**Human DNA glycosylase enzyme TDG repairs thymine mispaired with exocyclic
etheno-DNA adducts**

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Highlight

- TDG can remove T mispaired with alkanone substituted etheno-DNA adducts *in vitro*.
- TDG in human cells promoted cell death caused by etheno-DNA adducts inducers.
- TDG-knockdown cells exhibited lower repair activity for ϵ C.
- TDG-knockdown cells showed a modest acceleration of mutations caused by ϵ C.

Keywords: lipid peroxidation, etheno-DNA adducts, base excision repair enzyme, TDG,
3,*N*⁴-ethenocytosine

Abstract

Lipid peroxidation directly reacts with DNA and produces various exocyclic etheno-base DNA adducts, some of which are considered to contribute to carcinogenesis. However, the system for repairing them in humans is largely unknown. We hypothesized that etheno-DNA adducts are repaired by base excision repair initiated by DNA glycosylase. To test this hypothesis, we examined the activities of the DNA glycosylase proteins OGG1, SMUG1, TDG, NEIL1, MUTYH, NTH1, MPG and UNG2 against double-stranded oligonucleotides containing 1,*N*⁶-ethenoadenine (ϵ A), 3,*N*⁴-ethenocytosine (ϵ C), butanone-ethenocytosine (B ϵ C), butanone-ethenoguanine (B ϵ G), heptanone-ethenocytosine (H ϵ C) or heptanone-ethenoguanine (H ϵ G) using a DNA cleavage assay. We found that TDG is capable of removing thymine that has mispaired with ϵ C, B ϵ C, B ϵ G, H ϵ C or H ϵ G *in vitro*. We next examined the effect of TDG against etheno-DNA adducts in human cells. TDG-knockdown cells exhibited the following characteristics: (a) higher resistance to cell death caused by the induction of etheno-DNA adducts; (b) lower repair activity for ϵ C; and (c) a modest acceleration of mutations caused by ϵ C, compared with the rate in control cells. All these characteristics suggest that TDG exerts a repair activity against etheno-DNA adducts in human cells. These results suggest that TDG has novel repair activities towards etheno-DNA adducts.

Introduction

Lipid peroxidation (LPO) can be generated by reactive oxygen species, nitric oxide synthases, lipoxygenases, or cyclooxygenases on polyunsaturated fatty acids (PUFAs) [1-3]. Omega-3 and omega-6 are two different kinds of PUFAs, and their peroxidation can give rise to 4-oxo-2-hexenal (4-OHE) and to 4-hydroxy-2-nonenal (4-HNE) and 4-oxo-2-nonenal (4-ONE), respectively [4-6]. These aldehydes directly react with DNA and produce exocyclic etheno-base DNA adducts, such as 1,*N*⁶-ethenoadenine (ϵ A), 3,*N*⁴-ethenocytosine (ϵ C), butanone-ethenocytosine (B ϵ C), butanone-ethenoguanine (B ϵ G), heptanone-ethenocytosine (H ϵ C) and heptanone-ethenoguanine (H ϵ G) (Supplementary Fig. S1) [1,7-10]. Recently, these etheno-DNA adducts have been detected in several human organs [11-13].

Etheno-DNA adducts are considered to lead to mutagenesis and several diseases, including cancers. For instance, vinyl chloride (VC) is known to induce ϵ A and ϵ C [14], and workers exposed to VC reportedly have an increased risk of the onset of hepatocellular carcinoma [15,16]. Moreover, the level of etheno-DNA adducts in patients with familial adenomatous polyposis, Crohn's disease, ulcerative colitis or chronic pancreatitis, which predisposes an individual to cancer, is reportedly higher than that in each normal tissue [17].

A few kinds of etheno-DNA adducts are reportedly removed from DNA by base excision repair (BER) initiated by DNA glycosylase. For example, ϵ C in DNA is excised by the human DNA glycosylase enzyme TDG [18]. TDG is involved in the removal of thymine

and uracil mispaired with guanine, 5-fluorouracil (5-FU) paired with G and A as well as ϵ C from ϵ C:G base pairs [19]. TDG is also involved in the regulation of DNA methylation through the removal of oxidized products of 5-methylcytosine, such as 5-hydroxymethyluracil, 5-formylecytosine and 5-carboxycytosine [20-23]. ϵ C is excised also by the DNA glycosylase enzyme SMUG1, and ϵ A is removed by another DNA glycosylase MPG [24,25]. However, the repair mechanisms for many other LPO-related base lesions, including, in particular, 4-ONE-induced and 4-OHE-induced DNA adducts, are not fully understood. In this study, to elucidate previously unidentified repair mechanisms for etheno-DNA adducts, we examined the repair activities of eight human DNA glycosylase proteins (OGG1, SMUG1, TDG, NEIL1, MUTYH, NTH1 [also known as NTHL1], MPG and UNG2 [also known as CCNO and a nuclear form]) against six LPO-induced DNA adducts (ϵ A, ϵ C, B ϵ C, B ϵ G, H ϵ C and H ϵ G).

