Bioenergetic Metabolites Regulate Base Excision Repair–Dependent Cell Death in Response to DNA Damage

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Abstract

Base excision repair (BER) protein expression is important for resistance to DNA damage–induced cytotoxicity. Conversely, BER imbalance [DNA polymerase β (Polβ) deficiency or repair inhibition] enhances cytotoxicity of radiation and chemotherapeutic DNA-damaging agents. Whereas inhibition of critical steps in the BER pathway result in the accumulation of cytotoxic DNA double-strand breaks, we report that DNA damage–induced cytotoxicity due to deficiency in the BER protein Polβ triggers cell death dependent on poly(ADP-ribose) (PAR) polymerase activation yet independent of PAR-mediated apoptosis-inducing factor nuclear translocation or PAR glycohydrolase, suggesting that cytotoxicity is not from PAR or PAR catabolite signaling. Cell death is rescued by the NAD+ metabolite β-nicotinamide mononucleotide and is synergistic with inhibition of NAD+ biosynthesis, showing that DNA damage–induced cytotoxicity mediated via BER inhibition is primarily dependent on cellular metabolite bioavailability. We offer a mechanistic justification for the elevated alkylation-induced cytotoxicity of Polβ-deficient cells, suggesting a linkage between DNA repair, cell survival, and cellular bioenergetics. Mol Cancer Res; 8(1): 67–79. ©2010 AACR.

Introduction

Efficacy of chemotherapy or radiation treatment is intimately dependent on DNA repair capacity (1). Robust repair of therapeutically induced DNA damage can provide significant resistance, whereas tumor-specific defects in DNA repair or inhibition of specific DNA repair proteins can provide therapeutic advantage (2). In particular, inhibiting base excision repair (BER) can be an effective means to improve response to temozolomide, radiation, bleomycin, and cisplatin, among other treatments (3-10). As with most DNA repair pathways, BER is a multistep mechanism composed of >20 proteins depending on the initial base lesion (3). However, inhibiting each step in the BER pathway will have different outcomes. DNA glyco- lase inhibition or loss blocks BER initiation, leading to the accumulation of both cytotoxic (4) and mutagenic base lesions (5), the latter contributing to cellular dysfunction. In this regard, the preferred option is the inhibition of BER after repair initiation, promoting the accumulation of cytotoxic BER intermediates such as abasic sites and DNA single-strand breaks by inhibiting abasic site repair with methoxyamine and inhibiting the BER enzyme poly (ADP-ribose) polymerase 1 (PARP1) or by loss or inhibition of DNA polymerase β (Polβ; refs. 2, 6, 7). We refer to inhibition of the intermediate steps in BER as the induction of “BER failure” because repair is initiated yet is unable to be completed.

Importantly, understanding the mechanisms that are responsible for the increase in cell death due to BER inhibition or BER failure is critical in tailoring treatment as well as designing rational adjuvant or combination treatments that may further increase overall response. For example, inhibiting PARP1 has proven effective in improving temozolomide-induced cell death (8). Inhibition of PARP1 results in the accumulation of replication-mediated DNA double-strand breaks (DSB) and the onset of apoptosis (9, 10). This detailed understanding of the mechanism of cell death induced by combining a DNA-damaging agent (temozolomide) and a PARP1 inhibitor suggests that PARP1 inhibition would be effective against many tumors but may be ineffective against tumors that are resistant to apoptosis (11). Further, cell death induced by PARP1 inhibition suggested a requirement for homologous recombination in the cellular response to the accumulated DSBs.
promoting preclinical and clinical trials of PARP1 inhibitors in the treatment of homologous recombination–defective tumors (2).

There are several BER proteins essential for the repair of temozolomide-induced DNA lesions. Using a mouse embryonic fibroblast cell model, we have shown that loss of Polβ can significantly improve the cytotoxic effect of temozolomide (12), suggesting that inhibition of Polβ may improve response to temozolomide in human tumor cells. Temozolomide is currently used in the treatment of glioblastoma (13), and it is therefore critical to evaluate the role of Polβ in glioma cell response to temozolomide treatment. No previous studies have investigated the role of Polβ in the response of human glioma tumor cells to temozolomide. Further, there is no mechanistic explanation for the increase in alkylation-induced cell death observed in cells that are deficient in Polβ beyond the evidence that cell death in mouse cells is the result of accumulation of unrepaired BER intermediates (7, 12).

Acute alkylation damage has been suggested to induce cell death by multiple mechanisms, including necrosis (14), caspase-3 and caspase-9 activation and the onset of apoptosis (15), apoptosis-inducing factor (AIF) translocation from the mitochondria to the nucleus (16-18), ADP-ribose–induced activation of the Ca²⁺ channel TRPM2, or AMP-mediated inhibition of ATP transport (19-21). In most, if not all, cases, cell death has been attributed to the direct action of either PAR formed by PARP1 activation or PAR catabolites that accumulate after (19-21). In most, if not all, cases, cell death has been attributed to the direct action of either PAR formed by PARP1 activation or PAR catabolites that accumulate after PAR degradation by the catabolic enzyme PAR glycohydrolase (PARG). Polβ-deficient mouse cells are hypersensitive to the cell killing effects of alkylating agents due to failure to repair the 5′-deoxyribose phosphate (5′-dRP) BER intermediate (22). However, the exact downstream signaling events and mechanism of cytotoxicity specifically induced by the unrepaired 5′-dRP lesion remain unclear. Previous studies in mouse cells have not been conclusive. One report suggested that the absence of Polβ led to damage-induced cell death via apoptosis (23), whereas a later study proposed a necrotic form of cell death for both wild-type (WT) and Polβ-deficient cells (24), similar to what has been proposed as a general mechanism of alkylation-induced cell death in mouse fibroblasts (14). However, this latter study required the use of apoptosis-deficient cells to observe necrotic cell death (14). None of these previous studies have identified a mechanism of cell death specific to Polβ deficiency and BER failure or a failure to repair the cytotoxic BER intermediate 5′-dRP.

