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DNA mismatch repair protein MSH2 dictates cellular survival in response to low dose radiation in endometrial carcinoma cells

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ABSTRACT

DNA repair and G2-phase cell cycle checkpoint responses are involved in the manifestation of hyper-radiosensitivity (HRS). The low-dose radioresponse of MSH2 isogenic endometrial carcinoma cell lines was examined. Defects in cell cycle checkpoint activation and the DNA damage response in irradiated cells (0.2 Gy) were evaluated. HRS was expressed solely in MSH2+ cells and was associated with efficient activation of the early G2-phase cell cycle checkpoint. Maintenance of the arrest was associated with persistent MRE11, γH2AX, RAD51 foci at 2 h after irradiation. Persistent MRE11 and RAD51 foci were also evident 24 h after 0.2 Gy. MSH2 significantly enhances cell radiosensitivity to low dose IR.

1. Introduction

Ionizing radiation (IR) induces a spectrum of lesions, of which, DNA double strand breaks (DSBs) are considered the most deleterious. Cells have evolved protective mechanisms to repair DNA that link cell cycle checkpoints to repair processes that rejoin broken DNA. Five major DNA repair pathways co-ordinate the fidelity of DNA repair, these are non-homologous end joining (NHEJ), homologous recombination (HR), DNA mismatch repair (MMR), base excision repair (BER) and nucleotide excision repair (NER).

MMR is primarily involved in repairing errors that arise during DNA replication. Inherited mutations in MMR genes (MSH2, MLH1) give rise to a cancer predisposition syndrome called hereditary non-polyposis colorectal cancer (HNPCC) [4,13,23]. The role that MSH2 and MLH1 proteins play in the DNA damage response to IR remains controversial. Conflicting studies report that MMR-proficiency confers radiosensitivity, radioresistance, or has no effect on cellular radiosensitivity (reviewed in [29]). Emerging data suggest that MMR-proficiency indeed confers sensitivity to high dose IR but the impact of MMR-proficiency on radiosensitivity at low dose IR remains unknown. We have previously implicated MMR-dependent processes in low-dose hyper-radiosensitivity (HRS), i.e. the increase in cell kill per unit dose observed following doses below ~0.5 Gy [28]. While the underlying mechanism for HRS remains to be elucidated, defective early cellular response mechanisms, in particular evasion of the early G2-M cell cycle checkpoint [20,22], and defective HR have been implicated (reviewed in [24]).

The early G2-phase cell cycle checkpoint can be initiated by the MRN complex, consisting of the highly conserved proteins MRE11, RAD50, and NBS1, which are essential for activation of the ATM kinase [3,6]; the latter activates the checkpoint effector kinases Chk1 and Chk2 [7], which ultimately control entry into mitosis. The delay afforded by the arrest in proliferation is thought to allow vital time for the repair of mutagenic lesions created by DNA damaging agents, prior to cell entry and transit through mitosis [15]. MRE11 assists in this process via recruitment of repair factors following resection of damaged DNA. The arrest is only released when repair is completed. Where repair is not possible, damaged cells are removed by programmed cell death (apoptosis or autophagy) [17].

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MMR is essential for the activation of the early G2-M-phase cell cycle arrest following treatment with a variety of anti-cancer agents [19,27,35]. Accumulating evidence indicates that efficient G2-M checkpoint activation following exposure to IR also requires MMR. Proteomic studies have revealed that following exposure to IR, components of the MMR system interact with ATM both directly [5], and possibly indirectly, via localization of MRE11 [14], thus facilitating the phosphorylation of Chk2 [5]. Defects in IR-triggered activation of the G2-M-phase cell cycle checkpoint (1–8 Gy) have thus been attributed to dysregulated MSH2-dependent localization of MRE11, as well as incomplete activation of checkpoint kinases Chk1 and Chk2 [14].

DNA MMR components also participate in HR via abortion of strand exchange between divergent sequences [1,8,10,42]. A role for MSH2 in particular has been indicated in HR [10,36,40] via suppression of RAD51. Recent results suggest that MSH2 may also coordinate with p53 to monitor the fidelity of HR during S phase [43].

