The reason is not fully understood yet; however, ABAQ administration routes might affect its *in vivo* genotoxicity. In the present study, i.p. injection was used for the comet and micronucleus assays, and i.g. intubation for the *gpt* and Spi$^-$ mutation assays. In general, the absorption rates following i.p. administration were more rapid than i.g. intubation. This may account for the higher genotoxicity of ABAQ observed in the micronucleus and comet assay compared with the results of the mutagenicity test in *gpt* delta mice. Both *gpt* and Spi$^-$ MFs in the kidneys observed in ABAQ-treated mice tended to increase, but this was not statistically significant (Figs. 4 and 5). We suggest, therefore, that ABAQ dosing using i.g. intubation may not be high enough for robust genotoxicity.

The *gpt* mutations induced in the liver are summarized in Table 2. Because our control samples were limited in number, we included and compared the data from a previous report (Control-2 [16]). In the mutation spectrum analysis, the most prominent mutation induced by ABAQ was G:C → A:T ($P < 0.05$) compared with both control groups. A previous study showed that ABAQ is mutagenic for *S. typhimurium* TA98 and YG1024 with S9 mix [8]. The sensitivity was much higher in YG1024 than in TA98, suggesting that *O*-acetyltransferase activity is required to activate ABAQ, as for other food-borne mutagenic compounds, such as heterocyclic amines (HCAs). The exocyclic amino group of HCAs binds guanine bases to form DNA adducts [19–22]. No data are available regarding the chemical structures of ABAQ-DNA adducts, except for the mutational spectral data for ABAQ. Therefore, we may conclude that the guanine base may be involved, to form ABAQ-DNA adducts. In addition, the G:C → A:T transition commonly occurs in spontaneous mutants, and deamination of 5-methylcytosine or alkylation of guanine might be involved in these mutations [23,24]. Moreover, inflammation may be involved [25]. In contrast, the frequencies of A:T → C:G transversions were also significantly different between ABAQ-treated and control groups. Even though its specific MF was low, this type of mutation is rare (almost none in the control cases). Therefore, it might be diagnostic for ABAQ-exposure. Further studies are required to determine the nature of the reactions that produce ABAQ-DNA adducts, and the resulting genotoxic mechanisms.

DM is a risk factor for various types of cancers [2–5], and researchers have focused on the relation between type-2 diabetes and cancer incidence. Evidence indicates that alterations in signal transduction pathways that promote cell proliferation caused by hyperglycemia, or insulin resistance and hyperinsulinemia associated with DM, promote oncogenesis [26–28]. However, whether diabetes initiates tumorigenesis is unknown. *In vivo* Maillard reactions are increased under diabetic conditions [29] and reaction products such as ABAQ may play a role in cancer etiology. A study on *in vivo* formation of ABAQ in diabetic model animals and diabetic patients is in progress in our laboratory. To understand the effect of ABAQ on DM-related cancer, it is important to evaluate the carcinogenicity of ABAQ using animal models. Moreover, epidemiological studies to evaluate the relation between ABAQ and DM-related cancer will also be required.

Conflict of interest statement

None.

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A novel aromatic mutagen, 5-amino-6-hydroxy-8*H*-benzo[6,7]azepino[5,4,3-*de*]quinolin-7-one (ABAQ), induces colonic preneoplastic lesions in mice

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ABSTRACT

The benzoazepinoqunolinone derivative, 5-amino-6-hydroxy-8*H*-benzo[6,7]azepino-[5,4,3-*de*]quinolin-7-one (ABAQ), which is produced in a mixture of glucose and tryptophan incubated at 37 °C under physiological conditions in the presence or absence of hydroxyl radicals caused by the Fenton reaction, is a novel aromatic mutagen. In the current study, we determined the tumor-initiating potency of ABAQ using an inflammation-relate, two-stage mouse colon carcinogenesis model. Male Crj: CD-1 (ICR) mice were treated with the single intragastric administration (100 or 200 mg/kg body weight) of ABAQ followed by subsequent 1-week oral exposure to 2% dextran sodium sulfate (DSS) in drinking water. The ABAQ treatment alone resulted in high-grade dysplasia, which is a precursor to colorectal cancer, in the colon. Following the administration of DSS after ABAQ treatment, the incidence and frequency of high-grade dysplastic lesions increased; the values were highest in the mice treated with 200 mg/kg body weight of ABAQ followed by DSS. The lesions expressing β-catenin in their nuclei and cytoplasm exhibited high proliferation activity without the expression of programmed cell death 4. These findings indicate that ABAQ has a tumor-initiating activity in the mouse colon, with or without inflammation, although the potential pro-inflammatory effect of high doses of ABAC should be investigated.

