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The Ku-dependent non-homologous end-joining but not other repair pathway is inhibited by high linear energy transfer ionizing radiation

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ABSTRACT

Ionizing radiation (IR) induced DNA double strand breaks (DSBs) are repaired by both non-homologous end-joining (NHEJ) and homologous recombination repair (HRR) in mammalian cells. The NHEJ repair includes a Ku-dependent main pathway and a PARP-1-dependent complementary pathway. Compared with low linear energy transfer (LET) IR (X or γ ray) at the same doses, high LET IR (high-charge particles) induces more cell death because of ineffective DNA repair. However, it remains unclear whether high LET IR inhibits all repair or specifically one repair pathway. By combining the assays of clonogenic survival, G2M checkpoint and γH2AX in the cell lines with deficiencies in different repair genes, we show here that high LET IR inhibits only the Ku-dependent main NHEJ pathway and does not inhibit either the HRR pathway or the PARP-1-dependent complementary NHEJ pathway. In addition, by developing an assay to detect small fragments of DSB (<400 bp) and by detecting the binding abilities of purified Ku and PARP to different sized dsDNA, we present a possible link for explaining the phenotypes. When compared with low LET IR at the same dose, high LET IR might induce similar yields of DNA DSBs in total but it might induce more small fragments of DNA DSBs (<40 base pairs) that prevent Ku binding efficiently to two ends of one DSB fragment at the same time, thus delaying Ku-dependent repair.

1. Introduction

High linear energy transfer (LET) ionizing radiation (IR) kills more cells at the same dose compared to low LET IR. High LET IR is induced by high-charge (HZE) particles (a component in space radiation), high energy ions or by a special clinical radiotherapy machine. Low LET IR includes X or γ ray (a major component of IR from standard clinical radiotherapy machines), etc. The more cell death induced by high LET IR than by low LET IR at the same dose reflects a higher relative biological effectiveness (RBE). RBE on all kinds of quantitative effect induced by IR in X-ray exposed cells is 1. The RBE of cell killing induced by high LET IR is ~2–4 in cultured cells (killing ~2–4 times the number of cells at the same dose) depending on cell type. The induction of DNA double strand breaks (DSBs) either by high LET IR or by low LET IR is a threat to cell survival. Two major kinds of DSB repair exist in eukaryotic cells, homologous recombination repair (HRR) and non-homologous end-joining (NHEJ). HRR is the major pathway for repairing DSBs in yeast, but both NHEJ and HRR are required for

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DSB repair in mammalian cells. NHEJ repair includes a main pathway (requires Ku80, Ku70, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), ligase IV and XRCC4) and a complementary backup pathway (requires poly(ADP-ribose) polymerase-1 (PARP-1) and XRCC1/DNA ligase III) [1–3]. The HRR pathway requires Rad51, Rad52, Rad54 as well as the Rad51 paralogs including XRCC2, XRCC3, Rad52B, Rad51C and Rad51D. It is believed that the higher RBE on cell killing by high LET IR is because of ineffective rejoining of DNA DSBs [4–7], which occurs mainly by the Ku-dependent NHEJ pathway [8]. However, it remains unclear whether high LET IR inhibits all kinds of repair or specifically inhibits a single repair pathway. By using different cell lines that are genetically deficient in different repair pathways, we show here that LET IR inhibits only Ku-dependent repair but does not inhibit other kinds of repair including HRR or PARP-1-dependent NHEJ. In addition, based on the SSB measurement described by Dr. Rydberg [9], we developed a new method by combining the assays of asymmetric field inversion gel electrophoresis (AFIGE) with PCR amplification of IR-induced DNA DSBs to measure IR-induced small fragments of DNA DSBs with lengths of <400 bp. These results provide a possible link to explain why high LET IR when compared with low LET IR inhibits only Ku-dependent repair.

2. Materials and methods

2.1. Cell lines and irradiation

Ku80$^{-/-}$ cells (deficient in the main NHEJ pathway) and Ku80$^{+/+}$ cells are transformed mouse embryo fibroblasts (MEF) obtained from Dr. Gloria Li's laboratory [10]. PARP-1$^{-/-}$cells (deficient in the complementary NHEJ pathway) and PARP-1$^{+/+}$ cells are transformed MEF obtained from Dr. Zhao-Qi Wang's laboratory [11]. irs1SF cells (deficient in the HRR pathway) and AA8 cells (the wild type counterpart) are CHO cells obtained from Dr. Larry Thompson's laboratory [12]. These cells were adapted to grow in DMEM supplemented with 10% iron-supplemented calf serum (Sigma–Aldrich Co., USA) at 37 °C in an atmosphere of 5% CO_2 and 95% air. High LET IR was carried out using the alternating gradient synchrotron (AGS, Fe ions, 1 GeV/amu) at Brookhaven National Laboratory. X-ray irradiation was carried out using an X-ray machine (Pantak, East Haven, CT) (310 kV, 10 mA, 2-mm aluminum filtration) in our laboratory. The dose rates for both high LET IR and low LET IR were about 1 Gy/min.

