Effect of hypothermia on radiation-induced micronuclei and delay of cell cycle progression in TK6 cells

Halina Lisowska1, Karl Brehwens2, Friedo Zölzer3, Aneta Wegierek-Ciuk1, Joanna Czub4, Anna Lankoff1,5, Siamak Haghdoost2 & Andrzej Wojcik1,2

1Department of Radiobiology and Immunology, Institute of Biology, Jan Kochanowski University, Kielce, Poland, 2Centre for Radiation Protection Research, MBW Department, Stockholm University, Sweden, 3Department of Radiology, Toxicology and Civil Protection, University of South Bohemia in Budweis, Czech Republic, 4Department of Medical Physics, Institute of Physics, Jan Kochanowski University, Kielce, Poland, and 5Institute of Nuclear Chemistry and Technology, Warsaw, Poland

Abstract
Purpose: Low temperature (hypothermia) during irradiation leads to a reduced frequency of micronuclei in TK6 cells and it has been suggested that perturbation of cell cycle progression is responsible for this effect. The aim of the study was to test this hypothesis.

Materials and methods: Human lymphoblastoid TK6 cells were treated by a combination of hypothermia (0.8°C) and ionizing radiation in varying order (hypothermia before, during or after irradiation) and micronuclei were scored. Growth assay and two-dimensional flow cytometry was used to analyze cell cycle kinetics following irradiated of cells at 0.8°C or 37.0°C.

Results: The temperature effect was observed at the level of micronuclei regardless of whether cells were cooled during or immediately before or after the radiation exposure. No indication of cell cycle perturbation by combined exposure to hypothermia and radiation could be detected.

Conclusions: The protective effect of hypothermia observed at the level of cytogenetic damage was not due to a modulation of cell cycle progression. A possible alternative mechanism and experiments to test it are discussed.

Keywords: Hypothermia, temperature, cell cycle, micronuclei

Introduction
In many radiobiological experiments mammalian cells are exposed to radiation on ice in order to inhibit cellular processes such as DNA repair (for examples, see Lisowska et al. 2006, Padjas et al. 2012). It is assumed that all processes inhibited by hypothermia are restored at 37°C. However, hypothermia has been shown to act in a radioprotective manner (this is referred to as the ‘temperature effect’ [TE]) indicating that the cooling of cells does not only lead to a transient inhibition of cellular process, but somehow influences the cellular radiosensitivity. The TE was detected using various endpoints such as clonogenic survival (Belli and Bonetti 1963, Elmroth et al. 2000), survival of mice (Levan et al. 1970), enzyme activity (Kempner and Haigler 1982), frequency of chromosomal aberrations (Bajerska and Liniecki 1969, Gumrich et al. 1986, Virsik-Peuckert and Harder 1986, Lisowska et al. 2013), frequency of micronuclei (MN) (Brzozowska et al. 2009, Brehwens et al. 2010, Dang et al. 2012) and DNA supercoiling unwinding (Elmroth et al. 1999a, 1999b). At first glance, the TE might seem trivial, but it demonstrates that variation of temperature at radiation exposure may have serious implications for the outcome of the experiment. Although the TE is well documented, its underlying mechanisms remain largely unknown.

In a recent study (Dang et al. 2012) we investigated the mechanisms of hypothermia in human lymphoblastoid TK6 cells. We analyzed DNA damage signaling by inhibiting the ataxia telangiectasia mutated (ATM) kinase with the inhibitor KU55933, the role of chromatin conformation by inhibiting histone deacetylation using trichostatin A, the frequency of MN in sequentially harvested cells, the kinetics of DNA repair by the gamma-H2AX (activated histone 2AX) assay as well as clonogenic cell survival. The TE was evident only at the level of micronuclei at a single fixation time, was not influenced by the inhibition of ATM kinase activity, and was not seen at the level of gamma-H2AX foci and cell survival. It was completely abolished by the histone deacetylase inhibitor which led us to suggest that the TE is based on a temporary cell cycle shift that could lead to the reduced micronucleus frequency.

