Effects of targeted phosphorylation site mutations in the DNA-PKcs phosphorylation domain on low and high LET radiation sensitivity

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Abstract. The present study investigated the effect of targeted mutations in the DNA-dependent protein kinase catalytic subunit and phosphorylation domains on the survival of cells in response to different qualities of ionizing radiation. Mutated Chinese hamster ovary V3 cells were exposed to 500 MeV/nucleon initial energy and 200 keV/µm monoenergetic Fe ions; 290 MeV/nucleon initial energy and average 50 keV/µm spread-out Bragg peak C ions; 70 MeV/nucleon initial energy and 1 keV/µm monoenergetic protons; and 0.663 MeV initial energy and 0.3 keV/µm Cs137 γ radiation. The results demonstrated that sensitivity to high linear energy transfer radiation is increased when both S205 -phosphorylated G6 clusters (5-9) and the functional Ku subunit; however, the kinase activity is also affected by the loss of DNA-PKcs (2,3).

Introduction

DNA double-strand breaks (DSB) are the primary lesion induced by ionizing radiation and are most commonly associated with cell death (1). The cell has developed two major pathways of response to DSBs: Non-homologous end-joining (NHEJ) and homologous recombination (HR) (1). NHEJ is active throughout the cell cycle and is the repair pathway that deals with the majority of radiation-induced DSBs. NHEJ relies on a large protein complex, DNA-dependent protein kinase (DNA-PK), to bind to the ends of broken DNA and bring them together for direct ligation. DNA-PK is composed of the Ku70/80 end-binding proteins and the DNA-PK catalytic subunit (DNA-PKcs). The kinase activity of DNA-PK is directly dependent on the functional Ku subunit; however, the kinase activity is also affected by the loss of DNA-PKcs (2,3).

It appears that the phosphorylation activity of the DNA-PKcs contributes to the DSB repair capability of DNA-PK. DNA-PKcs not only have the ability to phosphorylate various NHEJ proteins, it is also able to autophosphorylate itself (4-6). DNA-PKcs has various phosphorylation sites throughout its protein structure, with the most critical sites located in the T2609 and S2056 clusters (5-9). Site-directed mutants involving phosphorylation sites in the T2609 and S2056 clusters result in cell lines with various levels of radiosensitivity, ranging from a DNA-PKcs and Ku null phenotype to very mild sensitivity. This varying sensitivity was previously demonstrated in a synchronized G1 population of cells exposed to low linear energy transfer (LET) radiation Cs137 γ radiation (10).

High LET radiation is more effective at killing cells than low LET radiation. This increase in cell death can be observed as an increased relative biological effectiveness (RBE) when comparing the D0 value of cells exposed to 250 KeV X-rays or γ irradiation to the D0 value of cells exposed to high LET radiation. High LET radiation creates various types of complex DNA damage in small clusters within the DNA strand (11-14), including DBS, single-stranded breaks (SSBs) and base damage. Due to its complexity, the cell takes considerably longer to repair this high LET radiation-induced DNA damage (15,16). In contrast to the types of DNA damage caused by high and low LET radiation, cisplatin induces
inter- and intrastrand cross-linking and DNA adducts (17-19). Cisplatin-induced damage is often resolved by the nucleotide excision repair pathway (20); however, occasionally, cisplatin can result in DSBs in dividing cells, and these DSBs require the NHEJ and HR pathways for complete repair (21).

The aim of the present study was to determine the relative sensitivity to high LET radiation of site-directed mutant cells, containing phosphorylatable residues in the T2609 cluster, S2056 cluster (19,20) and carboxy-terminus phosphoinositide 3 kinase (PI3K) domain of DNA-PKcs. Furthermore, the current study expands upon a previous study conducted by Chen et al (8), which focused only on the sensitivity of these DNA-PKcs mutants to low LET radiation.

Materials and methods

Cell lines and culture. The present study utilized a wild-type Chinese hamster ovary (CHO) cell line (CHO10B2) provided by Dr. Joel Beford, Department of Environmental & Radiological Health Sciences, Colorado State University (Fort Collins, CO, USA); NHEJ-deficient xrs-5 (Ku80 mutated) and V3 cells; HR-deficient 51D1 (Rad51D mutated) cells provided by Dr. Larry Thompson, Biosciences and Biotechnology Division, Lawrence Livermore National Laboratory (Livermore, CA, USA); and 14 cell lines derived from DNA-PKcs null V3 cells with complementary human DNA-PKcs containing amino acid substitutions at specific positions (shown in Table I). Cells were cultured in minimal essential medium-α (Gibco Life Technologies, Indianapolis, IN, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA), and 1% penicillin, streptomycin and amphotericin B (Gibco Life Technologies, Carlsbad, CA, USA), and maintained at 37°C in a humidified atmosphere of 5% CO₂ in air.

