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The Relative Biological Effect of Spread-Out Bragg Peak Protons in Sensitive and Resistant Tumor Cells

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Abstract

Purpose: Variations in the radiosensitivity of tumor cells within and between tumors impact tumor response to radiation, including the dose required to achieve permanent local tumor control. The increased expression of DNA-PKcs, a key component of a major DNA damage repair pathway in tumors treated by radiation, suggests that DNA-PKcs–dependent repair is likely a cause of tumor cell radioresistance. This study evaluates the relative biological effect of spread-out Bragg-peak protons in DNA-PKcs–deficient cells and the same cells transfected with a functional DNA-PKcs gene.

Materials and Methods: A cloned radiation-sensitive DNA-PKcs–deficient tumor line and its DNA-PKcs–transfected resistant counterpart were used in this study. The presence of functional DNA-PKcs was evaluated by DNA-PKcs autophosphorylation. Cells to be proton irradiated or x-irradiated were obtained from the same single cell suspension and dilution series to maximize precision. Cells were concurrently exposed to 6-MV x-rays or mid 137-MeV spread-out Bragg peak protons and cultured for colony formation.

Results: The surviving fraction data were well fit by the linear-quadratic model for each of 8 survival curves. The results suggest that the relative biological effectiveness of mid spread-out Bragg peak protons is approximately 6% higher in DNA-PKcs–mediated resistant tumor cells than in their DNA-PKcs–deficient and radiation-sensitive counterpart.

Conclusion: DNA-PKcs–dependent repair of radiation damage is less capable of repairing mid spread-out Bragg peak proton lesions than photon-induced lesions, suggesting protons may be more efficient at sterilizing DNA-PKcs–expressing cells that are enriched in tumors treated by conventional fractionated dose x-irradiation.

Introduction

The radiosensitivity of tumor cells varies between as well as within tumors and significantly impacts tumor response to radiation, including the dose required to achieve permanent local control [1–9]. The factor or factors governing tumor cell sensitivity, while not fully understood, likely modulate the induction and/or repair of radiation-induced DNA damage and especially DNA double-strand-breaks. Nonhomologous end-joining (NHEJ) is the principal repair pathway of radiation-induced DNA double-strand breaks. It is

therefore of interest that clinical studies have reported an increased expression of a key component of NHEJ, that is, DNA-PKcs, in tumors that fail conventional fractionated dose x-ray treatment [10–13]. In contrast to the response of cells that are relatively sensitive and resistant to x-rays, exposure of the same cells to high linear energy transfer radiation substantially reduces the difference in response, that is, the relative biological effectiveness (RBE) of high linear energy transfer irradiation is larger in x-ray–resistant cells than in x-ray–sensitive cells [14–18].

Charged particles are increasingly being used for the treatment of patients with cancer, largely owing to the range-limited property of particle beams and resultant reduced exposure of normal tissues. Current clinical uses and future perspectives are reviewed and found in an particle by Durante et al [19]. This study examines the RBE of spread-out Bragg peak (SOBP) protons in tumor cells that are sensitive to x-rays owing to a defective DNA-PKcs gene, and the same cells, which are resistant owing to their transfection with a proficient DNA-PKcs gene. A review of the literature suggests a trend for a higher proton RBE in x-ray–resistant versus x-ray–sensitive cells [20–23]. However, in studies using wild-type and DNA-PKcs–deficient or DNA-PKcs–inhibited cells (or other key constituents of NHEJ), a significant proton RBE difference between sensitive and resistant cells has generally not been observed [15, 16, 24, 25].

