The role of DNA polymerase \( \beta \) in determining sensitivity to ionizing radiation in human tumor cells

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ABSTRACT

Lethal lesions after ionizing radiation are thought to be mainly unrepaired or misrepaired DNA double-strand breaks, ultimately leading to lethal chromosome aberrations. However, studies with radioprotectors and repair inhibitors indicate that single-strand breaks, damaged nucleotides or abasic sites can also influence cell survival. This paper reports on studies to further define the role of base damage and base excision repair on the radiosensitivity of human cells. We retrovirally transduced human tumor cells with a dominant negative form of DNA polymerase \( \beta \), comprising the 14 kDa DNA-binding domain of DNA polymerase \( \beta \) but lacking polymerase function. Radiosensitization of two human carcinoma cell lines, A549 and SQD9, was observed, achieving dose enhancement factors of 1.5–1.7. Sensitization was dependent on expression level of the achievable dose enhancement factors of 1.5–1.7.

INTRODUCTION

Damage to DNA is considered to be the main event resulting in cell death or mutations after ionizing radiation. Ionizing radiation simultaneously produces different types of lesions, such as base modifications and loss, DNA–DNA intrastrand and interstrand crosslinks, DNA–protein crosslinks, single-strand breaks (SSBs) and double-strand breaks (DSBs). The pivotal role of DSB repair after exposure to ionizing radiation by non-homologous end joining (NHEJ) and recombinational repair has been well documented. Numerous examples of rodent cell lines or mice showing a defect in these repair pathways have been reported, all exhibiting a highly radiosensitive phenotype. This includes cells displaying a deficiency in Ku80, Ku70 and DNA-PKcs, proteins crucial to NHEJ (1–4).

Other lesions produced by ionizing radiation, such as base modifications, apurinic/apyrimidinic (AP) sites and SSBs, produced either directly or secondarily by oxidative attack, are thought to be repaired by the base excision repair (BER) pathway (5). Studies on mice or cell lines deficient in proteins/ enzymes involved in BER have failed to show the same clear increase in radiosensitivity as seen in DSBR repair-deficient cells. DNA glycosylases specific for different types of base damage remove the modified bases and generate abasic sites. There are no reports on radiosensitivity in DNA glycosylase mutant cells. Knocking out individual glycosylases leads to a mild or no phenotype, for example with respect to alkylating agent sensitivity, probably explained by partial redundancy between the different glycosylases (6–8).

During BER, AP endonucleases incise the DNA backbone at abasic sites resulting from glycosylase action or after spontaneous hydrolysis. One study reported increased sensitivity to \( \gamma \)-radiation in AP endonuclease-deficient blastocysts (9), indicating its role in processing radiation damage. When BER is initiated from a SSB, PARP and XRCC1 recognize the break and polynucleotide kinase (PNK) trims the ends for repair synthesis (10). Both PARP and XRCC1 mutant cells have been shown to display increased sensitivity to ionizing radiation (11–13). In contrast, cells deficient in DNA polymerase \( \beta \), an enzyme considered to play an essential role in the correct restitution of the DNA after base excision, are surprisingly not more radiosensitive than their wild-type counterparts (14,15). DNA polymerase \( \beta \) fills the gap created by the upstream BER enzymes, using the opposite complementary base as a template followed by phosphodiesterase removal of the remaining sugar–phosphate moiety (16). It has also been shown to repair SSBs \textit{in vitro} in a multiprotein complex with PNK, together with other proteins such as XRCC1 and ligase III (17).

The DNA polymerase \( \beta \) protein and enzymatic activity have been well characterized and its function and role in the repair of alkylating damage \textit{in vitro} and \textit{in vivo} has been described (16,18). This enzyme performs mainly a 1 nt gap filling reaction in mammalian cells, termed the ‘short patch’ repair pathway of BER (19). The minor long patch repair pathway involves...
polymerase δε and proliferating cell nuclear antigen (PCNA) (20,21) or DNA polymerase β (22). This pathway leads to a repair synthesis of 2–10 nt. Consistent with its role in BER, polymerase β-deficient cells show hypersensitivity to alkylating agents like methyl methanesulfonate (MMS) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNNG) (15,23).

