Analysis of Cell Cycle in Mouse Bone Marrow Cells Following Acute in vivo Exposure to $^{56}$Fe Ions

Kanokporn Noy RITHIDECH1*, Marc GOLIGHTLY1 and Elbert WHORTON2

HZE particles/$^{56}$Fe ions/Cell cycle/Mouse bone marrow/in vivo.

A pilot study was conducted to examine the magnitude of cell-cycle delay and apoptosis in bone marrow (BM) cells collected at 18, 42 and 66 hr from radiosensitive CBA/CaJ mice and radioresistant C57BL/6J mice following a whole-body in vivo exposure to 1 GeV/amu $^{56}$Fe ions or $^{137}$Cs $\gamma$ rays. At each sacrifice, BM cells were collected from three mice of each strain per dose of $^{56}$Fe ions (0, 10 and 100 cGy) and two mice of each strain per dose of $^{137}$Cs $\gamma$ rays (0, 100 and 300 cGy). A significant G1-arrest (ANOVA, $p < 0.05$) was observed at 18 hr after exposure of mice to 100 cGy of $^{56}$Fe ions or 300 cGy of $^{137}$Cs $\gamma$ rays, relative to their corresponding sham-controls, resulting in a significant decrease in the percentage of cells cycling into S-phase in both strains. The percentage of S-phase cells subsequently increased and persisted up to 66 hr post-irradiation. Significant numbers of G2/M cells were found at 18 and 66 (but not at 42) hr post-irradiation, regardless of radiation-type or mouse-strain. It is likely that BM cells have undergone at least one cell cycle at 66 hr after exposure of mice to either 100 cGy $^{56}$Fe ions or 300 cGy $^{137}$Cs $\gamma$ rays. Our study is the first to investigate the in vivo effects of $^{56}$Fe ions (1 GeV/amu) on the cell cycle of mouse BM cells using flow cytometry. The cell-cycle distribution (but not the number of apoptotic cells) was dependent on radiation-dose and harvest-time.

INTRODUCTION

A drastic cell cycle arrest and mitotic delay by $^{56}$Fe ions are well established in in vitro studies using synchronized cells, as compared to low LET-radiation (e.g. gamma or X rays).1,2 Such effects severely interfere with the expression time of chromosome aberrations (CAs) and can lead to an underestimation of chromosomal damage if samples were collected at a single sampling time following irradiation of cells (e.g. human lymphocytes, human fibroblasts, and V79 Chinese hamster cells).3–9 It has been found that the frequencies of CAs per metaphase cell (in their first cycle) increased sharply with time after exposure (from 48 to 72 hr) to $^{56}$Fe-ion-irradiation, indicating a delay in the entry of cells with $^{56}$Fe-ion-induced CAs into mitosis. Such effects were not found after X-irradiation.9 Based upon these findings, multiple harvest times are recommended in order to accurately estimate the potential cytogenetic hazards of $^{56}$Fe ions.9 It also has been found that time-dependent increases in the frequencies of CAs directly correlate with the dosage levels of $^{56}$Fe ions.

In contrast to the in vitro studies mentioned above, information on in vivo cell cycle arrest by $^{56}$Fe ions is limited and incomplete. In a study by Brooks et al10 a marked delay in the entry of cells into mitosis has been observed in BM cells collected at 8 hr from Wistar rats exposed to different doses of $^{56}$Fe ions (0, 50, 100, and 200 cGy). In that study, mitotic index (MI) decreased with increasing $^{56}$Fe ion-dose. Due to an extremely low MI in animals exposed to 200 cGy of $^{56}$Fe ions, it was possible to score CAs in metaphase cells only from animals exposed to doses below 200 cGy. The majority of chromosomal damage observed by Brooks et al10 at 8 hr post-exposure were the chromatid-type aberrations. This finding suggests that only cells in the late S and G2 phases of the cell cycle at the time of exposure were available for scoring. However, the in vivo data from the study conducted by Brooks et al10 do not provide information on the effects of radiation on cells in other stages of the cell cycle at the time of exposure. The availability of in vivo effects on cell cycle by $^{56}$Fe-ion-data would assist in determining the harvest times following irradiation so that a sufficient number of the first post-irradiation metaphase cells will be available for the analysis of CAs. In this study, we used a flow cytometry technique to examine the magnitude of cell cycle delay and apoptosis in BM cells collected at different times from two strains of mouse after an acute whole-body in vivo expo-
sure to different doses of 1 GeV/amu $^{56}$Fe ions (0, 10, or 100 cGy) or $^{137}$Cs $\gamma$ rays (0, 100, or 300 cGy, as a source of reference radiation). These strains of mouse are known to be sensitive (CBA/CaJ)$^{11,12}$ or resistant (C57BL/6J)$^{13}$ to radiation-induced CAs and acute myeloid leukemia (AML).

