004). Moreover, synergistic depression of tumour growth by combined radiation and HS treatment has been found only in *p53*^{wt} tumours (Ohnishi 2005). Here, we have evaluated cell cycle perturbations and apoptosis after the action of radiation or HS treatment in cell lines exhibiting either wild-type *p53* or deleted *p53* expression. These results explain the previous findings of thermal enhancement of radiosensitivity (Yasumoto *et al.* 2003; Takahashi *et al.* 2004), which at least partially takes place as a result of heat inactivation of cell survival systems through induced alterations in cell cycle regulation. Nevertheless, in agreement with previous suggestions (Takahashi *et al.* 2004), we may conclude that *p53* status could be a useful indicator in predictive assays for HS anticancer therapy and hyperthermia treatment (in combination with radiation and/or chemotherapy). Based on such predictive assays, this might improve the outcome and efficiency of cancer treatment in the future.

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REFERENCES

- Bryant PE (1997) DNA damage, repair and chromosomal damage. *Int. J. Rad. Biol.* **71**, 675–680.
- Camplejohn RS, Rutherford J (2001) p53 functional assays: detecting p53 mutations in both the germline and in sporadic tumours. *Cell Prolif.* **34**, 1–14.
- Eicheler W, Zips D, Dorfler A, Grenman R, Baumann M (2002) Splicing mutations in TP53 in human squamous cell carcinoma lines influence immunohistochemical detection. *J. Histochem. Cytochem.* **50**, 197–204.
- Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Ann. Rev. Physiol.* **61**, 243–282.
- Gabai VL, Sherman MY (2002) Interplay between molecular chaperones and signalling pathways in survival of heat shock. *J. Appl. Physiol.* **92**, 1743–1748.
- Gutzeit HO (2001) Interaction of stressors and the limits of cellular homeostasis. *Biochem. Biophys. Res. Commun.* **283**, 721–725.
- Hasbold J, Gett AV, Rush JS, Deenick E, Avery D, Jun J, Hodgkin PD (1999) Quantitative analysis of lymphocyte differentiation and proliferation *in vitro* using carboxyfluorescein diacetate succinimidyl ester. *Immunol. Cell Biol.* **77**, 516–529.
- Helmbrecht K, Zeise E, Rensing L (2000) Chaperones in cell cycle regulation and mitogenic signal transduction: a review. *Cell Prolif.* **33**, 341–365.
- Higashikubo R, White RA, Roti Roti JL (1993) Flow cytometric BrdUrd-pulse-chase study of heat-induced cell-cycle progression delays. *Cell Prolif.* **26**, 337–348.
- Higashikubo R, Ragouzis M, Roti Roti JL (1996) Flow cytometric BrdUrd-pulse-chase study of X-ray-induced alterations in cell cycle progression. *Cell Prolif.* **29**, 43–57.
- Jolly C, Morimoto RI (2000) Role of the heat shock response and molecular chaperones in oncogenesis and cell death. *J. Natl. Cancer Inst.* **92**, 1564–1572.
- Kang KA, Zhang R, Lee KH, Chae S, Kim BJ, Kwak YS, Park JW, Lee NH, Hyun JW (2006) Protective effect of triphloretol-A from *Ecklonia cava* against ionizing radiation *in vitro*. *J. Radiat. Res. (Tokyo)* **47**, 61–68.
- Kuhl NM, Kunz J, Rensing L (2000) Heat shock-induced arrests in different cell cycle phases of rat C6-glioma cells are attenuated in heat shock-primed thermotolerant cells. *Cell Prolif.* **33**, 147–166.
- Lyons AB (1999) Divided we stand: tracking cell proliferation with carboxyfluorescein diacetate succinimidyl ester. *Immunol. Cell Biol.* **77**, 509–518.
- McNally NJ, Wilson JD (1986) Cell kinetics of normal and perturbed populations measured by incorporation of bromodeoxyuridine and flow cytometry. *Br. J. Radiol.* **59**, 1015–1022.
- Molinari M (2000) Cell cycle checkpoints and their inactivation in human cancer. *Cell Prolif.* **33**, 261–274.
- Morimoto RI (1998) Regulation of the heat shock transcriptional response: cross-talk between a family of heat shock factors, molecular chaperones and negative regulators. *Gen. Dev.* **12**, 3788–3796.
- Ohnishi T (2005) The role of the p53 molecule in cancer therapies with radiation and/or hyperthermia. *J. Cancer Res. Ther.* **1**, 147–150.
- Pellegata NS, Antoniono RJ, Redpath JL, Stanbridge EJ (1996) DNA damage and p53-mediated cell cycle arrest: a re-evaluation. *Proc. Natl. Acad. Sci. USA* **93**, 15209–15214.
- Rice G, Laszlo G, Li G, Gray J, Dewey W (1986) Heat shock proteins within the mammalian cell cycle: relationship to thermal sensitivity, thermal tolerance, and cell cycle progression. *J. Cell Physiol.* **126**, 291–297.
- Sinclair WK (1968) Cyclic X-ray responses in mammalian cells *in vitro*. *Radiat. Res.* **33**, 620–643.
- Smith DF, Whitesell L, Katsanis E (1998) Molecular chaperones: biology and prospects for pharmacological intervention. *Pharmacol. Rev.* **50**, 493–513.
- Szymanska K, Hainaut P (2003) TP53 and mutations in human cancer. *Acta Biochim. Pol.* **50**, 231–238.
- Takahashi A, Matsumoto H, Nagayama K, Kitano M, Hirose S, Tanaka H, Mori E, Yamakawa N, Yasumoto J, Yuki K, Ohnishi K, Ohnishi T (2004) Evidence for the involvement of double-strand breaks in heat-induced cell killing. *Cancer Res.* **64**, 8839–8845.
- Takayama S, Reed JC, Homma S (2003) Heat-shock proteins as regulators of apoptosis. *Oncogene* **22**, 9041–9047.
- Tokalov SV (1990) The analysis of postradiative cytodynamic of normal and tumour tissues by flow cytometry. PhD Thesis, Central Research Institute of Roentgenology and Radiology, Ministry of Public Health, Leningrad, Russia.
- Tokalov SV, Gutzeit HO (2003) The heat shock-induced cell cycle arrest is attenuated by weak electromagnetic fields. *Cell Prolif.* **36**, 101–111.
- Tokalov SV, Gutzeit HO (2004) Weak electromagnetic fields (50 Hz) elicit a stress response in human cells. *Environ. Res.* **94**, 145–151.
- Tokalov SV, Pieck S, Gutzeit HO (2003) 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. *J. Appl. Biomed.* **1**, 85–92.