Irradiation and cell treatment. Logarithmic phase cells were irradiated aerobically at room temperature. The radiation source was a JL Shepherd and Associates (San Fernando, CA, USA) irradiator that emitted Cs³⁷ γ-rays at a rate of 2.5 Gy/min, and the cells were irradiated using accelerated Fe ions, C ions and protons at the National Institute of Radiation Sciences (Chiba, Japan). The LET of the radiation used were as followed: 500 MeV/nucleon initial energy and LET 200 keV/μm monoenergetic Fe ions; 290 MeV/nucleon initial energy and average LET 50 keV/μm spread-out Bragg peak (SOBP) C ions; 70 MeV/nucleon initial energy and LET 1 keV/μm monoenergetic protons; and, 0.663 MeV initial energy and LET 0.3 keV/μm of Cs³⁷ γ-ray radiation, and were immediately sub-cultured and plated for colony formation assays. As shown in Table II, the D₀ values obtained from the Prism⁵™ software indicated marginal variation between the DNA-PKcs mutants when exposed to proton radiation, similar to the D₀ values observed when the cells were exposed to γ-rays. Furthermore, the xrs-5 cells demonstrated the highest sensitivity and the control cells demonstrated the highest resistance in these two groups. The majority of cell lines exhibited sensitivities similar to or more resistant than V3. The complete survival curves shown in Fig. 1 highlight the differing sensitivities between the DNA-PKcs mutant cell lines.

Effect of site-specific mutations on sensitivity to low LET charged particle radiation (γ-rays and protons). To investigate the role of DNA-PKcs in cellular sensitivity to low LET charged particles, the D₀ values of the various DNA-PKcs mutants exposed to proton radiation were compared to the D₀ values of the same cell lines exposed to γ-rays. Asynchronized cells were exposed to 70 MeV/nucleon initial energy and LET 1 keV/μm protons or 0.663 MeV initial energy and LET 0.3 keV/μm of Cs³⁷ γ-ray radiation, and were immediately sub-cultured and plated for colony formation assays. As shown in Table II, the D₀ values obtained from the Prism⁵™ software indicated marginal variation between the DNA-PKcs mutants when exposed to proton radiation, similar to the values observed when the cells were exposed to γ irradiation. Furthermore, the xrs-5 cells demonstrated the highest sensitivity and the control cells demonstrated the highest resistance in these two groups. The majority of cell lines exhibited sensitivities similar to or more resistant than V3. The complete survival curves shown in Fig. 1 highlight the differing sensitivities between the DNA-PKcs mutant cell lines.

Effect of site-specific mutations on sensitivity to high LET radiation (C and Fe ions). Considering that the results for low LET charged particles were similar to those for low LET γ-rays, the effect of site specific mutations on the cells' sensitivity to high LET radiation (Fe and C ions only) was investigated. Asynchronized cells were exposed to 500 MeV/nucleon initial energy and 200 keV/μm monoenergetic Fe ions or 290 MeV/nucleon initial energy and average LET 50 keV/μm SOBP C ions, and were immediately subcultured and plated for colony formation assays. As Table II indicates, all DNA-PKcs mutants exhibited D₀ values similar to the V3 cells, with the exception of L-5 and L-6. L-5 and L-6 demonstrated similar D₀ values when compared with the L-1 cell line.

Effect of site-specific mutations on RBE. Following calculation of the D₀ values for each DNA-PKcs mutant and the control CHO10B2 cells at each exposure, the values were compared. As shown in Table III, the RBE values for all DNA-PKcs mutants are similar, demonstrating that the single point mutations do not increase the effectiveness of high LET radiation.