**MATERIALS AND METHODS**

**Animals**

Both male CBA/CaJ and C57Bl/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and were allowed two weeks to acclimate prior to their irradiation (at 10–12 weeks of age). They were housed in an AAALAC approved facility at both Brookhaven National Laboratory (BNL) for HZE particle-exposure and Stony Brook University for $^{137}$Cs $\gamma$-ray-exposure. The protocols for studies were approved by both the BNL and the Stony Brook University Institutional Animal Care and Use Committee. Mice used for HZE particle-exposure were delivered directly from the vendor to BNL.

**Exposure of mice to HZE particles**

In each strain, three groups of mice (nine in each) were given a whole body dose of 0, 10 or 100 cGy of 1GeV/amu $^{56}$Fe ions (HZE particles) at a dose rate of 1Gy/min. These radiation doses were the average total-body doses. The highest dose of 1 GeV $^{56}$Fe ions (100 cGy) is comparable to those used in previous in vivo studies.$^{10,14,15}$ Mice exposed to 0 cGy served as sham controls. The 1 GeV/amu $^{56}$Fe ions were delivered by a 20 cm × 20 cm beam. The dose-average LET at the sample position was 159.5 KeV/μm. The uniformity (± 2%) of the beam was confirmed by densitometric analysis of an exposed X-ray film.$^{16}$ At irradiation, each unanesthetized mouse was placed in the ventilated holder to minimize its movement during exposure so the whole body would receive the radiation dose uniformly. The holder allowed the mouse to sit in a normal position but did not allow the animal to turn around. The total time each animal was confined in the holder was approximately 4 min. All mice included in the study were transported back to Stony Brook University in a climate-controlled vehicle within 2 hr post-exposure. At each harvest time (18, 42 and 66 hr post-irradiation) for cell cycle and apoptosis, three mice of each strain per dose were used.

**Exposure of mice to $^{137}$Cs $\gamma$ rays**

Three groups of mice (six in each) of each strain were given a whole body dose of 0, 100, or 300 cGy of $^{137}$Cs $\gamma$ rays (at the dose rate of 72 cGy/min) using a Gamma Cell40 located at Stony Brook University. Similar to the $^{56}$Fe-ion-exposed group, these radiation doses were the average total-body doses. The dose of 300 cGy of $^{137}$Cs $\gamma$ rays was selected as the highest dose because it is the dose that induces a high frequency of complex exchanges after exposure of human lymphocytes in vitro.$^{17}$ In addition, our data showed that a single dose of 300 cGy of $^{137}$Cs $\gamma$ rays induces a 20–25% incidence of AML in CBA/CaJ mice.$^{12}$ As with the HZE particle-exposure, each un-anesthetized mouse was placed in the ventilated holder at irradiation to minimize its movement during exposure so the whole body would receive the radiation dose uniformly. Two mice of each strain per dose were used for BM collection at 18, 42 and 66 hr post-exposure.

**Collection of bone marrow cells**

At each sacrifice time, we collected BM cells from each mouse by flushing both femurs and tibiae with 10 mL of McCoy’s 5A medium (Invitrogen, Grand Island, NY) using a 23-gauge needle and syringe. Immediately after collection, BM cells from each individual mouse were used for the analyses of cell cycle and apoptosis by flow cytometry.