Abbreviations: ABAQ, 5-amino-6-hydroxy-8*H*-benzo[6,7]azepino[5,4,3-*de*]quinolin-7-one; AOM, azoxymethane; DSS, dextran sodium sulfate; HCA, heterocyclic amine; H&E, hematoxylin and eosin; i.g., intragastric; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; PAH, polycyclic aromatic hydrocarbons; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine.

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1. Introduction

Human people are continuously exogenously exposed to a variety of chemicals that have been shown to have mutagenic or carcinogenic properties in experimental systems [1]. Cooking meat and fish at a high temperature (above 180 °C) forms mutagenic and carcinogenic heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs). HCAs are formed by the pyrolysis of creati(ni)ne with sugars with specific amino acids. Since a high temperature is needed, only fried, broiled or barbecued meat contains a significant amount of HCAs [2]. Experimental studies of HCAs began with Dr. Sugimura's discovery that cooked meat and fish contain potent mutagens [2]. Some HCAs were later shown to be complete carcinogens that induce liver, colon, mammary and prostate tumors in rodents and monkeys [2,3]. Certain HCAs are consistently identified in well-done meat products consumed in the North American diet. Although a causal link has not been fully established, a majority of epidemiology studies have linked the consumption of well-done meat products to cancer of the colon and breast. Several HCAs thus represent an important class of carcinogens in foods and have been classified as "possibly carcinogenic to humans (Group 2B)" or "probably carcinogenic to humans (Group 2A)" by the IARC [4] and "Group 2: reasonably anticipated to be human carcinogens (R)" by the NTP [5]. Similar to most other chemical carcinogens, HCAs must be metabolized by CYP1A2 or CYP1B1 to chemically reactive electrophiles prior to reacting with DNA in order to exert their carcinogenic potency in both rats and humans [2].

Certain compounds that are mutagenic and carcinogenic in cooked foods are formed by the Maillard reaction of reducing sugars and amino acids. Indeed, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) are mutagenic and carcinogenic HCAs formed through the Maillard reaction in meat and fish cooked at a high temperature [2]. These compounds are suggested to be formed by the reaction of creatine with Maillard reaction products from glucose and amino acids by heating at a high temperature of 128 °C [2,3,6]. The Maillard reaction can also occur at physiological temperatures. In a series of our study performed to clarify the formation of mutagens during the Maillard reaction in glucose and amino acids, we recently identified a novel aromatic mutagen, 5-amino-6-hydroxy-8*H*-benzo[6,7]azepino[5,4,3-*de*]quinolin-7-one (ABAQ, $C_{16}H_{11}N_3O_2$, MW = 277.28, Fig. 1a), formed in the mixture of glucose and tryptophan incubated at 37 °C and a pH of 7.4 in the presence or absence of hydroxyl radicals produced by the Fenton reaction [7]. ABAQ exhibits a strong mutagenic activity toward *S. typhimurium* TA98 and YG1024 with S9 mix [7]. The mutagenic potency of ABAQ is comparable to that of PhIP [7]. ABAQ also revealed mutagenicity in the liver of *gpt* delta transgenic mice [8].

In order to understand the effects of ABAQ on human health, it is important to elucidate its tumor-initiating ability in rodents. The current study thus aimed to determine whether the novel mutagen ABAQ possesses a tumor-initiation activity in the colon in *in vivo* experiment in mice. We used an experimental model employing a colitis-inducing agent, dextran sodium sulfate (DSS), which has a powerful tumor-promotion activity [9], since the tumor-initiation activity of PhIP [10] and aminophenylnorharman [11] can be detected within a short-term period in this model. The single administration with ABAQ in two doses *via* gavage resulted in development of high-grade dysplasia in the inflamed colon induced by DSS in male mice. The immunohistochemical analysis revealed the lesions with a high proliferative activity and cytoplasmic and/or nuclear expression of β-catenin to be negative for programmed cell death 4 (PDCD4), suggesting the tumor-initiation ability of ABAQ.