The studies described herein were designed to specifically define the mechanism of cell death in human tumor cells resulting from failure to repair the BER intermediate 5′-dRP due to “inhibition of” or a “deficiency in” Polβ (BER failure). We have hypothesized that PARP1 functions in BER as both a complex coordinator and a molecular repair sensor. As a BER molecular sensor, we suggest that PARP1 facilitates cell death in response to incomplete BER or BER failure. In support of this hypothesis, we show that a specific BER intermediate, a single-strand DNA strand break containing a 3′-OH and 5′-dRP, is an in vivo substrate in human cells that activates PARP1 in the context of BER and that elevated cytotoxicity observed in Polβ-deficient human cells is controlled by the activation of PARP1. Further, we provide clear evidence that following BER failure human cells die independent of RIP1 activation or AIF translocation, thus ruling out PAR as the cell death signal that is initiated on BER failure. Further, we show that the observed cell death in Polβ-deficient cells is unrelated to the accumulation of PAR catabolites, such as ADP-ribose or AMP, yet is dependent on NAD⁺ metabolite bioavailability or the bioenergetic capacity of the cell.

This study provides mechanistic insight into why Polβ deficiency leads to cell death, defines the mode of death, and offers a mechanistic link between BER failure and energy metabolism—the novel finding that DNA damage–induced cytotoxicity mediated via BER inhibition is primarily dependent on cellular metabolite bioavailability. Finally, we offer a mechanistic justification for the elevated alkylation-induced cytotoxicity of Polβ-deficient cells, suggesting a linkage between DNA repair, cell survival, and cellular bioenergetics.

**Materials and Methods**

**Cell Culture and Cell Line Development**

The cell line LN428 is an established glioblastoma-derived cell line with mutations in p53 and deletions in p14ARF and p16 and is WT for PTEN (25, 26). LN428 cells were kindly provided by Ian Pollack (University of Pittsburgh, Pittsburgh, PA) and cultured in α-Eagle’s MEM supplemented with 10% heat-inactivated fetal bovine serum, glutamine, antibiotic/antimycotic, and gentamicin. MDA-MB-231 cells and derivatives were described previously (27). Cell lines expressing human MPG (WT), human MPG (N169D), FLAG-Polβ(WT), and FLAG-Polβ(K72A) were developed by transfection using FuGene 6 transfection reagent (Roche Diagnostic Corp.) according to the manufacturer’s protocol. Transfected cell lines were cultured in G418 and/or puromycin for 2 wk, and individual clones stably expressing human MPG or Polβ were selected. It was recently suggested that p14ARF deficiency results in proteosome-mediated degradation of Polβ (28). Although LN428 cells are deficient in p14ARF (26), we note that the expression levels of Polβ are stable. Lentiviral particles were generated by cotransfection of plasmid pCDFl-MCS1-EF1-copGFP (control) or pSIF-H1-hPOLB1-copGFP (Polβ shRNA) together with pFIV-34N and pVSV-G into 293-FT cells (29) using FuGene 6 transfection reagent. Forty-eight hours after transfection, lentivirus-containing supernatant was collected and passed through 0.45-μm filters to isolate the viral particles. Lentiviral transduction was done as described earlier (27). Briefly, 6.0 × 10⁵ cells were seeded into six-well plates 24 h before transduction. Cells were transduced for 18 h at 32°C and cultured for 72 h at 37°C. Cells expressing copGFP only or both copGFP and Polβ-specific shRNA

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were isolated by fluorescence-activated cell sorting, and Polβ-KD was confirmed by immunoblot analysis. All the cell lines developed and used in this study are described in Supplementary Table S1.

Chemicals and Reagents
α-Eagle’s MEM was from Mediatech. RPMI 1640 and DMEM were from Cambrex Bioscience Group and Bio-whittaker, respectively. Fetal bovine serum, heat-inactivated fetal bovine serum, penicillin/streptomycin/amphotericin, glutamine, and antibiotic/antimycotic were from Invitrogen. Temozolomide (NSC 362856; IUPAC name: 3-methyl-2-oxo-1,3,4,5,8-pentazabicyclo[4.3.0]nona-4,6,8-triene-7-carboxamide; CAS number: 856622-93-1; ref. 30) was purchased from Calbiochem. FK-866 ((E)-[4-(1-benzoylpiperidin-4-yl)butyl]-3-pyridin-3-yl)acrylamide] was obtained from the National Institute of Mental Health Chemical Synthesis and Drug Supply Program (NIMH F-901; ref. 31). NMN was prepared in DMSO at 100 mmol/L. MMS was from Sigma-Aldrich. Purymycin, gentamicin, and neomycin were purchased from Clontech Laboratories, Irvine Scientific, and Invitrogen, respectively. PJ34 was purchased from Calbiochem. Twenty micrograms of protein were loaded on a precast 4% to 12% precast NuPAGE T ris-glycine gel for immunoblot assay.

