Abundance of BER-related proteins depends on cell proliferation status and the presence of DNA polymerase β

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ABSTRACT

In mammalian cells, murine N-methylpurine DNA glycosylase (MPG) removes bases damaged spontaneously or by chemical agents through the process called base excision repair (BER). In this study, we investigated the influence of POL β deficiency on MPG-initiated BER efficiency and the expression levels of BER-related proteins in log-phase and growth-arrested (G₀) mouse embryonic fibroblasts (MEFs). G₀ wild-type (WT) or POL β–deficient (Pol β–KO) cells showed greater resistance to methyl methanesulfonate than did log-phase cells, and repair of methylated bases was less efficient in the G₀ cells. Apex1 mRNA expression was significantly lower in Pol β–KO or G₀ WT MEFs than in log-phase WT MEFs. Moreover, although Mpg mRNA levels did not differ significantly among cell types, MPG protein levels were significantly higher in log-phase WT cells than in log-phase Pol β–KO cells or either type of G₀ cells. Additionally, proliferating cell nuclear antigen protein levels were also reduced in log-phase Pol β–KO cells or either type of G₀ cells. These results indicated that MPG-initiated BER functions mainly in proliferating cells, but less so in G₀ cells, and that POL β may be involved in regulation of the amount of intracellular repair proteins.

KEYWORDS: BER, MPG, mouse, cell proliferation, POL β

INTRODUCTION

In mammalian cells, DNA is damaged by various endogenous and environmental factors [1, 2]. Under physiological conditions, base damage is the most common DNA lesion and is estimated to occur spontaneously in a single proliferating mammalian cell about 10 000 times each day [3]. This damage can cause cell death and mutation via induction of stalled replication forks and base mispairing. The base excision repair (BER) mechanism is highly conserved from prokaryotes to mammalian cells, but the intricate relationships among various repair proteins have hindered complete documentation of the BER mechanism. Also, the amount of spontaneous base damage has been estimated for ‘proliferating’ cells, but information on this amount in non-proliferative cells is relatively limited.

Sykora and colleagues recently showed that differentiated human neural cells exhibit resistance to methyl methanesulfonate (MMS) and that MMS-induced DNA damage is repaired slightly less efficiently in terminally differentiated cells than in differentiating cells [4]. MMS has been used to induce base damage in many studies to investigate BER mechanisms because the product spectrum is well established, with >90% of the products being the toxic 3-methyladenine (3meA, 11%) or the less harmful 7-methylguanine (7meG, 83%) [5]. In our previous study, the yield of methylated bases induced by MMS treatment in log-phase HeLa cells is estimated to be 7–8/10⁷ nucleotides/mM [6]. Even in rapidly growing cells, repair of methylated bases is slow, and about half of the MMS-induced damage is unrepaird 24 h after treatment [7]. In rat liver, the most abundant product, 7meG, has a half-life of about 48 h in non-proliferative cells [8], and accumulated damage increases with rat age [9]. The differences in removal rates between these cells may be ascribed to their growth states.

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Most bases that are methylated by MMS are exclusively removed by N-methylpurine DNA glycosylase (MPG), which initiates single-nucleotide BER (SN-BER). MPG has a broad substrate specificity, including 3meA, 7meG, hypoxanthine (Hx), xanthine and ethenoadenine [10–13]. In cells overexpressing MPG, the number of BER intermediates, such as AP sites and single-strand breaks, increases after treatment with alkylating agents as a result of imbalanced BER, and these BER intermediates can lead to chromosomal aberrations and sister chromatid exchange [14, 15]. These results may be attributable to uncontrolled MPG-mediated conversion of 7meG into more toxic BER intermediates [16]. In contrast, MPG-deficient HeLa cells display hypersensitivity to alkylating agents, and MPG-deficient mouse ES cells have higher levels of chromosome damage than do WT cells [17, 18]. Taken together, these results show that control of BER expression level during BER is critical for genetic integrity.

