Cell Cycle and LET Dependence for Radiation-induced Mutation: A Possible Mechanism for Reversed Dose-rate Effect

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A previous study of the mutagenic action of ²⁵²Cf radiation in mouse L5178Y cells showed that the mutation frequency was higher when the dose was chronic rather than acute, which was in sharp contrast to the effects reported for γ-rays (Nakamura and Sawada, 1988). A subsequent study using synchronized cells revealed that the cells at the G2/M stage were uniquely sensitive to mutation induction by ²⁵²Cf radiation but not to γ-rays (Tauchi et al., 1993). A log phase cell population was first subjected to conditioning gamma or ²⁵²Cf radiation doses at different dose-rates. The cell cycle distribution of these cells was then observed, and they were then exposed to ²⁵²Cf radiation, and the mutation rate was determined. The G2/M fraction increased by 3- to 4-fold when the conditioning doses (2 Gy of gamma or 1 Gy of ²⁵²Cf radiation) were delivered chronically over 10 h, but only slightly when the same doses were delivered over a 1 h period or less. Subsequent ²⁵²Cf irradiation gave higher mutation frequencies in the cells pre-irradiated with γ-rays over a protracted period of time than in those exposed with the higher dose-rate γ-rays. These results suggest that the radiation-induced G2 block could be at least partly (but not totally) responsible for this reverse dose-rate effect (Tauchi et al. 1996). Possible factors which cause the hyper-sensitivity of G2/M cells to mutation induction by neutrons will be discussed.
INTRODUCTION

The reversed dose-rate effect of fission spectrum neutrons was first reported for in vitro carcinogenesis by Hill et al in 1982\(^1\). This phenomenon for somatic mutation induction was reported by Nakamura and Sawada for \(hp\) deficiency mutations in mouse leukemia L5178Y cells using a californium-252 (\(^{252}\)Cf) fission neutron source at the Research Institute for Radiation Biology, Hiroshima University, Hiroshima, Japan\(^2\). Since the cells were exposed to radiation at 37°C and were continuously growing, changes in the cell cycle distribution may affect the expression of the reversed dose-rate effect. Several hypotheses have been proposed to explain the reversed dose-rate effect\(^3-5\). Among those, Rossi & Kellerer proposed that a hyper-sensitive stage for mutation induction or transformation exists at some point in the cell cycle, and that the cells could accumulate at this sensitive stage in response to radiation\(^3\). We previously documented that mutation induction by fission neutrons was cell cycle dependent during low dose-rate irradiation\(^6,7\). In this report, we will discuss the main factor responsible for the reversed dose-rate effect for in vitro mutagenesis.

MATERIALS AND METHODS

Cell cycle dependence of 6-thioguanine-resistant mutations in mouse L5178Y cells

Mouse leukemia L5178Y cells were used in this study. The cells were grown in Fischer’s medium (GIBCO) supplemented with 10% horse serum (GIBCO), 100 units/ml penicillin and 100 \(\mu\)g/ml streptomycin at 37°C.

Exponentially growing L5178Y cells were synchronized by successive treatments with excess thymidine and colcemid at the G2/M phase of the cell cycle\(^8\). About 95–97% of the cells were synchronized and one generation time was estimated to be about 7.5 h. The cell cycle stages were analyzed by flow cytometry, and we estimated the stages as G2/M at 0 h after release from the colcemid block, G1 phase at 1 h, G1/S boundary at 2 h, and S phase from 2 to 6 h. Synchronized cells were allowed to grow until the desired cell cycle stage was reached, and then irradiated with \(\gamma\)-rays or neutrons at 2–4°C. Immediately after irradiation, the surviving fraction was determined using a portion of the cells, and the rest of the cells were cultured for seven days to allow expression of the mutant phenotype. After 7 days culture, the induced mutation frequencies were estimated by the colony formation rate in medium containing 6-thioguanine (6-TG, 5 \(\mu\)g/ml). The induced mutation frequency was estimated as previously described\(^6\).

