7,8-dihydro-8-oxoadenine, a highly mutagenic adduct, is repaired by Escherichia coli and human mismatch-specific uracil/thymine-DNA glycosylases

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ABSTRACT

Hydroxyl radicals predominantly react with the C8 of purines forming 7,8-dihydro-8-oxouguanine (8oxoG) and 7,8-dihydro-8-oxoadenine (8oxoA) adducts, which are highly mutagenic in mammalian cells. The majority of oxidized DNA bases are removed by DNA glycosylases in the base excision repair pathway. Here, we report for the first time that human thymine-DNA glycosylase (hTDG) and Escherichia coli mismatch-specific uracil-DNA glycosylase (MUG) can remove 8oxoA from 8oxoA-T, 8oxoA-G and 8oxoA-C pairs. Comparison of the kinetic parameters of the reaction indicates that full-length hTDG excises 8oxoA, 3,N4-ethenocytosine (εC) and T with similar efficiency (k_{max} = 0.35, 0.36 and 0.16 min^{-1}, respectively) and is more proficient as compared with its bacterial homologue MUG. The N-terminal domain of the hTDG protein is essential for 8oxoA-DNA glycosylase activity, but not for εC repair. Interestingly, the TDG status had little or no effect on the proliferation rate of mouse embryonic fibroblasts after exposure to γ-irradiation. Nevertheless, using whole cell-free extracts from the DNA glycosylase-deficient murine embryonic fibroblasts and E. coli, we demonstrate that the excision of 8oxoA from 8oxoA-T and 8oxoA-G has an absolute requirement for TDG and MUG, respectively. The data establish that MUG and TDG can counteract the genotoxic effects of 8oxoA residues in vivo.

INTRODUCTION

Accumulation of oxidative damage to DNA has been implicated in diverse human diseases, including cancer, neurological disorders and premature aging. Hydroxyl radicals (OH•) preferentially react with C8 atom of purines to generate 7,8-dihydro-8-oxouguanine (8oxoG), 7,8-dihydro-8-oxoadenine (8oxoA) and formamido-pyrimidines (Fapy) residues in DNA (1–2). Although 8oxoA is present at approximately one-tenth level of 8oxoG (1), 8oxoA and FapyA are the most abundant oxidized adenine adducts in irradiated DNA samples (3). The presence of 8oxoA in duplex DNA does not perturb its B-helix conformation and DNA synthesis (4). Structural studies have shown that both oxidized purines are present predominantly in keto form and can adopt ‘syn’ conformation (5). In vitro, DNA polymerases almost exclusively incorporate dTMP opposite to 8oxoA in the presence of all four dNTPs (6). In Escherichia coli, no mutagenesis was observed on transfection of the single-stranded M13 phage DNA containing 8oxoA (7). The studies on mutagenesis in mammalian cells, however, showed that 8oxoA can induce mutations at a significant rate (8–9). Kamiya et al. (8) reported that in 3T3 mouse fibroblasts, 8oxoA induces A→C and A→G mutations when incorporated in the sequence context 5′-CCXAG-3′ of an exogenous double-stranded c-Ha-ras gene and that the mutagenic potency of 8oxoA was similar to the one of 8oxoG. However, Tan et al. (9) compared the mutagenic property of 8oxoA in two different sequence contexts in single-stranded DNA vectors in simian kidney cells. At variance with the previous study, they detected mainly A→C transversion with frequencies at least four times lower than for 8oxoG.
The majority of oxidative DNA base damage is removed by the base excision repair (BER) pathway. BER is initiated by a DNA glycosylase that cleaves the N-glycosyl bond between the abnormal base and the deoxyribose, generating either an abasic site or single-stranded break in DNA. The *E. coli* Fpg and Nei DNA glycosylases excise 8oxoA poorly, whereas the eukaryotic 8oxoG→DNA glycosylases (OGG1) and mammalian endonuclease VII-like protein 1 (NEIL1) can efficiently remove 8oxoA, but only when it is paired with cytosine (10–11). Based on these observations, it was hypothesized that in eukaryotes, 8oxoA•C base pairs may occur by misincorporation of the oxidized DNA precursor 8-oxo-2′-deoxyadenosine 5′-triphosphate (8oxodATP) opposite to cytosine during DNA replication. Indeed, the human (2′-deoxy)ribonucleoside 5′-triphosphate pyrophosphohydrolase (MTH1) protein, a dNTPs pool sanitizing enzyme, hydrolyses with good efficiency 8oxodATP in contrast to its *E. coli* counterpart MutT protein, which lacks activity on this oxidized precursor (12). Interestingly, an unidentified mono-functional mammalian DNA glycosylase, distinct from OGG1, which can remove 8oxoA when paired with guanine, has been also described (13). However, so far, no DNA glycosylase excising 8oxoA from its naturally occurring pair with thymine has been reported.

