

Chromosomal Damage in Two X-ray Irradiated Cell Lines: Influence of Cell Cycle Stage and Irradiation Temperature

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Abstract. *The aim of this study was to investigate if irradiation with X-rays in different cell cycle phases resulted in a different response as measured with the micronucleus technique. In addition, the influence of irradiation temperature was investigated. Materials and Methods: Cells from a non-transformed human fibroblast cell line, HS2429, and a human breast cancer cell line, MCF-7, were synchronized by thymidine block and irradiated at either 2°C or 37°C in the G1-, S- and G2/M-phases. After cytokinesis-block by cytochalasin B, the frequency of micronuclei was determined. Results: Clear dose-response relationships were found. More micronuclei were detected in fibroblast cells irradiated in G1 and S than in G2/M, while the differences were not as prominent in MCF-7 cells. The irradiation temperature had no significant influence on the formation of micronuclei in either of the cell lines. Conclusion: The formation of micronuclei varies with the cell cycle stage at the time of irradiation.*

It has long been known that the cell cycle stage strongly influences the radiosensitivity of irradiated cells (1). Chromatin organization influences DNA damaging processes, e.g. induction of double-strand breaks (DSB) (2), which may directly affect secondary cellular events such as formation of chromosomal aberrations and survival of irradiated cells. Since the chromatin structure varies during the cell cycle, we investigated whether chromosomal damage varies in irradiated cells synchronized in G1, S and G2/M phases of the cell cycle. The endpoint chosen, the formation of micronuclei (MN), is a well-established endpoint for chromosomal damage after the first mitosis

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and measures a response when processes involving DNA damage induction and repair have been completed.

We have shown that several types of DNA damage and the clonogenic survival are reduced on hypothermic irradiation of human cells (3-5). The induction of double-strand breaks after hypothermic irradiation is reduced and this decrease is correlated to the chromatin organization (2). Therefore, we examined whether the irradiation temperature influences chromosomal damage.

The specific aim of this study was to investigate if the number of MN induced after irradiation depends on cell cycle stage in normal (diploid fibroblasts) and transformed (breast adenocarcinoma MCF-7 cell line) human cells and whether the irradiation temperature could modify the number of MN induced.

Materials and Methods

Cell lines and culture conditions. The human foreskin fibroblast cell line HS2429 was purchased from ECACC, UK. The cells were cultured at low passage number in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum, 4 mM Glutamax™ and 2% sodium bicarbonate. The human breast adenocarcinoma MCF-7 cell line (ECACC) were maintained as cultures in Eagle's Minimal Essential Medium, supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% non-essential amino acids and 1% sodium pyruvate. All cell reagents were purchased from Gibco (Scotland, UK).

Synchronization of cells. In order to obtain cultures enriched with cells in G1, S and G2/M phase, cells were synchronized using the thymidine-block method (6), slightly modified by incubation with serum-free medium for 24 h in order to pre-accumulate cells in G1 before thymidine incubation, thereby increasing the synchronization rate. On day 1, 1.2x10⁵ cells/dish were seeded in 60-mm petri dishes. Twenty-four hours after plating, the medium was replaced with 5 ml serum-free medium (day 2). The serum-free medium was replaced 24 h later (day 3) with 5 ml complete medium containing 2 mM thymidine (Sigma-Aldrich, Sweden). Twenty-four hours later (day 4), cells were released from thymidine-induced block by rinsing the dishes three times with medium and were then incubated in complete medium for cell