Material and methods

Synthesis of lipid peroxidation products and etheno-DNA adducts

Lipid peroxidation products, such as 4-OHE and 4-ONE, were respectively synthesized from the corresponding 2-alkylfurans according to previously reported methods; i.e. 2-ethylfuran (or 2-pentylfuran) was respectively oxidized by *m*-chloroperbenzoic acid in ice-cold methylene chloride to obtain 4-OHE (or 4-ONE) [26]. Successive reactions with

2'-deoxycytidine and 2'-deoxyguanosine were then respectively performed according to previously reported methods; namely, 2'-deoxycytidine and about 2.8-fold molar excess amount of 4-OHE (or 4-ONE) was reacted at 60°C in EtOH-H₂O for about 1 day, whereas in the case of the reaction of 2'-deoxyguanosine with about 3.5-fold molar excess amount of 4-OHE (or 4-ONE), the adduct formation was conducted at 37°C in phosphate buffer (pH 7.4) and EtOH for about 4.5 days [8,9]. Ethenodeoxycytidine (εdC) was prepared by mixing with a 50% aqueous solution of chloroacetaldehyde (CAA), according to a previously reported procedure [27]. ¹⁵N₅-labeled εdC was prepared using a procedure similar to that described above for non-labeled εdC. The more detailed synthetic procedures of the etheno-DNA adducts, including their transformations to the corresponding phosphoramidites, will be published in due course.

Plasmid construction

The *Escherichia coli* (*E. coli*) expression vectors containing human base excision repair genes were constructed by inserting the human SMUG1 and TDG cDNA sequences into a pGEX-2T plasmid vector (GE Healthcare Bio-Science Corp., Piscataway, NJ) and the human NTH1, UNG2 and MPG cDNA sequences into a pET25b(+) plasmid vector (Novagen, Madison, WI). The *E. coli* expression vectors containing the human OGG1, NEIL1 and MUTYH cDNA sequence were used in previous papers [28-30]. All the plasmid vectors were

confirmed by DNA sequencing.

Preparation of the recombinant base excision repair proteins

MUTYH and NEIL1 proteins were expressed and purified as described previously [29,30]. For the preparation of the other proteins, *E. coli* BL21-CodonPlus (DE3)-RP competent cells (Stratagene, La Jolla, CA) were transformed with the NTH1-pET25b, UNG2-pET25b and MPG-pET25b vectors, and *E. coli* BL21 competent cells (Stratagene) were transformed with the OGG1-pGEX-1 λ T, SMUG1-pGEX-2T and TDG-pGEX-2T vectors. Transformed cells were cultured at 37°C until an A₆₀₀ of 0.6, and protein expression was induced by incubation with 0.1 mM IPTG at 15°C or 20°C. OGG1-GST, SMUG1-GST and TDG-GST proteins were purified using Glutathione Sepharose 4B (Amersham Biosciences, Piscataway, NJ) and a polyprep chromatography column (Bio-Rad, Richmond, CA), and NTH1-His₆, UNG2-His₆ and MPG-His₆ proteins were purified using TALON metal affinity resins (Clontech, Palo Alto, CA) and a TALON 2-mL disposable gravity column (Clontech). The proteins were then dialyzed against a buffer containing 10 mM sodium phosphate (pH7.6), 50 mM NaCl, 0.5 mM DTT, 0.1 mM EDTA, 0.5 mM PMSF, 2 μ g/mL pepstatin, 2 μ g/mL leupeptin, 50 μ M chymostatin and 10% glycerol. The quality and concentration of each protein were determined using Image J software (National Institutes of Health, Bethesda, MD).

DNA cleavage activity assay

To prepare for the DNA cleavage activity assay, 30-mer oligonucleotides containing a single damaged base (5'-CTG GTG GCC TGA C[εA, εC, BεC, BεG, HεC or HεG]C ATT CCC CAA CTA GTG-3') were chemically synthesized and purified using high-performance liquid chromatography (HPLC) (Bex Co., Tokyo, Japan). The oligonucleotides were ³²P-labeled at the 5' terminus using a MEGALABEL kit (Takara, Osaka, Japan) and [γ-³²P]ATP (PerkinElmer, Tokyo, Japan), then annealed to a complementary strand containing an unmodified base opposite the damaged base. Complementary oligonucleotides containing an unmodified base opposite the damaged base were also labeled at the 5' terminus with [γ-³²P]ATP, then annealed to oligonucleotides containing a single damaged base. For the DNA cleavage activity assay, 300 fmole of purified OGG1, SMUG1, MPG or NEIL1 proteins were reacted in 20 μL of a mixture containing 20 mM sodium phosphate (pH7.6), 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 1.5% glycerol, 2.5 nM labeled oligonucleotide and 50 μg/mL BSA. Three hundred fmole of NTH1 or UNG2 proteins were reacted in 20 μL of a mixture containing 20 mM HEPES-KOH (pH7.5), 50 mM NaCl, 0.5 mM DTT, 1 mM MgCl₂, 1.5% glycerol, 2.5 nM labeled oligonucleotide and 50 μg/mL BSA. Three hundred fmole of TDG protein was reacted in 20 μL of a mixture containing 20 mM sodium phosphate (pH7.6), 1 mM DTT, 0.5 mM EDTA, 1.5% glycerol, 2.5 nM labeled oligonucleotide and 50 μg/mL

BSA. Three hundred fmole of MUTYH protein was reacted in 20 μ L of a mixture containing 20 mM HEPES-KOH (pH7.5), 50 mM NaCl, 0.5 mM DTT, 0.5 mM ZnCl₂, 1.5% glycerol, 2.5 nM labeled oligonucleotide and 50 μ g/mL BSA. Each reaction was performed at 37°C, and the samples were then treated with 0.1 M NaOH at 95°C for 4 min. After the addition of denaturing formamide dye, the mixture was heated at 95°C for 3 min and subjected to 20% PAGE. A ³²P-labeled marker oligonucleotide was used as a size marker for the cleavage products. The radioactivity of intact and cleaved oligonucleotides was quantified using an FLA-3000 fluoroimage analyzer (Fuji Film, Tokyo, Japan) or BAS-2500 Bio-image analyzer (Fuji Film), and ImageGauge software (Fuji Film) [31,32].