Plasmid Expression and RNA Interference Vectors
Human MPG (WT) was expressed using the plasmid pRS1422, as described previously (27). The MPG expression plasmid (pRS1422) was then mutated at residue N169 using the QuikChange XL Site-Directed Mutagenesis kit (Stratagene) to yield pRES-Neo-MPG-N169D. The expression plasmid for FLAG-tagged WT human Polβ was generated by PCR amplification of the human Polβ cDNA using a FLAG-containing forward oligonucleotide and cloned into pENTR/D-TOPO as we described previously (27). pENTER/FLAG-Polβ(WT) was then mutated at residue K72 as described above to yield pENTER/FLAG-Polβ(K72A). FLAG-Polβ(WT) and FLAG-Polβ(K72A) were subsequently cloned into a Gateway-modified pIRES-Puro vector by TOPO cloning, as we have described previously (27). The FIV-based lentiviral shRNA expression vector system specific for human Polβ was as described previously (27) but was modified for copGFP expression (pSIF-H1-hPOLB1-copGFP). Lentiviral particles for coexpression of PARG shRNA and TurboGFP were prepared by transfection of four plasmids [the control plasmid pLK0.1-Puro-tGFP or the human PARG-specific shRNA plasmid pLK0.1-Puro-PARGshRNA4 plus pMD2.g(VSVG), pRSV-REV, and pMDLg/pRRE] into 293-FT cells (29, 32) using FuGene 6 transfection reagent. Culture medium from transfected cells was collected 48 h after transfection to isolate the viral particles, passed through 0.45-μm filters, used immediately, or stored at −80°C in single-use aliquots. Transduction of LN428 cells with control lentivirus (GFP expression only) and human PARG-specific shRNA lentivirus was completed as follows: Briefly, 6.0 × 105 cells were seeded into six-well plates and incubated for 24 to 30 h at 10% CO₂ at 37°C. Cells were transduced for 18 h with virus at 32°C and cultured for 72 h at 37°C before isolation of the GFP-expressing population by fluorescence-activated cell sorting using the University of Pittsburgh Cancer Institute Flow Cytometry Facility. Cells were then cultured to expand the population and analyzed for expression of PARG by quantitative reverse transcription-PCR.

Quantitative Reverse Transcription-PCR Analysis
Expression of PARG and Polβ mRNA was measured by quantitative reverse transcription-PCR using an Applied Biosystems StepOnePlus system. Briefly, 80,000 cells were lysed and reverse transcribed using the Applied Biosystems Taqman Gene Expression Cells-to-CT kit. Each sample was analyzed in triplicate and the results are an average of all three analyses. Analysis of mRNA expression was conducted as per the manufacturer (ΔΔCₘethod) using Applied Biosystems Taqman Gene Expression Assays (human Polβ: Hs00160263_m1; human PARG: Hs00608254_m1) and normalized to the expression of human β-actin (part 4333762T).

Cell Extract Preparation and Western Blot
Nuclear extracts were prepared and protein concentration was determined as described previously (12). Twenty micrograms of protein were loaded on a precast 4% to 20% NuPAGE Tris-glycine gel (Invitrogen). For whole-cell extracts used in PAR formation assays, 3 × 10⁶ cells were seeded into a 100-mm cell culture dish 24 h before drug treatment. Cells were either treated with temozolomide only or preexposed to a PARP inhibitor (PJ34 or DR2313) followed by PARP inhibitor plus temozolomide treatment. After treatment, cells were washed twice with cold PBS, collected, and lysed with 400 μL of 2× Laemmlı buffer [2% SDS, 20% glycerol, 62.5 mmol/L Tris-HCl (pH 6.8), 0.01% bromphenol blue]. Samples were boiled for 8 min and extract from -1.5 × 10⁵ cells was loaded onto each lane on a 4% to 12% precast NuPAGE Tris-glycine gel for immunoblot assay.

The following primary antibodies were used in immunoblot assays: anti-human MPG monoclonal antibody (clone 506-3D; ref. 27), anti-Polβ monoclonal antibody (clone 61; Thermo Fisher Scientific), anti-APE1 (EMD Biosciences), anti–proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology), anti-FLAG (M2 monoclonal antibody; Sigma-Aldrich), anti-PAR (clone 10H, kindly provided by M. Ziegler, University of Bergen, Bergen, Norway), anti-PARP1 (BD Pharmingen), and anti-human HMGB1 (R&D Systems).

Cell Cytotoxicity Assay
Temozolomide-induced cytotoxicity was determined by an MTS assay, a modified MTT assay as described previously (12). Results were calculated from the average of three or four separate experiments and are reported as the percentage of treated cells relative to the cells without treatment (% control). For PJ34, cells were preexposed to the inhibitor.
for 30 min and then treated with temozolomide in the presence of the inhibitor for 48 h. For NA and NMN, cells were preexposed to each for 24 h (concentrations as indicated in the legend) and then treated with temozolomide (1.0 mmol/L) in the presence of NA or NMN for 48 h. The effect on cell growth and survival was determined by an MTS assay, as described previously (12).

**HMGB1 Release Assay**

Cells were pretreated with medium alone or with PARP inhibitor (PJ34) for 30 min before cotreatment with PJ34 (2 μmol/L) and temozolomide (1.5 mmol/L) for 12 h. Cell culture medium was then collected and passed through 0.45-μm filters. Immobilized heparin (Thermo Fisher Scientific) slurry (100 μL) and 1 mL of medium were mixed and rotated at 4°C for 2 h before centrifugation at 8,000 × g to pull down HMGB1 bound to immobilized heparin (33). Pellets were boiled with 100 μL of 2× Laemmli buffer, and supernatants were used for immunoblot assay after brief centrifugation.

**PAR Assay**

Cells (1.5 × 10^6) were seeded in 100-mm dishes 24 h before treatment. For the FK-866 experiments, cells were then incubated in the presence of FK-866 (10 nmol/L) or DMSO for an additional 24 h. Medium was then removed and replaced with fresh medium or medium supplemented with PJ34 (2 μmol/L). After 30 min, cells
were lysed immediately (0 time point) or medium was replaced with temozolomide for the times indicated in the figure legends. Extracts were prepared by washing the cells with PBS and preparing cell extract with 400 μL of 2x Laemmlı buffer. Cell extract (20 μL) was analyzed by immunoblot with a 4,000-fold dilution of an anti-PARP primary antibody (clone 10H) followed by a 5,000-fold dilution of the horseradish peroxidase–conjugated secondary goat anti-mouse antibody.