To analyze the changes in the cell cycle distribution and mutation frequency in the cell population exposed to low dose-rate irradiation, the exponentially growing L5178Y cells were exposed to a conditioning dose of 2 Gy \(\gamma\)-rays or of 1 Gy neutrons at a high or low dose-rate. The temperature during the conditioning irradiation was kept at 37°C to allow cell cycle progression. Changes in the cell cycle distribution were then measured by flow cytometric analysis. The cells exposed to conditioning doses were then irradiated with \(^{252}\)Cf neutrons at
2–4°C at a dose rate of 1.6 cGy/min, and the induced mutation frequencies were measured.

*Simulation of the mutation induction by irradiation with different dose-rates*

In order to calculate the mutation frequency, a simple formula (1) was used:

\[
\text{mutation frequency} = (\sigma) \cdot (\text{surviving fraction at cell cycle stage})
\]

where

\[
\Sigma = \Sigma(\text{Mutation frequency of } 10^{-5} \text{ at each cell cycle stage}),
\]

The surviving fraction for each cell cycle stage is the surviving fraction at the beginning of the 6-TG treatment. The surviving fraction for each cell cycle stage at the beginning of the 6-TG treatment is defined as:

\[
(\text{Survival at each cell cycle stage after irradiation = } A) \cdot (\text{Percentage of the population in that phase after irradiation = } B) / (\text{total number of surviving cells = } \Sigma (A \cdot B))
\]

The values used for the calculations are shown in Table 1. Cell cycle stages are classified into G1, S and G2/M, and the fractions in each stage were estimated by flow cytometry using the Multicycle program (Phoenix Flow Systems). The mutation frequencies for each cell cycle stage were obtained from our former publication, and the values used were, 1 h for G1, 4 h for S, and G2/M begins at 0 h immediately after synchronization (Table 1).

### Table 1. Values used for the calculation of mutation frequency induced by fission neutrons at different dose-rate

<table>
<thead>
<tr>
<th>Cell cycle stage</th>
<th>Mutation frequency (× 10^{-5}/Gy)^a</th>
<th>Survival after neutron irradiation (%)^b</th>
<th>Population during irradiation (%)^c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>High dose-rate (1.2 cGy/min)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Low dose-rate (0.16 cGy/min)</td>
</tr>
<tr>
<td>G1</td>
<td>6.2</td>
<td>36.9</td>
<td>18.0</td>
</tr>
<tr>
<td>S</td>
<td>3.7</td>
<td>50.6</td>
<td>69.7</td>
</tr>
<tr>
<td>G2/M</td>
<td>18.9</td>
<td>30.5</td>
<td>12.2</td>
</tr>
</tbody>
</table>

^a Tauchi et al (1993)^6
^b Tauchi et al (1996)^7
RESULTS

Cell cycle dependence of 6-thioguanine-resistant mutations in mouse L5178Y cells

We reported previously that there is a cell cycle influence on cell death and 6-TG resistant mutation induction in mouse L5178Y cells after irradiation with \(^{60}\text{Co}\) \(\gamma\)-rays or with \(^{252}\text{Cf}\) fission neutrons\(^5\). To test the effect of the cell cycle on radiation response, doses for each type of radiation were selected to result in nearly identical survival and mutation frequency levels in exponentially growing populations. The changes in cell survival as the cells progressed through the cell cycle displayed the same tendencies with both types of radiation, although G2/M phase cells were much more sensitive to \(\gamma\)-irradiation. In contrast, when examining mutation induction, the pattern of cell cycle dependence depended strongly on the type of radiation. G2/M phase cells were clearly sensitive to mutation induction from fission neutrons, but not from \(\gamma\)-rays. Differences in mutation induction rates after exposure to neutron- and \(\gamma\)-irradiation could not be observed at other parts of the cell cycle\(^6\).

For exponentially growing cells, cell survival and induced mutation frequencies are well correlated. Figure 1 shows a plot of mutation frequency versus cell survival for G2/M and G1 cells. The data for these figures was taken from the results of experiments examining the dose dependence of mutation induction after exposure to \(\gamma\)-rays or neutrons. The shaded area represents the relationship between cell survival and mutation frequency estimated for exponentially growing L5178Y cells. G2/M cells show a hyper-sensitivity to mutation induction by neutrons. However, no such difference can be seen for G1 cells after exposure to either type of radiation.