Cell survival assay

LN428-control and LN428-TDG knockdown cell lines were purchased from Trevigen (Gaithersburg, MD) and were cultured according to the manufacturer's instructions. Cells were exposed to fetal bovine serum (FBS)-free medium containing 0-700 μ M CAA (Wako, Tokyo, Japan), 0-50 μ M 4-ONE or 0-50 μ M 4-OHE for 2 h; the cells were then exchanged for fresh medium containing FBS. Cells were harvested 12 h after the medium change, and the percentage of cell survival was measured using a trypan blue-exclusion assay.

Treatment of plasmids using an etheno-DNA adducts inducer

One hundred micrograms of pEGFP-N1 plasmids and 25 µg of the shuttle vector plasmid pMY189 containing the bacterial suppressor tRNA (*supF*) gene were treated with buffer containing 300 mM sodium cacodylate (pH7.5) and 100 mM CAA at 37°C for 1 h. Because the CAA-treated plasmids contained an abundance of 3,*N*⁴-α-hydroxyethanocytosine (HEC), which is an intermediate of εC [33], after the removal of the buffer using ethanol precipitation, the CAA-treated plasmids or untreated plasmids were reacted with buffer containing 20 mM of sodium cacodylate (pH6.5) at 37°C for 72 h to convert the HEC to εC.

To prepare plasmids containing 4-ONE-derived and 4-OHE-derived DNA adducts, 100 µg of pEGFP-N1 plasmids were treated with buffer containing 20 mM sodium phosphate (pH7.0), 17% ethanol and 5 mM 4-ONE at 37°C for 3 h and with buffer containing 20 mM sodium phosphate (pH7.0), 17% ethanol and 20 mM 4-OHE at 37°C for 3 h, respectively. These plasmids were purified using the Amicon Ultra Centrifugal Filter (Millipore, Bedford, MA).

Measurement of the DNA adduct level

LN428-control and LN428-TDG knockdown cells were transfected with pEGFP-N1 vector containing etheno-DNA adducts using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Cells were harvested 24 h after transfection. The plasmids were extracted using a QIAprep Spin Miniprep Kit (QIAGEN,

Valencia, CA during this extraction procedure, deferoxamine was added to all the solutions at a final concentration of 0.1 mM.

The plasmids were enzymatically digested as follows: 1.6-3.2 µg pEGFP was mixed with 52 µL of digestion buffer containing 5 mM Tris-HCl (pH7.4), 8.8 units of DNase I (Wako) and internal standards. After incubating at 37°C for 3 h, 2 µL of 300 mM sodium acetate (pH5.3), 2 µL of 1 M ZnCl₂ and 3 µL of 1.6 units/µL nuclease P1 (Wako) were added and the mixtures were incubated for a further 3 h at 37°C. After this incubation, 2 µL of 500 mM Tris base and 140 units/µL of alkaline phosphatase (Wako) and 20 units/µL of phosphodiesterase I (Wako) were added and the mixture was incubated for 18 h at 37°C. Because the mass of εdC is consistent with that of deoxyadenosine and their peaks overlap [34], to convert adenosine to inosine, which has a different mass, 0.5 µL of adenosine deaminase (Sigma, St Louis, MO) was added to the reaction solution and the sample was incubated for 1 h at 25°C. The sample was then purified using Sep-pak C18 cartridges (Water, Milford, MA) and was collected as 20% methanol fractions. The fractions were evaporated to dryness and then resuspended in 66.7 µL of distilled water. Fifteen microliters of the eluate was subjected to liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The DNA adducts were quantified using LC-MS/MS, as described previously [35], and positive ions were acquired in multiple reaction monitoring (MRM) mode. The MRM transitions were monitored using the following cone voltages and collision energies:

ϵ dC [252.1→136.05, 35 V, 14 eV], $^{15}\text{N}_3$ - ϵ dC [255.1→139.05, 35 V, 14 eV], heptanone-ethenodeoxycytidine (H ϵ dC) [364.2→248.15, 35 V, 14 eV], $^{15}\text{N}_3$ -H ϵ dC [367.2→251.15, 35 V, 14 eV], butanone-ethenodeoxycytidine (B ϵ dC) [322.13→206.08, 35 V, 14 eV], $^{15}\text{N}_3$ -B ϵ dC [325.13→209.08, 35 V, 14 eV], butanone-ethenodeoxyguanosine (B ϵ dG) [362.13→246.08, 35 V, 14 eV] and $^{15}\text{N}_5$ -B ϵ dG [367.13→251.08, 35 V, 14 eV].

Quantification was performed from standard curves constructed using the ratio of known amounts of authentic standards and internal standards. The adduct levels were normalized to the amounts of DNA.