- Turman MA, Kahn DA, Rosenfeld SL, Apple CA, Bates CM (1997) Characterization of human proximal tubular cells after hypoxic preconditioning: constitutive and hypoxia-induced expression of heat shock proteins HSP70 (A, B, and C), HSC70 and HSP90. *Biochem. Mol. Med.* **60**, 49–58.
- Wang SM, Khandekar JD, Kaul KL, Winchester DJ, Morimoto RI (1999) A method for the quantitative analysis of human heat shock gene expression using a multiple RT-PCR assay. *Cell Stress Chaperones* **4**, 153–161.
- Yasumoto J, Takahashi A, Ohnishi K, Ohnishi T (2003) The role of p53 molecule in radiation and hyperthermic therapies. *J. Health Sci.* **49**, 273–277.
- Zolzer F, Streffer C (1993) Induction of quiescent in S-phase cells by irradiation and/ or hyperthermia. I. Time and dose dependence. *Int. J. Radiat. Biol.* **63**, 69–76.
- Zolzer F, Streffer C (2000) Quiescence in S-phase and G₁ arrest induced by irradiation and/ or hyperthermia in six human tumour cell lines of different p53 status. *Int. J. Radiat. Biol.* **76**, 717–725.
- Zugel U, Kaufmann SHE (1999) Role of heat shock proteins in protection from and pathogenesis of infectious diseases. *Clin. Microbiol. Rev.* **12**, 19–39.