![Fig. 1. Induced mutation frequency plotted against the cell survival in mouse L5178Y cells irradiated with \(^{60}\text{Co}\) gamma-rays or \(^{252}\text{Cf}\) fission neutrons at G2/M or G1 stage. The shaded area represents the relationship between cell survival and mutation frequency in exponentially growing cells. G2/M cells are distinctively sensitive to neutron induced mutation.](image-url)
Changes in cell cycle distribution during irradiation with different dose-rates

Figure 2 shows the changes in the cell cycle distribution in response to the conditioning radiation dose using a high or low dose-rate from the $^{252}\text{Cf}$, $^{60}\text{Co}$, or $^{137}\text{Cs}$ source. The conditioning doses (2 Gy of $\gamma$-rays or 1 Gy of neutrons) were delivered chronically (over 10 h) or acutely (1 h or less). When the cells were irradiated with low dose-rate $\gamma$-rays or neutrons, the G2/M fraction clearly increased; in contrast, the accumulation of G2/M cells was much less pronounced after high dose-rates of either $\gamma$-rays and neutrons. In the population exposed to low dose-rates of $\gamma$-rays or neutrons, the G2/M fraction increased 3 to 4-fold compared with unirradiated cell populations, and the S phase fraction decreased. We also measured the mitotic index in the cell population exposed to the conditioning doses. The decrease in the mitotic index after the low-dose-rate conditioning dose was much smaller than that for high-dose rate$.^{5}\) These findings suggest that the populations which are exposed to low-dose rate radiation have a large fraction of cells in the G2/M phase, and that cells are still progressing through the cell cycle, even during irradiation. We examined the neutron-induced mutation frequency of cells exposed to the conditioning radiation doses. Cells exposed to the low-dose-rate conditioning dose were much more sensitive to mutation induction than the cells exposed to the high-dose rate when the conditioning doses were $\gamma$-rays. However, a small difference in the mutation frequency between the two types of neutron conditioned populations could be seen only when the cells were exposed to an increased dose of 1.5 Gy. This might be due to the higher mutation frequency caused by the conditioning dose itself$.^{7}\) The mutation frequencies induced by high- or low-dose rate neutrons were calculated using the equation (1) described above and utilizing the values shown in Table 1. The calculated mutation frequencies were 10.6 $6\text{-TGr cells}/10^5$ survivors with a high dose rate and 14.9 $6\text{-TGr cells}/10^5$ survivors with a low-dose rate. These values were in close agreement with the experi-

![Fig. 2. Changes in cell cycle distribution by 2 Gy of gamma-rays or 1 Gy of fission neutrons with short period (less than 1 h) or long term exposure (over 10 h).]
Fig. 3. Comparison between calculated and experimental data for mutation induction by gamma-rays or neutrons with different dose-rate. Experimental data were obtained from Nakamura & Sawada\textsuperscript{2}, and calculation was performed using the formula (1) (see text). Plots are represent as follows: experimental data for $^{252}$Cf with 1.2 cGy/min (□) or 0.16 cGy/min (□); experimental data for $^{60}$Co gamma-rays with 50 cGy/min (▲) or 1.3 cGy/min (▲); calculated values for $^{252}$Cf with 1.2 cGy/min (△) or 0.16 cGy/min (△); calculated values for $^{60}$Co gamma-rays with 50 cGy/min (▲) or 1.3 cGy/min (▲).

Experimental data: i.e., 9.9 ± 1.7 for the high dose-rate cells and 18.6 ± 4.0 for the low dose-rate cells. Figure 3 shows a comparison between the calculated values and the experimental data for mutation induction. The experimental data were obtained from Nakamura & Sawada\textsuperscript{2}. The calculated results for neutron irradiation were in close agreement with the experimental data. However, the calculation could not simulate the saturation of the mutation frequency observed after exposure to high dose-rate neutrons. In addition, the calculated values did not coincide with the typical dose-rate effects curve seen for $\gamma$-irradiation.