## Materials and Methods

Cells and Culture

The cloned DNA-PKcs– and NHEJ-deficient cell line and its DNA-PKcs–transfected isogenic counterpart (both lines being homologous recombination proficient) used in this study have been previously described [6–8]. Briefly, the parental sensitive DNA-PKcs–defective line FSC1-3 was cloned from an induced tumor in severe combined immunodeficient mice and transfected with a human DNA-PKcs gene. The stable genomic integration and expression of the DNA-PKcs transgene was confirmed by polymerase chain reaction and reverse transcription polymerase chain reaction. Both tumor lines exhibit the same colony-forming efficiency, in vitro population doubling time, and tumor doubling time. Frozen replicates of the DNA-PKcs–defective parental line and transfected DNA-PKcs line were thawed and used in the present study. The presence of functional DNA-PKcs in the transfected T53 cells, and its absence in the parental FSC1-3 cells, was confirmed by autophosphorylation at site Ser2056 when radiation challenged (**Supplementary Figure 1**) [26]. The cells were cultured in Dulbecco's modified Eagle's medium containing 12% fetal calf serum and adjusted to pH 7.4 in 5% CO_2.

Colony Formation Assay

To reduce the variability in results associated with each step in the dose response assay procedure, that is, trypsinizing, counting, preparation of a range of cell dilutions for plating cells, and any differences in multiplicities at the time of irradiation, each of which pertains to cells receiving a different dose of radiation, the following procedure was followed. A single flask of exponentially growing FSC1-3 and T53 cells was trypsinized, counted, diluted, and then plated into 25-cm^2 tissue culture flasks for irradiation approximately 25 hours later. Cells to be proton irradiated or x-irradiated were obtained from the same cell suspension and cell dilution series. At each radiation dose level (0, 1, 2, 4, 6, and 8 Gy) the number of cells plated into each flask was adjusted with the intent of obtaining approximately 20 to 100 colonies per flask following irradiation, based on prior studies. Four and generally 5 flasks were plated for colony formation at each dose. The fifth flask was periodically examined by phase contrast microscopy at approximately 6 to 11 days post irradiation (depending on the dose administered) to estimate when the number of surviving colonies per flask, with approximately 50 cells per colony or more, did not change with increased culturing time. Lethally irradiated feeder cells (15 Gy) were added to each flask immediately before plating live cells to maintain the same total number of cells per flask over the complete dose range, to preclude feeder cell effects [27]. Multiplicities were determined in flasks plated with 2000 unirradiated cells in "conditioned medium," that is, medium from cultures containing approximately 2 million cells in 5 mL medium for 24 hours. Multiplicities were determined at the time of irradiation approximately 24 hours after plating single cells. Following the random assignment of flasks to be proton irradiated or x-irradiated, cells were cultured 7 to 12 days for colony formation, depending on the dose administered. Following incubation, the colonies were rinsed with saline, fixed in 100% ethanol, stained with crystal violet, and manually counted with a variable power microscope. For the calculation of cell-surviving fraction (*Sf*), the ratio of number of colonies to number of cells plated (*f*) was corrected to obtain the surviving fractions, using the equation:

(1)
$$Sf = 1 - (1 - f)^{1/n},$$

Figure. Dose-response curves of the DNA-PKcs–deficient tumor line FSC1-3, and its DNA-PKcs–complemented counterpart, T53 cells. FSC1-3 cells (open symbols) and T53 cells (closed symbols) were exposed to 6-MV x-rays (blue lines) or spread-out Bragg peak protons (red lines). Confidence intervals are 1 SD.
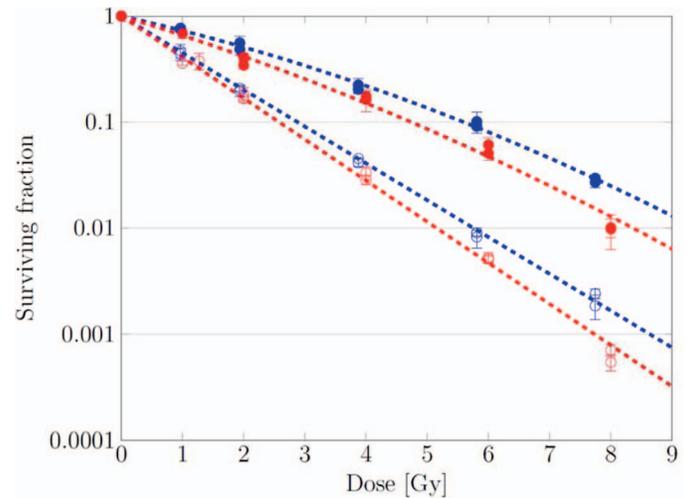
where n equals the average multiplicity at the time of irradiation. The resulting data were least-square fit to the following equation:

