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Mechanism of cell killing after ionizing radiation by a dominant negative DNA polymerase beta

Sari Neijenhuis[a], Manon Verwijs-Janssen[a], Ulla Kasten-Pisula[b], Gaby Rumping[a], Kerstin Borgmann[b], Ekkehard Dikomey[b], Adrian C. Begg[a,*], Conchita Vens[a,*]

[a] Department of Experimental Therapy, The Netherlands Cancer Institute, Amsterdam, The Netherlands
[b] Laboratory of Radiobiology and Experimental Radio-oncology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

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ABSTRACT

Several types of DNA lesion are induced after ionizing irradiation (IR) of which double strand breaks (DSBs) are expected to be the most lethal, although single strand breaks (SSBs) and DNA base damages are quantitatively in the majority. Proteins of the base excision repair (BER) pathway repair these numerous lesions. DNA polymerase beta has been identified as a crucial enzyme in BER and SSB repair (SSBR). We showed previously that inhibition of BER/SSBR by expressing a dominant negative DNA polymerase beta (polβDN) resulted in radiosensitization. We hypothesized increased kill to result from DSBs arising from unrepaired SSBs and BER intermediates. We find here higher numbers of IR-induced chromosome aberrations in polβDN expressing cells, confirming increased DSB formation. These aberrations did not result from changes in DSB induction or repair of the majority of lesions. SSB conversion to DSBs has been shown to occur during replication. We observed an increased induction of chromatid aberrations in polβDN expressing cells after IR, suggesting such a replication-dependence of secondary DSB formation. We also observed a pronounced increase of chromosomal deletions, the most likely cause of the increased kill. After H_2O_2 treatment, polβDN expression only resulted in increased chromatid (not chromosome) aberrations. Together with the lack of sensitization to H_2O_2, these data further suggest that the additional secondarily induced lethal DSBs resulted from repair attempts at complex clustered damage sites, unique to IR. Surprisingly, the polβDN induced increase in residual γH2AX foci number was unexpectedly low compared with the radiosensitization or induction of aberrations. Our data thus demonstrate the formation of secondary DSBs that are reflected by increased kill but not by residual γH2AX foci, indicating an escape from γH2AX-mediated DSB repair. In addition, we show that in the polβDN expressing cells secondary DSBs arise in a radiation-specific and partly replication-dependent manner.

1. Introduction

Several types of DNA lesion are induced after ionizing irradiation (IR) of which double strand breaks (DSBs) and crosslinks are expected to be the most lethal. Single strand breaks (SSB) and DNA base damages on the other hand are produced with considerably higher yields. Some of these lesions when unrepaired will cause replication fork stalling and mutations. In addition, unrepaired SSBs or base excision repair (BER) intermediates could be converted in DSBs. Repair of and/or response to these lesions would therefore be expected to determine the survival of an irradiated cell.

BER is the major pathway for repair of base lesions such as the products of deamination, oxidation and alkylation. A major part of DNA damages observed after IR is base lesions caused by reactive oxygen species, such as apurinic/apyrimidinic (AP) sites, formaminopyrimidine, thymine glycol and 8-OxoG. In BER, most of the damaged bases in the DNA will be removed by glycosylases, resulting in abasic sites substrates of AP endonucleases hydrolyzing the phophodiester bond and generating a SSB. In the short patch pathway of BER, DNA polymerase β (polβ) is thought to insert a single nucleotide in the repair gap, thereby removing the 5′-deoxyribose phosphate (dRP) left behind by the endonuclease. An alternative long patch pathway has been described that implicates polβ or polδ/ε or both in gap synthesis [1]. All glycosylases directed against oxidized base damages are bifunctional, such that they are capable of incising the strand 3′ of the lesion and are therefore thought to exclusively initiate polβ-dependent

Abbreviations: IR, ionizing radiation; DSB, double strand break; SSB, single strand break; BER, base excision repair; SSBR, single strand break repair; polβDN, dominant negative DNA polymerase β; AP, apurinic/apyrimidinic; polβ, DNA polymerase beta; dRP, deoxyribose phosphate; NHEJ, non-homologous end joining; CFGE, constant-field gel electrophoresis; FDR, fraction DNA released into the gel; DEF, dose enhancement factor.

* Corresponding authors at: Division of Experimental Therapy, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.
Tel.: +31 20512 2036; fax: +31 20512 2050.
E-mail addresses: a.begg@nki.nl (A.C. Begg), c.vens@nki.nl (C. Vens).

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short patch repair. Barnes and coworkers [2] showed that cells deficient in UNG and SMUG glycosylases were more sensitive to ionizing radiation, demonstrating the cytotoxicity of base damages induced by ionizing radiation. In addition to its role in BER, polβ is expected to be involved in single strand break repair (SSBR) complexed by XRCC1 and PNK, as demonstrated by *in vitro* assays [3].

Here we further investigated the role of polβ in repair of ionizing radiation damage, a subject of potential clinical relevance, since DNA polymerase beta has been shown to be altered in around 30% of tumors, suggesting a role in tumor formation [4]. These changes include overexpression, truncation, and mutations modulating the activity of the enzyme and are postulated to influence DNA repair and therefore possibly tumorigenesis [5–7].