DISCUSSION

We have investigated the effect of cell cycle position on mutation induction, and observed that cells at the G2/M stage were extremely sensitive to mutation induction after exposure to $^{252}$Cf neutrons, but not after exposure to $^{60}$Co $\gamma$-rays\textsuperscript{5}. We next observed changes in the cell cycle distribution after chronic irradiation. When the doses were delivered chronically over 10 h, the G2/M fraction of the irradiated population increased 3 to 4-fold. However, this effect
was not observed when the same doses were delivered over a short period of 1 h or less. A dose-response curve was calculated to describe mutation induction rates caused by chronic irradiation with\textsuperscript{252}Cf neutrons, and this curve was in close agreement with the experimental data. In addition, our earlier observations showed that G2/M cells are only slightly more sensitive to neutron-induced cell killing when compared with gamma irradiation. This causes an increase in the fraction of the cell population present at the G2/M stage at the time of irradiation and 6-thioguanine treatment.

Taking into consideration all of the findings reported here, we can conclude that the changes in the cell cycle distribution during irradiation and the hyper-sensitivity of the G2/M cells to mutation induction must be the main factors leading to the observed reversed dose-rate effect for neutrons. However, the calculated rates did not simulate the saturation of the mutation frequency observed with high dose-rate neutrons. In addition, the calculated values did not coincide with typical dose-rate effects seen for $\gamma$-irradiation. These observations suggest that there must be other factors which affect the dose-rate effect, such as repair activity or the type of damage caused by radiation.

These results may help to explain why G2/M cells are more sensitive to mutation induction by $^{252}$Cf radiation. There are two major differences between $\gamma$-rays and neutrons: first, neutrons are particles without an electric charge, while $\gamma$-rays are photons; second, the $^{252}$Cf fission neutrons have a high LET with an average LET of 80-100 keV/\mu m. To investigate the mechanism causing neutrons and $\gamma$-rays to produce different mutation induction rates over the cell cycle, we examined the effect of cell cycle position and LET on the rate of mutation induction using carbon beams with different LET values at HIMAC (Heavy Ion Medical Accelerator, Chiba). Synchronized mouse L5178Y cells were exposed to carbon-290 MeV/u beams with different LETs at the G2/M, G1, G1/S or S phases in the cell cycle, and the rate of induction of 6-thioguanine-resistant mutations was determined. For higher LET radiation (75.5 keV/\mu m), the maximum mutation frequency was observed at the G2/M stage, but for lower LET radiation (13.3 keV/\mu m) the maximal mutation rate was observed at the G1 stage (Tauchi et al, unpublished observation). This work found that carbon ion irradiation at high LET and fission neutrons both produce a similar cell cycle dependent spectrum of mutagenesis. In contrast, the carbon beam with a low LET value produced results similar to those seen with $\gamma$-rays. These observations support the conclusion that the difference in the cell cycle dependence of mutation induction caused by the two different types of radiation, $\gamma$-rays and neutrons, was due to the difference in LET values between these two types of the radiation, and not by the identity of the two different particles composing the radiation beams.

In summary, a reversed dose-rate effect of mutation induction rates was observed in mouse L5178Y cells when exponentially growing cells were irradiated with $^{252}$Cf fission neutrons. This phenomenon could be explained by the hyper-mutability of G2/M phase cells, and by changes in the cell cycle distribution during irradiation. In addition, the mutability of G2/M cells depends on the LET rather than on the identity of the particles composing the radiation. The mechanism(s) leading to mutations (DNA damage, damage repair systems, induction of genetic instability) at the G2/M stage by high LET radiation might also be different from mechanisms acting in response to low LET radiation. Other mechanisms could also be acting at other parts of the cell.
cycle because the observed mutation frequency at the G2/M phase caused by high LET radiation was much higher than what would have been expected from cell survival data. We are currently analyzing the \textit{hprt} locus in 6-TG resistant mutants induced at different stages of the cell cycle by radiation with different LET values, and these ongoing studies may provide more information to help elucidate the mechanisms of radiation induced mutagenesis.

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REFERENCES