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Homologous recombination mediates cellular resistance and fraction size sensitivity to radiation therapy

Navita Somaiah [a,b,*], John Yarnold [b], Anne Lagerqvist [c], Kai Rothkamm [d], Thomas Helleday [e,*]

[a] Gray Institute for Radiation Oncology & Biology, University of Oxford; [b] The Royal Marsden NHS Foundation Trust & The Institute of Cancer Research, Sutton, UK; [c] Department of Genetics Microbiology and Toxicology, Stockholm University, Sweden; [d] Health Protection Agency, Centre for Radiation, Chemical & Environmental Hazards, Didcot, UK; [e] Science for Life Laboratory, Division of Translational Medicine and Chemical Biology, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden

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ABSTRACT

Purpose: Cellular sensitivity to radiotherapy total dose and fraction size is strongly influenced by DNA double strand break (DSB) repair. Here, we investigate response to radiotherapy fraction size using CHO cell lines deficient in specific DNA repair pathways in response to radiation induced DNA double strand breaks (DSB).

Experimental design: We irradiated CHO cell lines, AA8 (WT), irs1SF (XRCC3-), V3-3 (DNA-PKcs-) and EM9 (XRCC1-) with 16 Gy in 1 Gy daily fractions over 3 weeks or 16 Gy in 4 Gy daily fractions over 4 days, and studied clonogenic survival, DNA DSB repair kinetics (RAD51 and 53BP1 foci staining) and cell cycle profiles (flow cytometry).

Results: In response to fractionated radiotherapy, wild-type and DNA repair defective cells accumulated in late S/G2 phase. In cells proficient in homologous recombination (HR), accumulation in S/G2 resulted in reduced sensitivity to fraction size and increased cellular resistance (clonogenic survival). Sensitivity to fraction size was also lost in NHEJ-defective V3-3 cells, which likely rely on functional HR. By contrast, HR-defective irs1SF cells, with functional NHEJ, remained equally sensitive to fractionation throughout the 3-week treatment.

Conclusions: The high fidelity of HR, which is independent of induced DNA damage level, is postulated to explain the low fractionation sensitivity and cellular resistance of cells in S/G2 phase. In conclusion, our results suggest that HR mediates resistance to fractionated radiotherapy, an observation that may help future efforts to improve radiotherapy outcome.

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Clinical radiotherapy (RT) is delivered as a sequence of fractional, usually daily, doses. Normal and malignant tissues differ in their responses to several treatment-related variables, including total dose, fraction size, inter-fraction interval and overall treatment time [1,2]. Fractionation was initiated in order to spare normal tissue by enabling repair of sublethal damage and repopulation from surviving cells and also to increase the damage to the tumour by re-oxygenation of hypoxic cells and redistribution of cells along the cell cycle. Repair and repopulation confer resistance to tissue between two radiation doses, while redistribution and re-oxygenation are expected to make the tissue more sensitive to a subsequent dose [3,4]. The α/β ratio describes the shape of the fractionation response. On average, most cancers have a high α/β ratio and are less sensitive to fraction size than the normal tissues responsible for dose-limiting adverse effects presenting months or years later [2,5,6]. In this setting, the use of small ($\leqslant$2 Gy) fractions spares the cancer less than the dose-limiting normal tissues, thereby increasing therapeutic gain [2]. Recent evidence suggests that breast and prostate cancers show, on average, comparable sensitivity to fraction size as the dose-limiting normal tissues [7–9]. If so, small fractions spare the cancer as much as the normal tissues, and there is no disadvantage in giving fewer, larger fractions to a lower total dose. This strategy is increasingly adopted for the adjuvant radiotherapy of women with early breast cancer, for example [10,11]. Given the evidence for variation in fraction size sensitivity between tumour types, it is also possible that significant variation in sensitivity exists within tumour types. It is therefore relevant to seek predictive biomarkers of sensitivity to fraction size that allow stratification of patients for treatment with the most appropriate fractionation regimen.