Because proteins involved in long-patch BER (LP-BER), such as proliferating cell nuclear antigen (PCNA), flap endonuclease 1 (FEN1), and DNA ligase I, are downregulated in non-dividing cells [4], the SN-BER pathway, in which POL β plays a pivotal role, is likely to be the predominant process responsible for repair of alkylated base lesions. Therefore, POL β deficiency should result in elevated sensitivity to MMS in non-proliferative cells, but less so in log-phase cells. During SN-BER, AP endonuclease 1 (APEX1) cleaves the DNA strand at the 5′-side of an AP site and leaves a 5′-deoxyribose phosphate (5′-dRP). POL β removes the 5′-dRP using its lyase activity and inserts a correct nucleotide. Exponentially growing POL β-deficient mouse embryonic fibroblasts (MEFs) are hypersensitive to MMS [19, 20], and expression of just the POL β 5′-dRP lyase domain in these cells abrogates this hypersensitivity [21]. The cytotoxicity of 5′-dRP created by MPG and APEX1 may result in the hypersensitive phenotype of POL β-deficient cells. Moreover, hypersensitivity to MMS or N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) does not occur in cells deficient for both POL β and MPG [22]. These results indicate that hypersensitivity of POL β-deficient cells to MMS depends on MPG. Using a comet assay, Pascucci et al. showed that POL β deficiency decreases the efficiency of SSB repair more significantly in G1 phase MEFs than in S phase MEFs [23].

These results may indicate that BER-related proteins are downregulated by a lack of POL β, because the activities of these proteins are thought to be strictly controlled. However, more direct evidence is needed to convincingly document these relationships. In this study, we investigated the mRNA and protein levels from BER-related genes in log-phase and non-proliferating (G0) mouse cells to understand the contribution of the BER system to cell viability.

MATERIALS AND METHODS

Antibodies

Anti-MPG antibody (Proteintech, Chicago, IL, USA), anti-XRCC1 and anti-APEX1 antibodies (Abcam, Cambridge, England), anti-POL β antibody (Trevisgen, Gaithersburg, MD, USA), anti-β-Actin antibody (Sigma-Aldrich, St Louis, MO, USA) and anti-PCNA antibody (Cell Signaling Technology, Danvers, MA, USA) were used in the study.

Cell culture and MMS treatment

MBl6tsA and MBI9tsA MEFs, which were kindly provided by Dr Masahiko Miura (Tokyo Medical and Dental University), were used as wild-type (WT) and POL β-deficient (Pol β-KO) cells, respectively [19, 20]. Cells were cultured in Eagle’s MEM (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) containing 10% fetal bovine serum (FBS; Thermo Scientific, Waltham, MA, USA), 1% sodium pyruvate, and MEM non-essential amino acids (Life Technologies, Carlsbad, CA, USA) at 37°C in a humidified atmosphere containing 5% CO2. To generate cultures with cells in G0 phase, 80% confluent cultures were incubated in FBS-free Eagle’s MEM for 24 h. Cell cycle distribution was assessed with propidium iodide (PI) and FACS CaliburTM (Becton, Dickinson, Franklin Lakes, NJ, USA). Log-phase and G0 cells were incubated in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, 0.9 mM CaCl2, 0.49 mM MgCl2) containing MMS (Sigma–Aldrich) at 37°C for 1 h. After MMS treatment, cells were immediately washed twice with Hank’s balanced salt solution (HBSS; Sigma–Aldrich). For each ARP assay, DNA was extracted immediately and 24 h after MMS treatment.

ARP assay

Each ARP assay was performed as described previously with slight modifications [6]. Cells were harvested by trypsinization, and DNA was extracted via the neutral guanidine thiocyanate-phenol-chloroform method [24, 25]. The prepared DNA was treated with 5 mM alkaline-reactive probe (ARP; Dojindo Molecular Technologies, Kumamoto, Japan) in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) at 37°C for 1 h. DNA recovered by ethanol precipitation was dissolved in TE buffer, and the DNA concentration was adjusted to 1 μg/ml. DNA solution (200 μl) was added to each well of a protein- coated plate and incubated at 37°C for 1 h. After washing the plate with TTBS (PBS (−) containing 1% Tween 20), 100 μl of 1:500 diluted ABC solution (avidin-biotinylated horseradish peroxidase complex; Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA) was added, and the mixture was incubated at room temperature for 30 min. After washing with TTBS, 200 μl of HRP substrate solution (0.2 M Na2HPO4, 0.1 M citric acid, 0.7 mg/ml o-phenylenediamine, 0.5% H2O2) was added into each well and mixtures were incubated at room temperature for 30 min in the dark. Each reaction was stopped by adding 40 μl of 8 N H2SO4, and the absorbance in each well was measured at 495 nm with a Model 680 Microplate Reader (Bio-Rad, Irvine, CA, USA).

Depurination of methylpurines

In order to quantify the total amount of methylpurines, DNA was dissolved in neutral depurination buffer (0.1 M NaCl, 10 mM citric acid, 10 mM K2HPO4, pH 7.5) and incubated at 80°C for 15 min to convert methylated bases to AP sites [6]. The depurinated DNA was treated with 5 mM ARP for the ARP assays described above.