**Immunofluorescence and Confocal Microscopy**

Cells were cultured on glass coverslips for 24 h before treatment with MMS or medium control. One hour after treatment, cells were washed and allowed to recover in medium for 5 h. Cells were then fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.5% Triton X-100 for 15 min, and blocked with 2% bovine serum albumin for 1 h, all at room temperature. AIF was detected by incubating 1 h at room temperature with an anti-AIF antibody (Santa Cruz Biotechnology) at 1:100 dilution followed by goat anti-mouse Alexa Fluor 488 (Molecular Probes) at 1:500, Alexa Fluor 647 phalloidin actin stain at 1:250 (Molecular Probes), and 5 μmol/L DRAQ5 nuclei stain for 1 h at room temperature. Slides were mounted and imaged on the Olympus FluoView 500 confocal microscope.

**NAD+ and ATP Measurements**

Cells were seeded in 96-well plates 24 h before treatment with MMS or medium control. NAD+: 1 h after treatment, cells were trypsinized and counted and 1 × 10^5 cells were pelleted. NAD+ lysates were prepared and NAD+ measurements were obtained using the EnzyChrom NAD+/NADH Assay kit (Bio-Rad) at 1:100 dilution followed by goat anti-mouse Alexa Fluor 488 (Molecular Probes) at 1:500, Alexa Fluor 647 phalloidin actin stain at 1:250 (Molecular Probes), and 5 μmol/L DRAQ5 nuclear stain for 1 h at room temperature. Slides were mounted and imaged on the Olympus FluoView 500 confocal microscope.

**FK-866 Cytotoxicity Assay**

Cells were seeded in 96-well plates 24 h before treatment. Cells were pretreated with 10 nmol/L FK-866 or DMSO control for 24 h and then exposed to MMS for 1 h. The cells were then washed with medium and allowed to recover for 48 h before assaying for cytotoxicity by an MTS assay previously described (12, 27). Results shown are the average of three independent experiments and reported as percent survival of MMS-treated cells compared with control wells.

**Results**

**Hyperactivation of PARP due to Polβ Deficiency and Failure to Repair the BER Intermediate 5′-dRP**

BER is a finely tuned process that requires balanced expression of several proteins to avoid accumulation of mutagenic or cytotoxic repair intermediates (3). To understand how alterations in BER enzyme activity in human tumor cells lead to DNA damage–induced cell sensitivity, we developed human glioma (LN428) cell lines with a functional deficiency in Polβ by increasing expression of N-methylpurine DNA glycosylase (MPG) and depleting the cell of Polβ by stable lentiviral-mediated expression of short hairpin RNA (shRNA). As we have reported, human cells with elevated expression of MPG are sensitive to alkylation damage due to a deficiency in Polβ (27), a phenotype that is enhanced by Polβ knockdown (Polβ-KD). Conversely, reexpression of Polβ eliminated the alkylation hypersensitive phenotype (Supplementary Figs. S1 and S2). These cells (LN428/MGP and LN428/MGP/Polβ-KD cells) are therefore functionally deficient in Polβ and were used to determine the mechanism that mediates the enhanced DNA damage–induced cell death resulting from Polβ deficiency.

The DNA binding and signaling molecules PARP1 and PARP2 have each been implicated in BER (3). PARP1 facilitates BER complex formation, and it has been postulated that local strand break–induced activation of PARP1 and the resultant synthesis of PAR mediate recruitment of the BER proteins XRCC1 and Polβ to stimulate DNA repair (34). We therefore have hypothesized that in cells that fail to complete BER (e.g., when 5′-dRP lesions are not repaired; herein referred to as BER failure), PARP1 is hyperactivated and functions as a DNA damage signaling protein that triggers cell death. To determine whether PARP is activated by the BER intermediate (5′-dRP) in vivo, we exposed the control (LN428) and corresponding BER-defective cells (Polβ-deficient LN428/MGP and LN428/MGP/Polβ-KD cells) to temozolomide for up to 90 minutes. Whole-cell extracts were probed by immunoblot for PAR accumulation following temozolomide exposure (Fig. 1A). The level of PAR accumulation was shown to correlate with the extent of the BER defect. PARP activation was elevated in the LN428/MGP cells (an intermediate level of sensitivity), with the highest level of PAR observed 30 minutes following exposure to temozolomide, whereas essentially no PARP activation was observed in LN428/MPG cells (an intermediate level of sensitivity), whereas essentially no PARP activation was observed in LN428 cells (Fig. 1A). In the more sensitive cell line (LN428/MGP/Polβ-KD), PARP activation was more robust and rapid as compared with that of the LN428/MGP cell line (Fig. 1A), as PAR reached its highest level at 15 minutes after exposure to temozolomide. Comparable results were also observed in a Polβ-defective breast cancer cell line, where elevated temozolomide–induced PARP activation is restricted to the cells with Polβ deficiency (Supplementary Fig. S2B and C). Conversely, exposure to etoposide resulted in a low level of PARP activation at all time points for all three cell lines LN428, LN428/MGP, and LN428/MGP/Polβ-KD (Supplementary Fig. S2D). Thus, PARP activation is elevated in BER-defective (Polβ-deficient) cells following alkylation damage.