Effect of site-specific mutations on the sensitivity of mutants to cisplatin. Cisplatin-induced DNA damage, unlike radia-
Table I. Cell lines derived from DNA-PKcs null V3 cells with human DNA-PKcs complementary DNA, containing amino acid substitutions at various positions in the DNA-PKcs constructs.

| Cell line | Altered DNA-PKcs mutant | S2023 | S2029 | S2041 | S2051 | S2056 | T2609 | S2612 | T2620 | S2624 | T2638 | T2647 | Y3715 | L3750 | K3752 | D3921 |
|-----------|-------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| L-1       | Wild-type               |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| L-2       | V3-7A                   | A     |       |       |       |       |       | A     |       |       |       |       |       |       |       |       |
| L-3       | V3-6A                   | A     |       |       |       |       |       | A     |       |       |       |       |       |       |       |       |
| L-4       | V3-2A                   | A     |       |       |       |       |       | A     |       |       |       |       |       |       |       |       |
| L-5       | V3-S2056A               |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| L-6       | V3-T2609A               |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| L-8       | V3-KA4                  |       |       |       |       |       |       |       | R     |       |       |       |       |       |       |       |
| L-9       | V3-KB20                 |       |       |       |       |       |       |       |       | R     |       |       |       |       |       |       |
| L-10      | V3-KC23\(^a\)           |       |       |       |       |       |       |       |       |       |     Δ   |       |       |       |       |       |
| L-11      | V3-KD51                 |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| L-12      | V3-5A                   | A     | A     | A     | A     | A     |       |       |       |       |       |       |       |       |       |       |
| L-14      | V3-3A                   |       |       |       |       |       |       |       |       |       |       |       | A     | A     | A     |       |

\(^a\)V3-KC23 mutant carries a frame-shift mutation at amino acid 3715 that resulted in truncation of the protein after 10 amino acids and loss of the entire PBK kinase domain. DNA-PKcs, DNA-dependent protein kinase catalytic subunit; PI3K, phosphoinositide 3 kinase; A, mutated alanine; R, mutated arginine; N, mutated asparagine; Δ, deletion.
translation-induced DNA damage, rarely causes DSBs. The various types of CHO cell were exposed to cisplatin, subcultured and plated for a survival assay. As shown in Table II, the DNA-PKcs mutants exhibit varying sensitivities to cisplatin. All the DNA-PKcs mutants investigated in the present study were more sensitive than the control CHO10B2 cells, however, no statistically significant difference between the sensitivity of the CHO10B2 and V3 cell lines was identified.

Comparison between radiation and cisplatin sensitivity. To better understand the role of DNA-PKcs, the sensitivity of cisplatin exposure was compared with each of the low and high LET radiation treatments.
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As demonstrated in Fig. 2, radiation sensitivities between γ-ray and Fe ion radiation were correlated with the wild-type and DNA repair-deficient cell lines. However, the bubble chart indicated a lack of correlation between cisplatin and radiation sensitivity. L-11, one of the most sensitive mutants to γ-rays

Table II. \( D_{10} \) values of control and mutant cell lines to ionizing radiation and cisplatin.

| Cell line | \( \gamma \)-rays, Gy | Proton, Gy | C ion, Gy | Fe ion, Gy | Cisplatin, \( \mu M \) |
|-----------|----------------|-----------|-----------|-----------|----------------|
| CHO10B2   | 6.37 (5.87-6.87) | 5.31 (4.86-5.77) | 3.16 (2.94-3.32) | 1.89 (1.52-2.1) | 29.29 (23.15-35.12) |
| XRS5      | 1.18 (1.05-1.31) | 1.19 (0.90-1.46) | 1.11 (0.94-1.28) | 1.00 (0.95-1.05) | 9.79 (8.85-10.70) |
| V3        | 1.98 (1.76-2.19) | 2.21 (1.98-2.44) | 0.97 (0.89-1.06) | 1.07 (0.93-1.21) | 11.86 (10.96-12.75) |
| 51D1      | 3.95 (3.69-4.16) | 3.57 (3.24-3.79) | 1.71 (1.53-1.87) | 1.35 (1.31-1.40) | 6.15 (5.40-6.88) |
| L-1       | 5.01 (4.52-5.46) | 3.82 (3.52-4.24) | 2.38 (2.17-2.53) | 1.61 (1.46-1.72) | 19.41 (14.21-24.30) |
| L-2       | 2.35 (2.00-2.69) | 1.79 (1.24-2.30) | 1.42 (1.25-1.60) | 1.01 (0.93-1.09) | 15.45 (14.31-15.67) |
| L-3       | 3.09 (2.87-3.30) | 1.84 (1.63-2.04) | 1.42 (1.25-1.58) | 1.14 (1.01-1.26) | 12.31 (9.27-15.18) |
| L-4       | 2.47 (2.29-2.64) | 2.15 (1.91-2.37) | 1.09 (1.00-1.18) | 1.02 (0.98-1.06) | 11.81 (10.55-13.02) |
| L-5       | 3.88 (3.62-4.07) | 3.54 (2.98-3.85) | 2.08 (1.93-2.19) | 1.61 (1.32-1.79) | 14.09 (12.03-16.08) |
| L-6       | 4.01 (3.35-4.37) | 3.35 (2.58-3.73) | 2.14 (1.93-2.29) | 1.50 (1.39-1.59) | 11.67 (10.77-12.56) |
| L-8       | 2.18 (2.00-2.35) | 2.40 (2.21-2.59) | 1.33 (1.13-1.53) | 1.02 (0.90-1.13) | 13.27 (11.19-15.26) |
| L-9       | 2.67 (2.43-2.91) | 2.48 (2.09-2.86) | 1.35 (1.05-1.63) | 0.93 (0.80-1.05) | 17.76 (12.15-23.03) |
| L-10      | 2.09 (1.86-2.31) | 2.10 (1.70-2.49) | 1.12 (1.02-1.22) | 0.93 (0.82-1.04) | 11.30 (8.85-13.63) |
| L-11      | 2.08 (1.90-2.26) | 2.40 (2.24-2.56) | 1.14 (1.01-1.27) | 0.86 (0.81-0.92) | 14.37 (11.01-17.55) |
| L-12      | 2.97 (2.45-3.46) | 1.68 (1.51-1.85) | 1.16 (0.92-1.40) | 1.05 (0.90-1.20) | 11.71 (8.98-14.29) |
| L-14      | 3.01 (2.67-3.34) | 2.27 (1.88-2.65) | 1.40 (1.19-1.60) | 1.14 (1.05-1.23) | 16.17 (13.52-18.71) |