Preparation of standard DNA containing AP sites

Preparation of standard DNA containing AP sites was performed according to Lindahl and Nyberg’s procedure [3]. Calf thymus DNA (Sigma–Aldrich) was dissolved in TE buffer and purified by phenol–chloroform extraction and ethanol precipitation. The purified DNA was treated with 100 μg/ml RNase A (Sigma) at 37°C for 1 h and then subject to phenol–chloroform extraction and ethanol precipitation. The DNA solution was dialyzed against AP buffer (10 mM sodium citrate, 100 mM NaCl, pH 5.0) and diluted at 1 mg/ml; dia-
lyzed DNA was incubated at 70°C for 0–50 min and recovered by ethanol precipitation; this procedure is reported to form 1 AP site/105
Measurement of cell viability
Log-phase or G0 cells were treated with 0–7.5 mM MMS at 37°C for 1 h; cells were then incubated in growth medium for 24 h. Cells were harvested and stained with PBS(-) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄) containing 0.5% Trypan blue, and >500 cells were counted under a microscope. EC₅₀ was determined by probit regression analysis using R [26].

Real-time PCR
Total RNA was prepared with an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). cDNA was prepared via reverse transcription with 5 μg of total RNA and ReverTra Ace (Toyobo, Osaka, Japan) and an MJ Mini Personal thermal cycler (Bio-Rad). Real-time PCR was performed with primer sets shown in Table 1, SYBR Green Realtime PCR Master Mix (Toyobo, Tokyo, Japan). After SDS-PAGE, proteins were transferred to Immobilon-P (Merck KGaA, Darmstadt, Germany); membranes were then blocked, with 5% skim milk at 4°C for 16 h. Separated proteins were probed with primary antibodies, and antibody-bound antigens were detected with the appropriate secondary antibodies. The concentration of protein in each cell extract was measured with a Bio-Rad Protein assay (Bio-Rad). After SDS-PAGE, proteins were transferred to Immobilon-P (Merck KGaA, Darmstadt, Germany); membranes were then blocked, with 5% skim milk at 4°C for 16 h. Separated proteins were probed with primary antibodies, and antibody-bound antigens were detected with the appropriate secondary antibodies, ECL Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, UK), and LAS-3000 (Fujifilm, Tokyo, Japan).

RESULTS
Sensitivities to MMS of log-phase and G0 cells
First, we examined whether growth-arrested (G0) MEFs were resistant to MMS, as observed in differentiated human neural cells [4]. G0 cells were prepared by serum starvation for 24 h. Based on cytometry analysis, >80% and >70% of WT and Pol β–KO cells, respectively, were shown to be in a G0/G1 phase (Fig. 1A). To assess cell viability, log-phase and G0 cells were treated with 0–7.5 mM MMS and stained with 0.5% Trypan blue 24 h after treatment. At 3.75 or 5 mM MMS, G0 WT cells were more resistant to MMS than were log-phase WT cells (Fig. 1B); EC₅₀ values were 4.22 and 5.84 mM for log-phase and G0 WT cells, respectively. Among Pol β–KO cells, there were also significant differences between log-phase and G0 cells at MMS concentrations from 1.5 to 2.5 mM (Fig. 1C); the respective EC₅₀ values were 1.41 and 2.20 mM. As with results for differentiated cells [4], these findings indicated that non-proliferating cells were less sensitive to MMS than were proliferating cells, regardless of the presence or absence of POL β. The increased sensitivities to MMS resulting from the deficiency of POL β in log-phase and G0 cells (3.0 and 2.7-fold increases, respectively) indicated that POL β contributed to cell viability in both log-phase and G0 cells.

BER activities in log-phase and G0 cells
Findings from cell viability assays revealed that G0 cells, either WT or Pol β–KO, were resistant to MMS. In G0 cells, MMS may have difficulty reacting with DNA bases because chromatin may be more tightly condensed. Thus, there may be fewer bases with MMS-induced damage in G0 cells than in log-phase cells. DNA damage after MMS treatment was measured via ARP assays. The numbers of AP sites and methylated bases induced by 1-mM MMS in G0 cells were compared with those in log-phase cells (Table 2). In G0 cells, 1-mM MMS induced 0.07 (WT) and 0.09 (Pol β–KO) AP sites and 6.68 (WT) and 6.53 (Pol β–KO) methylated bases. In contrast, in log-phase, 1 mM MMS induced 1.06 (WT) and 1.30 (Pol β–KO) AP sites and 6.68 (WT) and 6.53 (Pol β–KO) methylated bases. These data indicated that both WT and Pol β–KO cells have more efficient repair of methylated bases than log-phase cells, but that they are more resistant to MMS than genotype-matched log-phase cells. It is conceivable that accumulation of AP sites in log-phase Pol β–KO cells may occur, because SP-BER does not proceed after MPG and APEX1 activity. However, for log-phase cells, significantly fewer AP sites were found in Pol β–KO cells than in WT cells at 0 h; additionally, significantly more methylated bases remained in the Pol β–KO cells at 24 h (P < 0.05, Table 2). Thus, BER might be suppressed in log-phase Pol β–KO cells.