The human thymine-DNA glycosylase (hTDG) was first biochemically characterized for its ability to remove T mispaired with G (14). More detailed characterization showed that hTDG exhibits a wide substrate specificity: it excises 3,N4-ethenocytosine (eC) (15,16), thymine glycol (17), 5-hydroxycytosine (18), mismatched uracil (19) and its derivatives with modifications at the C5 position (20). The hTDG protein exhibits an extremely low turnover rate for both BER and histone acetylation (24). The hTDG co-activator CBP/p300 (CREB-binding protein/p300), hTDG protein interacts with the transcriptional activator CBP/p300 (CREB-binding protein/p300), and the resulting hTDG/CBP/p300 complex is competent for both BER and histone acetylation (24). The hTDG protein enhances the CBP/p300 transcriptional activity and, in turn, CBP/p300 acetylates hTDG. Acetylation of hTDG regulates the recruitment of APE1. Thus, TDG functions are tightly regulated by post-translational modifications (SUMOylation and acetylation) and by protein–protein interactions. Interestingly, TDG knockout mice are embryonically lethal, possibly because of aberrant de novo DNA methylation of CpG-rich promoters of developmental genes, which results in failure to establish and/or maintain cell type-specific gene expression programs during embryonic development (25,26). Recently, it has been demonstrated that oxidized derivatives of 5-methylcytosine (5mC) in CpG sequence contexts are efficiently processed by human TDG, suggesting a direct involvement of TDG-initiated BER in protection of gene promoters from aberrant DNA methylation (27,28).

*Escherichia coli* mismatch-specific DNA glycosylase MUG was identified by sequence homology to mammalian TDG and was characterized as enzyme excising uracil from G•U mismatch with a possible role as a back up enzyme for the classical uracil–DNA glycosylase (UDG) (29). MUG is a small 18.6 kDa protein, able to remove various ε-bases, including εC (16,30) and 1,N2-ethenoguanine (31) and also with low efficiency hypoxanthine and 5-hydroxyctosine residues when present in DNA (32).

At present, the mechanisms of 8oxoA repair when paired with thymine are only poorly investigated. Here, we report that *E. coli* MUG and human TDG are able to excise 8oxoA in 8oxoA•T pairs. To address the physiological relevance of the MUG and hTDG-catalysed excision of 8oxoA, we measured kinetic parameters using various DNA substrates and characterized 8oxoA–DNA glycosylase activities in whole cell-free extracts from BER deficient cells. The role of MUG/TDG-initiated BER in counteracting oxidized adenine residues in duplex DNA is discussed.

**MATERIALS AND METHODS**

**Chemicals, reagents and proteins**

Cell culture media were purchased from Invitrogen (Life Technologies SAS, Saint Aubin, France). Restriction enzymes and T4 DNA ligase were from New England Biolabs France (Evry, France). The *E. coli* BL21 (DE3) cells were from Novagen-EMD4Biosciences (Merck Chemicals, Nottingham, UK). Collection of the purified DNA glycosylases and AP endonucleases was from the laboratory stock (33,34). The activities of various DNA repair proteins were tested using their principal substrates and were checked just before use (data not shown).

**Oligonucleotides**

All oligonucleotides containing modified bases and their complementary strands were purchased from Eurogentec (Seraing, Belgium) including the following: 40 mer d(AAT TGCTATCTAGCTCCGC) [supplementary Table S1].

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DNA repair assays

The standard reaction mixture (20 μl) used for screening of DNA repair activities contained 10 nM [32P]-labelled U·G, G·T, εC·G and 8oxoA·T oligonucleotide duplexes and limited amounts of enzymes for 10 min at 37°C, unless otherwise stated. Assays for the AlkA, Nth, MUG, UDG and Nfo proteins were performed in buffer containing 20 mM HEPES–KOH (pH 7.6), 50 mM KCl, 0.1 mM EDTA, 0.1 mg/ml benzamidine and 1 mM DTT. Assays for the Fpg, Nei, NEIL1 and NEIL2 proteins were performed in buffer containing 20 mM HEPES–KOH (pH 7.6), 1 mM KCl, 2 mM DTT, 0.1 mM EDTA, 0.3% non-ionic detergent Nonidet P-40 (NP-40) and protease inhibitor cocktail (Complete EDTA-free, Roche). Extracts were clarified by centrifugation at 20 000g for 15 min at 4°C.

