Differential DNA repair pathway choice in cancer cells after proton- and photon-irradiation

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Abstract:

Background and Purpose: Non-homologous end-joining (NHEJ) and homologous recombination (HR) contribute to the repair of irradiation-induced DNA double-strand breaks (DSBs). We investigated the impact of the two major DSB repair machineries for cellular survival of human tumor cells in response to proton- and photon-irradiation.

Materials and Methods: DNA damage repair and cell survival were analyzed in wildtype, HR- and NHEJ-repair-compromised and pharmacologically DNA-PKcs-inhibited human tumor cells in response to clinically relevant, low-linear energy transfer proton- and 200-keV photon-irradiation.

Results: Pharmacological inhibition of DNA-PKcs strongly radiosensitized lung adenocarcinoma and glioblastoma cells to photon- but to a much lower extent to proton-irradiation. Enhanced radiosensitization correlated with strongly delayed repair kinetics with elevated amounts of γ H2AX foci after photon-irradiation. Interestingly, we observed reduced phosphorylation of DNA-PKcs at Ser-2056 and Thr-2609 clusters after proton-irradiation compared to photon-irradiation. In contrast, A549 cells depleted of the RAD51 recombinase were markedly hypersensitive to proton-irradiation in comparison to control cells. Likewise, human BRCA2-deficient ovarian carcinoma cells were hypersensitive towards proton- in comparison to photon-irradiation.

Conclusion: A differential DNA damage response with enhanced susceptibility of HR-deficient tumor cells to proton-irradiation and increased sensitivity of photon-irradiated tumor cells to NHEJ inhibitors were demonstrated.

Introduction

Currently, a generic RBE value of 1.1 is used in the clinic for proton radiotherapy. This value can vary significantly depending on the tissue, cell line or endpoint investigated, and an increasing number of studies now investigates these differences in detail at the cellular and molecular level [1-8].

Un- and misrepaired DNA double-strand breaks (DSBs) are the major cause for IR-induced cell death [6-7]. Even though both types of ionizing radiation (IR) are regarded as low LET-irradiation, the complexity of the DNA damage in response to clinically-relevant proton- and photon-irradiation could be different [5,6]. Independent of the amount of IR-induced DSBs, the quality of DNA damage might then demand differential DNA repair capacities. We previously could not detect any significant differences in the amount of DSBs induced by the two types of irradiation, but demonstrated in a model study based on genetically-defined chinese hamster ovary cells that cells with compromised homologous recombination (HR) are more sensitive to treatment with low-LET proton-irradiation (136 MeV) compared to photon-irradiation [8].

Cells have evolved two major pathways to cope with potentially lethal IR-induced DSBs, HR and non-homologous end joining (NHEJ) [9,10]. HR is primarily active through the S/G₂-phase of the cell cycle and results in preserved sequence integrity [11]. On the other hand, NHEJ is active throughout the cell cycle and is responsible for the repair of most IR-induced DSBs in eukaryotic cells [12]. Although very efficient in a quantitative way, the quality of repair by NHEJ can steadily decrease with increasing amounts of DNA damage [13].

Here we investigated a differential treatment response to proton- versus photon-irradiation in human tumor cells on the mechanistic level and demonstrated a differential involvement of the two major DSB repair machineries also by pharmacological interference.

Materials and Methods

Cell lines. A549 cells (ATCC) were cultured in RPMI cell culture media. The glioblastoma cells M059K and M059J were maintained in 1:1 MEM/F12 Ham's mixture. All media were supplemented with 10% FBS, glutamine (2mM) and penicillin-streptomycin (100U/ml-100µg/ml) and cells were kept at 37°C at 5% CO₂ atmosphere.

Drug Treatment. NU7026 (10µM, Selleckchem) and Vorinostat (2µM, Selleckchem) were added 1 h and 24 h, respectively, to the cells prior to irradiation.