(2)
$$lnSf = -\alpha d - \beta d^2.$$

Irradiation

Attached cells in 25-cm^2 polystyrene tissue culture flasks under 0.2 cm medium were x-irradiated at room temperature at a dose rate of 6.0 Gy/min. X-irradiation was performed by using a 6-MV beam from an Elekta Infinity series accelerator (Stockholm, Sweden) at gantry $= 0°$ with the flasks placed on a custom tray at a distance of 100 cm from the source, using a 20×20-cm field size with 5 cm of solid water buildup. The flask provided an additional buildup of 0.15 cm. The dose per monitor unit of machine output under these conditions was subsequently measured and confirmed to be within 2.9% across the 5 flasks, using thermoluminescent dosimetry (TLD-100, Thermo Fisher Scientific, Waltham, MA), MicroStar nanoDot Optically stimulated luminesence (Landauer, Glenwood, IL), a MatriXX Evolution series 2D detector (IBA, Louvain-La-Neuve, Belgium), and a model 23343 Markus chamber (PTW-Freiburg, Germany). The TLDs and optically stimulated dosimeters were placed inside empty culture flasks during the measurement and then read out in our department after 24 hours. The source to cell and source to TLD distance, as well as the field size, backscatter, 5-cm water equivalent buildup (and 0.15 cm flask "roof" thickness), and air gap, were identical for the cell exposure and dose measurements. A small difference was the presence of 2 mm medium overlying the cells, whereas the TLDs were placed in a 0.025-cm-wall-diameter polyethylene sheath. Using the percentage depth dose data for the linear accelerator, for a 20×20–cm field, 5.18-cm buildup for TLDs and 5.35 cm for cells, this equates to a dose difference of 78.59% for cells versus 79.06% for TLDs or 0.47%. A slight underestimation or overestimation of the dose pertained equally to both compared cell lines and does not alter or reverse the results of the study. The machine output was checked and the response for each detector was calibrated by using reference conditions of 10-cm depth (isocentric) and 10×10 field size before each of the x-ray irradiation sessions. Cells were proton irradiated at the Francis H. Burr Proton Therapy Center (Boston, MA) by using a passive scattered 137-MeV beam with the beam range of 13.4 cm (90%) and modulation width of 7 cm (90% to 98%) at gantry $= 0°$. The largest snout was used with the effective field size of 22 cm in diameter and dose uncertainty within 2%. A Lucite phantom was used to position the cells in flasks in the mid-SOBP, that is, at 10.3-cm water equivalent depth from the front surface of the phantom to the surface of the medium in the flask. The output was calibrated as based on International Atomic Energy Agency, publication 398 and the dose rate was 1.7 Gy/min. For protons, the dose was verified by using a Markus chamber at the level of the cells inside the flask.

Results

For the assessment of radiation sensitivity, 2 experiments were performed, each generating 4 survival curves, that is, 2 cell lines, each of which was x-irradiated and SOBP proton irradiated. The results are shown in the **Figure** (initial and repeated

Table. Response parameters of sensitive FSC1-3 and resistant T53 cells to x-rays and protons.