Expression and purification of hTDG

The expression vectors pET28c–hTDG (for full-length protein) and pET28c–TDGcat (for catalytic domain protein) were previously described (37). Rosetta 2 (DE3) cells transformed with pET28c–hTDG and pET28c–TDGcat were grown at 37°C in Luria Broth medium, supplemented with 50 μg/ml kanamycin, on an orbital shaker to OD600 = 0.6–0.8. Then temperature was reduced to 30°C, and the proteins expression was induced by 0.2 mM isopropyl β-D-galactopyranoside (Sigma-Aldrich), and the cells were further grown for 2 h for induction of the hTDG or 15 h for induction of the hTDGcat proteins. Bacteria were harvested by centrifugation, and cell pellets were washed with ice-cold phosphate buffered saline and incubated 30 min in lysis buffer containing 80 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1 mM DTT and 100 μg/ml proteinase K. The mixture was incubated at 37°C for a varied period, and AP sites were cleaved by light piperidine treatment, unless otherwise stated.

The standard reaction mixture (20 μl) used for detailed kinetic characterization of DNA glycosylase activities contained 20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1 mM DTT and 100 μg/ml BSA, 50 nM [32P]-labelled duplex oligonucleotide and either 5 μM MUG or 500 nM hTDG and hTDGcat proteins. The mixture was incubated at 37°C for a varied period, and AP sites were cleaved by light piperidine treatment, unless otherwise stated.

Mouse embryonic fibroblast (MEF–WT and MEF–Tdg−/−) cell lines were obtained as previously described (36). MEF–WT and MEF–Tdg−/− cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in the presence of 5% CO2. To prepare whole-cell extracts, MEF cells were washed with ice-cold phosphate buffered saline and incubated 30 min in lysis buffer containing 80 mM HEPES–KOH (pH 7.6), 1 M KCl, 2 mM DTT, 0.1 mM EDTA, 0.3% non-ionic detergent Nonidet P-40 (NP-40) and protease inhibitor cocktail (Complete EDTA-free, Roche). Extracts were clarified by centrifugation at 20 000g for 15 min at 4°C.

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electrophoresis (PAGE), and fractions containing the pure His-tagged hTDG and hTDG\textsuperscript{cat} proteins were stored at −80°C in 50% glycerol. The concentration of purified proteins was determined by the method of Bradford.

**Single turnover kinetics**

Here, we used single turnover kinetics under large excess of enzyme over substrate (K\textsubscript{d}<[E]≫[S]) to obtain rate constants (k\textsubscript{obs}) that are not affected by enzyme–substrate association or by product inhibition, such that k\textsubscript{obs} reflects the maximal base excision rate (k\textsubscript{obs} ≈ k\textsubscript{max}) (38–40). The data were fitted by non-linear regression to one phase exponential association Equation 1 using GraphPad Prism 5 software.

\[ \text{Fraction product} = A(1 - \exp(-k_{\text{max}}t)) \]  

(1)

where \( A \) is the amplitude, \( k_{\text{max}} \) is the rate constant and \( t \) is the reaction time (in seconds).

The enzymatic assays were performed in large volume reaction mixture containing 50 nM duplex oligonucleotide and 5 μM MUG or 500 nM hTDG for varying periods at 37°C. At each time point, 20 μl of sample was withdrawn, and reaction was stopped by light piperidine treatment. Reaction products were analysed as described earlier in the text.

**In vitro reconstitution of 8oxoA\textsuperscript{T} repair by hTDG**

5 nM 8oxoA\textsuperscript{T} unlabelled duplex was incubated in the presence of 20 nM hTDG, 5 nM APE1, 2 nM flap-structure endonuclease 1 (FEN1), 0.1 U of DNA polymerase-β (POL-β) and 5 nM T4 DNA Ligase, 50 μM dNTPs, 20 μCi of [γ-\textsuperscript{32}P]dATP in buffer containing 50 mM HEPES–KOH (pH 7.6), 30 mM NaCl, 0.1 mg ml\textsuperscript{-1} BS A, 2 mM DTT, 2 mM ATP and 3 mM MgCl\textsubscript{2} for 5 and 30 min at 37°C, unless otherwise stated. Reaction products were analysed as described earlier in the text.