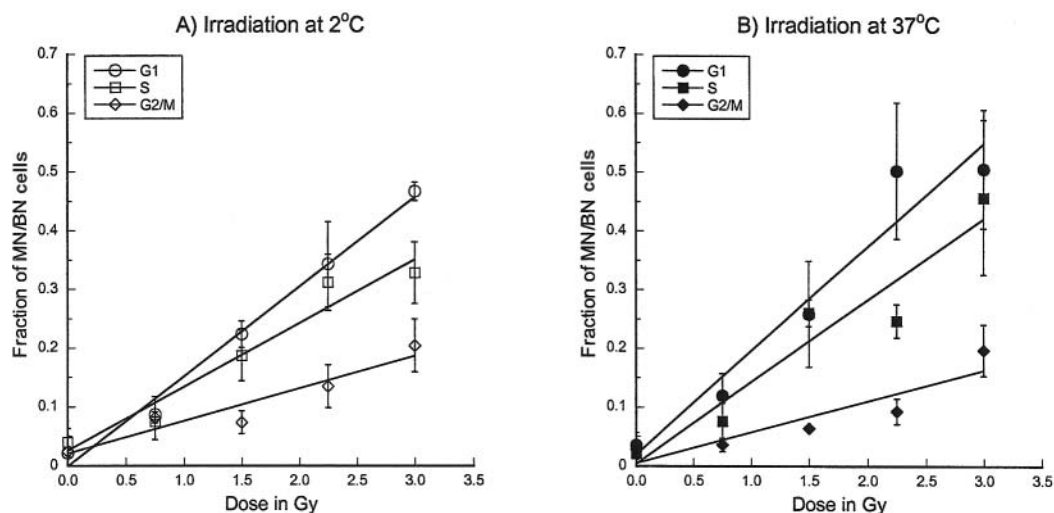


Figure 1. Fraction of micronuclei/bi-nucleated fibroblast HS2429 cells after irradiation in different cell cycle phases at 2°C (A) or 37°C (B). Data points represent the mean ± SEM from three independent experiments and the lines are linear regressions.

cycle analysis or irradiation experiments. In experiments where HS2429 cells were synchronized in G1 by serum starvation, 1.2x10⁵ cells/dish were seeded on day 1. Twenty-four hours after plating, cell cultures were treated with medium without serum for 24 h.

Flow cytometric analysis of DNA content. Flow cytometry was performed at different time points after thymidine release. Cells were harvested by trypsinization and centrifuged with 50 µl standard containing erythrocytes from chicken and trout with known DNA indices. Cells were fixed in 2 ml 70% ethanol (-20°C) and stored at -20°C overnight. The following day, cells were rinsed with PBS, centrifuged and then labeled in propidium iodide-nuclear isolating medium (50 µg/ml propidium iodide, 0.6% NP-40 and 100 µg/ml RNase in PBS) for 30 min at room temperature and stored for 24 h in a refrigerator. The DNA content per nucleus was analyzed using a Cytoron Absolute (Ortho Diagnostic Systems Inc., USA) and the cell cycle analysis software Multicycle (Phoenix Flow Systems, USA).

Irradiation procedure. Cells were seeded and synchronized as described above, and irradiated in G1, S and G2/M phase using a Philips RT 100 X-ray tube. Irradiations were performed with two applicators working at a distance of 10 cm (70 kV, 1.25 mm Al, 11.6 Gy/min) or 30 cm (100 kV, 1.7 mm Al, 1.35 Gy/min) depending on the dose. The irradiation at the two temperatures (2°C and 37°C) took place on a perfused glass plate connected to a circulating water-bath.

MN assay. Immediately after irradiation, the medium was replaced with fresh complete medium containing 1.1 or 1.8 µg/ml cytochalasin B (Sigma-Aldrich) for MCF-7 and HS2429 cells, respectively. The cells were fixed in 70% ethanol for 10 min and stained with 3% Giemsa for 20 min after 48 h (MCF-7) or 72 h (HS2429). The dishes were coded before scoring and examined at x1000 magnification using a light microscope (Axioskop, Zeiss, Germany). The number of MN in 500 bi-nucleated cells per dish were counted according to the criteria of Fenech *et al.* (7). MN formation was expressed as the ratio of the total number of MN to the number of bi-nucleated (BN) cells counted (MN/BN).

Table I. Cell cycle distributions of HS2429 and MCF-7 cells after different synchronization protocols.