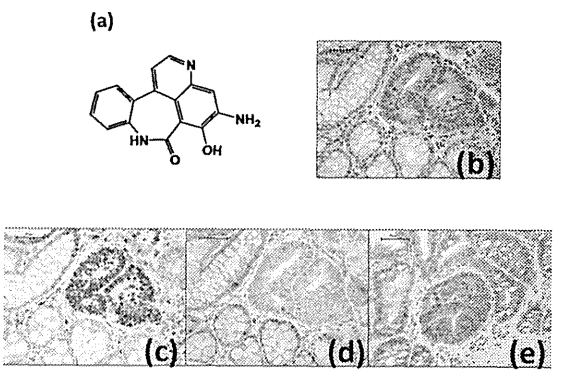

Fig. 1. (a) Chemical structure of ABAQ (ABAQ, $C_{16}H_{11}N_3O_2$, MW = 277.28). (b) A high-grade colonic dysplasia on H&E-stained section from a mouse in group 2 (200 ppm ABAQ + DSS). *Note*: Nuclear atypia in the crypts and Paneth's granules (red in color) in the cytoplasm of some dysplastic crypt cells. H&E stain; bar, 50 μm. (c) Many nuclei in the high-grade dysplasia in a serial section from (b) are positive for MCM2. MCM2 immunohistochemistry; bar, 50 μm. (d) Most nuclei in the high-grade dysplasia in a serial section from (b) are negative for PDCD4, whereas the nuclei in the surrounding normal crypts are positive for PDCD4. PDCD4 immunohistochemistry; bar, 50 μm. (e) The cytoplasmic expression of β-catenin is present in the high-grade dysplasia lesion developed in the colon of a mouse from group 2 (200 ppm ABAQ + DSS). Some nuclei are weakly positive for β-catenin. β-Catenin immunohistochemistry; bar, 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2. Materials and methods

2.1. Animals, chemicals and diet

Male Crlj: CD-1 (ICR) mice (Charles River Japan, Tokyo, Japan) 5 weeks of age were used. The mice were maintained at Gifu University Animal Facility according to the Institutional Animal Care Guidelines. All animals were housed in plastic cages (3 or 4 mice/cage) with free access to drinking water and a pelleted basal diet, CRF-1 (Oriental Yeast, Tokyo, Japan), under controlled conditions of humidity (50 ± 10%), light (12/12-h light/dark cycle) and temperature (23 ± 2 °C). After seven days of quarantine, they were randomized according to body weight into experimental and control groups. ABAQ was synthesized as previously described [7]; its purity was confirmed to be > 99% by HPLC. DSS with a molecular weight of 36,000–50,000 (Cat No. 160110) was purchased from MP Biochemicals, LLC (Aurora, OH, USA).

Table 1
Body, liver and relative liver weights and colon lengths in all groups.

Group no.	Treatment (no. of mice examined)	Body weight (g)	Liver weights (g)	Relative liver weight (g/100 g body weight)	Colon length (cm)
1	ABAQ [a] (100 mg/kg bw) → 2% DSS (15)	49.9 ± 7.29 [b]	2.5 ± 0.53 [c]	5.0 ± 0.48 [d]	16.5 ± 1.30 [c]
2	ABAQ (200 mg/kg bw) → 2% DSS (15)	44.8 ± 9.46	2.2 ± 0.56	4.8 ± 0.37 [c]	15.6 ± 1.31
3	ABAQ (200 mg/kg bw) (5)	42.0 ± 3.46	1.7 ± 0.23 [e]	4.1 ± 0.29	14.3 ± 0.42 [e]
4	Solvent → 2% DSS (5)	47.6 ± 1.62	2.1 ± 0.13	4.5 ± 0.35	15.9 ± 1.33
5	None (8)	51.6 ± 7.95	2.5 ± 0.45	4.8 ± 0.58	16.4 ± 1.21

[a] ABAQ, 5-amino-6-hydroxy-8*H*-benzo[6,7]azepino[5,4,3-*de*]quinolin-7-one; DSS, dextran sodium sulfate.
[b] Mean ± SD.
[c,d] Significantly different from group 3 ([c] $p < 0.05$ and [d] $p < 0.01$) according to a one-way ANOVA and the Tukey–Kramer Multiple Comparison test.
[e] Significantly different from group 5 ($p < 0.05$).