Antibodies. The following antibodies were used at the specified dilutions: rabbit monoclonal anti-H2AX-pSer139 (1:200, Abcam, Cambridge, UK); rabbit monoclonal anti-RPA32 (1:1000 Bethyl Science, Bethesda, USA); rabbit polyclonal anti-DNA-PKcs-pSer2056 (1:100, Abcam, Cambridge, UK); mouse monoclonal anti-DNA-PKcs-pThr2609 (1:200, Abcam, Cambridge, UK); mouse monoclonal anti-DNA-PKcs (1:500, Abcam, Cambridge, UK); mouse monoclonal anti-RAD51 (1:100 (IF) or 1:500 (WB) - Novus Biological, A-8 Littleton CO, USA); mouse monoclonal anti-β-actin (1:1000, Sigma-Aldrich, St. Louis MO, USA).

Irradiation procedures. Irradiation and dosimetry were essentially performed as described in [8]. Exponentially growing cultures were plated into 25 cm² TPP flasks 24 hours prior to irradiation (IR). Photon-irradiation was performed using an Xstrahl 200 kV X-ray unit at 1 Gy/min (kVp = 200kV; Amperage=15mA, HVL = 1.03mm Cu, Filter: 1mm Al and 0.45mm Cu). All proton-irradiations were delivered using the spot scanning approach [14] with the cells being placed in the centre of a Spread-Out-Bragg-Peak (SOBP) with a length of 5cm and maximum proton energy of 138 MeV. The field size orthogonal to the beam was 15x11cm.

Clonogenic survival. The clonogenic cell survival assay was performed in triplicates as described in detail in [8]. In order to calculate RBE and DMF values, α- and β-values for each experiment were determined. Data were pooled from at least 3 independent experiments. Survival data were fitted by weighted, stratified, linear regression to obtain the linear and quadratic parameters. Linear regression was performed in SPSS (Version 23) as described in

[15]. RBE and DMF values at the 10 % survival level (termed RBE₁₀ and DMF₁₀) were calculated according to [16].

Immunofluorescence microscopy and siRNA transfection. 24 hours prior to irradiation cells were plated onto Ibidi μ -slides VI0.4 (Ibidi, 80606, Munich), irradiated, fixed, permeabilized and incubated with the selected primary antibodies (see above) and incubated with the appropriate secondary antibody diluted 1:1000 (Alexa-488 or/and Alexa-546; Invitrogen) as described in detail in [8]. Foci of at least 50 cells/condition were counted and means of data were pooled from at least 3 independent experiments. siRNA transfection was performed as described in [8]. The following siRNA sequences were used: siLuc: 5'-CGTACGCGGAATACTTCGAdTdT-3'; siRad51: 5'-GAGCUUGACAAACUACUUCdTdT-3'. siDNA-PK_{cs}, Dharmacon SMARTpool against human PRKDC (GE Healthcare).

Western blotting. Cell lysates were prepared with lysis-buffer (2% SDS, 10% glycerol, 0.004% bromphenol blue, 62.5 mM Tris-HCl, pH 6.8), subjected to SDS-PAGE and blotted onto PVDF-membranes. Primary antibody detection (see above) was achieved by enhanced chemiluminescence according to the manufacturer's protocol (Amersham, Freiburg, Germany). Western blotting to control for siRNA-downregulated proteins was performed for each experiments. A representative example is shown.

Statistical Analysis. Data were presented as the mean $\pm$ standard deviation (SD) of at least three independent experiments. The results were tested for significance using the two-sided unpaired Student's t-test or the Wilcoxon-Mann-Withney-U Test. Results were considered statistically significant when $p < 0.05$ (*) or highly significant $p < 0.001$ (**) and $p < 0.0001$ (***).