Considering the critical enzymatic role of DNA polymerase β, the lack of a radiosensitive phenotype in knockouts is puzzling. Defining the role of BER in sensitivity to ionizing radiation has been difficult, however, since some deletions mutations would be lethal to the cell (e.g. polymerases δε) and the phenotype of others are affected by redundant pathways. Husain et al. previously described a fragment of DNA polymerase β that acts as a dominant negative inhibiting polymerase β in vitro, probably by its specific DNA binding properties (24). In order to clarify the role of BER in repair of DNA damage after irradiation, we introduced this 14 kDa fragment of DNA polymerase β into two different radio-resistant human tumor cells and tested the influence on radiosensitivity.

MATERIALS AND METHODS

Cell culture and cell lines

SQ20B cells isolated from human squamous cell carcinoma of the head and neck were obtained from J. B. Little (Harvard). SQD9 is a subclone of SQ20B and has been previously described (25). A549 human lung carcinoma cells were provided by the American Type Culture Collection. The amphotropic viral packaging cell line Phoenix-Ampho was obtained from A. Q. Bakker and H. Spits (Netherlands Cancer Institute) with permission of the G. P. Nolan laboratory (Stanford). All cells were grown as monolayers in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37°C in a 5% CO₂ humidified atmosphere. For growth assays, cells were plated at different initial concentrations on 6-well plates. Cell growth curves were determined by counting the cells daily until confluence. Doubling times were calculated from the exponential growth phase.

Construction of the polDN His-tagged protein-expressing LZRS vector

The LZRS vector has been previously described (26). The LZRS-MS-EGFP vector (a gift of J. Collard, Netherlands Cancer Institute) is a bicistronic retroviral vector expressing the gene of interest and the enhanced green fluorescent protein (EGFP) cassette linked via an IRES site under the control of the viral 3’-long terminal repeat (LTR) promoter (27). polDN was created by PCR amplification of the sequence corresponding to the 14 kDa N-terminal DNA-binding domain of DNA polymerase β. The plasmid pKSpolβ (obtained from B. Kaina, University of Mainz, Germany), a subclone of the pMTβpol clone (28), was used for this purpose. As 5’ primer, an oligonucleotide was synthesized containing a BamHI restriction site and the optimal Kozak sequence GCCACC. It included the polymerase β ATG start codon and the first 30 5’ nucleotides of the polymerase β cDNA sequence. As 3’ primer, an oligonucleotide containing a Has tag sequence and a stop codon, as well as a second restriction enzyme site for EcoRI, was synthesized. PCR was performed using the high fidelity Pwo DNA polymerase (Boehringer) and produced an ∼500 nt fragment that was isolated and purified by gel electrophoresis. The restriction enzyme sites at each end allowed ligation of the polDN PCR fragment into the LZRS-MS-EGFP vector. The resulting constructs were sequenced with the ABI Prism Big Dye Terminator kit (Perkin Elmer) and an error-free polDN-LZRS vector selected for transfecting cells (see below).

Transfection of the packaging cell line and transduction of human carcinoma cells

The transduction and transfection protocols followed in principal procedures described by the Nolan laboratory (www.stanford.edu/group/nolan/) (26). Briefly, Phoenix amphotropic retroviral packaging cells were transfected with LZRS or LZRS-polDN DNA (prepared with Endofree Maxi Prep; Qiagen) by a modified version of the calcium phosphate co-precipitation protocol (29). The viral supernatant was harvested 48 h post-transfection and used fresh on log phase A549 or SQD9 cells which were then grown to confluence. Using EGFP fluorescence marker expression, transduction efficiencies were determined by flow cytometry (FACSscan; Becton Dickinson). PolDN expression of transduced cells was verified by western blot analysis (see below). Cells with different levels of EGFP expression were sorted individually into 96-well plates with a FACSTAR Plus sorter (Becton Dickinson). Single cell clone lines were then established. EGFP-positive cells were also bulk sorted shortly after transduction (3–5 days) to produce a mixed cell population. EGFP and polDN expression levels were monitored at every passage for mixed cell populations and every second passage for single cell clones.

Irradiation and clonogenic survival assay

Cells were plated into 6-well plates with varying cell concentrations and irradiated after 24 h using a 137Cs irradiation unit with a dose rate of ∼1 Gy/min. Cells were allowed to grow for another 10–14 days before rinsing in phosphate-buffered saline (PBS), fixing in methanol and staining with 1% crystal violet, another 10–14 days before processing as mentioned above. Surviving colonies consisting of more than 50 cells were counted in 6–12 wells of 6-well plates and the survival of irradiated cells normalized to the plating efficiency of unirradiated controls. Data points on survival curves represent the mean surviving fraction of five or six independent experiments. In some experiments cells were irradiated when confluent when the fraction of G1/G0 and S cells were 80 and 10%, respectively, as determined by BrdU labeling and flow cytometry. After an incubation of 6 h at 37°C cells were then plated in 6-well plates and allowed to grow for another 10–14 days before processing as mentioned above.

