SHORT COMMUNICATION

Heavier Ions with a Different Linear Energy Transfer Spectrum Kill More Cells Due to Similar Interference with the Ku-Dependent DNA Repair Pathway

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Ionizing radiation kills cells mainly due to the generation of DNA double-strand breaks (DSBs). High-linear energy transfer (LET) ionizing radiation induces more cell death and generates a higher relative biological effect (RBE) than low-LET ionizing radiation (such as X or γ ray). Although it is known that interference with the Ku-dependent nonhomologous end-joining (NHEJ) pathway appears to be the major cause of iron-ion- and carbon-ion-induced cell death, it remains unclear whether other ions with a similar or different LET and higher RBE in terms of cell killing are controlled in the same way. In this study, we compared the clonogenic survival frequency of Ku80^{+/+} (NHEJ-proficient) and Ku80^{-/-} (NHEJ-deficient) cells after exposure to iron (175 keV/ μ m), silicon (75 keV/ μ m), oxygen (25 keV/ μ m) and X ray (low-LET). The results showed that Ku80^{-/-} cells had the same RBE value of 1 for cell killing for all types of ionizing radiation, whereas Ku80^{+/+} cells had different RBE values for cell killing that depended on the specific type of ionizing radiation. The results indicate that the Ku-dependent NHEJ is the major repair pathway that heavier ions interfere with, resulting in higher RBE for cell killing. These results provide useful information for followup studies that will focus on improving high-LET protection or heavier ion radiotherapy in the near future. © 2014 by Radiation Research Society

effectiveness (RBE) in terms of cell killing. The RBE range of 2–6 depends on cell type. Ionizing radiations kill cells mainly by inducing DNA double-strand breaks (DSBs), which if left unrepaired are a severe threat to cell survival. Since both high- and low-LET radiation generate similar numbers of initial DSBs per unit of absorption, the RBE for high-LET radiation for cell killing occurs because high-LET radiation-induced DSBs are more difficult to repair (1, 2). There are two major pathways in mammalian cells available for repairing DNA DSBs: nonhomologous end joining (NHEJ); and homologous recombination repair (HRR). The NHEJ pathway includes the Ku-dependent canonical NHEJ (cNHEJ) and the Ku-independent but Parp1-dependent alternative NHEJ (alt-NHEJ). Previously, we and other groups reported that high-LET radiation compared with low-LET radiation interfered with cNHEJ (3–5) but did not affect either alt-NHEJ (5) or HRR (5, 6). Similar results (RBE of 1 in cell killing for cNHEJ-deficient cells) were obtained after exposures to either carbon with LET of 75 keV/ μ m (4) or iron with LET of 150 keV/ μ m (5), suggesting that heavy ions with LET $\geq$ 70 keV/ μ m affect DNA repair in a similar way. However, it remains unclear whether other ions with similar LET or lower LET-generated DNA damage also share similar mechanisms for affecting DSB repair. To address this question, we examined the RBE of cell killing for different ions with different LET spectrum in cNHEJ-proficient or cNHEJ-deficient cells. The results should provide useful information for space radiation protection from cosmic rays, which contains different ions with a different LET spectrum. Furthermore, these data should be useful for sensitizing tumor cells to high-LET radiotherapy.

INTRODUCTION

When compared with low-linear energy transfer (LET) ionizing radiation (such as X or γ rays), high-LET ionizing radiation from either space radiation or a heavier ion radiotherapy machine generates higher relative biological

MATERIALS AND METHODS

Cells and Irradiation

cNHEJ-proficient (Ku80^{+/+}) and cNHEJ-deficient (Ku80^{-/-}) mouse embryonic fibroblast (MEF) cells were cultured as described previously (5). X-ray (low-LET ionizing radiation) or heavy ion (Fe,

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TABLE 1
Description of Radiation Quality