Despite polβ's demonstrated role in BER and SSBR, polβ-deficient cells do not show a radiosensitive phenotype [8,9], a surprising result considering the large amount of base damages and SSBs induced after IR, thus suggesting strong redundancy within the pathways thought to counteract the polβ repair deficiency [10]. The long patch pathway, involving polδ/ε, is among the candidates. We have recently shown that these backup pathways are predominantly acting in replicating cells, since polβ-deficient cells from confluent cultures deprived of S-phase cells or in G1 phase of the cell cycle are hypersensitive to ionizing radiation [11,12]. Interestingly, expression of a dominant negative DNA polβ (polβDN) in a polβ wild-type background also radiosensitized human tumour cells [13]. This dominant negative comprises the DNA binding domain of DNA polymerase β and is lacking the C-terminal polymerase activity. The polβDN is therefore thought to compete with the wild-type protein for binding sites on the DNA and within protein complexes, but lacks enzymatic activity, thus inhibiting repair of the lesion at the site. We confirmed BER/SSBR specificity of the polβDN by its XRCC1-dependence of radiosensitization [14]. Inhibition of BER/SSBR has been further suggested by data using *in vitro* assays [15]. Sensitization of polβ-deficient cells by the polβDN indicated additional persistent inhibition of redundant polβ-independent repair pathways involved in ionizing radiation damage [14]. We hypothesized that inhibition of BER/SSBR after ionizing radiation will lead to an increase of BER intermediates. These intermediates would in turn, either due to replication fork collapse [16] or during repair attempts at non-DSB clustered lesion sites, result in additional secondary DSBs [17,18], therefore leading to increased cell kill. Alternatively, increased kill could result from changes in repair efficiencies that result in increased numbers of unrepaired primary DSBs. Here we analysed DSB formation and repair in polβDN expressing cells further.

2. Materials and methods

2.1. Cells and cell culture

Human A549 (lung adenocarcinoma) cells were transduced with a retroviral construct carrying a HIS-tagged truncated version of DNA polymerase β (polβDN) and single cell clones were established. A549-polβDN-IIG10, expressing the DNA polymerase beta dominant negative (further referred to as A549-polβDN), and A549-LZRS-IIE2, transduced with the empty vector (referred to as A549-LZRS) were selected for further analysis. Both cell lines have been characterized extensively with respect to radiosensitivity [13]. Cells were grown as monolayers in DMEM (Gibco) supplemented with 10% foetal calf serum at 37 °C and 5% CO_2.

2.2. Radiation sensitivity and colony formation assay

Radiation experiments with proliferating cells were performed as previously described [14]. For radiation experiments in confluence, cells were plated, cultured for 4 days to reach confluence and maintained under this condition for another 3 days. Cells were then irradiated using a ^{137}Cs irradiation unit with a dose rate of 0.66 Gy/min at room temperature. After an additional culture period of 24 h to allow repair, cells were plated for colony formation. Preliminary time course experiments were performed to establish changes in cell cycle phase distribution during confluent culture and to further define the optimal time point of radiation. For each experiment, independent identical flasks were prepared to determine S-phase content of the cells by BrdUrd labelling and flow cytometry at time of irradiation.

2.3. Survival curve modelling

In order to assess if a particular subpopulation was sensitized by the dominant negative, survival curves from the colony assays were fit to a simple linear-quadratic model. The total population was assumed to comprise two populations, an S-phase population and a non-S-phase population (G1, G2, M), each with different cell survival parameters. Survival of each subpopulation was assumed to conform to the equation: $-\ln \mathrm{SF} = \alpha D + \beta D^2$, where SF is the surviving fraction, D is dose and α and β are the polynomial coefficients representing the dose and dose squared terms, respectively. Survival for a subpopulation was multiplied by the fraction of cells in the population, and the survivals for the two populations then summed to obtain a value of the total population. The fractions of cells in S for each population in each growth stage were taken from the BrdU incorporation data. Data for both exponential and confluent cells were analysed, and for both LZRS and polβDN cells. Goodness of fit was tested by the root-mean-square difference between the curve fit and the data points, summed over all conditions (growth stage, genotype). Graphs shown in Fig. 3C apply α values of 0.195 and 0.95 for LZRS and polβDN S-phase cells, respectively and 0.425 for the remainder subpopulation (β remains 0.01 in both cell lines and fractions). In Fig. 3D survival was calculated using the same α and β values as in Fig. 3C but considering a reduction of the S-phase fraction from 45 and 55% to 14 and 17% for LZRS and polβDN, respectively. Cell cycle phase distribution values were experimentally determined by BrdUrd labelling and flow cytometry.

2.4. Fluorescence in situ hybridisation (FISH)

Whole chromosome-specific probes were prepared from plasmid libraries from Dr. J. Gray (University of San Francisco, CA). Probes were amplified by degenerate oligonucleotide-primed-polymerase chain reaction (DOP-PCR) and labelled in a second reaction with biotin labelled-dUTP [19]. Slides with metaphase spreads were hybridised with specific probes to the human chromosome 1, 2, and 4 after RNase/pepsin treatment as described previously [20]. The targeted chromosomes were detected by fluorescein-isothiocyanate (FITC) labelled streptavidin with an additional signal amplification step using biotinylated goat anti-avidin.

2.5. Chromosome and chromatid type aberration analysis

Translocations and acentric fragments per cell were determined after fluorescence *in situ* hybridisation with whole chromosome probes on metaphase chromosomes of cells in their first post-irradiation mitosis. Asynchronous exponentially growing cells were irradiated using a ^{137}Cs source at a dose rate of approximately 0.9 Gy/min. After approximately one cell cycle (22 h), metaphase cells were collected by overnight colcemid treatment. Colcemid (0.2 μg/ml, Gibco) was added to the culture medium for an additional 16 h and mitotic cells were then shaken off and collected by