Fraction size sensitivity is a cellular property reflecting, the ability to repair otherwise lethal DNA double-strand breaks (DSBs) prior to the next fraction of radiotherapy [12]. DSBs are rapidly

* Corresponding authors. Addresses: Department of Radiotherapy, The Royal Marsden, Downs Road, Sutton, Surrey SM2 5PT, UK (N. Somaiah). Science for Life Laboratory, Division of Translational Medicine and Chemical Biology, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Box 1031, S-171 21 Stockholm, Sweden (T. Helleday).

E-mail addresses: navita.somaiah@gmail.com (N. Somaiah), thomas.helleday@scilifelab.se (T. Helleday).

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repaired by non-homologous end-joining (NHEJ) in all phases of the cell cycle, in addition to which, homologous recombination (HR) requiring an intact sister chromatid, repairs a proportion of DSBs in S and G2 phases of the cell cycle [13–15]. The relatively high radioresistance of NHEJ-defective mutants in the S–G2 portion of the cell cycle further suggests that HR promotes survival when sister chromatids are present [16,17]. Rodent cell lines that are deficient in NHEJ and that rely disproportionately on HR to repair DSBs, show no dose-rate sparing, an indicator of insensitivity to fraction size [18]. We postulate that the high fidelity of DSB repair in replicated chromatin [19,20], explains the low fractionation sensitivity of cells reliant on HR.

In order to study the fractionation sensitivity of different DNA repair pathways we used Chinese hamster ovarian (CHO) cell lines, with well characterised defects in base excision repair (BER), NHEJ or HR, representing the most important DNA repair pathways for radiation-induced single strand breaks and DSBs, respectively. The parent wild type AA8 [21] and its derivatives irs1SF (XRCC3-) [22,23], V3-3 (DNA-PKcs-) [24] and EM9 (XRCC1-) [25] were irradiated with different fractionated schedules and tested for clonogenic survival, DNA DSB repair kinetics and cell cycle profiles.

Materials and methods

Cell culture

CHO cells were obtained from Larry H Thompson's lab, tested and authenticated at source. The parent wild type (AA8) [21], XRCC1 deficient (EM9) [25], XRCC3 deficient (irs1SF) [22,23] and DNA-PKcs deficient (V3-3) cells [24] were grown in Dulbecco's Modified Eagle Medium (D-MEM) (1X) containing 1000 mg/L glucose, 4 mM L-glutamine and 110 mg/L sodium pyruvate with 10% fetal bovine serum and penicillin–streptomycin. Cells were incubated in a well-humidified incubator at 37 °C with 5% CO_2. Exponentially growing cultures were used for all experiments.

Clonogenic survival assays

The experiments were initiated by seeding of 50,000–100,000 (depending on cell line) cells in 25 cm^2 flasks which were cultivated in 37 °C and 5% CO_2 in 8 mL D-MEM (1×). Six flasks were seeded for all groups including the control. The flasks were incubated for four hours followed by treatment with 1 Gy or 4 Gy ionising radiation (IR). The control was sham irradiated. Four hours after irradiation cells were counted and plated in triplicate onto 10 cm^2 petri dishes in appropriate numbers optimised by cell line and day of IR. On every Friday the AA8 and EM9 cell lines required reseeding to avoid overgrowth for the following week, which was done at the same concentration as in the beginning. Irs1SF required reseeding in the first week only, whereas V3-3 cells which were the most radiosensitive cells did not require to be reseeded throughout the 3 weeks. No irradiation or plating for survival was done over the weekend. When reseeding and seeding for survival all the cultivation media and washes were retained, centrifuged and the cell pellet pooled with the trypsinised ones. This was done to reduce any loss of cells that may have detached during IR. Once plated for survival, cells were stained with methylene blue in methanol (4 g/L) after 10 days of incubation and colonies of more than 50 cells scored. This procedure was repeated for 16 days with 1 Gy exposure each day (or 4 days for the 4 Gy experiment).