Radiation type	Energy	LET/keV/ μm	Dose rate (Gy/min)
X ray	320 KV	Low LET	0.5–1 Gy
Oxygen	600 MeV/u	25	0.5–1 Gy
Silicon	300 MeV/u	70	0.5–1 Gy
Iron	600 MeV/u	175	0.5–1 Gy

Silicon or Oxygen ions with different LET spectrum) exposure is shown in Table 1. Low-LET irradiation was performed using an X-ray generator (10 mA, 2 mm aluminum filtration, 320 kV, X-RAD 320, Precision X-ray, North Branford, CT) in our laboratory. High-LET irradiation was performed using an alternating-gradient synchrotron (range in water: 27 cm; beam area: 20×20 cm; uniformity: $\pm 5\%$) at the Brookhaven National Laboratory. The dose rates for both high- and low-LET irradiation were approximately 0.5–1 Gy/min.

Gamma-H2AX (γ -H2AX) Foci Assay in MEF Cells

The assay was performed as described previously in ref. (7). Briefly, cells were grown in chamber slides and collected at different times after irradiation. The cells were fixed in 4% paraformaldehyde for 15 min, permeabilized for 10 min in ice in 0.2% Triton X-100 and blocked in 10% normal goat serum. The cells were incubated with an anti- γ -H2AX antibody (Millipore) for 1 h, washed in phosphate buffered saline (PBS), 1% bovine serum albumin and incubated with Alexa Fluor[®] 488-conjugated goat anti-mouse secondary antibody (Invitrogen[™]) for 1 h at room temperature. Cells were washed in PBS and mounted using VECTASHIELD[®] mounting media containing 4,6 diamidino-2-phenylindole (Vector Laboratories Inc., Burlingame, CA). Fluorescent images were captured using a Carl Zeiss Axio Scope A1 epi-Fluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped with an MRm cooled digital camera. Axiovision software (version 4.8) was used for camera control, image acquisition, processing and multi-channel display.

Clonogenic Cell Survival Assay

Cellular sensitivity to radiation was determined by loss of colony-forming ability as described previously in ref. (5). Briefly, 2×10^5 cells were plated per T25 flask with 5 ml of growth media. In experiments using exponential growth cells, the cells were irradiated 2 days after plating. The cells were then collected and plated, at appropriate numbers to achieve 20–100 colonies per flask after irradiation. Two replicates were prepared for each datum point and were incubated for 7 days to allow colonies to develop. Colonies were fixed and stained with crystal violet (100% methanol solution) for counting.

Statistical Analysis

Data were analyzed using the χ^2 test or Student's *t* test. Statistical significance was determined as $*P < 0.05$ or $**P < 0.01$.

RESULTS AND DISCUSSION

cNHEJ Deficient Cells Showed Similar Sensitivity to Fe, Silicon, Oxygen and X Ray

To examine whether heavy ions with different LETs have a similar effect on DNA DSB repair, we compared the sensitivities of Ku80^{+/+} (cNHEJ-proficient) and Ku80^{-/-} (cNHEJ-deficient) cells to different ions with different LETs (Table 1). When compared with X ray (low-LET

radiation), all tested ions (oxygen, silicon and iron) killed more Ku80^{+/+} cells (Fig. 1A), and the order of these different types of radiations in terms of cell killing depended on an increase of LET: iron > silicon > oxygen > X ray (Fig. 1A). It is known that heavy ions with LET > 150 keV/ μm waste the extra energy on breaking DNA strands (8), and therefore do not generate more DNA DSBs or kill more cells. Notably, all tested ions (oxygen, silicon and iron) did not kill more Ku80^{-/-} cells than X ray, which resulted in Ku80^{-/-} cells showing the same sensitivity to all types of ionizing radiation and an RBE of 1 for these different heavier ions with a different LET spectrum (Fig. 1B). Previously, we reported that the RBE of MEF Ku80^{-/-} cells after exposure to iron ions (150 keV/ μm) is 1 in cell killing, which is similar to the results where the RBE of human 180BR cells [with mutant Lig4 and deficient in cNHEJ (9)] after exposure to carbon (75 keV/ μm) is 1 in cell killing (10). Combining these results with the data shown in Fig. 1C, we propose that the higher RBE for all these high-LET radiations in terms of mammalian cell killing are due to the same mechanism: interference with only cNHEJ. To verify this conclusion, we performed a γ -H2AX focus assay to detect the remaining γ -H2AX foci-positive cells in Ku80^{+/+} and Ku80^{-/-} cells after exposure to different types of ionizing radiation.