**Cell cycle**

The procedure for DNA cell cycle analysis and DNA content was performed according to routine standard protocols established by the manufacturer of the Cycletest DNA reagents (Becton Dickinson, Mountainview, CA). FACS instrument settings for data acquisition were optimized and set using DNA QC Particles and CELLQuest DNA Experiment software. Cell cycle analysis and DNA content measurement were performed on the experimental data set using the BD Modfit LT analysis package. Briefly, the cells were brought to $1 \times 10^9$ cells per mL, treated with trypsin for 10 minutes at room temperature, followed by incubation with trypsin inhibitor and RNase for an additional 10 minutes at room temperature. Finally, the cells were stained with the DNA dye Propidium Iodide at 4°C for 10 minutes in the dark. The cell solutions were strained through a 35 μm cell strainer and analyzed by the standard flow cytometric procedures and software.

**Apoptosis**

Apoptosis was analyzed in BM cells from the same mice used for cell cycle analysis and was done by means of flow cytometry using the AnnexinV/Propidium Iodide (PI) staining procedure previously published.$^{18}$ Briefly, the cells were washed twice with cold PBS and resuspended at $1 \times 10^6$ cells per mL in binding buffer [10mM Hepes/NaOH (pH 7.4) 140 mM NaCl, 2.5 mM CaCl$_2$] and incubated with 5 μL of Annexin V - FITC and 5 μL of PI for 15 minutes at room temperature. The cells were then analyzed within an hour by standard protocols. The percentage (%) of total cells for each of the quadrants was recorded. This method relies on the property of cells to lose membrane symmetry in the early phase of apoptosis resulting in a translocation of the membrane phospholipid phosphatidylserine from the inner leaflet of the plasma membrane to the outer leaflet. Annexin V has a high affinity for PS and is very useful for identifying early
apoptotic cells. Therefore, cells that are stained positively for Annexin V but negatively for PI (Annexin V+/PI–) are considered early apoptotic cells while Annexin V+/PI+ cells are late apoptotic cells or are undergoing necrosis, or are already dead. Cells that are stained negatively for both Annexin V and PI (AnnexinV–/PI–) are viable cells.

**Statistical analysis**

At each time post-irradiation, the percentage of cells in each stage of the cell cycle was obtained for each dose of each type of radiation for each mouse of each strain. These values were considered the response variables. The results were then analyzed separately for each type of radiation using three factor analysis of variance (ANOVA) methods to determine the significance of radiation related dose differences and trends (factor one), time related differences and trends (factor two), and mouse strain differences (factor three). Importantly, this particular analysis of variance methodology allowed us to utilize larger numbers of mice in each comparison for each factor. The results were similar to those obtained by using the inverse sine transformation on the observed percentages. The p-values < 0.05 (one-sided) were considered to be statistically significant. The apoptotic data were analyzed similarly.

**RESULTS AND DISCUSSION**

**Cell Cycle**

**56Fe ions**

The means and standard error of the means (S.E.) of the percentage of cells in different phases of the cell cycle (G0/G1, S and G2/M) of mouse BM cells collected from CBA/CaJ or C57Bl/6J at different times following in vivo exposure to 56Fe ions are shown in Table 1.

Our results demonstrated no strain differences and no time trend on cell cycle distribution in both strains of mouse after exposure to 0 or 10 cGy 56Fe ions. However, there was a significant decrease (P < 0.05) in the percentage of cells cycling through S-phase at 18 hr after exposure to 100 cGy 56Fe ions in both CBA/CaJ and C57BL/6J mice relative to their corresponding sham controls. The results suggest that G1-phase cells were being delayed or arrested from entering S-phase while S-phase cells were still cycling resulting in a significantly high number of G2/M cells at 18 hr post-irradiation in both strains of mouse. The percentage of S-phase cells then dramatically increased and persisted up to 66 hr post-exposure, regardless of the genetic background of the exposed mice. This increase was directly proportional to the decrease in number of G1-phase cells, reflecting a large number of G1-phase cells cycling into S-phase after 18 hr post-irradiation. Hence, our data indicated an inverse correlation between the percentages of G1-phase cells and S-phase cells. Our data also demonstrated significantly high numbers of G2/M cells at 18 and 66 (but not at 42) hr post-irradiation in the population of BM cells of mice (both strains) exposed to 100 cGy of 56Fe ions than those in sham control mice (p < 0.05). This set of data suggests that BM cells in all stages of the cell cycle at the time of exposure to 100 cGy of 56Fe ions would be available for scoring chromosome aberrations in metaphase cells. In contrast, metaphase cells harvested at 18 hr post-exposure are most likely to represent cells in late S and G2 phases of the cell cycle at the time of exposure.