Table 1. Primer sets used in this study

| Primer set       | Sequence                                                                 |
|------------------|--------------------------------------------------------------------------|
| Mpg forward      | 5′-TGAATTGTCTCTAGTCAAGGG-3                                               |
| Mpg reverse      | 5′-AGTGCTTTTTCGAGGGAGGT-3′                                              |
| Xrec1 forward    | 5′-GGGAACTCGCCATACAGGAA-3′                                               |
| Xrec1 reverse    | 5′-GGCTCCACAGATGAGAACAC-3′                                               |
| Polβ forward     | 5′-ACTGCCAGGAGTAGGAAACAC-3′                                              |
| Polβ reverse     | 5′-AGATGGTTCAATGCCAGTA-3′                                               |
| Apex1 forward    | 5′-TCTTGTGGCCTCAAGAGACC-3′                                              |
| Apex1 reverse    | 5′-TTTCTTCTCCTGCAATGGC-3′                                               |
| β-Actin forward  | 5′-AGCGCAAGTACTCTGTGGA-3′                                               |
| β-Actin reverse  | 5′-AAGCGAGCTCAGTAAACAGTC-3′                                            |
activity is stimulated by proteins such as APEX1 and XRCC1 that work late in BER [28, 29]. Therefore, to determine the steady-state levels of repair proteins, the expression levels of mRNAs participating in MPG-initiated BER were measured in log-phase and G0 cells (Fig. 2).

Log-phase WT and Pol β–KO cells differed significantly with regard to Apex1 mRNA levels (Fig. 2A). The amount of Apex1 mRNA in log-phase Pol β–KO cells was 43% of that in log-phase WT cells, although both cell types expressed Apex1 mRNA at similar levels in G0 cells (Fig. 2B). Expression levels of Apex1 and Pol β mRNAs in G0 WT cells were 34% and 48%, respectively, of those in log-phase WT cells (Fig. 2A and B). In contrast, Mpg and Xrcc1 mRNA levels did not differ significantly between WT and Pol β–KO cells for either log-phase or G0 cells (Fig. 2). These results indicated that expression of Apex1 and Pol β mRNAs depended on cell proliferation. Apex1 mRNA expression was also significantly suppressed in log-phase Pol β–KO cells, but it was not clear whether this difference was the result of the POL β deficiency (Fig. 2).
Expression of MPG and BER-related proteins in log-phase and G₀ phase

To determine whether the findings for mRNA expression were reflected in protein levels, the amount of each protein in log-phase and G₀ cells was examined on immunoblots (Fig. 3). Consistent with the amounts of mRNA expression, the amount of APEX1 protein in log-phase Pol β-KO cells was 48% of that in log-phase WT cells (Fig. 3C). For WT and Pol β-KO cells, APEX1 levels were significantly lower in G₀ cells than in the genotype-matched log-phase cells (60% and 78%, respectively, Fig. 3C). Reduced MPG levels were also observed in G₀ WT and G₀ Pol β-KO cells (55% and 50% of the genotype-matched log-phase cells, respectively; Fig. 3B), suggesting that MPG had a more important role in growing cells than in quiescent cells and that the amount of MPG was regulated depending on cell proliferation state. Unexpectedly, the MPG protein level in log-phase Pol β-KO cells was 52% of that in log-phase WT cells, although Mpg mRNA levels did not differ between these cell types (Figs 2, 3B). In contrast, the POL β protein levels did not differ between G₀ WT cells and log-phase WT cells, even though Pol β mRNA levels were lower in G₀ WT cells (Figs 2, 3E). The XRCC1 protein level was constant regardless of POL β or the cell proliferation status (Fig. 3D). These results indicated that low levels of MPG and APEX1 proteins in G₀ WT, G₀ Pol β-KO, and log-phase Pol β-KO cells were responsible for the low number of AP sites and the slow removal of methylated bases in each of these cell types.