UV irradiation

Cells were plated into 10 cm or 6-well plates. Before irradiation cells were washed with PBS and irradiated with UV light (Stratagene UV-C bulbs, 254 nm) at a dose rate of 25 J/m²/s. Cells were then grown in fresh medium for 10 days. After fixation and staining, colonies consisting of more than 50 cells were counted. Data points on survival curves represent the mean surviving fraction of three independent experiments.
Immunoblotting

Cell lysates were prepared in Horton buffer from logarithmic or confluent monolayer cells. An aliquot representing 0.5 × 10^6 cells was resolved by 14% SDS–PAGE and transferred to PVDF membrane using a semi-dry blot apparatus (Novablot; Pharmacia) as recommended by the manufacturer. Blots were incubated with either an 18S monoclonal anti-polerase β antibody (a generous gift from R. Prasad, NIH), which detected polerase β as well as the dominant negative 18 kDa form resulting from the His-tagged 14 kDa fragment, or with the BMG-His-1 monoclonal anti-His antibody (Boehringer). Immunoblots were carried out with secondary antibodies conjugated to alkaline phosphatase from Santa Cruz, detected by the NBT/BCIP color reaction. In some experiments the BM chemiluminescence kit (Boehringer) was used with an anti-mouse horseradish peroxidase-conjugated secondary antibody according to the manufacturer’s instructions.

Immunostaining

Cells were grown on CultureSlides (Falcon), fixed with 4% formaldehyde in PBS for 10 min, permeabilized with 1% SDS and incubated with blocking buffer containing BSA and saponin as described previously (30). Cells were then incubated with the monoclonal antibody to polerase β or with the monoclonal anti-His antibody in blocking buffer. After washing, appropriate species-specific, TRITC-conjugated secondary antibodies were applied as recommended by the manufacturer (Jackson Immunolab). Fluorescence was visualized by confocal microscopy.

Cytotoxicity assay

Cells were plated into 96-well plates and treated with MNNG after 1 day. Cultures were stopped by removing the medium after a further 6 (A549) or 8 (SQD9) days, before subconfluence was reached. Cells were then stained with the fluorescent nuclear dye CyQUANT GR (Molecular Probes) according to the manufacturer’s instructions. Fluorescence intensities were measured using a fluorescence microplate reader (Cytofluor). Values were normalized to the untreated controls. IC_{50} values were defined as the concentration of drug at which half the growth of the untreated controls was reached. IC_{50} values of four to five individual experiments were calculated.

Cell cycle phase determination

Logarithmically growing cells were pulse labeled with 1 µM BrdU by incubating them for 10 min at 37°C. Cells were then trypsinized, resuspended in 1 ml of PBS and fixed by adding the cell suspension to 5 ml of ice-cold 70% ethanol. Anti-BrdU staining was performed as described previously (31). Briefly, nuclei were isolated by pepsin treatment, subsequently incubated with 2 N HCl for 20 min at 37°C, neutralized with sodium borate and washed with PBS containing Tween-20. A mouse monoclonal anti-BrdU antibody (CLB) was used at a 1/1000 dilution for 1 h, followed by a FITC-conjugated goat anti-mouse IgG antibody (Sigma) at a 1/50 dilution for 30 min at room temperature. Cells were counterstained for DNA with propidium iodide. Samples were then measured using a FACScan flow cytometer (Becton Dickinson) and the data analyzed with the Cell Quest software package.

Mitotic shake-off experiment

A549 cell lines were synchronized by mitotic selection (32). Cells were seeded in T175 flasks at an appropriate density to obtain cultures at 50–80% confluence 4 days later. Flasks were slapped several times against the palm of the hand and the mitotic cells were then harvested by collecting the medium and washing the cells once with PBS. The cells were then counted and seeded into 6-well plates containing prewarmed medium and allowed to progress through the cell cycle under standard growth conditions. After 4 h, at which time most of the cells have progressed into G1, the cells were irradiated with 6 Gy. An aliquot of the mitotic cells was grown under identical conditions and labeled with 1 µM BrdU for 4 h to determine the cell cycle phase distribution. In parallel, standard colony assays were performed with exponentially growing, asynchronous cells irradiated to 6 Gy as controls.