2.2. Radiosensitivity survival assay

Cellular sensitivity to radiation was determined by loss of colony-forming ability. Briefly, 2×10^5 cells were plated per T25 flask with 5 ml of medium. In experiments using exponential growth cells, the cells were irradiated 2 days after plating. In experiments using plateau phase cells, the cells were irradiated 4–5 days after plating. The cells were then collected and plated, aiming at 20–100 colonies per flask following IR. Two replicates were prepared for each datum point and were incubated for 2 weeks to allow colonies to develop. Colonies were stained with crystal violet (100% methanol solution) before counting.

2.3. Measurements of nuclear area

The measurements of nuclear area were based on the method described by Hill et al. [7] with one modification. Briefly, 5×10^4 cells were plated on a slide chamber with 1 ml of medium and allowed to grow for 2 days, thus mimicking the conditions for detecting the radiosensitivity of exponential cells described in Section 2.2. The medium in the chamber was removed and the cells were washed with PBS followed with 3.7% formaldehyde fixation at room temperature for 5 min. The cells were washed with PBS again and in mounting solution containing 4′-6-diamidino-2-phenylindole (DAPI) (Vector Laboratory Inc.) and covered with a cover glass. Nuclear areas were analyzed with a Zeiss LSM510 confocal microscope.

2.4. Cell cycle distribution

Briefly, 2×10^5 cells were plated, per T25 flask with 5 ml of medium. Forty-eight hours later, cells were exposed to IR and returned to 37 °C. At different times thereafter, cells were trypsinized and fixed in 70% ethanol. Cells were stained in a solution containing 62 μg/ml RNase A, 40 μg/ml propidium iodide and 0.1% Triton X-100 in PBS buffer at room temperature for 1 h. The distribution of cells in the cell cycle was measured in a flow cytometer (Coulter Epics Elite).

2.5. Western blot for histone γH2AX measurement

After IR, the cells were returned to 37 °C. At different times, the cells were collected and counted. 1×10^6 cells were lysed in 50 μl of RIPA lysis buffer (50 mM Tris–HCl, pH 7.4; 1% NP40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EGTA; 1 mM PMSF; 1 μg/ml each aprotinin, leupeptin, pepstatin; 1 mM Na_3VO_4; 1 mM NaF) and mixed with 50 μl of 2× protein loading buffer. The proteins were separated by SDS-PAGE on 15% gel and transferred to a nitrocellulose membrane at 4 °C overnight. The antibody against H2A or phosphorylated H2AX (γ-H2AX) was purchased from Upstate Biotechnology, Inc.

2.6. Detection of IR-induced small dsDNA fragments

This assay included four major steps: (1) DNA DSBs generation: agarose plug containing G0/G1 cells preparation and irradiation. (2) Small dsDNA fragment collection. (3) Small dsDNA fragment amplification. (4) Small dsDNA fragment detection. The detailed procedures are described in the Supplemental data.

2.7. Electrophoretic mobility shift assay (EMSA)

Ku and PARP-1 for dsDNA end binding were assessed by EMSA. All oligonucleotides were chemically synthesized and purified by high pressure liquid chromatography (Operon). The blunt-ended DNA duplexes S20 (GAA-CGA AAA-CAT-CGG-GTA-CG and CG-TAC-CCG-ATG-TTT-TCG-TTC), S35 (GAA-CGA-AAA-CAT-CGG-GTA-CGA-GGA-CGA-AGA-CTG-AC and GT-CAG-TCT-TCG-TCC-TCG-TAC-CCG-ATG-TTT-TCG-TTC) and S75 (GAA-CGA-AAA-CAT-CGG-GTA-CGA-GGA-CGA-AGA-CTG-ACC-ACG-ACA-TAC-TAA-CAG-GGA-CAT-GAC-TCA-CAG- AAC-AGA-GCG and CGC-TCT-GTT-CTG-TGA-GTC-ATG-TCC-