2.2. Experimental procedure for evaluating the tumor-initiating activity

The present study was approved by the Experimental Animal Research Committee of Gifu University. A total of 38 male ICR mice were divided into five experimental and solvent control groups. Groups 1 through 3 were treated with the single intragastric (i.g.) intubation of ABAQ at a dose of 100 or 200 mg/kg body weight. Starting one week after the ABAQ treatment, the animals in groups 1 ($n = 15$) and 2 ($n = 15$) were given 2% (w/v) DSS in drinking water for seven days, followed by no further treatments for 14 weeks. Groups 3 ($n = 5$) and 4 ($n = 5$) were given ABAQ (200 mg/kg body weight) alone and 2% DSS alone, respectively. Group 5 ($n = 8$) was given solvent (physiological saline) alone and served as an untreated control group. All animals were sacrificed *via* CO_2 asphyxiation at week 16. The colons were flushed with saline, excised, measured for length (from the ileocecal junction to the anal verge), cut open longitudinally along the main axis and washed with saline. After carefully macroscopically inspecting the colons, the tissues were cut and fixed in 10% buffered formalin for at least 24 h. A histological examination was performed on paraffin-embedded sections following hematoxylin and eosin (H&E) staining. The presence or absence of mucosal ulceration, dysplasia and colonic neoplasms was examined according to our previous report [9]. A histopathological examination was also performed for other organs.

2.3. Immunohistochemistry of minichromosome maintenance protein 2 (MCM2), β-catenin and programmed cell death 4 (PDCD4)

We used paraffin-embedded sections of the colons of the mice in all groups for the immunohistochemical analysis. Serial histological sections (4 μm thickness) were made from each paraffin wax block. Immunohistochemical staining was performed automatically (Ventana Benchmark XTsystem; Ventana, Touchstone, Arizona, USA), according to the manufacturer's instructions. The primary antibodies were anti-MCM2 rabbit monoclonal antibody (no. 3619, anti-MCM2 (D7611)XP, 1:400 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-β-catenin rabbit polyclonal antibody (#9661, 1:200 dilution; Cell Signaling Technology) and anti-PCDC4 rabbit polyclonal antibody (ab51495, 1:500 dilution; Abcam, Inc. Cambridge, MA, USA). In each case, the positive and negative controls were run concurrently. As the final step, the sections were lightly counterstained with Mayer's hematoxylin (Merck, Tokyo, Japan).

Immunoreactivity for antibodies against MCM2, PDCD4 and β-catenin was assessed in the lesions (high-grade dysplasia) that developed in groups 1 through 3 using a microscope (Olympus BX41, Olympus Optical Co., Tokyo, Japan). The intensity and localization of the immunoreactivity against the primary antibodies were determined by a pathologist (T.T.) who was unaware of the treatment group to which the slide belonged.

2.4. Statistical analysis

All measurements were compared using a one-way ANOVA with either Tukey's correction or Fisher's exact probability test (GraphPad Instat version 3.05, GraphPad Software, San Diego, CA), with a value of $p < 0.05$ as the criterion for significance.

3. Results

3.1. General observations

As listed in Table 1, the mean liver ($p < 0.05$) and relative liver weights ($p < 0.01$), and mean colon length ($p < 0.05$) in group 1 (100 mg/kg ABAQ + 2% DSS) were significantly larger than those observed in group 3 (200 mg/kg ABAQ alone). The mean liver weight and colon length of group 3 were significantly lower than that of group 5 (solvent control) ($p < 0.05$ for each comparison).

3.2. Pathological findings of the liver and colorectum of mice treated with ABAQ and/or DSS

There were no tumors in any organs, including the colorectum, in all groups. Fatty changes were observed in the liver of two mice (13%) in group 1, but not in other groups. As indicated in Table 2, colonic dysplasia (high-grade, Fig. 1b) developed in groups 1 through 3. DSS exposure increased the multiplicity of high-grade dysplasia induced by ABAQ. There were no dysplastic lesions in the mice in groups 4 and 5.

Table 2
Incidence and multiplicity of colonic lesions in the mice in each group.