RESULTS

DNA polymerase β dominant negative expression

A recombinant 14 kDa N-terminal fragment (residues 1–140) of polerase β has been shown to be capable of binding to both single- and double-stranded DNA but lacks DNA polymerase activity, thus inhibiting DNA polerase β in an in vitro assay (24). An equivalent His-tagged fragment was generated here by PCR and checked by sequencing, as described in Materials and Methods. This fragment was introduced into the human tumor cell lines SQD9 and A549 by retroviral transduction. The bicistronic retrovirus LZRS-MS-EGFP, encoding EGFP as the reporter gene together with the 14 kDa His-tagged fragment, was used to achieve high and stable expression levels of the polDN protein. Transduction efficiencies were high, up to 70% for A549 and 50% for SQD9 cells. EGFP expression can be rapidly monitored by flow cytometry and allowed efficient selection of transduced cells. Single cell sorting into individual wells of 96-well plates allowed development of single cell clones with different EGFP expression. PolDN expression levels of individual cell clones, determined by western blot analysis with polyclonal and monoclonal anti-polerase β antibodies, correlated well with EGFP expression levels determined by flow cytometry. Transduction with LZRS or polDN expression did not change expression of the endogenous wild-type DNA polerase β. Several clones with high and medium expression levels of polDN were selected for further analysis.

Nuclear localization of 14 kDa DNA polymerase β dominant negative

The nuclear localization signal of polerase β has not been described. To determine whether the 14 kDa N-terminal domain accumulates in the nucleus we performed immunofluorescence analysis using an anti-His monoclonal antibody. Confocal images (Fig. 1) revealed the presence of punctate nuclear staining in both polDN-transduced cell lines A549-polDN-II-G10 (ADN-IIG10) and SQD9-polDN-II-G3 (SQDN-IIG3) with the anti-HIS antibody, in contrast to the vector LZRS-transduced control cell lines. The intensity of staining was higher in the polDN-transduced single cell clones compared with the parental cell lines or controls when using a
monoclonal antibody against polymerase β. This was expected since the antibody recognizes the endogenous full-length protein as well as the polDN fragment.

**Dominant negative DNA polymerase β radiosensitizes squamous cell carcinoma cells**

In order to evaluate the role of base excision repair in determining radiosensitivity in tumor cell lines, we introduced the dominant negative form in human tumor cell lines having a radioresistant phenotype. The SQD9 cell line, a subclone derived from the well-known SQ20B human squamous carcinoma cell line (25), is radioresistant, with an SF2 (survival after 2 Gy) of 60% and a D10 (dose for 10% survival) of 8 Gy. Four polDN-transduced single cell clones and two corresponding LZRS empty vector control cell lines were selected according to their polDN expression levels. We observed that high levels of EGFP alone after retroviral transduction led to slightly increased survival (radioprotection) after irradiation in several independent experiments (data not shown). The LZRS empty vector control cell clones were therefore selected for comparable EGFP expression to their polDN counterparts.

As shown in Figure 2B, the polDN-expressing cell clones exhibited decreased cell survival after irradiation compared with the empty vector control. The extent of the radiosensitization was dependent on the amount of dominant negative expression, as determined by western blot analysis (Fig. 2D). The calculated average dose-effect factor (DEF) at 10% survival increased from 1.3 to 1.7 in the SQDN-IIG3 clone compared with the SQLz-IIE2 control. To determine if the observed differences resulted from non-specific clonal effects not connected to polDN expression (e.g. integration locus of the virus, other mutations), we examined the clonogenic survival in a second triplet of single cell clones derived from an independent transduction experiment. The same polDN dose-dependent sensitization pattern was observed in these clones, reaching an average DEF of 1.75 calculated at 10% survival for the highest polDN-expressing clone (Fig. 2A). Clonogenic assays performed by irradiating these cells in the plateau phase confirmed the radiosensitization effect observed with survival assays on log phase cells (DEF 1.5).

We conclude that expression of the 14 kDa N-terminal DNA polymerase β dominant negative protein significantly enhanced cell death after irradiation of SQD9 cells and postulate that this could be due to interference in BER.