We have now carried out a series of experiments to test this hypothesis. First, TK6 cells were treated by a combination of hypothermia and ionizing radiation in varying order (hypothermia before, during or after irradiation) and MN were scored. Second, cells were irradiated at 0.8°C (ice water) or...
Materials and methods

Cells and irradiation
The experiments were performed with the human lymphoblastoid cell line TK6 (American Type Culture Collection, USA, mycoplasma screened). Cells were grown in suspension in RPMI (Roswell Park Memorial Institute) 1640 medium (Sigma Aldrich, St Louis, MO, USA) supplemented with 10% defined bovine calf serum (HyClone, Thermo Fisher Scientific, Waltham, MA, USA), 1% PenStrept (10,000 U penicillin and 10 mg streptomycin/ml, Sigma Aldrich) and 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) buffer (Applichem, Darmstadt, Germany) at 37.0°C, 5% CO₂. All experiments were performed with exponentially growing cells.

Prior to, or following irradiation, cells were incubated for 20 min in small water cups made of polystyrene foam at the respective irradiation temperature: 0.8°C (ice water) or 37.0°C. This time has previously been shown to induce the TE both in TK6 cells (Dang et al. 2012) and human peripheral blood lymphocytes (Brzozowska et al. 2009, Lisowska et al. 2013). Irradiation was always carried out at the temperature at which cells were kept for the last 20 min. The same cups were used to irradiate cells with 1 Gy of gamma radiation from a Cs¹³⁷ source (0.4 Gy/min, Scanditronix, Uppsala, Sweden). Sham-exposed cells were kept at the appropriate temperature for the corresponding period of time.

Micronucleus assay
Cell suspensions of 140,000 cells per ml were prepared in six centrifuge tubes and treated according to the scheme shown in Figure 1. Cytochalasin B (Sigma Aldrich) was added at a final concentration of 5.6 μg/ml. Cells were cultured for 27 h at 37.0°C, 5% CO₂ and harvested as described previously (Dang et al. 2012). Briefly, cells were centrifuged and resuspended in 0.14 M KCl (Sigma Aldrich). Following a 5-min incubation time at room temperature, the cells were centrifuged again and fixed in fixative I (methanol: 0.9% NaCl: acetic acid (Sigma Aldrich); 12:13:3) and subsequently in fixative II (methanol: acetic acid; 4:1). The cells were washed in fixative 2 until the supernatant was clear, and were dropped onto clean, dry slides. The slides were stained with 4′,6-diamidino-2-phenylindole (vectashield-DAPI, Vector Laboratories Ltd, Peterborough, UK) and analyzed with an automated image acquisition system Metafer (Metasystems, Altusheim, Germany) (Rossnerova et al. 2011). Three independent experiments were performed. Between 450 and 1100 binucleated cells (BNC) per sample were scored. Three independent experiments were performed but due to technical problems slides from only two experiments could be analyzed for micronuclei (MN) in the ‘1 Gy 37.0°C during’ group (see Figure 1 and Table I).

Cell growth
For the analysis of growth rate, TK6 cells were incubated for 20 min at 0.8°C and 37.0°C and then exposed to 1 Gy of gamma rays. Cell growth was monitored by regular counting of cell numbers during a period of 14 days. Two independent experiments were performed.