SupF forward mutation assay

LN428-control and LN428-TDG knockdown cells were trypsinized and washed in PBS(-); the cells were then suspended in Gene Pulser Electroporation Buffer (Bio-rad). A total of 5×10^6 cells plus 5 μg of shuttle plasmids were placed in a Gene Pulser Cuvette for a 0.4-cm electrode (Bio-rad), and electroporation was performed using a Gene Pulser II apparatus (Bio-rad) at 220 V and 950 μF . The cells were incubated at 37°C for 72 h. The propagated plasmids were then extracted from the cells and digested with *DpnI* to eliminate unreplicated plasmids. The plasmids were introduced into the KS40/pKY241 indicator *E. coli* strain using electroporation. The transformants were plated onto LB agar plates containing nalidixic acid, ampicillin, chloramphenicol, IPTG and X-gal. White colonies on this plate

were counted as *supF* mutants. The mutation frequencies were calculated as the number of *supF* mutants per the total number of transformants, which were counted on LB plates containing ampicillin, chloramphenicol, IPTG and X-gal. The mutations in the *supF* gene were then analyzed as described previously [36].

Results

DNA glycosylase activity against oligonucleotide containing etheno-DNA adducts

To investigate the repair mechanism of the LPO-induced DNA adducts ϵ A, ϵ C, B ϵ C, B ϵ G, H ϵ C and H ϵ G, we first prepared eight purified recombinant DNA glycosylase proteins: OGG1, SMUG1, TDG, NEIL1, MUTYH, NTH1, MPG and UNG2 (Supplementary Fig. S2A). To confirm that the DNA glycosylase proteins prepared using our system actually possessed enzymatic activity, we examined the repair activity of each protein towards an oligonucleotide containing a previously known substrate using a DNA cleavage assay [25,28-31,37,38]. The oligonucleotides that reacted with DNA glycosylase protein were analyzed on a denaturing polyacrylamide gel and their mobility was compared with that of a marker oligonucleotide. Substantial repair activity was shown for all the proteins (Supplementary Fig. S2B). Next, we prepared double-stranded oligonucleotides containing ϵ A, ϵ C, B ϵ C, B ϵ G, H ϵ C or H ϵ G paired with unmodified A, C, G or T. For each double-stranded oligonucleotide, [γ - 32 P]ATP labeling at the 5' terminus was performed for

etheno-adduct-containing oligonucleotides or unmodified oligonucleotides, meaning that we can evaluate DNA glycosylase activity towards the etheno-DNA adduct itself and an unmodified base paired with an etheno-adduct. When an oligonucleotide containing an ϵ A adduct was reacted with the DNA glycosylases, the cleavage product was detected in the lanes of the reaction of the MPG protein and an oligonucleotide containing ϵ A:A, ϵ A:T, ϵ A:C or ϵ A:G, which agreed with previous findings [37] (Fig. 1A). When an oligonucleotide containing an ϵ C adduct was reacted, the cleavage product was detected in the lanes of the reaction of the SMUG1 protein and an oligonucleotide containing ϵ C:A, ϵ C:T, ϵ C:C or ϵ C:G and in the lane of the reaction of the TDG protein and an oligonucleotide containing ϵ C:G (Fig. 1B). Although the cleavage activity of SMUG1 and TDG to the ϵ C:G substrate has been previously reported [24,25], the effects of SMUG1 on the ϵ C:A, ϵ C:T and ϵ C:C substrates are novel findings. Since the DNA cleavage assay against ϵ C and H ϵ C showed signal background at the cleavage product position to some extent, there was some difficulty in judging the cleavage activity of some proteins (Fig 1B and 3A). So, we extended a reaction time to 1 h, and examined DNA glycosylase activity towards an oligonucleotide containing ϵ C and H ϵ C again (Supplementary Fig. S3). The background was still detected not only in the lanes of oligonucleotides reacted with DNA glycosylases but also in the lane of an oligonucleotide treated only with alkali. The background levels in the lanes of the reaction of OGG1, NTH1, UNG2, MPG, MUTYH or NEIL1 with ϵ C-containing oligonucleotide and those in the lanes

of the reaction of all the DNA glycosylases with HεC-containing oligonucleotide were nearly identical to the signal seen in the lane of εC- or HεC-containing oligonucleotide treated only with alkali. These results mean that OGG1, NTH1, UNG2, MPG, MUTYH and NEIL1 do not have the activity against εC, and all the eight DNA glycosylase proteins do not have the activity against HεC. Furthermore, cleavage product was also detected in the lane of the reaction of the TDG protein and the oligonucleotide containing an unmodified T mispaired with εC (Fig. 1B). Interestingly, and most unambiguously, when an oligonucleotide containing a BεC, BεG, HεC or HεG adduct was reacted, the TDG protein also showed cleavage activity against unmodified T mispaired with BεC, BεG, HεC or HεG (Figs. 2 and 3). These TDG activities towards unmodified T mispaired with etheno adducts have not been reported, meaning that they are novel findings. Next, to further investigate the cleavage activity of TDG, TDG protein was reacted with substrate for various time periods (i.e., a time-course assay) and the percentage of cleaved products per total oligonucleotide was calculated and expressed as the % incision. The time-course assay clearly demonstrated that the εC glycosylase activity of TDG against oligonucleotides containing an εC:G base pair and the unmodified T cleavage activity of TDG against the oligonucleotides containing T:εC, T:BεC, T:BεG, T:HεC or T:HεG mispairs increased in a time-dependent manner (Fig. 4). Meanwhile, no clear cleavage products were detected when an oligonucleotide containing an unmodified C:G and T:A base pairs were reacted with the

TDG protein. Interestingly, the level of cleavage activity of TDG against T is strongly influenced by the kind of opposite etheno-adduct. These results suggested that TDG is capable of excising T paired with ϵ C, B ϵ C, B ϵ G, H ϵ C or H ϵ G as well as ϵ C in the ϵ C:G pair.