cNHEJ Deficiency Results in a Similar Ratio of Remaining γ -H2AX Foci-Positive Cells after Exposure to Fe, Silicon, Oxygen or X Ray

At 1 h after exposure to radiation (X ray, oxygen, silicon and iron) almost 100% of the cells (Ku80^{+/+} and Ku80^{-/-}) had γ -H2AX signals (Fig. 2A). After exposure to high-LET radiation, at 4 and 24 h, there were more γ -H2AX foci-positive cells remaining in Ku80^{+/+} cells, the order of the radiation type to generate a high ratio of residual γ -H2AX foci-positive cells was: iron > silicon > oxygen > X ray (Fig. 2B), which indicates that DNA DSB repair is affected more by radiation with higher LET. These results match the order of radiation response to induced cell killing (Fig. 1), supporting that unrepaired DNA DSBs are the major reason for cell death and interference with the cNHEJ pathway is the major reason for different types of heavier ions with different LET spectrum having higher RBE for cell killing. The underlying mechanism is believed to involve high-LET radiation-induced small DNA fragments (≤ 40 bp) that prevent the Ku molecule from efficiently binding to the two ends of one small fragment at the same time (5, 11), which dramatically decreases the cNHEJ efficiency after high-LET irradiation of cells. By contrast, Ku80^{-/-} cells with normal alt-NHEJ and HRR were not affected by the length of DNA DSBs but only by the yield of DNA DSBs, and therefore these cells showed the same sensitivity (Fig. 1) to different ions with different LETs at the same dose as well as a similar ratio of remaining γ -H2AX foci positive cells at 4 or 24 h after different types of radiation exposure (Fig. 2C).

FIG. 1. cNHEJ-deficient cells showed similar sensitivity to Fe, silicon, oxygen and X ray. Panel A: Ku80^{+/+} and panel B: Ku80^{-/-} cell sensitivities to different types of radiation (X ray, iron, silicon and oxygen) exposure at different doses as labeled were examined using a clonogenic assay. Data are the mean and standard deviations (SD) obtained from three independent experiments. **P* < 0.05; ***P* < 0.01. Panel C: The RBE of different ions for cell killing with different LET spectrum in Ku80^{+/+} cells (NHEJ-proficient) and Ku80^{-/-} cells (NHEJ-deficient).

Taken together, our results indicate that heavier ions with a different LET spectrum have a higher RBE in terms of cell killing due to the same mechanism, interference with cNHEJ and not with other DNA repair pathways including HRR and alt-NHEJ. These results provide useful information for followup studies that may improve high-LET radiation protection and heavier ion radiotherapy in the near future.

FIG. 2. cNHEJ deficiency results in a similar ratio of remaining γ-H2AX foci-positive cells after exposure to Fe, silicon, oxygen and X ray. Panel A: Image of γ-H2AX foci in Ku80^{+/+} or Ku80^{-/-} cells at 1 h after 1 Gy of high- or low-LET irradiation. Panel B: Percentage of γ-H2AX foci-positive Ku80^{+/+} cells at different times after 1 Gy of high- or low-LET irradiation. Each point represents the mean ± SD from two separate experiments (300 cells/experiment were examined) **P* < 0.05. Panel C: Ku80^{-/-} cells at different times after 1 Gy of high- or low-LET irradiation. Each point represents the mean ± SD from two separate experiments (300 cells/experiment were examined) **P* < 0.05.

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