Because the combination of alkylating agent treatment and Polβ deficiency triggers PARP activation, we next validated the significance and specificity of this finding by reexpression of Polβ in the LN428/MGP and LN428/MGP/Polβ-KD cells. We find that the BER-deficient phenotype (increased cellular sensitivity to alkylating agents) observed
in both the LN428/MPG and the LN428/MPG/Polβ-KD cells was reversed by complementation (expression) of FLAG-Polβ(WT) (Fig. 1B, top; Supplementary Fig. S1E) but not the 5′-dRP lyase–deficient (K72A) mutant of Polβ (Fig. 1C, top). Similarly, we find that complementation with FLAG-Polβ(WT) but not with the Polβ 5′-dRP lyase mutant eliminated the temozolomide-induced activation of PARP observed in BER-defective cells (Fig. 1B and C, bottom). These data therefore suggest that the Polβ-specific BER intermediate (5′-dRP lesion) triggers rapid and robust PARP1 activation in vivo, triggering the onset of cytotoxicity.

The correlation between PARP activation and alkylation sensitivity prompted us to determine if inhibition of PARP reverses the cellular hypersensitivity of Polβ-deficient human tumor cells. We inhibited activation of PARP by pretreatment and cotreatment with the PARP1/PARP2 inhibitors PJ34 or DR2313. Inhibition of PARP by PJ34 significantly reduced the level of temozolomide-induced PARP activation in the Polβ-deficient cells (LN428/MPG; Fig. 2A, lanes 3 and 4 and lanes 7 and 8). We next assayed if PARP inhibition can rescue the alkylation-sensitive phenotype of LN428/MPG cells, as determined by an MTS assay 48 hours after temozolomide exposure. Most importantly, we find that PARP inhibition by either PJ34 or DR2313 treatment converted the LN428/MPG cells from a sensitive phenotype to a resistant phenotype (Fig. 2B; Supplementary Fig. S3A). Rescue by PARP inhibition was also observed in Polβ-deficient MDA-MB-231 cells (Supplementary Fig. S3B). It remains to be determined if this resistant phenotype is long lived.

Unrepaired BER Intermediates (5′-dRP Lesions)
Trigger Cell Death via Energy Depletion in the Absence of PAR or PAR Catabolite–Mediated Signaling

Several different mechanisms have been attributed to PARP1 activation–induced cell death. We first evaluated the involvement of caspase-dependent cell death in control cells as compared with the corresponding Polβ-deficient cells following temozolomide treatment. These experiments (Supplementary Fig. S4A and B) rule out a caspase-dependent response due to BER failure, in line with our previous report (27). Although it has been shown that an autophagic response contributes to temozolomide-induced cell death in some cells (38), temozolomide hypersensitivity of Polβ-deficient cells is not affected by the autophagy inhibitor 3-methyladenine (Supplementary Fig. S4C). In support of this observation, we did not observe increased LC3 puncta in BER-defective cells following temozolomide exposure (27).

A major mechanism that has been attributed to PARP activation–induced cell death is direct PAR signaling to the mitochondria, where PAR mediates translocation of AIF from the mitochondria to the nucleus to induce caspase-independent cell death (16-18) via a mechanism that requires receptor (tumor necrosis factor receptor superfamily)–interacting serine-threonine kinase 1 (RIP1) activation (see model; Fig. 3A; ref. 39). RIP1 can be inhibited here persists or if the BER failure–induced single-strand breaks lead to the formation of DSBs and the onset of apoptosis after several rounds of replication (9). Regardless, these studies support our hypothesis that PARP hyperactivation mediates the alkylation-sensitive phenotype of Polβ-deficient cells.

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by necrostatins, small-molecule inhibitors shown to inhibit cell death (40, 41). Therefore, we investigated the role of RIP1 in the PARP-mediated cell death we observed by inhibiting RIP1 with necrostatin-1 (41) and evaluating the effect of RIP1 inhibition on DNA damage–induced cell survival in both control and Polβ-deficient cells. However, inhibition of RIP1 did not prevent cell death in either the parental or the Polβ-deficient cells (Supplementary Fig. S5), suggesting but not proving that AIF translocation may not be related to the observed cell death.

We therefore next evaluated the subcellular localization of AIF in control and Polβ-deficient cells following exposure to the alkylating agents methyl methanesulfonate (MMS) or temozolomide as compared with vehicle (medium) by immunofluorescent staining and confocal microscopy (Fig. 3B) or by subcellular fractionation and immunoblot analysis (Supplementary Fig. S6). In line with the RIP1 inhibition data above, alkylating agent treatment of Polβ-deficient cells did not alter the subcellular localization of AIF (Fig. 3B; Supplementary Fig. S6). All the detectable AIF was localized to the mitochondria in both cell lines regardless of agent or time of exposure (up to 12 hours), thus ruling out PAR as a cell death signal on BER failure.

In the absence of a PAR-mediated cell death process (AIF translocation), it is possible that cell death is initiated via the rapid breakdown of PAR (see Fig. 1A) by the degradative enzyme PARG and the accumulation of the PAR catabolites ADP-ribose, ribose-5-phosphate, and/or AMP (see model; Fig. 3A; ref. 42). ADP-ribose acts as a second