Role of TDG on cell death caused by inducers of etheno-DNA adducts in human cells

CAA, which is a VC metabolite, induces etheno adducts (including ϵ C) into genomic DNA, similar to VC [39]. To investigate whether the TDG protein has any effect on the viability of cells exposed to an etheno-DNA adduct inducer, an LN428-TDG-knockdown cell line and its control cell line were treated with CAA, 4-ONE or 4-OHE for 2 h; after 12 h, cell survival was measured. A higher survival was shown in TDG-knockdown cells than in control cells when exposed to 450-700 μ M of CAA (Fig. 5A). The survival of cells exposed to 5-30 μ M of 4-ONE or 4-OHE tended to decrease similarly in both control cells and TDG knockdown cells, whereas the cell survival of TDG-knockdown cells was significantly higher than that of control cells when treated with 50 μ M of 4-ONE or 4-OHE (Figs. 5B and C). These data suggested that TDG promotes cell death induced by etheno-DNA adducts.

Activity of TDG for the repair of etheno-DNA adducts in human cells

To determine whether TDG is capable of repairing etheno-DNA adducts in human cells, we attempted to investigate the ability of TDG to suppress etheno-DNA adduct levels in

human cells. First, we treated pEGFP-N1 plasmid with CAA, 4-ONE or 4-OHE and measured the levels of ϵ dC, H ϵ dC or butanone-etheno adducts, respectively, using an LC-MS/MS analysis. A dramatic increase was observed in the levels of ϵ dC, H ϵ dC, and butanone-etheno adducts (Supplementary Fig. S4). TDG-knockdown cells and control cells were then transfected with plasmids treated using CAA, 4-ONE or 4-OHE; after 24 h, the plasmids were collected and their adduct levels were measured using an LC-MS/MS analysis. The adduct levels of ϵ dC, H ϵ dC, B ϵ dC and B ϵ dG were significantly reduced in both the TDG-knockdown cells and the control cells (Fig. 6), indicating the repair of these adducts in human cells. No significant differences in the levels of H ϵ dC, B ϵ dC and B ϵ dG were seen between the TDG-knockdown cells and the control cells (Figs. 6B-D); however, remarkably, the ϵ dC level of plasmids collected from the TDG-knockdown cells was significantly higher than that of plasmids collected from the control cells (Fig. 6A). This result suggests that TDG has the capacity to remove ϵ C in human cells.

Effect of TDG on mutation spectrum of etheno-DNA adducts in human cells

Next, to study the functional characteristics of TDG against mutations caused by etheno-DNA, we prepared a shuttle plasmid pMY189 treated or not treated with 100 mM of CAA; the mutation frequency was then compared between TDG-knockdown cells and their control cells using a *supF* forward mutation assay and both kinds of plasmids. The mutation

frequency in the *supF* of CAA-treated pMY189 was significantly higher than that of non-treated pMY189 in both the control and TDG-knockdown cells (Fig. 7A, $P < 0.05$). When the mutation frequency in CAA-treated pMY189 was compared between the control and the TDG-knockdown cells, the mutation frequency in the TDG-knockdown cells ($20.5 \pm 15.2 \times 10^{-3}$) was 1.5-fold higher than that in the control cells ($14.0 \pm 6.8 \times 10^{-3}$), but the difference did not reach statistical significance. Next, the mutation spectrums of the *supF* gene in plasmids treated at CAA in the control and the TDG-knockdown cells were examined using PCR and subsequencing analyses (Fig. 7B). First, the PCR-amplified products of the *supF* region were subjected to agarose gel electrophoresis; almost all the products showed the same mobility as a wild-type *supF* clone, indicating that most of the mutations were likely to be base substitutions or small insertions or deletions. Further sequencing analysis of the PCR products revealed that almost all the products showing the same mobility as a wild-type *supF* clone were actually base substitution-type mutations, but the mutation spectrums differed between the control and the TDG-knockdown cells. Interestingly, modest increases of G:C to T:A transversion and G:C to A:T transition were found in TDG-knockdown cells (i.e., the proportions of G:C to T:A transversions in TDG-knockdown and control cells were 46.4% and 42.3%, respectively, and the proportions of G:C to A:T transitions in TDG-knockdown and control cells were 32.1% and 26.9%, respectively), but this difference was not statistically significant. Thus, the role of TDG in suppressing mutations caused by ϵ C in

human cells may be modest.