**Radiosensitization of A549 human lung carcinoma cells**

To confirm the radiosensitizing effect, we selected a second human carcinoma line with a different genetic background. p53 has been demonstrated to interact directly with polymerase β and AP endonuclease, stabilizing the binding of DNA polymerase β to abasic DNA and markedly stimulating BER in vitro (33). The authors proposed a direct involvement of p53 as a facilitator of BER after induction through stress factors such as γ-rays and alkylating damage. The expression of the DNA polymerase β N-terminal domain could possibly interfere with this interaction. We addressed this issue by choosing cell lines with different p53 status but exhibiting a radioresistant phenotype. Analysis of p53 status by FASAY and PCR revealed expression of mutant p53 in SQD9 (34), whereas the A549 cell line has been reported to have wild-type p53 expression (35,36).

We transduced the A549 human lung carcinoma cells with the polDN retroviral vector and generated several single cell clones. One of these was selected (ADN-IIG10) that exhibited comparable expression levels of polDN to the SQD9 cell line SQDN-IIG3 (Fig. 2D). The LZRS control cell line (ALz-II-E2) showed slightly decreased cell death compared with the parental A549 cell line, as found previously with SQD9. This small effect can be seen in Figure 2C (dashed line versus closed circles). Two cell clones were therefore chosen in which EGFP expression levels (flow cytometry) of the polDN-expressing clone and the LZRS empty vector control cell clone were equal. PolDN expression was also found to render the A549 cells significantly more sensitive to irradiation (Fig. 2C). Average DEFs were 1.7 calculated at a survival of 10%. As both cell lines were radiosensitized by polDN to a similar extent, this suggests that the inhibitory effect on repair was not dependent on p53 status or tissue of origin.
Radiosensitization of mixed cell populations

Although several single cell clones were analyzed and showed increased cell death after irradiation, we wanted to further exclude possible artifactual clonal effects by analyzing freshly transduced cell populations without any clonal selection (which we term mixed cell populations). Two days after transduction with empty or polDN-containing retroviruses, SQD9 and A549 tumor cells were sorted for EGFP positivity. Irradiation experiments were then performed as soon as possible. The use of low passage numbers after sorting were then critical, since EGFP and polDN expression levels decreased significantly with every passage, with concomitant decreases in radiosensitization. The clonogenic survival curves shown in Figure 3 demonstrate a radiosensitizing effect of the polDN protein in both SQD9 and A549 cells compared with their empty vector controls. The average polDN protein expression levels in the mixed cell population were lower than in the single cell clones, probably explaining the lower DEFs of around 1.2. The mixed cell population data do, however, confirm the radiosensitization effects observed in the single cell clones.

The effect of cell growth stage and cell cycle phase

Changes in cell growth or cell cycle phase distribution can result in differences in radiosensitivity. It is known that cells in G1 are generally more sensitive to irradiation than cells in S. An increase in the relative number of G1 cells by DN expression, due to an unknown cause, could then lead to radiosensitization; an essentially trivial explanation. We therefore studied the cell growth rate and the cell cycle phase distribution of A549 and SQD9 single cell clones. As shown in Table 1, there were no significant differences in the doubling times of polDN-expressing cells versus LZRS empty vector EGFP-expressing cells. Relative numbers of cells in G1/G0, S and G2 were determined by BrdU labeling and flow cytometry of exponentially
Sensitivity to UV

The cells expressing a dominant negative to polymerase β are not expected to be more sensitive to UV irradiation. DNA damage resulting from UVC irradiation is primarily pyrimidine dimers that are repaired by the nucleotide excision repair (NER) pathway, which does not involve polymerase β. No significant difference in UVC sensitivity could be detected between ALz-IIE2 and SQDN-IIG10 cells (Fig. 4). A slight increase in sensitivity was observed in comparison with the parental A549 cells (dashed line in Fig. 4), although the effect was small. These results confirm the expectation that the presumed dominant negative of polymerase β does not interfere with NER. Moreover, these data indicate, like the cell growth studies, that dominant negative expression does not globally affect viability in such a way that the response to any genotoxic insult is increased.