HS2429		MCF-7	
Treatment	% cells ± SEM ^a	Treatment	% cells ± SEM
0 h ^b	G1 41±2.0	0 h ^b	G1 90±0.64
	S 44±2.8		S 5.3±0.39
	G2/M 15±1.1		G2/M 5.0±0.31
2 h ^b	G1 26±3.3	7 h ^b	G1 38±4.4
	S 71±1.2		S 51±3.9
	G2/M 29±2.5		G2/M 11±0.63
6 h ^b	G1 0	13 h ^b	G1 23±0.61
	S 9.7±1.1		S 17±0.54
	G2/M 90±2.4		G2/M 60±1.1
Serum starvation ^c	G1 81±2.1	Serum starvation ^c	G1 NA
	S 7.6±1.9		S NA
	G2/M 12±1.1		G2/M NA
Asynchronous ^d	G1 38±2.2	Asynchronous ^d	G1 62
	S 43±3.0		S 28
	G2/M 19±1.5		G2/M 10

^aCalculated on the fraction of cycling cells, which was calculated by subtracting the fraction of G1 cells that did not enter the S-phase after 8 h; ^bTime after thymidine release; ^c24 h serum starvation and incubation in complete medium for 3 h; ^d 24 h after subcultivation.

Results

The fraction of BN cells after irradiation with 3 Gy in G1, S or G2/M was about 10% and 50% for HS2429 and MCF-7 cells, respectively. This difference in dividing

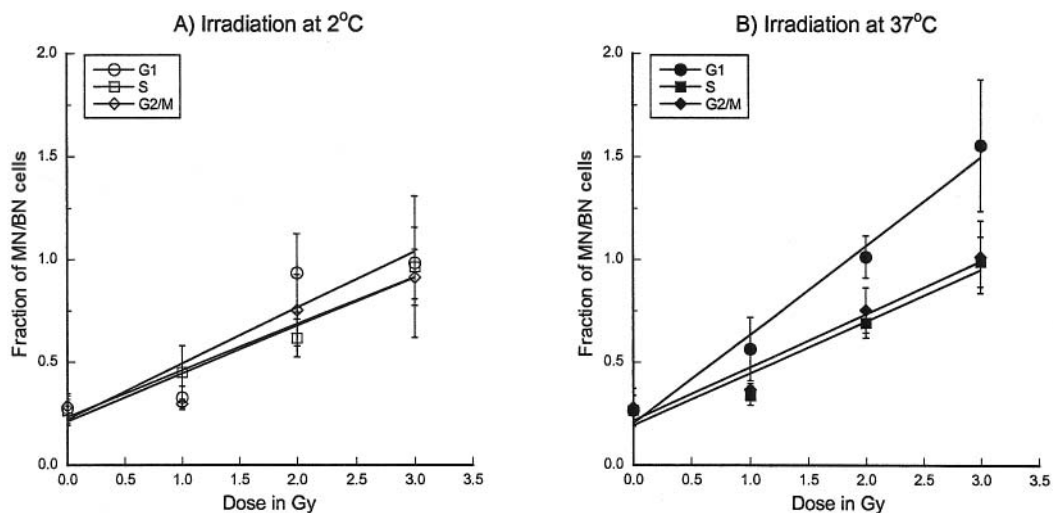


Figure 2. Fraction of micronuclei/bi-nucleated MCF-7 cells after irradiation in different cell cycle phases at 2°C (A) or 37°C (B). Data points represent the mean \pm SEM from three independent experiments and the lines are linear regressions.

fraction was expected since normal fibroblasts respond to ionizing radiation by G1 arrest, thereby decreasing the mitotic index.

Cell cycle distributions for different cell cultures are shown in Table I. The dose-response relationships for HS2429 cells, irradiated in different cell cycle phases at 2°C or 37°C is shown in Figure 1. For each data point, a total of 1500 BN cells from three independent experiments were analyzed. More MN were formed after irradiation in the G1 and S phases compared with the G2/M phase. The dose-response relationships for MCF-7 cells, established from analysis of 1500 cells/data point from three independent experiments, are shown in Figure 2. There was a spontaneous formation of MN in unirradiated cells, which was higher in MCF-7 cells compared with HS2429 cells. For MCF-7 cells, the highest induction of MN occurred upon irradiation at 37°C in the G1-phase.