Discussion

ϵ A, ϵ C, $N^2,3$ -ethenoguanine ($N^2,3$ - ϵ G) and $1,N^2$ -ethenoguanine ($1,N^2$ - ϵ G) in etheno-DNA adducts is reported to be repaired in *E. coli* and human BER pathway by several groups [40]. However, repair system of B ϵ C, B ϵ G, H ϵ C and H ϵ G recently found in human tissues have not been investigated yet. In addition, although a BER enzyme MUTYH is known to catalyze the removal of adenine mispaired with 8-hydroxyguanine (8OHG), which is an oxidized form of guanine and is highly mutagenic, in double-stranded DNA and to prevent G:C to T:A mutations at 8OHG site, the repair function of DNA glycosylase proteins against opposite bases of etheno-DNA adducts have not been fully examined. In this study, we newly found that in addition to its direct repair activity against ϵ C, the human BER enzyme TDG is also capable of removing thymine mispaired with ϵ C, B ϵ C, B ϵ G, H ϵ C or H ϵ G *in vitro*. We also revealed that TDG in human cells is involved in the promotion of cell death caused by inducers of etheno-DNA adducts, the removal of ϵ C and the suppression of G:C to T:A and G:C to A:T mutation by comparing the TDG-knockdown cell line and its control cell line. These results suggested that TDG is involved in the repair of etheno-DNA adducts in human cells *in vivo*.

In our cell survival assay, the proportion of cells that survived was significantly lower

in the control cells than in the TDG-knockdown cells when exposed to a higher dose of CAA, 4-ONE and 4-OHE. This result raises the possibility that TDG may be involved in the induction of the plural cell death mechanism. Kunz *et al.* [41] previously reported that immortalized mouse embryo fibroblasts (MEFs) derived from *Tdg*^{-/-} mice and TDG-knockdown cells from a human cancer cell line exhibited an increase in resistance of 5-FU that is one of substrate on TDG. The loss of TDG induces XRCC1 foci, which enhances single-stranded DNA breaks repair activity, after 5-FU treatment and is thought to reduce lethal 5-FU processing. Our finding that TDG-knockdown cells are resistant to cell death after treatment with an inducer of etheno-DNA adducts is also suspected to be caused by a mechanism similar to the suppression of cell death observed in 5-FU treatment. In addition, TDG interacts with p53 and its family proteins, and this interaction promotes the transcription of the p53 family downstream genes when p53 and its family proteins are activated [42]. Since the p53 family proteins regulate apoptosis, the decreased cell death in TDG-knockdown cells, compared with control cells, is likely to be partly caused by the fact that p53 family proteins cannot interact with the TDG protein. From the result that TDG-knockdown cells were more viable than control cells when exposed to an etheno-DNA adduct inducer, we assumed that those cells with a lower TDG expression, abundant etheno-DNA adducts, and subsequent abundant mutations may have greater chance to survive or to gain disease-causing mutations. Thus, the maintenance of the TDG expression level is

likely to be important in human cells, especially during exposure to severe oxidative stresses. LN428 cells with or without TDG expression began to die when treated with comparatively low dose of 4-ONE or 4-OHE. We do not know the exact underlying mechanism, but it is known that the unmodified base mispaired with exocyclic base is recognized by mismatch repair (MMR) enzymes as well as by BER enzymes [43]. Since MMR proteins reportedly interact with translesion DNA synthesis (TLS) polymerase [44], this polymerase may also play a direct role in the repair process of etheno-DNA adducts. Exocyclic DNA adducts, such as HεC, can inhibit function of TLS DNA polymerase and strongly block DNA synthesis [45]. Thus, the repair of etheno-DNA adducts by MMR system may also create a persistent single-strand break and induce cell death.

TDG was shown to have two kinds of functions against εC repair in this study: the excision of εC from εC:G base pairs in double-stranded DNA, and the removal of thymine mispaired with εC in double-stranded DNA. εC is highly mutagenic and causes C:G to A:T transversions or C:G to T:A transitions in *E. coli* and mammalian cells [46,47], thus it may wrongly pair with thymine or adenine bases during DNA replication. In the case of εC:T mispairing, TDG is predicted to remove T, but the consequence of this process is different depending on which base is opposed to εC after T excision. For example, when DNA polymerase works and fills the gap with G, εC:T is changed to εC:G, and finally, restored to the normal C:G base pair by authentic function of TDG or SMUG1. This TDG activity of

removal of the base opposing to the adducted base, here reported, reminds us of the behavior of MUTYH, which removes the A opposing to 8-OHG. This function implies that TDG plays an important role in preventing mutations against ϵ C, especially C:G to A:T transversions. When the DNA polymerase inserts T, DNA containing ϵ C falls into a futile repair cycle, and suffers from persistent single-strand break. If the polymerase inserts A and C, ϵ C:T base pairs may cause C:G to T:A mutation and C:G to G:C mutation, respectively. An assay employing shuttle vector plasmid containing H ϵ C has been reported that H ϵ C blocked DNA synthesis and brought many mutations in human cells [45]. The miscoding frequency was higher than 90% and T and A were preferentially inserted to the place opposite to the adduct. Therefore, we assume that the mutations caused by H ϵ C can also be prevented by TDG. The position where the bases removed by TDG are reportedly reconstituted by BER pathway-related enzymes including DNA polymerase beta [48]. In the future studies, we would like to clarify the details how TDG and its related enzymes work after T against etheno-DNA adduct was removed.

In this paper, *supF* forward mutation assay showed modest increase in the proportion of G:C to T:A (C:G to A:T) mutations and G:C to A:T (C:G to T:A) mutations in CAA-treated TDG-knockdown cells than in control cells with the same treatment (Fig. 7B), and these mutation spectra are known to be caused by ϵ C. The observation that the increase was not statistically significant in our experiment is thought to have been due to the effect of other

DNA adducts generated by CAA, because it has been reported that CAA treatment generates not only ϵ C DNA adducts, but also other DNA adducts, such as ϵ G, in DNA [49].

