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Activity of ribonucleotide reductase helps determine how cells repair DNA double strand breaks

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ABSTRACT

Mammalian cells can choose either nonhomologous end joining (NHEJ) or homologous recombination (HR) for repair of chromosome breaks. Of these two pathways, HR alone requires extensive DNA synthesis and thus abundant synthesis precursors (dNTPs). We address here if this differing requirement for dNTPs helps determine how cells choose a repair pathway. Cellular dNTP pools are regulated primarily by changes in ribonucleotide reductase activity. We show that an inhibitor of ribonucleotide reductase (hydroxyurea) hypersensitizes NHEJ-deficient cells, but not wild type or HR-deficient cells, to chromosome breaks introduced by ionizing radiation. Hydroxyurea additionally reduces the frequency of irradiated cells with a marker for an early step in HR, Rad51 foci, consistent with reduced initiation of HR under these conditions. Conversely, promotion of ribonucleotide reductase activity protects NHEJ-deficient cells from ionizing radiation. Importantly, promotion of ribonucleotide reductase activity also increases usage of HR in cells proficient in both NHEJ and HR at a targeted chromosome break. Activity of ribonucleotide reductase is thus an important factor in determining how mammalian cells repair broken chromosomes. This may explain in part why G1/G0 cells, which have reduced ribonucleotide reductase activity, rely more on NHEJ for DSB repair.

1. Introduction

Efficient and accurate repair of DNA double strand breaks (DSBs) is essential for cell survival. Eukaryotic cells employ two major pathways for DSB repair: nonhomologous end joining (NHEJ) and homologous recombination (HR) (reviewed in [1]). NHEJ religates broken ends with minimal or no requirement for DNA synthesis, and is active during the whole cell cycle [2–6]. In contrast, HR has extensive requirements for DNA synthesis and is primarily employed for DSB repair in S and G2 phases [3–6]. A key step in HR, and the point where cells commit to repair by HR over repair by NHEJ [6,7], involves resection of 100 s to 1000 s of nucleotides from 5′-ends to produce long single stranded 3′-overhangs. These 3′-overhangs then invade the sister chromatid or homologous chromosome and serve as primers for re-synthesis of the previously degraded sequence around the break (reviewed in [8]). HR's need for extensive DNA synthesis suggests it will be much more dependent than NHEJ on the presence of sufficient dNTPs.

Availability of dNTPs is primarily reliant on the *de novo* pathway and specifically activity of ribonucleotide reductase (RNR). RNR activity has been additionally linked to the cellular capacity to survive DNA damage [9,10]. RNR possesses a large subunit (Rl) and one of two possible small subunits (R2 or p53R2) [11]. RNR activity is regulated over the cell cycle by limiting transcription of the primary version of the small subunit, R2, to S and G2 [12], as well as destruction of this protein in M phase [13]. As a consequence, RNR activity rises in early S, and falls after G2 – a fluctuation that correlates well with the extent cells perform HR.

Cells nevertheless retain some ability to generate nucleotide pools *de novo* in Gl by using a complex of Rl and the alternative version of the small subunit, p53R2 [14]. p53R2 is expressed at low levels throughout the cell cycle [15], but expression can be further augmented after DNA damage through a p53-dependent mechanism [16]. p53R2 protein is additionally stabilized after DNA damage through an ataxia telangiectasia mutated (ATM) dependent mechanism [17]. This up-regulation and stabilization after DNA damage is consistent with a specific role for p53R2 in providing nucleotides for DNA repair [14].

Here we address whether manipulation of cellular capacity to generate dNTPs *de novo* by RNR has an impact on whether cells repair chromosome breaks by HR or by NHEJ. We show that treatment with hydroxyurea (HU), which inhibits RNR activity,

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suppresses HR; conversely, stimulation of nucleotide synthesis promotes HR. We conclude that *de novo* nucleotide production is an important determinant of repair pathway choice.

2. Materials and methods

2.1. Cell culture and colony formation assays

All cell lines were grown at 37 °C and 5% CO_2 in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), l× MEM non-essential amino acids (Gibco), penicillin, and streptomycin. The Brca2-/-" cell line (V-C8) and the matching parental line (V79) was the kind gift of Dr. M.Z. Zdzienicka. Cells were synchronized essentially as previously described [4], except that cells were grown in presence of 5 mM *N*-acetyl-cysteine (Sigma) and 10 mM HEPES (Gibco) for synchronization in G1/G0. For enrichment in G1/G0, 8×10^4 cells/cm^2 were plated out and grown to confluency during 3 days. For enrichment in S phase, G1/G0 cells were replated at a density of 4×10^4/cm^2, incubated for 16 h in the presence of aphidicolin (1 μg/ml, Sigma), and released by medium change. 3 h after release (3.5 h for HR-deficient cell line irslSF), the majority of the cells reached mid-S phase. Synchronization was verified by analysis of cell cycle profiles by flow cytometry (Dako Cyan ADP) after propidium iodide staining (Roche). Only experiments with at least 80% pure populations were analyzed (e.g. Supplemental Fig. 1).

Xrs6 was complemented by generating a subclone that stably integrated a cDNA containing the *C. griseus* gene encoding Ku80 (the kind gift of Dr. D.B. Roth) and that was grown in presence of 400 μg/ml Geneticin (Invitrogen). In order to measure repair by homologous recombination, pDR-GFP [18] (the kind gift of Dr. M. Jasin) was stably integrated into the Chinese hamster ovary (CHO) cell line K1, resulting in the cell line K-DR, which was grown in presence of 10 μg/ml puromycin (Sigma). The murine gene encoding p53R2 was inserted into pcDNA6/myc-His A (Invitrogen) and stably integrated into the CHO cell lines K1 and Xrs6, resulting in lines K + P and X + P. p53R2 overexpressing lines were grown in presence of 10 μg/ml blasticidin (Invitrogen). Expression of myc-tagged p53R2 was verified by Western blot with the monoclonal mouse antibody 9B11 (Cell Signaling). An actin-specific polyclonal rabbit antibody (A2066; Sigma) was used for the loading control.