**Proliferation assay of MEF cells exposed to γ-irradiation**

When the exponentially growing WT and Tdg\textsuperscript{−/−} MEFs cell density reached ≈80% confluence, cells were trypsinized, harvested and resuspended in phosphate buffered saline at a concentration of 10\textsuperscript{6} cells/ml. The cell suspensions were exposed to a single dose ranging from 0, 1, 2, 3, 5, 10, 15 and 20 Gy using a \textsuperscript{137}Cs source (IBL 637, Institut Gustave Roussy, Villejuif, France) with a dose rate of 2.9 Gy/min. Cells were plated in triplicate for each dose (100 000 cells per well in 24-well plate) with fresh Dulbecco’s modified Eagle’s medium medium; the non-irradiated control cells were plated in the same way. Cells were incubated at 37°C in humidified atmosphere containing 5% of CO\textsubscript{2} for indicated period; at regular intervals, cells were trypsinized, stained with trypan blue (Sigma-Aldrich) and counted under a microscope. At each time point, number of viable WT and Tdg\textsuperscript{−/−} MEFs cells after irradiation were reported to number of the corresponding viable non-irradiated control cells for each radiation dose. The results were expressed as the percentage of viable cells in irradiated samples relative to that in corresponding control non-irradiated cells. Kinetics of WT and Tdg\textsuperscript{−/−} MEFs proliferation were presented as fold increase in cell numbers depending on time. The data were calculated from three independent experiments and presented as the mean ± standard error. Statistical analyses were performed using GraphPad Prism 5 software.

**RESULTS**

**Mono-functional mismatch-specific uracil/thymine DNA glycosylases MUG and hTDG excise 8oxoA residue paired with thymine**

We examined whether 8oxoA\textsuperscript{T} pair is a substrate for previously characterized BER enzymes from E. coli and human. For this, a \( 5'[^{32}P] \)-labelled 40 mer oligonucleotide duplex containing a single 8oxoA\textsuperscript{T} pair was challenged with a variety of highly purified AP endonucleases and DNA glycosylases. As not all DNA glycosylases possess an AP lyase activity, the samples incubated with mono-functional DNA glycosylases were treated with light piperidine to cleave the potential abasic sites generated by the excision of 8oxoA. As shown in Figure 1, only incubation with E. coli MUG, human full-length TDG (hTDG) and catalytic domain TDG (hTDG\textsuperscript{cat}) led to the cleavage of the labelled oligonucleotide at the position of 8oxoA and generation of 19 mer product fragment (lanes 6, 15 and 16). As expected, human OGG1 and NEIL1 cleave the 8oxoA\textsuperscript{T} duplex with extremely low efficiency (lanes 12 and 13). It should be noted that 19 mer cleavage fragments generated by MUG/hTDG coupled to light piperidine treatment and OGG1 contain a 3’-phosphoradjaldehyde terminus and migrate at the same position (lanes 6, 12 and 15,16), whereas that of NEIL1 generated fragment contains 3’-phosphate terminus and migrates faster than that of OGG1 (lane 13 versus 12). Despite being used in excess amount, the pure AlkA, Fpg, Nth, Nei, MutY, UDG, Nfo, APE1,
ANPG, NEIL2, MBD4, SMUG1, Ung2 and MYH proteins did not act on 8oxoA•T (lanes 2–5, 7–11, 14 and 17–20).

Kinetic parameters of the excision of 8oxoA, U, T and εC residues when present in duplex DNA by hTDG and MUG

Previously, it was demonstrated that MUG and hTDG are product inhibited; they remain bound to the produced AP site opposite guanine with a higher affinity than for the substrate U•G and T•G DNA duplexes (21,39). As MUG and hTDG fail to turnover during reaction, which implies that each molecule of enzyme can only remove one uracil or thymine residue, comparing the steady-state kinetic parameters, such as $K_M$ and $k_{cat}$ of these DNA glycosylases for different DNA substrates is not useful. Therefore, we further characterized substrate specificity of hTDG, hTDGcat and MUG by measuring the cleavage rates of 8oxoA•T, U•G, T•G and εC•G duplexes under single-turnover conditions, using a molar excess of enzyme over DNA substrate, which provide the maximal rate of base excision ($k_{max}$) for a given substrate (38–40) (Supplementary Figure S1 and Supplementary Table S1). It should be noted that the kinetic parameter $k_{max}$ determined under single-turnover conditions measures the maximal rate of AP site product formation after DNA binding and before product release; therefore, it does not reflect product release as in case of $k_{cat}$ (40). The time course of the cleavage product generation shows that U•G is the most preferred substrate for hTDG followed by εC•G and then by 8oxoA•T and T•G, which are cleaved with similar efficiency (Supplementary Figure S1A and B). Importantly, MUG reaches a plateau of only 15% of 8oxoA•T cleaved after 2 h of incubation, indicating that the bacterial DNA glycosylase has low affinity for the DNA substrate (Supplementary Figure S1C). Under the experimental condition used, the truncated version of human enzyme hTDGcat exhibits lower activity on all DNA substrate tested (Supplementary Figure S1B), which is in agreement with a previous report (37). The $k_{max}$ constants were determined by fitting the single-turnover data to one phase exponential association using non-linear regression equation using GraphPad Prism 5.