The synchronization rate in G1 after thymidine block was poor for HS2429 cells and to obtain G1-accumulated cell population, HS2429 cells were serum starved for 24 h and then irradiated at 2°C and 37°C, as shown in Figure 3. Dose-response relationships for asynchronous cells irradiated at different temperatures are shown in Figure 4. Neither of the situations described above revealed a statistical difference in temperature dependence using Student's *t*-test of the difference in *k*-values of the regression lines.

In another set of experiments asynchronous HS2429 cells were also irradiated with 4 Gy at different temperatures and assessed for G2 arrested cells using flow cytometric analysis. Maximal G2 arrest was obtained 9 h post-irradiation. The

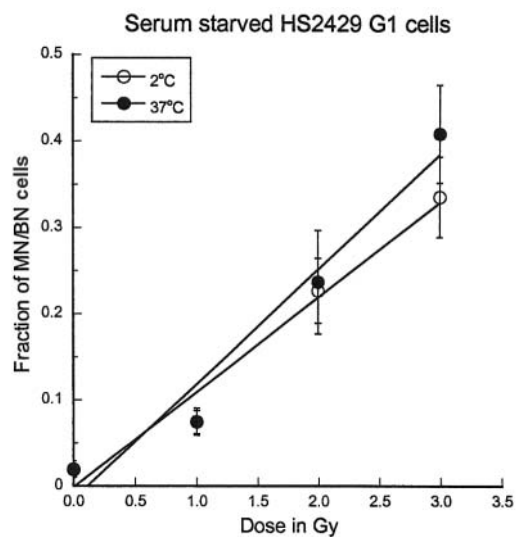


Figure 3. Fraction of micronuclei/bi-nucleated HS2429 cells after irradiation at 2°C (open symbols) or 37°C (closed symbols) in G1, obtained by serum starvation. Data points represent the mean \pm SEM from three independent experiments and the lines are linear regressions.

relative increase in G2 cells compared with cell cultures analyzed immediately after irradiation was calculated (data not shown). The number of cells in G2 increased by a factor of two (2.4 ± 0.23 and 2.3 ± 0.30 for 2°C and 37°C, respectively), showing no difference between the two irradiation temperatures thus supporting the results from the MN experiments.

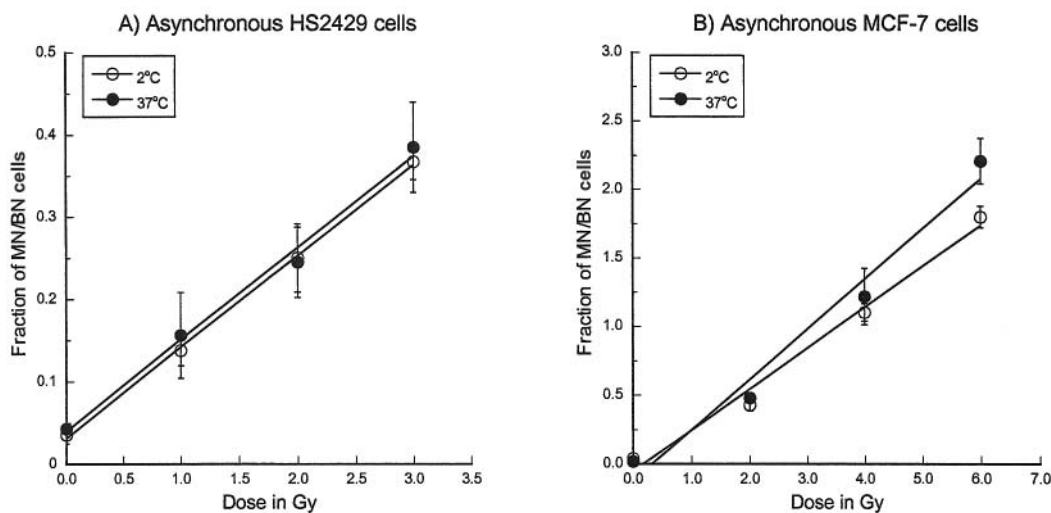


Figure 4. Fraction of micronuclei/bi-nucleated cells from asynchronous cultures of HS2429 cells (A) or MCF-7 cells (B) after irradiation at 2°C or 37°C. Data points represent the mean \pm SEM from three independent experiments and the lines are linear regressions.