Irradiation

γ-irradiations were performed at room temperature using a GSR D1 137Cs γ-irradiator (Gamma-Service Medical GmbH, Leipzig, Germany) at a dose rate of ~1.9 Gy/min. Cells were either irradiated with 1 Gy each weekday to a total of 16 Gy over 3 weeks or at a higher dose per fraction of 4 Gy daily for 4 days to the same total dose. For acute dose survival curves, cells were irradiated with 0, 1, 2, 3, 4, 6 and 8 Gy and counted after 10 days of incubation. Before and after irradiation, cells were incubated in a well-humidified incubator at 37 °C with 5% CO_2, situated next to the irradiator room in order to minimise detachment during transportation.

Flow cytometry

Cells were harvested with trypsin from columns 2, 4, 6, 8, 10 and 12 of duplicate 96 well plates (Fig. 1), 24 h after the previous dose of RT for each cell line separately. The cells were fixed with ice-cold ethanol at 4 °C for a minimum of 30 min. For cell cycle analysis the fixative was removed by centrifugation at 250 g and the cells were resuspended in PBS containing propidium iodide (Sigma, Dorset, UK) at a final concentration of 10 μg/mL. The samples were run on a Becton–Dickinson FACScan. Analyses were carried out using Modfit (Verity Software House, ME, USA).

Foci staining for IN Cell analysis

Twenty-three 96 well plates were seeded with optimised cell numbers for each of the 4 CHO cell lines and allowed to attach for at least 4 h prior to IR (as per the template in Supplementary Fig. 1). 3 plates served as controls (sham irradiated) and were fixed 24 h, 1 week and 2 weeks after seeding. The remaining twenty 96 well plates were irradiated each day with 1 Gy from Monday to Friday for 2 weeks (total of 10 Gy) with 2 plates removed each day for fixing cells and studying DSB repair kinetics at each 1 Gy incremental dose level. For each cell line alternate columns were fixed at 0.5, 1, 2, 4, 6 and 24 h after irradiation (Supplementary Fig. 1). Whilst each column was being fixed the remaining wells were covered with adhesive plate sealing film (BD Falcon, Catalogue number 353073) so that the living cells would be protected from paraformaldehyde (PFA). The plates were placed on a hot plate at 37 °C whilst fixing so as not to slow DSB repair. The cells from the desired column were washed with phosphate-buffered saline (PBS), fixed with 4% PFA for 15 min and then washed with PBS. The plates were returned back to the incubator after removing the adhesive film till the next time point. After all the columns in the plates were fixed, the cells were permeabilised with 0.3% TritonX-100 in PBS for 10 min followed by blocking with 3% bovine serum albumin (BSA) in PBS for 40 min. RAD51 antibody (rabbit polyclonal, sc-8349, Santa Cruz) was added at 1:1000 in 3% BSA and incubated at 4 °C overnight. For dual staining with γH2AX, the RAD51 antibody was removed and γH2AX antibody (mouse, JBW301 clone, 05-636, Millipore) added at 1:1000 for 1.5 h at room temperature. Single staining with 53BP1 antibody (rabbit polyclonal, A300–272A, Bethyl labs) was done at 1:500 for 1.5 h at room temperature. After washing thrice with PBS, appropriate secondary antibodies Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, 1:500) and Alexa Fluor 555-conjugated goat anti-mouse IgG (Invitrogen, 1:500) was applied for 1 h in the dark at room temperature. Cells were counterstained with 4,6 diamidino-2-phenylindole (DAPI) for 5 min and washed in PBS. Fluorescence images were captured with the IN Cell Analyzer 1000 automated epifluorescence microscope (GE Healthcare) using a 40× objective. Fifteen images were taken per well and at least 100 cells were analysed for each time point. Automated foci analysis for percentage positive cells and mean foci number per cell was carried out using the IN Cell analyser 1000 workstation software (v3.5) as previously described [26,27]. The cut-off for 53BP1 positivity was taken as >5 foci and for RAD51 as >4 foci based on the control un-irradiated levels and previous optimisation studies [26].