Table 1. Pre-steady-state kinetic parameters of the hTDG, hTDGcat and MUG proteins acting on various DNA substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>hTDG</th>
<th>hTDGcat</th>
<th>MUG</th>
</tr>
</thead>
<tbody>
<tr>
<td>8oxoA•T</td>
<td>0.35±0.05</td>
<td>ND</td>
<td>0.085±0.003</td>
</tr>
<tr>
<td>U•G</td>
<td>6.1±3.7</td>
<td>0.19±0.04</td>
<td>0.101±0.003</td>
</tr>
<tr>
<td>T•G</td>
<td>0.16±0.01</td>
<td>0.056±0.003</td>
<td>ND</td>
</tr>
<tr>
<td>εC•G</td>
<td>0.36±0.05</td>
<td>0.27±0.09</td>
<td>15.0±5.8</td>
</tr>
</tbody>
</table>

$^a$kmax constants were calculated by one phase exponential association equation using GraphPad Prism 5.

To further characterize 8oxoA repair, we examined the base pair specificity of MUG and hTDG DNA glycosylase activities using 8oxoA•T, 8oxoA•A, 8oxoA•C and 8oxoA•G oligonucleotide duplexes. Similar excision efficiencies were observed for full-length hTDG when 8oxoA was paired with T, C or G, whereas no activity was observed on 8oxoA•A (Figure 2A). For *E. coli* MUG, excision of 8oxoA was more efficient when paired to either C or G than to T or A (Figure 2B). In summary, the relative order for opposite base-dependent incision of 8oxoA•N oligonucleotide duplexes was G>T>C>>A for hTDG and C≥G>T>A for MUG (Supplementary Table S1).

To examine whether excision of 8oxoA residues depends on the neighbouring sequence context, we used DNA duplexes with varying bases 5’ and 3’ to the oxidized adenine (Supplementary Table S2). At variance to the
previously described context specificity for thymine excision (41), the rate of 8oxoA excision by hTDG varied little among seven sequence contexts tested (Supplementary Figure S2), the maximum difference being only 2-fold between the most (5'-CpAmpC-3'/5'-GpTpG-3') and least (5'-GpAmpC-3'/5'-GpTpC-3') preferred one (Supplementary Figure S2).

**In vitro reconstitution of the BER pathway for 8oxoA paired with thymine**

As shown earlier in the text, the hTDG-catalysed excision of 8oxoA generates an AP site, which may be further processed by either the short-patch or the long-patch BER pathway. To examine the contribution of both downstream BER processes, we have reconstituted the BER pathway for the 8oxoA*T pair in **in vitro** using purified proteins (Figure 3 and Supplementary Figure S3). Incubation of a 40 mer 8oxoA*T oligonucleotide duplex in the presence of hTDG, APE1, FEN1, DNA POL-β, all dNTPs except for dATP and [α-32P]-dATP for 5 min generated labelled major 20 mer and minor 40 mer DNA products (lane 2). Continued incubation of the reaction mixture resulted in accumulation of the labelled full-length 40 mer DNA product (lane 3). These results indicate that APE1 cleaves AP sites generated by hTDG and allows POL-β to remove the 5'-deoxyribose phosphate residue and either insert one nucleotide (lane 2) and/or initiate strand-displacement (lane 3). Addition of T4 DNA ligase completes the restoration of the full-length 40 mer duplex and results in disappearance of 20 mer cleavage product (lane 4). In addition, we have studied in **in vitro** reconstitution of 8oxoA repair when any one of these five proteins was omitted from the reaction (Supplementary Figure S3). As expected, no significant incorporation of [α-32P]-dATP was observed in the absence of either hTDG or APE1 or POL-β. Although in the absence of either DNA ligase or FEN1, POL-β-catalysed strand-displacement synthesis can efficiently generate the full-length 40 mer DNA repair product (Supplementary Figure S3). These data demonstrate that 8oxoA residues can be processed by both the short-patch and long-patch BER pathways.