Flow cytometric analysis of cell cycle distribution and progression
Two types of analyses were carried out: (1) Analysis of cell cycle distribution by BrdU (5-bromo-2-deoxyuridine, Sigma Aldrich) labelling, and (2) analysis of cell proliferation with the help of a BrdU pulse-chase schedule and calculation of the relative movement of S-phase cells. Two independent experiments were performed for each approach. For (1): 24 ml of cell suspension with 140,000 cells per ml were incubated for 20 min at 0.8°C and 37.0°C, exposed/sham exposed to 1 Gy of gamma rays, warmed to 37.0°C and split to 12 centrifuge tubes, 2 ml per tube. Cells were incubated at 37.0°C and fixed at 2-h intervals (between 2 and 24 h). BrdU was added to a tube for the last 30 min of incubation time at a final concentration of 10 μM. (2): 24 ml of cell suspension with 140,000 cells per ml were incubated for 20 min at 0.8°C and 37.0°C and exposed/sham exposed to 1 Gy of gamma rays. Immediately after radiation BrdU at a final concentration of 10 μM was added for 30 min. Thereafter cells were washed 2 times with medium, split to 12 centrifuge tubes, incubated at 37.0°C and fixed at 2 h intervals (between 2 and 24 h).

Flow cytometric analysis of cell debris indicative of apoptosis/necrosis
Levels of apoptosis/necrosis were estimated from results of cell cycle distribution (type 1 analysis in the above section) by analyzing the number of fragments (debris) with a DNA content lower than G₁ (Darzynkiewicz et al. 1997). The percent values of debris where obtained with the help of the ModFit LT 3.0 software (Becton Dickinson Polska, Warszawa, Poland).

Two-parameter flow cytometry
Cells were centrifuged, washed with a washing buffer (phosphate buffered saline (PBS, Sigma Aldrich) containing 0.5% bovine serum albumin (BSA, Sigma Aldrich) and
incubated with a denaturing solution (2 M HCl, POCH, Poznan, Poland) for 20 min at room temperature (RT). After that, cells were washed with washing buffer and resuspended in 0.1 M sodium borate (Na₂B₄O₇, Sigma Aldrich), pH 8.5 for 2 min at RT. After washing again in washing buffer, cells were incubated with 20 μl FITC (fluorescein isothiocyanate)-conjugated anti-BrdU mouse antibody (Beckton Dickinson) for 20 min at RT. Next, the cells were washed with washing buffer, resuspended in propidium iodide (PI, Sigma Aldrich, 10 μg/ml in PBS) and incubated for 30 min at RT in the dark.

Cytofluorometric measurements were performed with a LSR II cytometer (Becton Dickinson). Green (FITC) and red (PI) fluorescence was plotted in two-parameter scattergrams. Ten thousand events were recorded per measurement. Cell cycle fractions were quantified by setting gates on scattergrams as described in (Zölzer et al. 1993a, 1993b).

**Statistical analysis**

Numbers of cell doublings were calculated by dividing the time between seeding and passaging by the doubling time $T_d$. $T_d$ was calculated by the formula

$$T_d = \frac{\ln(2)}{\ln(C_2/C_1)} \ast h,$$

where $\ln$ is the natural logarithm, $C_1$ the cell density at seeding, $C_2$ cell density at passaging and $h$ the time between seeding and passaging. For each experiment and treatment group, the numbers of cell doublings plotted against incubation time were fitted to linear (0 Gy treatments) and linear-quadratic (1 Gy treatment) functions by the method of least-squares, using the software SigmaPlot 12.5 (Systat Software Inc., USA). The reason for the fitting was that the