Discussion

The purpose of this study was to investigate if the cell cycle stage during irradiation with X-rays influences the formation of MN in human normal fibroblasts and the adenocarcinoma cell line MCF-7. We also wanted to determine whether the irradiation temperature had any impact on the number of MN detected.

Several criteria for the synchronization procedure had to be fulfilled. First, we wanted to use an identical protocol for both cell lines with as high synchronization rates as possible and second, the procedure had to be reversible to allow cells to continue cycling and successfully pass through mitosis in order to express MN after first division. The protocol that met these two criteria was serum starvation followed by thymidine block and release. Still, the synchronization was not complete and a substantial fraction (29%) of HS2429 cells was permanently trapped in G1 after thymidine treatment. It is reasonable, however, to believe that they did not pass through mitosis as required for MN assessment at a later time point and therefore, those cells were not falsely classified as S or G2 cells in the assay.

The MN assay is widely used in radiobiology. It is a fast and relatively simple method for quantification of chromosomal damage and the use of the cytokinesis inhibitor cytochalasin B allows a specific tagging of cells that have passed mitosis only once after irradiation (8). Optimization of cytochalasin B concentration and incubation time were made for the two cell lines (data not shown). Induction of MN was expressed as the number of micronuclei per bi-nucleated cell. As expected, the spontaneous formation of MN in unirradiated cells was

higher in the transformed cell line MCF-7 due to a larger and more unstable genome (9), compared with the normal fibroblasts, and there were more cells with a large number of MN (5 or 6) in MCF-7 cells than what would be expected from a Poisson distribution (data not shown). The data presented here shows that in the case of the fibroblast cell line, cells that were irradiated in G2/M expressed a 50-70 % lower frequency of MN compared with cells irradiated in G1, S or a mixture of both. The situation in MCF-7 cells was different: here the most sensitive cell cycle phase was G1 while cells irradiated in S and G2/M exhibited equally fewer MN, but this was only found for cells irradiated at 37°C. The literature on cell cycle effects on chromosomal damage is conflicting. It was reported that cells irradiated in G2 are more sensitive than cells irradiated in G1 and S (10, 11) but it was shown that cells in early G2 exhibit far fewer MN than cells in late G2 (11), supporting our data on the fibroblast cell line. On the other hand, MN in MCF-7 cells irradiated in S were found to occur more frequently than in cells irradiated in G1 (12). These contradictory findings could possibly be explained by differences in choice of synchronization protocols, which may have a large impact on the cellular response, as well as the cell origin and genetic background.

In previous publications, we have shown that the temperature effect on DSB induction correlates with the scavenging capacity and chromatin organization (2, 5). The protective effect of low temperature during irradiation decreased with increased chromatin organization and, therefore, it would have been expected that the most temperature sensitive phase as measured by the MN assay would be the S phase. Here the chromatin structure is more

open and susceptible to radical attacks, a process that has been shown to depend on the irradiation temperature (2, 5). Similarly, the most insensitive phase would be the G2/M phase where the chromatin is compacted and prepared for chromosome condensation. Moreover, we have shown that the clonogenic survival of MCF-7 cells increased after irradiation at 2°C compared with 37°C by a factor of 1.2 (13), as well as an increased growth of a glioma cell line, irradiated at 28°C (14). Furthermore, there are some early publications on the protective effect of hypothermic irradiation temperature on chromosomal damage in human lymphocytes (15, 16) with a dose modifying factor (DMF_{temp}) between 1.5 and 2.0. In the present study we found no statistically significant difference between the two irradiation temperatures for any of the cell cycle phases. Thus, for chromosomal damage we did not find a DMF_{temp} of a magnitude similar to that we have revealed in DNA or chromatin (2) for DSB induction, but numerically close to what we found for clonogenic survival (13). For the normal fibroblast cells, the DMF_{temp} in S>G1>G2/M as expected but again, this difference was not statistically significant. Based on our observations, we conclude that early radiochemical DNA damaging events that are temperature sensitive do not necessarily correlate to chromosomal damage assessed as MN formation.

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