**Repair activities on DNA containing εC>G and 8oxoA*N pairs in E. coli and mouse cell-free extracts**

Data obtained with the purified DNA repair proteins show that 8oxoA paired with thymine, cytosine and guanine is mainly repaired by MUG and hTDG. To ascertain the role of these DNA glycosylases in **in vivo**, we examined εC- and 8oxoA-DNA glycosylase activities in cell-free extracts from E. coli and MEFs cells lacking MUG and TDG, respectively. It should be recalled that we and others have previously demonstrated that E. coli **mug** strain completely lacks εC-DNA glycosylase activity and that hTDG is the most efficient εC-DNA glycosylase among other mammalian repair enzymes (16,35). To measure DNA glycosylase activities in the extracts, we used 5'-[α-32P]-labelled εC>G and 8oxoA*N oligonucleotide duplexes as DNA substrates. As expected, we observed robust εC-DNA glycosylase activities in cell extracts from WT MEFs and E. coli (Supplementary Figure S4), whereas no εC-activity was observed in cell extracts from TDG knockout MEFs (Tdg<sup>−/−</sup>) and the E. coli **mug** strain (Supplementary Figure S4), indicating that MUG and TDG are major εC-DNA glycosylases in E. coli and MEF, respectively. As shown in Figure 4, cell extracts from Tdg<sup>−/−</sup> MEFs completely lack 8oxoA*T- and 8oxoA*N-specific DNA glycosylase activity (Figure 4A, lanes 5 and 11 and B), whereas extracts from WT MEFs exhibit robust cleavage activity (lanes 4 and 7). Interestingly, cleavage of the 8oxoA*C duplex is dramatically reduced in the Tdg<sup>−/−</sup> MEF extract (Figure 4A, lane 8) when compared with the WT MEF extract (lane 7), suggesting that TDG also significantly contributes to the repair of 8oxoA when paired with C (Figure 4B). The 8oxoA*A duplex is poorly processed in both MEF extracts, and the marginal cleavage activity is not dependent on TDG (Figure 4A, lanes 1–2, and B). Taken together, these results suggest that TDG represents the main 8oxoA-DNA glycosylase activity in mammalian cells. Unlike mouse cell extracts, E. coli WT and **mug** cell-free extracts did not exhibit significant 8oxoA*A-specific cleavage activities under the experimental conditions used here (Figure 4C, lanes 16–17). However, strong
and moderate dependences on presence of MUG were detected for cleavage of 8oxoA/C15G and 8oxoA/C15C, respectively (Figure 4C lanes 22–23, 19–20 and D). Only weak processing activities were found for 8oxoA/C15A, which were independent from the MUG status (Figure 4C lanes 13–14 and D). These results are in agreement with data obtained with the purified protein and suggest that MUG can efficiently excise 8oxoA when paired with C and G.

Role of the TDG protein in the cellular response to ionizing radiation

A previous study has demonstrated that the TDG status did not affect cell survival after ionizing radiation (IR) and H₂O₂ exposures (25). We, therefore, used the cell proliferation assay as a functional end point to investigate whether absence of TDG would affect the proliferation rate after IR exposure. The cells were irradiated with single doses ranging from 1 to 20 Gy of γ-irradiation and then analysed on days 1, 2, 3, 6 and 8 post-irradiation. It should be noted that under unchallenged conditions (no irradiation) the growth rate was much higher for Tdg+/C0/C0 MEFs than for WT MEFs (Figure 5A). Compared with the respective untreated cells, proliferation rates of both WT and Tdg+/C0/C0 MEFs were drastically reduced (<20% of control cells) by high doses (10–20 Gy) of IR even until the 8th day after exposure (Figure 5F–H). Importantly, no effect of the TDG status on the proliferation rate was detected at high doses of IR. At lower doses of 1–5 Gy, Tdg+/C0/C0 MEFs showed reduced growth rates over an 8-day period after IR exposure (Figure 5B–E), whereas this reduction was only transient for treated WT MEFs and recovered after 6 and 8 days post-irradiation (Figure 5C–E). Note that we observed an abnormal increase in the number of WT MEFs at the 6th day after irradiation for doses of 1–3 Gy (Figure 5B–D). However, this increase in the growth rate of irradiated WT MEFs was
not observed at the 8th day. Taken together, these data suggest that the TDG status may affect growth rate of MEFs for a long period after exposure to low doses of IR.