For colony formation assays, synchronized cells were plated out in presence or absence of 0.2 mM hydroxyurea or 1 μg/ml aphidicolin (both Sigma). After 1 h, cells were irradiated with 1 Gray (Gy) in a Gammacell 40 irradiator (^{137}Cs). 7 h after irradiation cells were washed with l× phosphate buffered saline (PBS) and provided with fresh medium without drug. The number of colonies was assessed 7 days later by Comassie staining (50% methanol, 5% Comassie) and counted using ImageJ (NIH) as software. All experiments were repeated at least 3 times, and the mean and standard error of the mean for each experiment were calculated with Prism 4.0c (Graphpad).

2.2. Repair substrate assay

1×10^6 exponentially growing wild type CHO cells containing the recombination substrate DR-GFP, K-DR, were transfected with 2 μg plasmid DNA (Amaxa, Kit T (VCA-1002), program H-014): empty vector alone (pcDNA6/LacZ-myc) in combination with expression vectors for p53R2 or p53R2–Y138V, a catalytic mutant, respectively. In parallel reactions, expression vectors for I-Scel and p53R2 or p53R2–Y138V, respectively, were delivered by electroporation.

GFP expression was analyzed by flow cytometry 48 h later. All experiments were repeated 3 times and means as well as standard errors of the mean were calculated with Prism 4.0c (Graphpad).

2.3. Immunofluorescence

G1/G0 or S phase enriched cells were seeded onto collagen-coated coverslips (Becton-Dickinson) in medium containing 0.2 mM hydroxyurea, 1 μg/ml aphidicolin, or no drug. Cells were irradiated 1 h later with 8 Gy. Cells were then fixed with 4% buffered paraformaldehyde in PBS at indicated times; no apoptotic cells could be detected up to 10 h after irradiation (Supplemental Fig. 2). Fixed cells were then permeabilized with 0.2% Triton X-100 for 3 min and analyzed with primary antibodies (Santa Cruz; catalogue numbers and dilutions noted for each antibody in parenthesis) against R2 (sc-10848; 1:200), Rad51 (sc-8349; 1:150), and Cyclin A (sc-751; 1:100) and secondary antibodies Alexa Fluor 488 conjugated donkey anti-goat (Molecular Probes; 1:1000) or Cy3 conjugated donkey anti-rabbit (Jackson ImmunoResearch; 1:1000). After three washing steps with PBS, whereof the first contained DAPI (5 μg/ml), coverslips were mounted onto glass slides with Fluorescent Mounting Medium (DakoCytomation). Specimens were analyzed in an AxioScope II (Zeiss) using a 40× objective and Openlab software. At least 100 cells were analyzed for each condition and experiments were repeated at least three times. Cells with >3 Rad51 foci were considered to be focus-positive. The mean and standard error of the mean was calculated with Prism 4.0c as software.

3. Results

3.1. Nucleotide synthesis influences survival after damage

We first tested whether inhibition of nucleotide synthesis impairs cell survival after ionizing radiation (IR) in a pattern consistent with HR's requirement for extensive DNA synthesis. For this analysis we varied the ability of cells to use the two pathways by making use of well-established Chinese hamster cell lines with mutations in various genes essential for efficient HR or NHEJ.

We manipulated the cellular ability to synthesize dNTPs by treating cells with hydroxyurea, which specifically blocks production of nucleotides *de novo* through inhibition of the enzyme ribonucleotide reductase [11,16]. In order to focus on the impact of HU on repair of radiation-induced breaks, we targeted G1/G0 enriched cells, so that HU alone could not introduce damage (e.g. during replication). We also limited both the amounts of HU used (200 μM; 5–10-fold less than used in most protocols) as well as the contact time to the hour immediately prior to irradiation and the additional 7 h following. Cells were then washed extensively and returned to normal growth conditions. The frequency of cells that survive this treatment was determined by assessing their capacity to form colonies relative to untreated controls (Table 1). To further summarize this data and focus on the impact of genotype, we then additionally compared surviving fractions for each deficient cell line to its parental line (Fig. 1)

Gl/G0 enriched cells deficient in HR (irslSF and V-C8) [19,20] were not significantly sensitive to 1 Gy of IR (Table 1A, Fig. 1A), relative to matched wild type controls (AA8 and V79, respectively). This result is consistent with prior studies indicating that in Gl/G0, IR-induced breaks are primarily repaired by NHEJ [3–5]. However, we were able to detect significant radiosensitivity in G1/G0 enriched V-C8 cells at higher doses (5 Gy; data not shown). Critically, our data indicated neither wild type nor HR-deficient cells were made significantly more radiosensitive at either dose when also treated with HU (Table 1A; Fig. 1A). We conclude that blocking the ability to generate dNTPs *de novo* does not significantly impact how well NHEJ proficient, G1/G0 enriched cells repair IR-generated DSBs, consistent with our predictions.

In contrast, HU treatment significantly increased the radiation sensitivity of G1/G0 enriched cells deficient in NHEJ (Xrs6 and XR-1 [21,22]; Table 1A, Fig. 1A). Survival of NHEJ-deficient cells was