In our quantitative analysis of adduct level in transfected plasmids (Fig. 6A), the ϵ dC levels of plasmids transfected into TDG-knockdown cells were higher than those of plasmids transfected into control cells. The reduced repair activity of TDG towards ϵ C in TDG-knockdown cells may compensate the ordinary power to reduce ϵ dC levels in the cells. Molatore et al. [46] and Turco et al. [47] have reported that the 8OHG levels in immortalized MEFs derived from *Mutyh*^{-/-} mice were higher than in wild-type MEFs after exposure to KBrO₃, a base damage inducer. The results of both papers suggest that a difference in DNA glycosylase activity towards unmodified bases paired with modified bases in cells can lead to the differences in the modified adduct levels in the cells. Like the findings in their reports, if TDG repairs the mispairing of ϵ C:T to ϵ C:G, the loss of this function of TDG itself may also contribute to the remaining ϵ dC levels in TDG-knockdown cells.

In our DNA cleavage assay with control lane in which oligonucleotide was treated only with alkali (Supplementary Fig. S3), it was found that background signal due to alkali treatment was seen in the lanes of reaction of DNA glycosylase and substrate oligonucleotide. It is known that etheno-DNA adducts are decomposed under alkaline conditions and converted into ring-opened structures [52-54]. Since the background bands are found in the lane of an oligonucleotide treated only with alkali, decomposition of etheno-DNA adducts

can be presumed to generate apurinic/apyrimidinic site. Interestingly, alkali treatment against etheno-DNA adducts seems to influence more strongly on the cytosine-DNA adducts, in particular on ϵ C, than on guanine-DNA adducts.

There is nothing simple in the repair of etheno-DNA adducts. ϵ A and ϵ C are repaired by not only BER systems but also by *E. coli* AlkB family proteins and their human homologues ABH2 and ABH3 proteins [55-57]. AlkB enzymes directly reverse etheno-DNA adduct to normal base in DNA [55,58]. DNA glycosylase proteins did not show the removal activity against butanone-DNA adducts and heptanone-DNA adducts in our paper. At present, it has not been reported whether AlkB and ABH proteins have the repair activity against these etheno-DNA adducts, so investigation of this point is one of future experimental plans.

Repair activity of TDG may not be limited to the exocyclic etheno-DNA adducts. *O*⁶-Methylguanine (*O*⁶-meG), one of the alkyl adducts, pairs preferentially with T, and causes G:C to A:T transitions [59]. TDG is known to also excise T mispaired with *O*⁶-meG [60,61], and this function is similar to the repair activity against etheno-DNA adducts found in the present paper. This phenomenon together with that we report here indicates the possibility that TDG removes T mispaired with a wide range of modified cytosine or guanine in DNA. Thus, search for the modified bases against T as a TDG substrate is likely to be important to comprehend the extensive biological roles of TDG.

A genetic polymorphism in the *TDG* gene has recently been reported to be associated

with an increased risk of the development of nonmelanoma skin cancer plus other cancer and esophageal squamous cell carcinoma [62,63]. In addition, the loss of heterozygosity of the *TDG* gene, which is located at chromosome 12q24.1, has been detected in gastric cancers [64]. These genetic alterations of the *TDG* gene in cancer suggest that TDG plays important roles in preventing carcinogenesis in various human organs. The DNA repair activity of TDG, shown in previous papers [48,65] and in the current study, as well as other TDG functions, such as DNA demethylation, may be involved in such cancer-preventing roles.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at XXXX.

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Figure captions

Fig. 1. Evaluation of repair activities of 8 DNA glycosylase proteins against 1,*N*⁶-ethenoadenine (εA) and 3,*N*⁴-ethenocytosine (εC). The abilities of the DNA glycosylase proteins OGG1, SMUG1, NTH1, UNG2, MPG, TDG, MUTYH and NEIL1 to repair double-stranded DNA containing εA (A) or εC (B) were examined using a DNA cleavage assay. Each BER protein (300 fmole) was allowed to act on a double-stranded oligonucleotide containing εA or εC at 37°C for 30 min. The asterisks show the 5'-labeled ³²P-labeled oligonucleotides, consisting of εA-containing and εC-containing oligonucleotides in the upper panels of (A) and (B), respectively, and unmodified oligonucleotides in the lower panels of (A) and (B). Numbers 1, 2, 3, 4, 5, 6, 7 and 8 indicate OGG1, SMUG1, NTH1, UNG2, MPG, TDG, MUTYH and NEIL1, respectively. A ³²P-labeled marker oligonucleotide was used as a size marker for the cleavage products. The intact 30-mer oligonucleotides (substrate) and cleavage products are indicated by S and P, respectively. The reference numbers for OGG1, SMUG1, NTH1, UNG2, MPG, TDG, MUTYH and NEIL1 proteins are NP_002533.1, NP_001230716.1, NP_002519.1, NP_550433.1, NP_002425.2, NP_003202.3, NP_001041639.1 and NP_078884.2, respectively.