*Calculated using Prism 5™.

Table III. Relative biological effectiveness.

| Cell line | \( \gamma \)-rays | Protons | C ions | Fe ions |
|-----------|----------------|--------|--------|--------|
| CHO10B2   | 1.20           | 2.02   | 3.37   |
| XRS5      | 1.00           | 1.06   | 1.18   |
| V3        | 0.90           | 2.04   | 1.85   |
| 51D1      | 1.11           | 2.31   | 2.93   |
| L-1       | 1.31           | 2.11   | 3.11   |
| L-2       | 1.31           | 1.65   | 2.32   |
| L-3       | 1.68           | 2.18   | 2.71   |
| L-4       | 1.15           | 2.27   | 2.42   |
| L-5       | 1.10           | 1.87   | 2.41   |
| L-6       | 1.20           | 1.87   | 2.67   |
| L-8       | 0.91           | 1.64   | 2.14   |
| L-9       | 1.08           | 1.98   | 2.87   |
| L-10      | 1.00           | 1.87   | 2.25   |
| L-11      | 0.87           | 1.82   | 2.42   |
| L-12      | 1.77           | 2.56   | 2.83   |
| L-14      | 1.33           | 2.15   | 2.64   |

*Relative biological effectiveness (RBE) was calculated using the following equation: RBE = (\( D_{10} \gamma \) / \( D_{10} \) test radiation).
as indicated in Fig. 2, the correlation was identified, indicating that the contribution of cross-linking DNA damage, as a result of ionizing radiation, to cell death is minor. High LET radiation creates complex DNA damage consisting of DSB, SSBs, cross-linking and various other types of single nucleotide damage (11,13,14). Based on the $D_{10}$ values of the L-5 and L-6 cell lines, single point mutations in the S2056 or T2609 clusters exhibited partial sensitivity to low LET radiation but appear to be insufficient for creating a V3 phenotype upon exposure to low LET radiation; however, L-5 and L-6 demonstrated a V3-like phenotype when exposed to cisplatin. This is in contrast to low LET-induced damage or cisplatin-induced damage, which requires a single mutation among three clusters to induce a V3 phenotype.

In conclusion, the present study demonstrated that the entire DNA-PKcs protein is required for repair of low LET radiation and cisplatin-induced DNA damage. However, a single mutation in the PI3K domain, multiple mutations within the S2056 or T2609 clusters, or two mutations in the S2056 and T2609 clusters, are required for the repair of high LET radiation-induced DNA damage. These results indicate that the interaction of two clusters may synergistically contribute to the repair of high LET radiation-induced DNA damage. However, further studies are required to investigate high LET-induced DNA damage and the associated molecular repair mechanisms.

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