---

**Table I. Frequencies and distributions of micronuclei in TK6 cells exposed to gamma-rays and temperatures of 0.8°C or 37.0°C concomitantly or sequentially. See Figure 1 for the meaning of ‘during’, ‘before’ and ‘after’.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Exp</th>
<th>BNC with MN</th>
<th>Sum of BNC</th>
<th>Sum of MN</th>
<th>MN per 1000 BNC</th>
<th>DI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Gy 0.8°C</td>
<td>1</td>
<td>990 17 3 0 0</td>
<td>1010</td>
<td>23</td>
<td>23</td>
<td>1.24</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1000 10 0 0 0</td>
<td>1010</td>
<td>10</td>
<td>10</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1005 11 0 0 0</td>
<td>1016</td>
<td>11</td>
<td>11</td>
<td>0.99</td>
</tr>
<tr>
<td>0 Gy 37.0°C</td>
<td>1</td>
<td>994 11 1 0 0</td>
<td>1006</td>
<td>13</td>
<td>13</td>
<td>1.14</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1003 8 1 0 0</td>
<td>1012</td>
<td>10</td>
<td>10</td>
<td>1.19</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1008 8 0 0 0</td>
<td>1016</td>
<td>8</td>
<td>8</td>
<td>0.99</td>
</tr>
<tr>
<td>1 Gy 37.0°C during</td>
<td>1</td>
<td>920 77 13 1 0</td>
<td>1011</td>
<td>106</td>
<td>105</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>904 91 11 0 0</td>
<td>1006</td>
<td>113</td>
<td>112</td>
<td>1.08</td>
</tr>
<tr>
<td>1 Gy 0.8°C before</td>
<td>1</td>
<td>434 34 1 0 0</td>
<td>470</td>
<td>36</td>
<td>77</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1303 93 11 2 1</td>
<td>1410</td>
<td>125</td>
<td>89</td>
<td>1.28</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>935 59 10 1 1</td>
<td>1006</td>
<td>86</td>
<td>86</td>
<td>1.36</td>
</tr>
<tr>
<td>1 Gy 0.8°C during</td>
<td>1</td>
<td>945 65 6 1 0</td>
<td>1017</td>
<td>80</td>
<td>79</td>
<td>1.15</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>750 26 6 0 0</td>
<td>782</td>
<td>38</td>
<td>48</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>932 70 8 0 0</td>
<td>1010</td>
<td>86</td>
<td>86</td>
<td>1.10</td>
</tr>
<tr>
<td>1 Gy 0.8°C after</td>
<td>1</td>
<td>866 59 9 1 0</td>
<td>935</td>
<td>80</td>
<td>85</td>
<td>1.22</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>404 24 2 0 0</td>
<td>429</td>
<td>26</td>
<td>61</td>
<td>1.08</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>937 67 7 0 0</td>
<td>1011</td>
<td>81</td>
<td>81</td>
<td>1.09</td>
</tr>
</tbody>
</table>

Exp, experiment; BNC, binucleated cells; MN, micronuclei; DI, dispersion indices.
for irradiated or sham-irradiated cells. No effect of temperature at exposure was detected for exposed or sham-exposed cells.

Relative movements are shown in Figure 5. Due to short cell cycle duration of the cells, a meaningful calculation of RM was only possible up to 6 h post exposure. No effect of temperature at exposure was detected for exposed or sham-exposed cells.

The relative levels of debris as a function of time after treatment are shown in Figure 6. While a clear effect of radiation is evident, no effect of temperature at exposure was detected for exposed or sham-exposed cells.

**Discussion**

The protective effect of hypothermia, referred to as the temperature effect (TE), on the level of radiation-induced chromosomal damage is a puzzling but repeatedly observed phenomenon (Bajerska and Liniecki 1969, Gumrich et al. 1986, Brzozowska et al. 2009, Brehwens et al. 2010, Dang et al. 2012, Lisowska et al. 2013). The aim of the present study was to verify if the sequence of exposing cells to hypothermia and radiation plays a role in the TE. Moreover, using flow cytometry and monitoring cell growth we tested the earlier hypothesis that the TE is related to perturbations of the cell cycle (Dang et al. 2012). The results clearly show that in exponentially growing TK6 cells, the TE is observed at the level of MN regardless of whether cells are cooled during or immediately before or after the radiation exposure. No indication of cell cycle perturbation by combined exposure to hypothermia and radiation could be detected.