**Table 1.**

<table>
<thead>
<tr>
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<th>0 cGy</th>
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<tr>
<td></td>
<td>CBA/CaJ</td>
<td>C57BL/6J</td>
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<tr>
<td>18 hr</td>
<td>G0/G1</td>
<td>G0/G1</td>
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<tr>
<td>Mean</td>
<td>83.12</td>
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<td>S.E.</td>
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<tr>
<td>42 hr</td>
<td>Mean</td>
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<tr>
<td>S.E.</td>
<td>0.67</td>
<td>0.11</td>
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<tr>
<td>66 hr</td>
<td>Mean</td>
<td>84.67</td>
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<tr>
<td>S.E.</td>
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<td>0.66</td>
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<tr>
<td></td>
<td>CBA/CaJ</td>
<td>C57BL/6J</td>
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<tr>
<td>18 hr</td>
<td>G0/G1</td>
<td>G0/G1</td>
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<tr>
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<td>86.11</td>
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<td></td>
<td>CBA/CaJ</td>
<td>C57BL/6J</td>
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<tr>
<td>18 hr</td>
<td>G0/G1</td>
<td>G0/G1</td>
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<tr>
<td>Mean</td>
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Table 2 shows the means and standard error of the means (S.E.) of the percentage of cells in different phases of the cell cycle in the population of BM cells collected from CBA/CaJ or C57BL/6J mice at different times following in vivo exposure to 137Cs γ-rays.

Similar to the 56Fe-exposed group, our results demonstrated no strain differences and no time trend on cell cycle distribution in both strains of mouse in the sham control groups. We also found that a single dose of 300 cGy 137Cs γ-rays exhibited similar effects on the cell cycle of BM cells to those exposed to a single dose of 100 cGy 56Fe ions (data shown in Table 1). At 18 hr post-irradiation, there was a significant decrease in the number of cells in the S-phase of the cell cycle (p < 0.05). This dose of 137Cs γ-rays (300 cGy) also induced a statistically significant (p < 0.05) G1 arrest in BM cells of CBA/CaJ mice and in C57BL/6J mice, collected at 18 hr after in vivo exposure. The percentage of G2/M phase in BM cells collected from mice exposed to 300 cGy 137Cs γ-rays at 66 hr post-irradiation was higher than that in their sham controls (p < 0.05), reflecting more S-phase cells cycling into the G2/M phase. Additionally, a significant reduction in G1-phase cells (p < 0.05) was found in BM cells collected at 66 hr post-irradiation from mice (both strains) exposed to 100 cGy of 137Cs γ-rays, related to sham control mice. This decrease was proportional to the increase in S-phase cells. These effects, however, were less than those found in mice exposed to 300 cGy 137Cs γ-rays.

**Apoptosis**

Figures 1.1 to 1.4 show the percentage of apoptotic (either early or both early and late) cells as a function of 56Fe ion-dose and time after exposure of mice; while Figs. 2.1 to 2.4 demonstrate the percentage of apoptotic (either early or both early and late) cells as a function of 137Cs-γ-ray-dose and time after exposure of mice.