Results

To examine activation of the NHEJ machinery in response to clinically relevant high-energy, low-LET proton irradiation (136 MeV) and photon-irradiation (200kV), we first monitored the formation of DNA-PKcs foci in non-small cell lung cancer A549 cells using phospho-specific antibodies directed against Ser-2056 and Thr-2609. Remarkably, proton-irradiated cells displayed fewer phospho-DNA-PKcs foci compared to photon-irradiated cells (Fig. 1A-B), which suggested a differential requirement of NHEJ in response to the two types of IR. We therefore investigated the radiosensitizing effect of the selective DNA-PKcs inhibitor NU7026 in combination with either type of IR. NU7026 sensitized A549 cells in response to both types of irradiation, yet to a higher extent for photon irradiation with a DMF_{10} of 1.91 ± 0.05 compared to proton-radiation (1.49 ± 0.06) (Fig. 1C). Of note, we could not determine a differential sensitivity of wtA549 cells to the two types of IR alone, but the RBE determined *in vitro* can vary significantly from the generic RBE 1.1 value used in the clinics. Treatment of A549 cells with either type of irradiation resulted in the same amount of initial γ H2AX foci and similar kinetics of γ H2AX focus removal. Surprisingly, however, γ H2AX focus removal after proton-irradiation was only minimally affected by NU7026 pretreatment, whereas removal was strongly delayed after photon-radiation (Fig. 1D). Moreover, we observed similar kinetics of focus formation and removal for 53BP1, another marker for the presence of DSBs (Suppl. Fig. 1-2).

To extend these findings, we investigated the radiosensitivity of A549 cells following siRNA-mediated downregulation of DNA-PKcs. Interestingly, and in contrast to chemical inhibition of DNA-PKcs, clonogenic survival of DNA-PKcs-depleted cells was reduced to the same extent in response to both types of IR (Fig. 2A). Consistently, slower kinetics of γ H2AX focus removal in DNA-PKcs-knockdown cells was similar after proton- and photon-irradiation (Fig. 2B). Delayed γ H2AX foci disappearance in NU7026-treated cells after photon-irradiation

might be caused by DNA-PKcs stuck to the DNA damage site, thereby preventing efficient repair by both NHEJ and HR [17].

Reduced localization of activated DNA-PKcs after proton-induced DNA damage, as measured by phospho-DNA-PKcs foci (see Fig. 1A), suggests reduced blockade of the DNA damage site caused by NU7026 in response to proton-irradiation, which would facilitate DNA-end resection and HR-mediated repair of DSBs. Therefore, we analyzed RPA foci formation, a known marker for single-stranded DNA in response to both types of IR (Fig. 2C and Suppl. Fig.3). In the absence of NU7026, we observed a similar, time-dependent increase in RPA foci per cell after photon- and proton-irradiation. Interestingly, DNA-PKcs-inhibition specifically impaired RPA foci formation in response to photon-irradiation, while NU7026 had no significant effect on RPA recruitment following proton-irradiation, indicating that photon-induced lesions are more susceptible to undergo NHEJ.

To corroborate our findings, we analyzed cell survival and γ H2AX focus formation in DNA-PKcs-deficient M059J cells and their isogenic but DNA-PKcs-proficient counterpart, M059K [18]. Similarly to DNA-PKcs downregulation in A549 cells, M059J cells were equally sensitive to proton- and photon-radiation and showed similar kinetics of γ H2AX foci removal in response to both types of IR (Fig. 3A-B). Furthermore, treatment of the DNA-PKcs-proficient M059K cells with NU7026 resulted in a stronger radiosensitizing effect and delayed γ H2AX focus removal after photon- compared to proton-irradiation, with a DMF₁₀ of 1.49 ± 0.02 and 1.2 ± 0.11 , respectively (Fig. 3C-D).

We reasoned that in response to proton-irradiation, reduced dependence on NHEJ could be linked to a specific requirement for HR for DNA repair and enhanced cell survival. We first analyzed RAD51- and RPA-foci formation in response to proton- and photon-irradiation, two major components of the HR pathway, but did not observe any quantitative difference between the two types of IR (see above). However, siRNA-mediated downregulation of RAD51 rendered A549 cells markedly more hypersensitive to proton- versus photon-irradiation (Fig.