The rational for testing if the sequence of exposing cells to hypothermia and radiation plays a role in the TE was that our earlier results (Brzozowska et al. 2009) obtained after treating human peripheral blood lymphocytes with the radical scavenger dimethylsulfoxid (DMSO) indicated that TE may be related to modulation of chromatin conformation resulting in protecting the DNA from attacks by reactive oxygen species. Falk et al. (2008) showed that condensation of chromatin has a radioprotective effect on the DNA and it could be assumed that cooling cells in ice water leads to chromatin condensation. In a later study with TK6 cells (Dang et al. 2012), we tested this hypothesis by inhibiting chromatin deacetylation with trichostatin A (TSA – an inhibitor of histone deacetylation) prior to treatment of cells with hypothermia and radiation. This treatment indeed abolished the TE and could suggest that chromatin, forced into an open conformation, does not condense at low temperature and is thus unable to protect the DNA from the attacks of free radicals. We assumed and tested the option that in such case, the TE should be seen not only at the level of MN but also of DNA damage and clonogenic cell survival (Dang et al. 2012). This however was not the case leading us to suggest that TE is not related to a reduced level of DNA damage after radiation exposure under the conditions of hypothermia (Dang et al. 2012). The present results confirm this conclusion in that the TE was seen even when hypothermia treatment and radiation exposure were separated in time.

The present results also weaken another hypothesis put forward by Brzozowska et al. (2009), that the TE could be related to inhibition of the bystander effect, thus leading to a reduced level of DNA damage. Although it could be
imagined that the bystander effect, that was shown to be responsible for 20–50% of DNA damage observed after radiation exposure (Ryan et al. 2008), is inhibited in cells cooled down in ice water and irradiated at the same time, it can unlikely explain the TE observed after sequential exposure of cells to hypothermia and radiation.

An intriguing feature of the TE is that it was predominantly observed at the level of cytogenetic damage
to a changed intrinsic radiosensitivity of cells, but to a perturbation of the cell cycle. This interpretation also allowed explaining why the TE was not seen with the gamma-H2AX focus assay (Dang et al. 2012), the clonogenic cell survival test (Dang et al. 2012) and the alkaline comet assay (Brzozowska et al. 2009). The present investigation was undertaken to identify any changes in cell cycle progression between cells irradiated at 37°C and 0.8°C. We analyzed the kinetics of cell growth during one week after irradiation, the distribution of cells in the cell cycle during 24 h and the relative movement of cells during 6 h after irradiation. In none of the endpoints did we see any indication of a difference in cell cycle progression between cells irradiated at the two temperatures. These results indicates that the TE observed at the level of MN in TK6 cells is either not related to modified cell cycle kinetics or that the modifications are below the resolution power of the applied measurement techniques.

If perturbation of cell cycle kinetics is not responsible for the TE, what then is the mechanism behind the reduced frequency of MN in cells exposed at 0.8°C? We think that the only plausible explanation is an elimination of damaged cells from the pool of cells analyzed for MN. If the responsible mechanism is not a transient arrest of damaged cells in the cell cycle, it could be due to cell death. The analysis of DNA content on flow cytometric scattergrams allows identifying cell debris, which is characterized by DNA content lower than that of cells in the G1 phase. Cell debris is regarded as manifestation of apoptotic or necrotic cell death (Darzynkiewicz et al. 1997). We checked if the level of cell debris in our experiments suggests different kinetics of death in cells treated at the different temperatures. The results are negative, similarly as those of cell cycle kinetics. Again, it is possible that the resolution power of the ‘debris assay’ is too low to detect subtle differences in the level of cell death. Experiments are now underway where apoptosis is detected by the Annexin V assay.

In conclusion, our results again confirm the existence of the cytogenetic TE in TK6 cells at the level of MN. However, its mechanisms remain unclear. It is important to stress that the effect is not an artefact, since it is repeatedly detected both in human peripheral blood lymphocytes (Bajerska and

Figure 5. Relative movement of cells through the cell cycle following treatment at different temperatures. Error bars represent standard deviations. Circles: no radiation. Triangles: dose of 1 Gy.

Figure 6. Relative levels of debris indicative of apoptosis following treatment at different temperatures. Error bars represent standard deviations. Circles: no radiation. Triangles: dose of 1 Gy. Error bars represent standard deviations.
Acknowledgements

This study was partly financed by the Swedish Radiation Safety Authority (SSM).

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References