**FIGURE 3.** Absence of PAR or PAR catabolite–mediated cell death following BER failure. A. Model depicting the nexus of BER, the synthesis of PAR, and the generation of PAR catabolites in response to BER failure–induced PARP1/PARP2 hyperactivation. B. Absence of mitochondria to nucleus translocation of AIF due to BER failure as determined by confocal microscopy. BER-deficient cells (LN428/MPG) were treated with medium (left) or 1.5 mmol/L MMS (right) for 1 h and then washed and allowed to recover in medium for 5 h before fixation and staining for AIF (green), actin (red), and nucleus (blue). C. PARG-KD prevented degradation of DNA damage–induced PAR. Left, immunoblot of PAR to determine the degradation of PAR in LN428/MPG/PARG-KD cells following treatment with 1.5 mmol/L temozolomide. PCNA protein expression level was shown as a loading control. Right, preventing generation of PAR catabolites from degradation of PAR via PARG-KD enhances temozolomide-induced cytotoxicity. LN428 and LN428/MPG cells with (black columns) or without (white columns) PARG-KD were exposed to temozolomide (1 mmol/L) or vehicle control (DMSO) for 48 h. Viable cells were determined as in Fig. 1B and reported as percentage relative to vehicle control (% control). Columns, mean of three independent experiments; bars, SE. D. HMGB1 released into the cell culture medium, as shown by immunoblot. LN428 and LN428/MPG cells were pretreated with PJ34 (4 μmol/L) or vehicle control for 30 min and then exposed to temozolomide (1.5 mmol/L) with or without PJ34 (2 μmol/L) for 12 h. HMGB1 was then captured using immobilized heparin and analyzed by immunoblot, as described in Materials and Methods.
messenger to activate the cation channel TRPM2 to trigger Ca\(^{2+}\) influx, resulting in cell death (19, 20), or inhibits ATP-binding cassette transporters (43), whereas elevated AMP can block ATP transport, leading to ATP depletion and cell death (21). To investigate the possibility that PAR catabolites contribute to PARP-mediated cell death in Polβ-deficient cells, we first blocked Ca\(^{2+}\) influx with BAPTA-AM, shown recently by Bentle et al. (44) and Bey et al. (45) to

**FIGURE 4.** BER failure–induced cell death depends on NAD\(^{+}\) availability. **A.** Alkylation damage promotes NAD\(^{+}\) and ATP depletion in BER-defective cells. Left, NAD\(^{+}\) content. Cells were treated with medium (white columns) or 0.5 mmol/L MMS (black columns) for 1 h before collection for NAD\(^{+}\) content analysis via enzymatic assay as described in Materials and Methods. Right, ATP content. Cells were treated with medium (white columns), 0.5 mmol/L MMS (gray columns), or 1.5 mmol/L MMS (black columns) for 1 h. ATP content was measured after 1-h recovery in normal medium via the luminescence ATP assay described in Materials and Methods. NAD\(^{+}\) levels or ATP levels shown are the average of three independent experiments and are reported as percent control of the untreated control cell line. **B.** PARG-KD does not rescue alkylation damage–induced NAD\(^{+}\) and ATP depletion in BER-defective cells. Left, NAD\(^{+}\) content. PARG-KD cell lines were treated with medium (white columns) or 0.5 mmol/L temozolomide (black columns) for 1 h before collection for NAD\(^{+}\) content analysis as described in Materials and Methods. Right, ATP content. PARG-KD cells were treated with medium (white columns), 0.5 mmol/L temozolomide (dotted columns), 1.0 mmol/L temozolomide (gray columns), or 1.5 mmol/L temozolomide (black columns) for 1 h. ATP content was measured after 1-h recovery in normal medium via the luminescence ATP assay described in Materials and Methods. NAD\(^{+}\) levels or ATP levels shown are the average of three independent experiments and are reported as percent control of the untreated control cell line. **C.** Bioenergetic metabolites rescue Polβ-deficient cells from DNA damage–induced cell death. LN428 and LN428/MPG cells were pretreated with NMN, NA, or vehicle control (medium) for 24 h and then exposed to temozolomide (1 mmol/L) in the presence or absence of NMN or NA for 48 h. Viable cells were measured as in Fig. 1B and reported as percentage relative to vehicle control–treated cells (% control). Columns, mean of three independent experiments; bars, SE. **D.** NAD\(^{+}\) biosynthesis inhibition augments BER failure–induced cell death. Cells were pretreated for 24 h with a nontoxic 10 nmol/L dose of FK-866 (black columns) or DMSO (white columns). Cells were then exposed to medium control or MMS (0.5 mmol/L) for 1 h. Viable cells were determined as in Fig. 1B. Columns, mean of three independent experiments; bars, SE.
abrogate PARP1 activation–induced cell death. Unlike that observed following DNA damage from reactive oxygen species or oxidative stress, BAPTA-AM did not prevent the elevated damage-induced cell death in Polβ-deficient cells (Supplementary Fig. S7). However, as there may be multiple mechanisms of PAR catabolite–induced cell death, we next knocked down expression of PARG by stable transduction of both cell lines with a lentivirus-expressing shRNA specific to PARG. Expression of PARG mRNA is reduced to 35% as compared with the green fluorescent protein (GFP) control cells when determined by quantitative reverse transcription-PCR (data not shown). Importantly, we found no evidence for PAR-degrading activity in the cells with stable depletion of PARG (Fig. 3C, left). When exposed to an alkylating agent, BER-deficient PARG-KD cells accumulate significant levels of PAR with no evidence for PAR degradation (Fig. 3C, left, lanes 2–4). This is in contrast to the presence of PARG when the PAR molecule is degraded within 60 to 90 minutes (Fig. 1, lanes 7–12). These data show that these PARG-KD cells do not degrade PAR and, hence, do not accumulate PAR catabolites, providing an opportunity to determine if PAR catabolites contribute to cell death in these cells. As shown in Fig. 3C (right), PARG-KD did not rescue or reverse the enhanced damage–induced cell death phenotype of Polβ-deficient (LN428/MPG) cells. In fact, PARG-KD cells (black columns) were more sensitive to the cell killing effect of the alkylating agent temozolomide as compared with the PARG-expressing cells (white columns, Fig. 3C, right). The inability of necrostatins to abrogate the PARP1 activation–induced cell death damage (e.g., a failure to mediate caspase-independent cell death, together with the specific loss of NAD+ and ATP (Fig. 4A)) even when the formation of PAR catabolites is prevented (Fig. 4B), suggests that the BER failure response is linked to the cellular bioenergetic capacity of the cell.