DISCUSSION

Our results show for the first time that *E. coli* and human mismatch-specific uracil/thymine DNA glycosylases can excise 8oxoA residues in duplex DNA when paired with T, C and G (Figures 1 and 2). Analysis of kinetic parameters for the full-length hTDG protein showed that it excises 8oxoA, εC and T residues from 8oxoA•T, εC•G and G•T base pairs, respectively, with similar efficiencies (Table 1), suggesting that 8oxoA is a physiologically relevant substrate for the human DNA glycosylase. The catalytic domain of hTDG (111–308), hTDGcat, has extremely low activity on 8oxoA•T, but it excises εC with an efficiency similar to the full-length protein, suggesting that
the N-terminal and C-terminal parts of hTDG are essential for the recognition of damaged purines but not of exocyclic DNA adducts (Table 1). In agreement with our previous observation, hTDG\textsuperscript{cat} exhibits lower activity on U\textsubscript{5}G and T\textsubscript{4}G duplexes as compared with the full-length protein (Supplementary Figure S1 and Table 1) (37). Importantly, both hTDG\textsuperscript{cat} and bacterial MUG, which shares sequence homology with the core domain of human enzyme, exhibit similar DNA substrate preference.

It should be stressed that 8oxoA residues can arise in DNA either by direct oxidation of chromosomal DNA (1) or by incorporation into the nascent strand during replication as 8-oxo-2'-deoxyadenosine-5'-monophosphate (8oxodAMP) (12). Recently, it has been shown that 8oxodATP can be misincorporated opposite guanine and adenine by various DNA polymerases, resulting in the generation of 8oxoA\textsubscript{G} and 8oxoA\textsubscript{A} base pairs (42). Study of the base pair specificity of hTDG in the present work revealed that the DNA glycosylase can also excise 8oxoA from 8oxoA\textsubscript{C} with similar efficiency and from 8oxoA\textsubscript{G} with ~5-fold higher rate as compared with 8oxoA\textsubscript{T}, but it has no activity on 8oxoA\textsubscript{A} pair (Figure 2 and Supplementary Table S1). Interestingly, the hTDG-catalysed excision of 8oxoA is barely dependent on the neighbouring base pairs context (Supplementary Figure S3). Taken together, these data demonstrate that the substrate specificity of MUG and hTDG depends more on the base pairing partner rather than on the sequence context of the lesion. This is in contrast to the observations made on family-1 UDGs for which the sequences flanking the target uracil have a greater effect on DNA glycosylase activity than the partner base of the uracil (43,44). Overall, the effects of sequence context and opposite base on excision of 8oxoA by hTDG are similar to that of eC and are dramatically different from that of T and U, suggesting that the human DNA glycosylase can recognize 8oxoA and eC in a global genome context. Finally, the removal of 8oxoA paired with T can be fully reconstituted \textit{in vitro} using the purified BER proteins (Figures 3 and Supplementary Figure S3).

Crystal structures of MUG and hTDG\textsuperscript{cat} in complex with DNA substrates have been resolved (45–47). The structure of MUG with a non-hydrolysable deoxyuridine analogue reveals an essentially non-specific pyrimidine-binding pocket that could accommodate bulky exocyclic DNA adducts, thus explaining wide substrate specificity of the bacterial DNA glycosylase (45). The structures of hTDG\textsuperscript{cat} in complex with DNA containing an AP site, 5caC or its fluorinated analogue have also been solved (46,47). The structure of hTDG\textsuperscript{cat} bound to DNA containing an abasic site reveals that hTDG\textsuperscript{cat} crystallized in a 2:1 complex with 22 mer duplex oligonucleotide DNA. One molecule of protein was bound at the AP site, whereas the other was bound to an undamaged DNA site (46). Apparently, formation of 2:1 complex has no effect on DNA glycosylase activity of hTDG and hTDG\textsuperscript{cat}. The structures of hTDG\textsuperscript{cat} with 5caC-containing DNA showed that the DNA glycosylase specifically recognizes 5caC in its well organized carboxyl binding active site pocket (47). Based on these DNA-ligated structures, it is still not clear how hTDG can accommodate oxidized purine residues in the active site pocket. Previously, we and others have demonstrated that TDG enzymes can excise hypoxanthine paired with guanine, suggesting that compared with other mismatch-specific uracil-DNA glycosylases, TDG has a spacious active site pocket, able to accommodate bulky purine lesions (20,48). Yet, the precise structural basis of 8oxoA recognition by MUG/TDG enzymes needs further investigations.