4A). In addition, RAD51 depletion strongly delayed γ H2AX focus removal after proton-irradiation but only minimally affected RAD51 foci kinetics after photon-irradiation, indicating a preferential requirement for HR-mediated DSB repair after proton-irradiation (Fig. 4B). Enhanced sensitivity of HR-deficient cells to proton- compared to photon-irradiation was also demonstrated in BRCA2-deficient PEO1 cells (Suppl. Fig. 4).

While direct pharmacological targeting of the HR pathway has proven to be largely unsuccessful in the past years, several chemotherapeutic agents exist that eventually downregulate RAD51 protein levels, including the broad-range histone deacetylase inhibitor SAHA (Vorinostat)[19]. Continuous exposure with 2 μ M SAHA for 24 hours was sufficient to reduce RAD51 levels down to approximately 10 % of its basal level, without affecting Ku80 or DNA-PKcs protein levels (Fig. 5A). Pretreatment of A549 cells with SAHA led to increased sensitivity following proton- but not photon-irradiation (Fig. 5B). We further investigated the effect of cellular pretreatment with SAHA on γ H2AX and DNA-PKcs (Thr-2609) foci formation after both proton- and photon-irradiation (Fig. 5C). Similar to the results obtained in RAD51-knockdown cells, γ H2AX focus removal was strongly delayed after proton-irradiation, but only minimally after photon-irradiation.

Discussion

Here we investigated a differential requirement of the two major DNA DSB repair pathways for survival of irradiated human tumor cells in response to photon- and proton-irradiation. We demonstrated that IR-induced DNA-PKcs activation is lower in response to proton-irradiation compared to photon-irradiation. Furthermore, pharmacological inhibition of the catalytic subunit of DNA-PK by the DNA-PKcs-specific inhibitor NU7026 sensitizes tumor cells to both types of irradiation yet to a higher extent to photon-irradiation. The kinetics of γ H2AX-focus removal in NU7026-pretreated cells strongly correlated with increased dependence on NHEJ in response to photon-irradiation-induced DNA-damage. On the other hand, lack of an intact HR-machinery rendered tumor cells more sensitive towards proton- than towards photon-

irradiation.

Several studies have shown that phosphorylation of DNA-PKcs promotes release of DNA-PKcs from DNA and that blocking DNA-PKcs phosphorylation using small molecule inhibitors results in reduced rates of DSB repair and reduced survival after photon-irradiation. However, the effect of chemical inhibition of DNA-PK on HR remains controversial. Our own results on the level of γ H2AX-focus removal and reduced RPA32-foci formation strongly correlate with a model that NU7026-inhibited DNA-PKcs is blocking the DSB site not allowing repair machineries other than NHEJ to promote DNA-repair in response to photon-irradiation. In contrast, γ H2AX focus removal after proton-irradiation was not disturbed in tumor cells pretreated with NU7026 indicating reduced affinity of proton-induced DNA-damage towards NHEJ as major repair pathway. Consequently, depletion or genetic deficiency of DNA-PKcs rendered tumor cells more sensitive to both types of IR though without increased sensitivity towards proton-irradiation and without delayed γ H2AX focus removal after photon-irradiation. Furthermore, our results suggest a differential sensitization by DSB repair pathway-specific inhibitors for photon- *versus* proton-irradiation, respectively. Tumor cells depleted of the RAD51 recombinase, carrying BRCA2 mutations or treated with the chemotherapeutic agent SAHA were significantly more sensitive to proton-irradiation than to photon-irradiation. Thus, while inhibitors of NHEJ more specifically sensitize to photon-irradiation, pharmacological agents that suppress HR might more specifically sensitize to proton-irradiation. Unfortunately, no inhibitors directly and specifically inhibiting HR entities are available so far.

We previously determined that the initial amounts of DNA damage induced by the two types of IR are comparable [8]. As such, a differential requirement for HR and NHEJ after clinically-relevant proton- and photon-irradiation points towards a differential quality of DNA damage induced by the two types of IR. Our results now demonstrate a clear preference of proton-induced DNA damage towards HR in human cancer cells, which might become relevant for clinical stratification of patients carrying mutations in this repair pathway.