For this paradigm to hold, we hypothesized that the availability of bioenergetic metabolites would affect the survival of Polβ-deficient cells exposed to an alkylating agent. In line with this hypothesis, we find that supplementation of the cells with either β-nicotinamide mononucleotide (NMN; ref. 54) or nicotinic acid (NA) reversed the DNA damage–induced phenotype, rendering the Polβ-deficient cells (black columns) completely (NMN) or 80% (NA) resistant to the cell killing effects of the alkylating agent as compared with the BER-proficient cells (white columns; Fig. 4C). Conversely, we anticipated that the hypersensitive phenotype of Polβ-deficient cells would be exacerbated by a reduction in the cellular level of NAD+ and related bioenergetic metabolites. We therefore evaluated the effect of transient NAD+ depletion on the observed BER failure response by pretreating cells with FK-866, a highly specific noncompetitive small-molecule inhibitor of nicotinamide phosphoribosyltransferase, a critical enzyme in the NAD+ biosynthetic salvage pathway that catalyzes the synthesis of NMN (31). Most importantly, the sensitivity of control cells to alklylation damage was not altered by FK-866 treatment. However, the BER-deficient cells are 9-fold more sensitive to MMS following a nontoxic (10 nmol/L) treatment with FK-866 as compared with the untreated cells (Fig. 4D), although PAR synthesis after the combined FK-866 + MMS treatment is attenuated (Supplementary Fig. S8). These results support our overall hypothesis that the BER failure phenotype of Polβ-deficient cells is mediated by BER intermediate (5′-dRP)–induced PARP1 activation and induction of caspase-independent cell death that is uniquely dependent on the availability of bioenergetic metabolites such as NMN and NAD+

**Discussion**

The requirement for BER in general and Polβ more specifically in the repair of genomic DNA base damage, particularly DNA damage induced by alkylating agents such as the chemotherapeutic temozolomide and the SN1 and SN2 alkylating agents N-methyl-N′-nitro-N′-nitrosoguanidine (MNNG) and MMS, respectively (7, 12), elevates the significance of characterizing the mechanism responsible for Polβ deficiency–induced cell death (e.g., a failure to
complete repair of the BER intermediate 5′-dRP in the absence of Polβ. As evidenced recently by the development of clinically significant PARP1 inhibitors, identifying BER proteins critical for response to DNA-damaging agents (e.g., chemotherapy) can have broad human health implications. Equally important is a clear understanding of the mechanism(s) that contributes to the enhanced cell death observed on DNA repair inhibition. For example, PARP1 inhibition triggers apoptosis via the accumulation of DSBs (9, 10) and a requirement for homologous recombination proteins such as BRCA1 and BRCA2 (2). To this end, we have developed a unique series of genetically modified human tumor cell lines as models of Polβ deficiency that accumulate the cytotoxic BER intermediate 5′-dRP following exposure to alkylating agents (remazolomide, MMS, and MNNG). By directly comparing BER-defective (Polβ-deficient) and BER-competent isogenic human cell lines, the cellular, biochemical, and signaling responses to DNA base damage can be defined as either global (non-specific) or BER (Polβ)-specific effects, the latter resulting from a cellular response to the inability to complete BER, referred to herein as BER failure. We have then used this system to define the mechanism of cell death resulting from Polβ loss/inhibition or BER failure and propose and test paradigms to enhance the cell death response.

From these studies, we find that the unrepaired BER intermediates that accumulate on DNA-damaging agent exposure when Polβ is deficient will activate PARP1, leading to a rapid onset of PARP1-dependent, caspase-independent cell death with little or no role for a caspase-dependent or autophagy-dependent process in the response. It remains to be determined if the BER failure–induced cell death observed herein is dependent on extracellular signal-regulated kinase 1/2–mediated PARP1 phosphorylation (55) and SIRT1–regulated deacetylation of PARP1 (56) or if the observed PARP1–induced cell death requires BAX, calpain, and c-Jun NH2-terminal kinase activation (57). Coincident with damage-induced necrosis in Polβ-deficient cells is PARP1–dependent HMGB1 secretion (49), a hallmark of caspase-independent cell death and inflammation signaling. HMGB1 functions in the extracellular space as a robust RAGE ligand and inflammatory cytokine or damage-associated molecular pattern molecule (48), suggesting that BER failure and the resulting PARP1 activation may trigger an inflammatory response in tissues with a BER imbalance such as ulcerative colitis (58).

There are multiple PARP1 activation–induced cell death mechanisms, as outlined in the diagram shown in Fig. 3A. In one, it is suggested that PAR, the product of PARP1 activation, is a cell death molecule. In this process, PAR initiates the translocation of AIF from the mitochondria to the nucleus by a RIP1–dependent mechanism (Fig. 3A; refs. 16-18, 39). Uniquely, PAR generated due to BER failure does not seem to trigger cell death via RIP1 activation nor does PAR function as a signal to initiate AIF translocation. PARP1 is involved in many DNA repair processes, including homologous recombination and nonhomologous end joining, in response to DSBs and has a role in telomere maintenance (59, 60). The question remains if PAR generated via BER failure is of a unique chemical makeup as compared with PAR generated from DSB-induced PARP1 activation. One possible explanation for the absence of a role for AIF in this study is the concentration of DNA-damaging agents used. In this report, we have used temozolomide or MMS at a maximum concentration of 1.5 mmol/L or MNNG at a concentration of 5 μmol/L, resulting in 90% to 95% cell death in the BER-deficient cells with little or no cell death in the control cells (Supplementary Fig. S2A). Many reports of PAR-induced AIF translocation include MNNG concentrations of 100 and 500 μmol/L (35, 57, 61). Such high concentrations of DNA-damaging agents (e.g., MNNG at 20× and 100× that used herein) have the potential to directly induce DNA DSBs and create overwhelming levels of both nuclear and mitochondrial genome damage (62) as well as the possibility of direct protein alkylation. Regardless, it is clear that the cell death initiated by BER failure is independent of RIP1 activation and AIF translocation, thus ruling out PAR as the cell death signal that is initiated on BER failure.