Experiment	$A \pm SD\alpha$	$B \pm SD\beta$	$D(Sf = 0.01) \pm SD\ D$	$RBE \pm SD\ RBE$
E1 FSC1-3 x-ray	0.7992 ± 0.0032	0	2.882 ± 0.012	1.113 ± 0.013
E1 FSC1-3 proton	0.8893 ± 0.0095	0	2.590 ± 0.028	
E1 T53 x-ray	0.281 ± 0.018	0.0231 ± 0.0029	5.605 ± 0.037	1.187 ± 0.049
E1 T53 proton	0.432 ± 0.062	0.012 ± 0.012	4.72 ± 0.19	
E2 FSC1-3 x-ray	0.8230 ± 0.0036	0	2.798 ± 0.012	1.091 ± 0.019
E2 FSC1-3 proton	0.898 ± 0.015	0	2.564 ± 0.044	
E2 T53 x-ray	0.313 ± 0.032	0.0185 ± 0.0044	5.55 ± 0.10	1.161 ± 0.044
E2 T53 proton	0.376 ± 0.048	0.0222 ± 0.0076	4.78 ± 0.16	
Pooled FSC1-3 x-ray	0.8005 ± 0.0027	0	2.8763 ± 0.0099	1.117 ± 0.012
Pooled FSC1-3 proton	0.8942 ± 0.0089	0	2.575 ± 0.026	
Pooled T53 x-ray	0.297 ± 0.016	0.0206 ± 0.0024	5.588 ± 0.042	1.181 ± 0.030
Pooled T53 proton	0.403 ± 0.036	0.0177 ± 0.0062	4.73 ± 0.11	

Abbreviations: RBE, relative biological effectiveness; E1, experiment 1; E2, experiment 2.

experiments not labeled separately). Examination of the curves in the **Figure** shows that for both x-rays and protons, over the dose range examined, FSC1-3 cells were well fit by the equation:

$$(3) \qquad \ln(Sf) = -\alpha d.$$

In contrast to the DNA-PKcs–defective FSC1-3 cells, the efficiency of cell killing per unit dose increased with increasing dose of both x-rays and protons in T53 cells; these data were well fit to by the linear-quadratic equation:

$$(4) \qquad \ln(Sf) = -\alpha d - \beta d^2.$$

The initial and repeated experiment survival curves were fit individually; furthermore, a separate fit was made on the pooled data from the 2 experiments (**Figure** shows the results of the fits, using the pooled data). All fits were weighted in inverse proportion to the variance of the surviving fractions. All fit parameters were determined together with their covariance matrix, and the uncertainties coming from the parameter covariance matrices were propagated to all derived estimates by using standard error propagation techniques. The response parameters are summarized in the **Table**.

For all 8 individual survival curves, the value of the parameter α was larger in cells receiving mid-SOBP proton irradiation than x-irradiation regardless of the cells' DNA-PKcs status ($P = .016$, paired t test). The pooled results show the same, α for mid SOBP protons is higher than for x-irradiation, with the 2 values well outside each other's confidence intervals ($P = .0397$, paired t test).

For calculation of the RBE of mid-SOBP protons, the α and β values of each experiment listed in the **Table** were substituted into the LQ equation, and the dose yielding a surviving fraction of 0.100 was calculated, yielding RBE values of 1.113 and 1.187 for FSC1-3 and T53 cells, respectively, in experiment 1; and 1.091 and 1.161 for FSC1-3 and T53 cells, respectively, in experiment 2. At the 5% level, the RBE difference is significant according to both the paired ($P = .0156$) and the unpaired ($P = .0493$) 2-sample t tests. When the surviving fraction data of experiments 1 and 2 are pooled before statistical analysis, the results also suggest that the RBE values for T53 are higher than for FSC1-3 cells, that is, RBE = 1.181 ± 0.030 versus 1.117 ± 0.012 respectively, with an RBE difference of 0.064 ± 0.032, ($P = .107$). To summarize, the proton RBE values are larger in DNA-PKcs–dependent resistant cells than in their DNA-PKcs–deficient counterparts by factors of 1.066 and 1.064 in experiments 1 and 2, respectively, and by a factor of 1.057 in an analysis of the pooled data.

Discussion

Transfection of the NHEJ double-strand break repair gene DNA-PKcs into DNA-PKcs–defective FSC1-3 severe combined immunodeficient tumor cells increases the cells' resistance to both x-rays and protons. However, the increased resistance to protons is less pronounced than the increased resistance to x-rays. The results indicate that DNA damage induced by protons is less amenable to DNA-PKcs–dependent repair than damage induced by x-rays, a result that is not inconsistent with studies showing that homologous recombination-defective cells (NHEJ competent) exhibited an increased proton RBE [24, 25]. Nevertheless, few studies have investigated, and none to our knowledge have reported, a difference in the proton RBE of