Lack of radiosensitization in G1-enriched populations

The two step pattern seen in the MNNG cytotoxicity assays, and data on irradiation experiments carried out at different growth stages, suggested a potential differential effect on mouse embryonic fibroblasts deficient in DNA polymerase β have been shown to be highly sensitive to alkylating drugs like MNNG. This drug induces lesions repaired primarily by BER, thus confirming the role of polymerase β in this repair pathway (15,23). The polymerase β dominant negative is thought to bind to glycosylase- and AP-endonuclease-treated DNA sites, thus blocking wild-type polymerase β access. If so, we would expect an increased sensitivity of polDN-expressing cells to MNNG. We therefore analyzed the ADN-IIG10 and SQDN-IIG3 clones in a 96-well cell growth assay for MNNG sensitivity and compared this with their corresponding vector controls ALz-IIE2 and SQLz-IIE2. A typical dose-response curve is shown in Figure 5A. All curves showed a characteristic two step pattern, with a subpopulation of cells showing the same sensitivity as the LZRS controls. The IC₅₀ values of four independent experiments were calculated (Fig. 5B). A significant increase in MNNG sensitivity was observed in the sensitized subpopulation in both cell carcinoma lines transduced with the polymerase β dominant negative, suggesting inhibition of BER.

### Table 1. Cell cycle phase distribution and cell growth

<table>
<thead>
<tr>
<th></th>
<th>G&lt;sub&gt;1&lt;/sub&gt;/G₀ (%)</th>
<th>S (%)</th>
<th>G₂ (%)</th>
<th>Doubling time (h)</th>
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<tbody>
<tr>
<td>SQLz-IIE2</td>
<td>30 ± 16</td>
<td>55 ± 15</td>
<td>13 ± 2</td>
<td>44.4</td>
</tr>
<tr>
<td>SQDN-IIG3</td>
<td>19 ± 14</td>
<td>68 ± 18</td>
<td>12 ± 7</td>
<td>43.2</td>
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<tr>
<td>ALz-IIE2</td>
<td>32 ± 3</td>
<td>57 ± 2</td>
<td>10 ± 3</td>
<td>21.8</td>
</tr>
<tr>
<td>ADN-IIG10</td>
<td>33 ± 8</td>
<td>50 ± 6</td>
<td>11 ± 3</td>
<td>22.3</td>
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G<sub>1</sub>/G₀, S and G₂ cell proportions were determined by BrdU labeling and flow cytometry of exponentially growing cells. The mean of the percentages of cell cycle phases of five (A549) or three (SQD9) independent experiments are shown with SD. Doubling times were determined as described in Materials and Methods.

Figure 4. Sensitivity to UV. UVC radiation survival curves of A549 parental cells and the polDN-expressing ADN-IIG10 and the empty vector control ALz-IIE2 cell clones. Each point represents the mean of three independent experiments; error bars, SD.

Figure 5. Sensitivity to MNNG. Cells were exposed to MNNG 24 h after seeding in 96-well plates. Cell growth was determined by a fluorescence assay after 6 or 8 days. (A) Representative growth response curves of two experiments are shown for the SQDN-IIG10 clone. For comparison, the mean ± SD of five individual experiments for SQLz-IIE2 vector controls are shown. (B) IC₅₀ values (mean ± SD) of five individual experiments for A549 (left) and SQD9 cells (right) transduced with polDN (DN) or empty vector (Lz). For the polDN-expressing clones, IC₅₀ values of the sensitized subpopulation are given. DN-expressing cells are 7–30 times more sensitive to MNNG than vector controls.

Sensitivity to MNNG

Mouse embryonic fibroblasts deficient in DNA polymerase β have been shown to be highly sensitive to alkylating drugs like MNNG. This drug induces lesions repaired primarily by BER, thus confirming the role of polymerase β in this repair pathway (15,23). The polymerase β dominant negative is thought to bind to glycosylase- and AP-endonuclease-treated DNA sites, thus blocking wild-type polymerase β access. If so, we would expect an increased sensitivity of polDN-expressing cells to MNNG. We therefore analyzed the ADN-IIG10 and SQDN-IIG3 clones in a 96-well cell growth assay for MNNG sensitivity and compared this with their corresponding vector controls ALz-IIE2 and SQLz-IIE2. A typical dose-response curve is shown in Figure 5A. All curves showed a characteristic two step pattern, with a subpopulation of cells showing the same sensitivity as the LZRS controls. The IC₅₀ values of four independent experiments were calculated (Fig. 5B). A significant increase in MNNG sensitivity was observed in the sensitized subpopulation in both cell carcinoma lines transduced with the polymerase β dominant negative, suggesting inhibition of BER.
different cell cycle phases. We therefore wished to study radiosensitization on synchronized cells. Since we ideally wanted to use a non-cytotoxic procedure, we chose to use mitotic shake off. Mitotic cells collected from an exponentially growing culture will proceed synchronously through S and G2. At the time of irradiation, the G1 cell population was enriched from 34% of the control to an average of 75%, with <20% S phase cells as determined by BrdU labeling and flow cytometry. We found that the sensitizing effect of polDN was abolished when irradiating cells enriched for G1 using this method (Fig. 6). We therefore conclude that the polDN sensitizing effect occurs largely in the S and G2 phases. It should be noted that a resistant subpopulation should be reflected in the survival curves for asynchronous populations. Figure 2A and B is consistent with this, although Figure 2C appears to be an exception. Why this is is not known at present and needs further study.