Group no.	Treatment (no. of mice examined)	Mucosal ulcer	High-grade dysplasia
1	ABAQ [a] (100 mg/kg bw) → 2% DSS (15)	10/15 (67%) 1.40 ± 1.18 [b]	4/15 (27%) [c, d] 0.53 ± 1.13
2	ABAQ (200 mg/kg bw) → 2% DSS (15)	11/15 (73%) 1.27 ± 1.16	10/15 (67%) [e] 1.60 ± 1.50
3	ABAQ (200 mg/kg bw) (5)	0/5 (0%) 0	1/5 (20%) 0.20 ± 0.45
4	Solvent → 2% DSS (5)	5/5 (100%) 1.80 ± 0.84 [f]	0/5 (0%) 0
5	None (8)	0/8 (0%) 0	0/8 (0%) 0

[a] ABAQ, 5-amino-6-hydroxy-8*H*-benzo[6,7]azepino[5,4,3-*de*]quinolin-7-one; DSS, dextran sodium sulfate.
[b] Mean ± SD.
[c,d,e] Significantly different from group 2 ([c] $p < 0.05$), group 3 ([d] $p < 0.05$) and group 4 ([e] $p < 0.05$) according to the Fisher's exact probability test.
[f] Significantly different from group 5 ($p < 0.05$) according to a one-way ANOVA and the Tukey–Kramer Multiple Comparison test.

3.3. Immunohistochemical expression of MCM2, β-catenin, and PDCD4 in the high-grade dysplasia lesions

The nuclei in the high-grade dysplasia lesions were positive for MCM2 (Fig. 1c), reflecting a high proliferation activity, and negative for PDCD4 (Fig. 1d). A cytoplasmic and nuclear expression of β-catenin was observed in the high-grade dysplasia lesions (Fig. 1e).

4. Discussion

In this study, we confirmed the tumor-initiating ability of ABAQ in an inflammation-associated, two-stage mouse carcinogenesis model. Importantly, ABAQ treatment alone produced lesions exhibiting high-grade dysplasia lesions that are preneoplastic for colorectal cancer, although the incidence and multiplicity were low and statistically insignificant from group 5 (untreated control). One week of exposure to DSS after the single i.g. administration of ABAQ increased the incidence and number of high-grade dysplasia lesions in the colorectum. The dysplasia-inducing potency was considered to be dose-dependent, although only two doses of ABAQ were applied in this study.

In this study, dosing of ABAQ (100 mg/kg) followed by DSS (group 1) increased the liver weight, while 200 mg/kg of ABAQ (group 2) did not. However, both doses of ABAQ increased the relative liver weight. Fatty degeneration observed in the liver of a few mice of group 1 may be related to these changes. As to the colon length, the treatment of ABAQ alone (group 3) shortened the colon, suggesting pro-inflammatory action of ABAQ, but the colon lengths of groups 1 and 2 were increased when given DSS. In order to proof the dose dependency and discard the possible pro-inflammatory effect at higher doses of ABAQ, which might contribute to the tumor development, it would be interesting to use three or four different doses (from 50 to 200 mg/kg, for instance). Such an experiment is planned in our laboratory.

The Maillard reaction *in vivo* is involved in aging [12] and a variety of chronic diseases, such as diabetes and related retinopathy and nephropathy [13]. Pyrraline is formed as an advanced glycation end product in the Maillard reaction between glucose and the ε-amino group of lysine under physiological conditions [14]. The serum [15] and urine [16] concentrations of pyrraline are known to be increased in diabetic patients, and pyrraline is detected in individuals with diabetic glomerulosclerosis [17]. Epidemiological [18,19] and experimental investigations [20,21] have indicated a positive association between diabetes and cancer development in several tissues, including the colorectum, suggesting that certain chemicals formed by the Maillard reaction in the body increase the risk of cancer in patients with diabetes. However, little is known about mutagens formed through the Maillard reaction *in vivo*.

In this study, we observed high-grade dysplasia in the colorectum of the mice treated with ABAQ alone and ABAQ followed by DSS exposure. Although no colorectal neoplasms developed, these findings are of importance since high-grade dysplasia is known to be a precursor lesion of inflammatory conditions in the colon, such as inflammatory bowel disease (IBD) [22]. In the fact, the high-grade dysplasia observed in this study increased the degree of proliferation, as estimated on MCM2 immunohistochemistry, and altered the expression of β-catenin in the cytoplasm and nuclei. More importantly, almost null expression of PDCD4 was observed in the high-grade dysplasia lesions. This finding is in accordance with the results showing a negative expression of PDCD4 in patients with sporadic colorectal cancer [23,24], IBD-related colorectal cancer [22,25] and dysplasia in IBD [22,25].