For each dose of 56Fe ions, there was no strain difference in the percentage of apoptotic cells at each harvest-time used in this study. Moreover, a decline of the percentage of apoptotic cells, either early apoptotic cells only (Figs. 1.1 and 1.2) or early plus late apoptotic cells (Figs. 1.3 and 1.4), as a function of time after exposure was observed in both strains of mouse exposed to 56Fe ions. This finding suggests a recovery from 56Fe-ion-induced apoptosis has already taken place at 18 hr following in vivo irradiation. In contrast, within the γ-ray exposed group, dose-dependent increases in the number of apoptotic cells (either early apoptotic cells only, shown in Figs. 2.1 and 2.2; or early plus late apoptotic cells, shown in Figs. 2.3 and 2.4) were found at each harvest time in both strains of mouse. Nevertheless, there was no significant change in the number of apoptotic cells within 18 to 66 hr after whole-body exposure to any dose of 137Cs γ-rays. Our data also demonstrated that the number of apoptotic cells in the CBA/CaJ mouse exposed to the highest dose (300 cGy) of 137Cs γ-rays was slightly higher than that in the C57BL/6J mouse (with one exception when the number of early and late apoptotic cells were combined at 42 hr post-irradiation). However, Kadhim et al. reported a higher number of apoptotic cells in the BM population of the C57BL/6J mouse than that of the CBA/H (an isogenic strain to CBA/CaJ) at 1 or 365 days after in vivo exposure to a single dose of 300 cGy of 0.2 keV/μm 60Co γ-rays. The use of radiation with different LETs (0.91 keV/μm 137Cs γ-rays vs 0.2 keV/μm 60Co γ-rays) and different methods for the enumeration of apoptotic cells (flow cytometry with live cells vs...
Fig. 1. Apoptosis as a function of time after in vivo exposure to different doses of $^{56}$Fe ions (▲0 cGy, ■10 cGy, ◆100 cGy). Figures 1.1 and 1.2 show the percentage of early apoptotic cells only, while Figs. 1.3 and 1.4 show the percentage of early apoptotic plus late apoptotic cells. Each point represents a mean percentage of apoptotic cells ± S.E.

Fig. 2. Apoptosis as a function of time after in vivo exposure to different doses of $^{137}$Cs γ rays (▲0 cGy, ■100 cGy, ◆300 cGy). Figures 2.1 and 2.2 show the percentage of early apoptotic cells only, while Figs. 2.3 and 2.4 show the percentage of early apoptotic plus late apoptotic cells. Each point represents a mean percentage of apoptotic cells ± S.E.
scanning laser with fixed cells) may contribute to this discrepancy.

It has been well recognized that the biological effects of HZE particles are not simply related to the absorbed dose averaged in an organ or tissue (Gy), but rather to the traversal pattern of the particles, reflecting the number of hit cells. Differences in responses after 10 and 100 cGy of 1 GeV/amu 56Fe ions may relate in part to differences in particle fluences and microdosimetry (related to the number of cells that are hit). The particle fluences were calculated using the equation suggested by Vazquez and Kirk, i.e. 6.24 × Dose (Gy)/LET × bone marrow cell cross section area (presumably 100 μm²) to be 0.39 per per 100 μm² and 3.91 per 100 μm² for 10 and 100 cGy of 1 GeV/amu 56Fe ions, respectively. Relating to the microdosimetric calculation for the number of “hit” cells, it is beyond the scope of our research described in this paper. However, a presumption can be made that a small number of BM cells were hit at the dose level of 10 cGy of 1 GeV/amu 56Fe ions, reflecting the finding of no effects on cell cycle by this low-dose level.

Our study is the first to use flow cytometry for determining the in vivo effects of 56Fe ions (1 GeV/amu) on the cell cycle of BM cells collected from exposed CBA/CaJ and C57BL/6J mice. The in vivo data from our study suggest that a decrease in the percentage of S-phase cells at 18 hr after exposure is caused by radiation-induced G1 arrest rather than radiation-induced apoptosis. This finding is similar to what was suggested previously in an in vitro study using human lymphocytes, in which cells were in G0/G1 at the time of irradiation. We recognize that our in vivo study was conducted on an asynchronous cell population that permits the detection of only relative differences between cell cycle stages because it is difficult to determine when cells at different stages of the cell cycle at the time of exposure would actually reach mitosis. Our in vivo data also suggest that a single dose of 100 cGy 56Fe ions or of 300 cGy 137Cs γ rays have similar effects on the cell cycle of the mouse BM population in both strains. These data therefore indicate that 56Fe ions are more effective in inducing cell cycle delay (per unit dose of radiation) than 137Cs γ rays.

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