Data obtained in cell-free extracts from \textit{E. coli} WT and \textit{mug} mutant cells indicate that MUG is the main DNA glycosylase involved in the repair of 8oxoA\textsubscript{G} and 8oxoA\textsubscript{C} in \textit{E. coli} (Figure 4). On the other hand, MUG-dependent activity on 8oxoA\textsubscript{T} is barely detectable in these extracts, which is in agreement with results obtained with the purified protein. In addition, mutagenesis studies \textit{in vitro} and \textit{in vivo} have shown that 8oxoA is weakly mutagenic in \textit{E. coli} (4,6,7). Consistent with these observations, \textit{E. coli} \textit{mug} mutant does not exhibit a mutator phenotype under normal growth conditions (49). Interestingly, when measuring spontaneous mutation frequencies based on Miller’s lacZ reversion assay system, we observed in average a 3-fold increase in \textit{A}\textsubscript{T}\textrightarrow\textit{C}\textsubscript{G} transversions in \textit{E. coli} \textit{mug} mutant when compared with WT strain; however, this increase was not statistically significant under the experimental condition used (35). Subsequent studies have shown that MUG expression strongly depends on growth phase, being specifically expressed in stationary phase cells (50). Furthermore, its inactivation elevated the frequency of rifampicin-resistant mutants by 6-fold when cells were maintained in stationary phase for >2 days (50). Taken together, these observations suggest a potential role of MUG-catalysed removal of 8oxoA residues from DNA in preventing mutations in non-dividing \textit{E. coli} cells.

The eC residues in DNA can be excised by three mammalian TDG, MBD4 and SMUG1 DNA glycosylases, although the two latter enzymes exhibit much weaker activity as compared with TDG (16,51,52). Repair of eC and 8oxoA residues in whole cell extracts from MEFs showed that cleavage of eC\textsubscript{G}, 8oxoA\textsubscript{G} and 8oxoA\textsubscript{T} duplexes have an absolute requirement for the intact Tdg gene, indicating that in mouse cells TDG is the main DNA glycosylase excising eC and 8oxoA paired to G and T, respectively (Figure 4 and Supplementary Figure S4). In agreement with a previous study, we detected the cleavage of 8oxoA\textsubscript{C} duplex in the cell extracts from MEFs Tdg\textsuperscript{−/−}, suggesting that the mouse OGG1 excises 8oxoA paired with C (13) (Figure 4A and B). We also observed barely detectable cleavage of 8oxoA\textsubscript{A} duplex in both WT and Tdg\textsuperscript{−/−} cell extracts, which could be because of unidentified DNA glycosylase activities (Figure 4A). Overall, studies of the 8oxoA-DNA glycosylase activities with purified enzymes and mammalian cell-free extracts support the major role of TDG in the repair of 8oxoA residues paired with T and G \textit{in vivo}.

The biological role of TDG in cellular response to oxidative stress conditions is supported by its ability to remove various oxidized bases generated by reactive oxygen species, including 5-hydroxymethyluracil (20),
thymine glycol (17), 5-hydroxyxycytosine (18) and 8oxoA (present study) residues when present in DNA. However, in the present work, we showed that the cellular response of WT and Tdg<sup>−/−</sup> MEFs to high doses (10–20 Gy) of γ-irradiation was not dependent on the TDG status (Figure 5). Yet, compared with Tdg<sup>−/−</sup> MEFs, WT MEFs exhibited a somewhat better recovery after lower doses of γ-irradiation (1–5 Gy) after a prolonged recovery time, although this difference was not seen during the first 3 days after irradiation and also at higher doses of IR (Figure 5B–E).

Given the major role of TDG in the repair of mismatched T, eC and 8oxoA residues, one might expect that TDG deficiency would be associated with higher rate of C→T and A→C mutations and increased cancer risk. However, a recent study on the embryonic lethal phenotype of TDG knockout mice demonstrated that mutation frequencies in a Big Blue transgenic MEFs Tdg<sup>−/−</sup> were similar to that of WT MEFs, suggesting that the biological role of TDG in the repair of oxidized and/or deaminated DNA bases is minor (25). To explain the inconsistencies between biochemical data and genetic evidences, we may propose that MBD4, a second mammalian TεG-specific DNA glycosylase, can efficiently back up TDG for the repair of GεU and GεT mismatches, which originate in CpG contexts through deamination of C and 5mC, respectively. However, it should be stressed that there is no efficient back up enzyme to repair eCεG and 8oxoAεT pairs in the absence of TDG, and this might lead to accumulation of these lesions in cellular DNA of Tdg<sup>−/−</sup> MEFs. Interestingly, recently, it has been demonstrated that loss of TDG in tumour cells of mismatch-repair deficient patient resulted in high number of somatic base substitution mutations, providing the first <i>in vivo</i> evidence for the role of TDG in mutation prevention (33). In conclusion, we suggest that the physiological role of TDG in the defence against the mutagenic and toxic effects of eC and 8oxoA residues in DNA might be more important at tissue, organ and whole body level rather than in cultured cell lines. Further studies are required to elucidate the role of various DNA repair activities of TDG <i>in vivo</i>.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2 and Supplementary Figures 1–4.

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**REFERENCES**