Our previous studies have suggested that direct MMR-dependent processes might be required for the expression of HRS [28]. The enhanced low-dose cell killing was likely attributable to an inactivation of the early G2 arrest and inefficient HR repair. Here, we test our hypothesis that MMR proteins may be involved in HRS by examining the low-dose radiation response of isogenic cell lines proficient and deficient in the expression of MSH2.

2. Materials and methods

2.1. Cell lines

HEC59 and HEC59 + chr2 cells were kindly provided by Dr. Thomas A. Kunkel and Alan Clark (NIEHS, National Institutes of Health, Research Triangle Park, NC), in which chromosome 2 (containing wild-type MSH2) was introduced into MSH2-deficient HEC59 human endometrial carcinoma cells by microcell fusion [39], to create HEC59 + chr2 cells. Cells were maintained in advanced DMEM/F-12 containing 10% FBS and 1% penicillin/streptomycin (Invitrogen, Dublin, Ireland). Chromosome corrected cells were supplemented with geneticin selective antibiotic (G418) (Sigma Aldrich, Wicklow, Ireland) at 400 μg/mL.

2.2. Irradiation parameters

Two different dose rates were used to deliver doses from 0 to 2 Gy. The change in dose rate was necessary to ensure accurate dosimetry at the lowest doses. Asynchronously-growing cell monolayers in 25 cm^2 flasks were irradiated at room temperature at a dose rate of 0.75 Gy min^{-1} (0–0.8 Gy) or at 3.25 Gy min^{-1} (1–2 Gy) using an Xstrahl RS225 molecular research system (Gulmay Medical Ltd., UK).

2.3. Clonogenic survival assay

All survival assays were performed as previously described in detail [26]. A total of 500–6000 flow cytometry sorted cells (MoFlo XDP, Beckman Coulter, Dublin, Ireland) were plated per 25 cm^2 flask for low to high doses of radiation (0–2 Gy). After incubation at 37 °C for 14 days the resultant colonies were stained with crystal violet in 95% ethanol, and those consisting of greater than 50 cells were scored as representing surviving cells using ColCount™ (Oxford Optronix Ltd., Oxford, UK). The surviving fraction was calculated using the plating efficiency (PE) of irradiated cells/unirradiated cells.

2.4. Data analysis for survival assays

Surviving fractions measured at the doses tested were fitted with the induced-repair equation (Eq. 1) as described previously [25].

$$S = \exp\left\{-\alpha_r\left(1 + (\alpha_s/\alpha_r - 1)e^{-d/d_c}\right)d - \beta d^2\right\} \quad (1)$$

where d is dose, and α_s represents the low-dose value of α (derived from the response at very low doses), α_r is the value extrapolated from the conventional high-dose response, d_c is the 'transition' dose point at which the change from the very low-dose HRS to the IRR response occurs (i.e. when α_s–α_r is 63% complete) and β is a constant as in the LQ equation. All parameters were fitted simultaneously and estimates of uncertainty were expressed as likelihood confidence intervals. The presence of low dose hyper-radiosensitivity is deduced by values of α_s and α_r whose confidence limits do not overlap and a value of d_c (the change from low to high dose survival response) significantly greater than zero To study HRS, cells were treated with a radiation dose of 0.2 Gy, the upper limit for the estimate of d_c (Table 1).

2.5. Preparation of protein extracts

Cell pellets of treated and untreated samples were washed with cold PBS and subsequently lysed in cold RIPA lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol [DTT], 0.25% sodium deoxycholate, 0.1% NP-40) (Santa Cruz Biotech., Santa Cruz, CA, USA.) containing 1 mM phenylmethysulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, and protease inhibitors cocktail (Roche, Indianapolis, IN). Cell lysis was performed on ice for 30 min. Clear protein extracts were obtained by centrifugation for 30 min at 4 °C.