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Conflict of interest statement: none

Figure Legends

Fig. 1. Radiosensitizing effect of DNA-PKcs inhibitor NU7026 on A549 cells. Time course of DNA-PKcs-phosphorylation at the Ser-2056 (A) and Thr-2609 (B) cluster sites in response to 1Gy of proton- and photon-irradiation. (C) Clonogenic survival of A549 cells pretreated with NU7026 followed by photon- and proton-irradiation. Clonogenic cell survival assays were performed in triplicates. Points represent mean $\pm$ SD. Data analysis was performed on pooled values from at least 3 independent experiments. Kinetics of γ H2AX (D) foci removal after 1Gy of proton- and photon-irradiation in A549 cells pretreated for 1 hour with NU7026 and fixed at the indicated time points. Foci of at least 50 cells/condition were counted and means of data were pooled from at least 3 independent experiments.

Fig. 2. Proton- and photon-irradiation-induced cell killing in DNA-PKcs-knockdown A549 cells. (A) DNA-PKcs protein levels of siDNA-PKcs-treated A549 cells, determined at the indicated time points, and clonogenic cell survival of proton- and photon-irradiated cell lines treated with control siRNA (siLuc) or siRNA against DNA-PKcs (siDNA-PKcs). Clonogenic cell survival assays were performed in triplicates. Points represent mean $\pm$ SD. (B) Kinetics of γ H2AX-foci removal after 1Gy of proton- and photon-irradiation in siLuc- and siDNA-PKcs-treated cells. (C) RPA32 recruitment in irradiated cells pretreated with NU7026. RPA32 foci were determined at 1 hour and 4 hour after irradiation with 10Gy. Foci of at least 50 cells/condition were counted and means of data were pooled from at least 3 independent experiments.

Fig. 3. Clonogenic survival and DNA DSB repair in cells pretreated with NU7026 or DNA-PKcs deficiency. Clonogenic survival (A) and γ H2AX-foci removal (B) of DNA-PKcs-proficient (M059K) and DNA-PKcs-deficient (M059J) cells in response to proton- and photon-irradiation. Clonogenic survival (C) and γ H2AX-foci removal (D) of M059K cells pretreated with NU7026 1 hour prior to irradiation. Clonogenic cell survival assays were performed in triplicates. Points represent mean $\pm$ SD. Data analysis was performed on pooled values from at least 3 independent experiments. Foci of at least 50 cells/condition were counted and means of data were pooled from at least 3 independent experiments.

Fig. 4. Proton- and photon-irradiation-induced cell killing in RAD51-knockdown cells. (A) RAD51 protein levels of siRAD51-treated A549 cells, determined at the indicated time points, and clonogenic cell survival of proton- and photon-irradiated cell lines treated with control siRNA (siLuc) or siRNA against RAD51. Clonogenic cell survival assays were performed in triplicates. Points represent mean $\pm$ SD. (B) Kinetics of γ H2AX-foci removal after 1Gy of proton and photon-irradiation in siLuc- and siRAD51-treated cells. Foci of at least 50 cells/condition were counted and means of data were pooled from at least 3 independent experiments.

Fig. 5. Radiosensitizing effect of SAHA (Vorinostat) in A549 cells. (A) Levels of DSB-repair proteins in irradiated A549 cells pretreated with SAHA. (B) Clonogenic survival of A549 cells pretreated with SAHA. Cells were incubated for 24 hour with 2 μ M SAHA, followed by irradiation at the indicated doses. Clonogenic cell survival assays were performed in triplicates. Points represent mean $\pm$ SD. (C) Quantification of initial and residual γ H2AX and pDNA-PKcs (Thr-2609) foci after 1Gy of proton- and photon-irradiation. Foci of at least 50 cells/condition were counted and means of data were pooled from at least 3 independent experiments.

Figure 1

A

B

C

D

Figure 2

Figure 3

Figure 4

A

B

Figure 5