DNA-PKcs–deficient and DNA-PKcs–proficient cells. Gerelchuluun et al [16] reported a mean ($\pm$95% confidence interval) proton RBE of 0.91 $\pm$ 0.02 in wild-type CHO cells versus 2 NHEJ-deficient CHO sublines: RBE = 1.00 $\pm$ 0.41 and 0.94 $\pm$ 0.61 at the 10% surviving fraction level. Although the RBE of the wild-type and NHEJ-deficient cells did not significantly differ, the results are not incompatible with the RBE difference observed in the present study. In studies reported by Grosse et al [24], the RBE values of wild-type and NHEJ-deficient cells were 1.20 $\pm$ 0.05 versus 1.09 $\pm$ 0.08, respectively, at the 0.10 surviving fraction level. Fontana et al [25] evaluated the response of A549 lung and M059K glioblastoma cancer cells to x-rays and protons in the presence and absence of the DNA-PKcs inhibitor NU70276. NU7026 had a significantly greater sensitizing effect on A549 cells exposed to x-rays than to protons. That is, a greater killing efficiency of protons versus photons was observed in normal (ie, not DNA-PKcs inhibited) cells than in radiation-sensitive DNA-PKcs–inhibited cells. These results are consistent with those observed in the present study. Unlike in the present study, a proton RBE difference was not observed between M059K and DNA-PKcs–deficient M059J glioblastoma cells. Nevertheless, as it pertains to A549 cells, NU7062 inhibition of DNA-PKcs sensitized M059K cells to both photons and protons, and the sensitization to photons was more pronounced that sensitization to protons.

As previously noted, tumors contain subpopulations of radioresistant cells [2, 3]. Overexpression of DNA-PKcs has been reported in a number of human tumor types [28, 29] and there is evidence that the greater resistance of tumor or tumor subpopulations is related to the expression of DNA-PKcs. Beskow et al [11] evaluated the percentage of DNA-PKcs–positive staining cells in the primary and residual tumor material at 4 weeks post irradiation in patients with cervical carcinoma. Residual tumors displayed a higher percentage of DNA-PKcs–positive cells than the primary tumor ($P = .037$), suggesting their resistance to and selection by radiation. Similar results were reported by Shintani et al [10] in oral squamous cell carcinoma. Bouchaert et al [12] reported a significant predictive relationship between DNA-PKcs staining and biochemical recurrence in patients treated for prostate cancer by external beam radiation therapy ($P = .01$). Molina et al [13] reported a predictive relationship between biochemical relapse and DNA-PKcs nuclear staining in patients treated for prostate cancer by interstitial brachytherapy on multivariate analysis ($P = .03$).

In related studies, Fu et al [22] selected cancer stemlike and nonstem cells from the breast cancer cell line MCF7, as based on the cells' expression of the stem cell markers CD44 and CD24. The response of the 2 cell populations to radiation was assessed by the cells' percentage increase in apoptosis and γ-H2AX foci. The marker-identified cells were more resistant to both photons and protons than nonstem cells, but the magnitude of the difference in sensitivity was significantly reduced in proton-irradiated cells. Zhang et al [23] investigated the response of parental and paclitaxel-resistant non–small cell lung cancer cells to x-rays and protons. Cell sensitivity was examined by the level of radiation-induced reactive oxygen species, apoptosis, and colony and sphere formation. Compared to the differential response of parental cells to x-rays and protons, chemoresistant and chemoresistant side-population cells were relatively sensitive to protons. While the above studies did not include an investigation of DNA-PKcs expression in the radiation-resistant population, the results show that protons may be relatively effective against resistant tumor cell subpopulations.

To summarize, the results suggest that the RBE of mid-SOBP protons is higher in tumorgenic cells containing a functional DNA double-strand break repair gene, DNA-PKcs, than in their isogenic DNA-PKcs–deficient counterpart. The results also suggest that protons may be more effective than x-rays for sterilizing cells that are relatively radiation resistant owing to DNA-PKcs–dependent repair of radiation damage.

ADDITIONAL INFORMATION AND DECLARATIONS

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