**DISCUSSION**

We have shown here that the expression of a 14 kDa N-terminal fragment of DNA polymerase β (polDN) is capable of sensitizing two different human tumor cell lines to ionizing radiation. The magnitude of radiosensitization was dependent on the expression level of polDN. In addition, the effects were seen not only in individual subclones but also in mixed populations of cells transduced with the retroviral vector containing the dominant negative. The LZRS vector alone appeared to induce slight radioprotection. Several studies have shown that LTR viral promoters bind API transcription factors (37), which can be stimulated by irradiating radiation (38). Whether this effect on the stress response influences radiosensitivity or whether there is some radical scavenging effect of EGFP is not known. Whatever the mechanism, the dominant negative more than compensated for this small effect, causing significant radiosensitization.

Based on the in vitro analysis by Husain et al. (24) we hypothesized that the polDN protein consisting of the N-terminal DNA-binding domain of polymerase β disrupts BER by binding to specific damaged DNA (AP sites). This will prevent the gap from being filled, since polDN lacks the ability to catalyze DNA synthesis. Binding to the gaps will probably also interfere with the binding of other alternative DNA polymerases. In this way the polDN protein competes with endogenous DNA polymerases β and δ/ε for the damage, thereby probably inhibiting both the short and long patch pathways. The fact that sensitization was still increasing with increasing amount of polDN indicates that binding of polDN at damaged sites in this dose range was probably not yet saturated. It is therefore possible that higher radiosensitizing effects are possible if higher levels of polDN could be achieved.

Our data suggesting interference of BER by polDN are consistent with other studies expressing a catalytically non-functional polymerase β mutant. A similar polymerase β dominant negative has been used in Saccharomyces cerevisiae (39,40). The authors showed that the DNA polymerase β mutant specifically interferes with the gap filling step in BER. This resulted in sensitivity to the DNA alkylating agent MMS but not to UV (40), indicating an involvement in BER and not in NER. Bhattacharyya and Banerjee (41) showed that colorectal carcinoma cells expressing a DNA polymerase β truncated variant without the catalytic domain from one allele exhibited substantially decreased BER activity and increased sensitivity to MNNG.

DNA damage produced by alkylating drugs are partly repaired by BER, as shown by the hypersensitivity to MMS and MNNG of polymerase β- and 3-methyladenine DNA glycosylase-deficient cells (15,23,42). The increased sensitivity to MNNG of cells expressing our dominant negative construct reported here is thus indirect evidence confirming the mechanism and suggests inhibition of BER. However, it should be noted that alkylated DNA is not exclusively repaired by the BER pathway. Mismatch repair is involved in the removal of damage resulting from alkylating agents, as demonstrated by the increased sensitivity to MNNG of mismatch-deficient cells (43). To date, however, cell lines deficient in MMR and NER have not shown significant changes in survival after ionizing radiation (44). In addition, strong binding of the polDN protein, derived from the highly specific DNA-binding domain of polymerase β, to possible intermediates in mismatch repair seems unlikely.

It is clear from in vitro studies that polymerase β is important in the repair of SSBs (17), oxidized bases (45,46) and AP sites (47,48), the major radiation lesions in DNA apart from DSBs. However, knocking out polymerase β has no effect on sensitivity to ionizing radiation (14,15). Knockout cells may repair such damage by using the alternative long patch BER pathway involving PCNA-dependent DNA polymerase δ or ε. This is supported by in vitro analyses showing an increase in long patch repair of 8-oxoguanine in DNA polymerase β-deficient cells (46). We propose that polDN inhibits not only the major short patch repair pathway of BER, in which polymerase β is a central player, but also blocks the DNA polymerase β-independent long patch repair pathway, resulting in higher levels of unrepaird nicked AP sites, ultimately leading to increased radiosensitivity. Knocking out polymerase β thus primarily eliminates the short patch pathway, leaving the long patch pathway intact and giving the cells an escape route for repair. The dominant negative construct on the other hand also blocks this escape route.