One explanation for the absence of PAR-mediated cell death is the rapid catalolism of PAR by PARG (42). In this study, we find that PAR synthesized due to PARP activation is degraded within 90 minutes (Fig. 1). As summarized in Fig. 3A, the breakdown products of PAR (PAR catabolites) are also likely mediators of cell death, including ADP-ribose (activator of the Ca2+ channel TRPM2) and AMP (inhibitor of ATP transport; refs. 19-21). However, PARG knockdown did not reverse the DNA damage–sensitive phenotype of Polβ-deficient cells (Fig. 3C), suggesting that damage-dependent cell death in Polβ-deficient cells is not initiated by PAR catabolites. Conversely, the PAR catabolite AMP may provide a protective phenotype by activation of AMP kinase, induction of autophagy, and enhanced ATP synthesis, as recently reported following reactive oxygen species–induced DNA damage and PARP1 activation (63). Although loss of AMP kinase activation and induction of autophagy on PARG-KD could explain, in part, the enhanced cell death observed in the PARG-KD cells (Fig. 3C), we suggest this is unlikely because in this study autophagy is not involved (Supplementary Fig. S4C) and the activation of AMP kinase, if any, does not seem to overcome the damage-induced cell death phenotype resulting from BER failure in the PARG-proficient cells. Regardless, it is interesting to speculate that PARG may regulate AMP kinase activation in response to reactive oxygen species–induced PARP1 activation (63). In all, these studies imply that the alkylation-sensitive phenotype of Polβ-deficient cells is unrelated to the accumulation of PAR catabolites, such as ADP-ribose or AMP, and is likely wholly dependent on the metabolite bioavailability or bioenergetic capacity of the cell.

The overriding response to the loss of Polβ and an inability to complete BER (BER failure) is energy failure or depletion of bioenergetic metabolites with no evidence for cell death triggered by PAR or the PAR catabolites ADP-ribose or AMP. The energy collapse or depletion of
NAD⁺ and ATP due to BER failure is offset by elevated levels of NMN (54) and is negatively affected by NAD⁺ biosynthesis inhibition (FK-866), suggesting that (a) FK-866 (APO866) and related clinically useful NAD⁺ biosynthesis inhibitors might be combined with temozolomide and BER inhibitors to improve temozolomide response and (b) any stress on or defects in the NAD⁺ biosynthesis pathway such as overactivation of SIRT1 (64) or attenuating defects in nicotinamide phosphoribosyltransferase, NMNAT1, or related NAD biosynthetic enzymes (65) may have significant effects on cell survival following BER failure.

Similar phenotypes (stress-induced PARP1 activation and cell survival dependent on NAD⁺ metabolites) have been observed in diverse human cell types and mammalian organ systems, stressing the significance of these findings. PARP1 activation and the resulting “NAD⁺ depletion”-mediated or ATP depletion-mediated cell death play critical roles in tissue injury from cerebral and myocardial ischemia (66–69). Analogous to the studies described herein, cellular protection from cerebral ischemia is provided by NAD⁺ metabolite supplementation (70, 71). Similarly, streptozotocin-induced diabetes results from PARP1 activation, energy imbalance, and cell death dependent on the BER enzyme MPG (72–75). Most importantly, cellular NAD⁺ metabolism plays an essential role in pancreatic β-cell viability and insulin secretion (76). With the observation that BER failure triggers NAD⁺ depletion, it is interesting to speculate if overall BER capacity controls susceptibility to ischemia or streptozotocin-induced diabetes in age-related diabetes, reperfusion injury or β-cell death from loss of bioenergetic metabolites after BER failure. The onset of these physiologically significant outcomes (stroke, neurodegeneration, ischemia, and diabetes) involves PARP1 activation, NAD⁺ depletion, and cell death, similar to that reported here. Although a portion of the environmental and endogenous stressors that induce these phenotypes via PARP1 activation will directly induce DNA single-strand breaks, it is reasonable to presume that a significant proportion of cell death related to stroke, reperfusion injury, ischemia, and diabetes may initiate from genomic DNA base damage, requiring repair by the BER machinery. As such, the failure to repair the DNA damage and the resulting accumulation of DNA repair intermediates (BER failure) may be the trigger of PARP1 activation and cell death.

In summary, these studies suggest that PARP1 functions as a BER molecular sensor protein to induce caspase-independent cell death following BER failure and provides mechanistic insight into why Polβ deficiency leads to cell death. Further, we show that the observed DNA damage–dependent cell death in Polβ-deficient cells is unrelated to the accumulation of PAR catabolites, such as ADP-ribose or AMP, yet is dependent on NAD⁺ metabolite bioavailability or bioenergetic capacity of the cell, suggesting a linkage between DNA repair capacity, cell survival, and cellular bioenergetic metabolites. Finally, these studies have potentially important implications for therapeutic development as it relates to a chemotherapeutic-induced synthetic lethality approach to cancer therapy involving the combination of a chemotherapeutic DNA-damaging agent, a DNA repair inhibitor, and a regulator or inhibitor of NAD⁺ biosynthesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

Regulation of Alkylating Agent–Induced Cell Death


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