2.6. Western blot analysis

The method used was previously described [32]. Protein (50 μg) from cell extracts was separated in 6%/15% SDS polyacrylamide gels and blotted onto a PVDF transfer membrane for 1 h. Membranes were blocked for 1 h at room temperature in 5% (wt/vol) fat-free milk powder in PBS containing 0.1% Tween 20, incubated overnight with the primary antibody (1:100–1:1000 dilution), washed three times with 0.1% Tween in PBS, and incubated for 1 h with a horseradish peroxidase-coupled secondary antibody 1:2000. The following primary antibodies were used: MLH1 (C-20), MSH2 (N-20), and goat anti-rabbit IgG-HRP (Santa Cruz Biotech.); and α/ß Tubulin, actin, Chk1, Chk2, phospho-Chk1(ser296), phospho-Chk2(Thr68) (Cell Signaling Technology, Wicklow, Ireland). After final washing with 0.1% Tween 20 in PBS (3 × 10 min each) blots were developed using SuperSignal chemiluminescent substrate (Pierce, Rockford, IL, USA). HeLa nuclear extract was used as a positive control for MMR protein expression.

2.7. High content screening

Cells were grown, treated, fixed and stained directly in 384 multiwell plates (Matrix Technologies, Cheshire, UK). Cells were fixed in 4% paraformaldehyde (Pierce Technologies, Dublin, Ireland) for 15 min, washed in PBS for 3 × 10 min, permeabilised with ice-cold 100% methanol for 10 min in a −20 °C freezer, and blocked in 5% normal goat serum (Santa Cruz Biotech. Santa Cruz, CA, USA) containing 0.3% Triton X-100 for 60 min at room temperature. The plates were incubated with anti-phospho-histone H3(ser28)(1:600 dilution)(Santa Cruz Biotech.), anti-MRE11(1:100)(EMD Biosciences, Nottingham, UK), anti-phospho histone H2AX(-ser139)(1:50) (Cell Signalling, Wicklow, Ireland), or anti-RAD51(1:50) (Abcam, Dublin, Ireland), overnight at 4 °C, washed in PBS for 3 × 10 min, and incubated with Alexa Fluor 488-conjugated goat anti rabbit secondary antibody (Invitrogen, Dublin, Ireland) (1:1000) containing Hoechst 33342 (Sigma Aldrich, Wicklow, Ireland), for 1 h at room temperature. Cells were washed in PBS, 3 × 10 min.

2.8. Automated image capture and analysis

The IN Cell Analyzer 1000 automated fluorescent imaging system (GE Healthcare, Piscataway, NJ) was used for automated image acquisition. Images were acquired with either a 10× or 20× objective using 340/40-nm and 480/40-nm excitation filters, a Q505LP dichroic mirror and 460/40-nm and 535/50-nm HQ emission filters. To score targets of interest in a high-content throughput, we used the Multi-Target Analysis (MTAs) algorithm (GE Healthcare, Investigator v3.5) to identify individual cells and mitotic cells (phospho-H3 positive). The algorithm chosen was validated using a mitotic spindle inhibitor as a positive control. The Dual-target Analysis algorithm was used to identify individual cells and MRE11 foci in these cells. The nucleus was segmented via a top-hat method (30 μm^2 minimum area). Only nuclei with >5 foci (MRE11, RAD51) or >10 foci (H2AX) were counted as positive. At least 8 fields were analysed in each well, with a 10x objective (phospho-H3) or 15 fields in each well with a 20× objective (MRE11, H2AX, RAD51), corresponding to at least 1600 cells counted. Experiments with secondary antibodies alone were performed to verify the specificity of the signals.

2.9. Determination of mitotic ratio

The mitotic ratio was determined by calculating the ratio of irradiated vs unirradiated cells that stained positive for phospho-H3 in matched cell cultures.

2.10. Statistical analysis

All experiments were performed in triplicate unless otherwise stated. Unpaired *t*-tests were used to compare means. A *p*-value of <0.05 was considered statistically significant.

3. Results

3.1. MSH2 confers sensitivity to low dose radiation

Western blotting confirmed the differential MSH2 expression status of the two isogenic cell lines (Fig. 1A). Addition of chromo-