Which repair pathway is predominantly used after radiation damage is not clear. Klungland and Lindahl (22) showed that
repair of γ-ray induced oxidized AP sites in plasmid DNA, in contrast to regular AP sites, required FEN-1, an enzyme involved in long patch repair. H_2O_2 causes oxidation at purines and pyrimidines, abasic sites and SSBs (49), similar to ionizing radiation. Fortini et al. (50) showed that sensitivity to H_2O_2 was increased in polymerase β-deficient cells. They also detected no differences between repair of SSB in H_2O_2-treated polymerase β-deficient and normal cells, indicating polymerase β-independent repair of the damage. Nakamura et al. (51) demonstrated that oxidized AP sites after H_2O_2 are not efficiently excised by polymerase β. These data all suggest that ionizing radiation-induced base damage is primarily repaired by the DNA polymerase β-independent long patch pathway, accounting for the absence of a radiation sensitivity phenotype in polymerase β-deficient cells.

Our data thus suggest a significant role of BER, probably via the polymerase β-independent long patch pathway, in the repair of radiation-induced damage that contribute to cytotoxicity. BER efficiency has been correlated with radiation sensitivity in other studies on human normal or tumor cells. Hu et al. showed that variants of the APE1 (AP endonuclease 1) and XRCC1 genes were associated with ionizing radiation sensitivity (52). It has also been reported that APE nuclear expression was associated with chemo-radiotherapy resistance in head and neck cancer (53), while others have found a correlation between HAP1 (APE1) levels and radiosensitivity in human cervix carcinoma (54). We observed DEFs of 1.5–1.7 here, which compare favorably with other reported genetic modifications of radiosensitivity. Dominant negative inhibition of PARP or MEK2 achieved DEFs of 1.3 and 1.4 in Chinese hamster (55) and HeLa cells (56), respectively. Marangoni et al. reported DEFs of 2.0 after transfection of a dominant negative mutant of Ku80 in CHO cells (57), while XRCC1 knockouts showed DEFs of 1.4 (58). The increased sensitivity observed in our DNA polymerase β dominant negative-expressing cells is therefore consistent with biologically significant reduction in repair of DNA damage.

The observed radiosensitization appeared to be dependent on cell growth status and cell cycle phase. Cell populations enriched for G1 cells of exponentially growing cultures showed no radiosensitization, in contrast to confluent cultures (also high in G1/G0), which exhibited radiosensitization by polDN. There are several possible explanations. (i) Greater expression of polymerase β in G1, for example, would compete more effectively with polDN, although such differential expression has not been reported (59–62). (ii) PolDN might inhibit the short patch pathway more efficiently than alternative pathways, and these alternative pathways may be more pronounced in G1. At the moment, there is little direct evidence to support this. (iii) Translesion (TL) and recombination repair offer alternative pathways for repair of DNA lesions remaining after polDN interference in BER (63). In Escherichia coli, repair of chromosomal AP sites involves all three pathways (64). We suggest that cells damaged in G1 that enter S phase can repair remaining damage through TL or recombination. Cells exhibiting a dominant negative effect have probably already replicated their DNA and will not be able to use the TL repair pathway. This is consistent with our MNNG data showing a subpopulation of cells with normal sensitivity, indicating that unrepaired alkylating damage, probably consisting of nicked AP sites, can also be repaired through TL or recombination.

Confluent cells do not enter replication within the first hours of repair after irradiation, precluding this repair route. A similar conclusion was made by Taylor et al., who stated that S phase DNA repair processes can remove strand breaks induced in S phase and those that persist from G1 and can in part compensate for lack of repair in G1 (65).

In summary, the results suggest that the polymerase β dominant negative construct radiosensitized human tumor cells by interfering with BER due to binding to gapped DNA, thus blocking appropriate repair enzymes. In contrast to polymerase β-deficient cells, the dominant negative efficiently interfered with BER of ionizing radiation-damaged DNA, most probably due to additional blocking of the alternative long patch repair pathway involving polymerases δ and ε. These studies support an important role for BER in determining radiosensitivity and suggest potential applications in diagnosis and possibilities to modulate radiosensitivity of tumor cells in radiotherapy.

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