Expression of PCNA protein in log-phase and G₀ phase

As shown in Fig. 3, MPG and APEX1 protein levels were lower in G₀ WT, G₀ Pol β-KO, and log-phase Pol β-KO cells than in log-phase WT cells, suggesting that SN-BER was not efficient in each of these three cell types. In log-phase Pol β-KO cells, SN-BER is unlikely to occur because POL β is also absent and because of low levels of MPG and APEX1. To investigate whether LP-BER, the other BER pathway, was affected in these cells, the amount of PCNA protein was measured. In log-phase Pol β-KO cells, the amount of PCNA was 29% of that in log-phase WT cells (Fig. 4A and B), whereas there was no significant difference in PCNA between G₀ WT and G₀ Pol β-KO cells. The PCNA protein level in G₀ WT cells was 50% of that in log-phase WT cells, but there was no statistically significant growth state-dependent change in PCNA in Pol β-KO cells (Fig. 4B). These results suggested that LP-BER was also less efficient in G₀ WT, G₀ Pol β-KO, and log-phase Pol β-KO cells than in log-phase WT cells and that POL β deficiency may affect PCNA expression, especially in proliferating cells.

DISCUSSION

MPG has a very prominent role in repair of spontaneously methylated or deaminated purines. The sensitivity to the MMS and DNA repair capacity of human neural cells depends on the level of cellular differentiation [4]; specifically, levels of SN-BER–related (APEX1, DNA Ligase III and XRCC1) and LP-BER–related enzymes are significantly lower in differentiated cells. MPG also interacts with PCNA and transcription activation factor estrogen receptor α [30, 31], and thus has a role in ensuring genomic integrity, mainly in proliferating cells. In this study, we examined the influence of POL β deficiency on MPG-initiated BER in proliferating and non-proliferating cells.

In good agreement with the previous findings [4], MMS resistance in G₀ cells occurred regardless of the presence or absence of POL β (Fig. 1). The amount of MMS-induced methylated bases in G₀ cells was similar to that in log-phase cells, but the number of AP sites in G₀ cells was 26–49% of that in log-phase cells at 0 h; additionally, more methylated bases remained in G₀ cells 24 h after removal of MMS (Table 2). The main MMS product, 7meG, is less cytotoxic than the AP sites formed from 7meG by MPG. It is conceivable that MMS resistance in G₀ cells resulted from a reduction in the number of AP sites. Thus, we concluded that the excision activity of MPG was higher in log-phase cells than in G₀ cells (Table 2) and consequently that MPG-initiated BER is more active in log-phase cells than in G₀ cells.

Immunoblot-based measurements of expression of proteins related to MPG-initiated BER showed that the amounts of MPG, APEX1 and PCNA were each affected by cell proliferative state and POL β deficiency (Fig. 3B and C and Fig. 4). BER proteins are regulated by protein–protein interactions [32]. Because MPG activity is stimulated
by APEX1 and PCNA [29, 30], decreased levels of MPG and these proteins may explain the low levels of BER activity in G0 cells.

Log-phase Pol β-KO cells, like G0 WT and Pol β-KO cells, had low levels of BER-related proteins and suppressed BER. Thus, the lack of POL β-dependent polymerase activity and/or dRP-lyase activity may have been primarily responsible for the hypersensitivity of Pol β-KO MEFs to MMS, presumably because these cells may not have been able to properly process BER intermediates.

The relative amounts of some proteins were not consistent with the relative amounts of the respective mRNA (Figs 2 and 3). Levels of APEX1 and POL β proteins are regulated by ubiquitination [33], which may explain the inconsistencies between mRNA and protein levels. Because an inconsistency was also observed for MPG protein and mRNA, it is likely that MPG is regulated in a similar way. We could not determine here whether the reduced levels of these proteins simply resulted from POL β deficiency. It is also conceivable that a clone with suppressed levels of BER enzymes was selected in the absence of POL β because the Pol β-KO cells retaining normal levels of MPG and APEX1 activity would be continuously threatened by accumulation of harmful BER intermediates.

Fig. 3. Amounts of MPG and MPG-related proteins in log-phase and G0 cells. The amount of each protein in WT and Pol β-KO cells (KO) in log-phase (Log) and G0 were measured by immunoblotting. (A) Proteins in cell lysate were separated by SDS-PAGE, and proteins were detected via antibody probes. (B–E) Quantification of immunoblot signals as shown in (A); each signal was normalized relative to the β-Actin signal. The amount in log-phase WT cells is shown as 1. *P < 0.05, **P < 0.01. Data are represented as the mean of three experiments, and error bars indicate SD.
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