Fig. 2. Evaluation of repair activities of 8 DNA glycosylase proteins against 4-oxo-hexenal adducts. The abilities of the DNA glycosylase proteins OGG1, SMUG1, NTH1, UNG2, MPG,

TDG, MUTYH and NEIL1 to repair double-stranded DNA containing butanone-ethenocytosine (BεC) (A) or butanone-ethenoguanine (BεG) (B) were examined using a DNA cleavage assay. Each BER protein (300 fmole) was allowed to act on a double-stranded oligonucleotide containing BεC or BεG at 37°C for 30 min. The asterisks show the 5'-labeled ³²P-labeled oligonucleotides, consisting of BεC-containing and BεG-containing oligonucleotides in the upper panels of (A) and (B), respectively, and unmodified oligonucleotides in the lower panels of (A) and (B). Numbers 1, 2, 3, 4, 5, 6, 7 and 8 indicate OGG1, SMUG1, NTH1, UNG2, MPG, TDG, MUTYH and NEIL1, respectively. A ³²P-labeled marker oligonucleotide was used as a size marker for the cleavage products. The intact 30-mer oligonucleotides (substrate) and cleavage products are indicated by S and P, respectively.

Fig. 3. Evaluation of repair activities of 8 DNA glycosylase proteins against 4-oxo-nonenal adducts. The abilities of the DNA glycosylase proteins OGG1, SMUG1, NTH1, UNG2, MPG, TDG, MUTYH and NEIL1 to repair double-stranded DNA containing heptanone-ethenocytosine (HεC) (A) or heptanone-ethenoguanine (HεG) (B) were examined using a DNA cleavage assay. Each BER protein (300 fmole) was allowed to act on a double-stranded oligonucleotide containing HεC or HεG at 37°C for 30 min. The asterisks show the 5'-labeled ³²P-labeled oligonucleotides, consisting of HεC-containing or

HεG-containing oligonucleotides in the upper panels of (A) and (B), respectively, and unmodified oligonucleotides in the lower panels of (A) and (B). Numbers 1, 2, 3, 4, 5, 6, 7 and 8 indicate OGG1, SMUG1, NTH1, UNG2, MPG, TDG, MUTYH and NEIL1, respectively. A ³²P-labeled marker oligonucleotide was used as a size marker for the cleavage products. The intact 30-mer oligonucleotides (substrate) and cleavage products are indicated by S and P, respectively.

Fig. 4. Time-course assay for cleavage of DNA containing an etheno adduct by TDG protein.

(A) Time-course assay for the cleavage of DNA containing εC:G and C:G base pairs by TDG protein. TDG protein (300 fmole) was incubated at 37°C for 0-120 min with double-stranded oligonucleotides containing εC:G and C:G base pairs (50 fmole). The amount of cleavage products as a proportion of the total oligonucleotides was calculated as the % incision. The % incision values are shown as the means ± standard deviations of data from three independent experiments. The lower panels show representative results of the DNA cleavage assays of TDG protein. (B) Time-course assay of the cleavage of DNA containing T:εC, T:BεC, T:BεG, T:HεC, T:HεG and T:A base pairs by TDG protein. TDG protein (300 fmole) was incubated at 37°C for 0-120 min with double-stranded oligonucleotides containing T:εC, T:BεC, T:BεG, T:HεC, T:HεG and T:A (50 fmole). The lower panels show representative results of the DNA cleavage assays for TDG protein.

Fig. 5. Cell survival of LN428-control and LN428-TDG-knockdown cells treated with CAA, 4-ONE or 4-OHE. Cells were treated with (A) 0-700 μ M CAA, (B) 0-50 μ M 4-ONE or (C) 0-50 μ M 4-OHE for 2 h; after the medium was exchanged with fresh medium, survival was measured after 12 h using a trypan blue-exclusion assay. The % of cell survival is the mean $\pm$ standard deviation of data from 2-3 independent experiments.

Fig. 6. Measurement of repair activity against etheno-DNA adducts in LN428-control and LN428-TDG-knockdown cells. Adduct-modified pEGFP plasmids were transfected into the cells; after incubation for 24 h, the plasmids were collected and the levels of ϵ dC (A), H ϵ dC (B), B ϵ dC (C) and B ϵ dG (D) were measured using LC-MS/MS. Values are the means $\pm$ standard deviations of three measurements. *P* values were calculated using a two-tailed Student *t*-test, **P* < 0.05. ND means not detected.

Fig. 7. *SupF* forward mutation assay using CAA-treated plasmid pMY189 in LN428-control and LN428-TDG knockdown cells. (A) The mutation frequency of the *supF* gene in pMY189 was measured using a *supF* forward mutation assay. The mutation frequency is shown as the mean $\pm$ standard deviation of data from 6 experiments. (B) Proportion of mutation spectrum of the *supF* gene in the CAA-treated pMY189 replicated in the control and TDG knockdown

Figure 1

A

B

Figure 2

A

B

Figure 3

A

B

Figure 4

Figure 5

Figure 6

A

B

C

D

Figure 7

A

B

Graphical abstract

X = etheno-DNA adducts

1 : OGG1 5 : MPG
2 : SMUG1 6 : TDG
3 : NTH1 7 : MUTHYH
4 : UNG2 8 : NEIL1

1,N⁶-thenoadenine (εA)

3,N⁴-thenocytosine (εC)

butanone-ethenocytosine (BεC)

butanone-ethenoguanine (BεG)

heptanone-ethenocytosine (HεC)

heptanone-ethenoguanine (HεG)