In conclusion, the results of the current study indicate the potential tumor-initiating activity of ABAQ in the colon, with and without inflammation. Although the ABAQ level has not been determined in healthy subjects, ABAQ is a potential novel endogenous mutagen and tumor-initiating compound, as shown in this study and a previous investigation [7]. Since the mutagenic activity of ABAQ is comparable to that of PhIP [7], additional studies of the carcinogenicity of ABAQ in the colon and other tissues are required. Our findings provide a scientific basis for further research on the involvement of ABAQ in human health.

Conflict of interest

The authors declare no financial or commercial conflicts of interests.

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Original Contribution

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

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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 (εA), 3,N^4-ethenocytosine (εC), butanone-ethenocytosine (BεC), butanone-ethenoguanine (BεG), heptanone-ethenocytosine (HεC), or heptanone-ethenoguanine (HεG) using a DNA cleavage assay. We found that TDG is capable of removing thymine that has mispaired with εC, BεC, BεG, HεC, or HεG *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 εC; and (c) a modest acceleration of mutations caused by εC, 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 toward 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 (εA), 3,N^4-ethenocytosine (εC), butanone-ethenocytosine (BεC), butanone-ethenoguanine (BεG), heptanone-ethenocytosine (HεC), and heptanone-ethenoguanine (HεG) (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 εA and εC [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 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, εC in DNA is excised by the human DNA glycosylase enzyme TDG [18]. TDG is involved in the removal of

Abbreviations: εA, 1,N^6-ethenoadenine; εC, 3,N^4-ethenocytosine; BεC, butanone-ethenocytosine; BεG, butanone-ethenoguanine; BER, base excision repair; CAA, chloroacetaldehyde; 5-FU, 5-fluorouracil; HεC, heptanone-ethenocytosine; HεG, heptanone-ethenoguanine; HEC, 3,N^4-α-hydroxyethanocytosine; 4-HNE, 4-hydroxy-2-nonenal; LPO, lipid peroxidation; MMR, mismatch repair; 4-OHE, 4-oxo-2-hexenal; 8OHG, 8-hydroxyguanine; 4-ONE, 4-oxo-2-nonenal; PUFAs, polyunsaturated fatty acids; VC, vinyl chloride.

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thymine and uracil mispaired with guanine, 5-fluorouracil (5-FU) paired with G and A as well as εC from εC: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-formylcytosine, and 5-carboxycytosine [20–23]. εC is excised also by the DNA glycosylase enzyme SMUG1, and εA 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 (εA, εC, BεC, BεG, HεC, and HεG).

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) were reacted at 60 °C in EtOH-H_2O 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]. $^{15}N_5$-labeled εdC was prepared using a procedure similar to that described above for nonlabeled 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-2 T 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-1λT, SMUG1-pGEX-2 T, and TDG-pGEX-2 T vectors. Transformed cells were cultured at 37 °C until an A_{600} of 0.6, and protein expression was induced by incubation with 0.1 mM IPTG at 15 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-His_6, UNG2-His_6 and MPG-His_6 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 (pH 7.6), 50 mM NaCl, 0.5 mM DTT, 0.1 mM EDTA, 0.5 mM PMSF, 2 μg/mL pepstatin, 2 μg/mL leupeptin, 50 μM 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 ^{32}P-labeled at the 5′ terminus using a MEGA-LABEL kit (Takara, Osaka, Japan) and [γ-^{32}P]ATP (PerkinElmer, Tokyo, Japan) and 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-^{32}P]ATP and then annealed to oligonucleotides containing a single damaged base. For the DNA cleavage activity assay, 300 fmol of purified OGG1, SMUG1, MPG, or NEIL1 proteins was reacted in 20 μL of a mixture containing 20 mM sodium phosphate (pH 7.6), 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 femtomoles of NTH1 or UNG2 proteins was reacted in 20 μL of a mixture containing 20 mM Hepes-KOH (pH 7.5), 50 mM NaCl, 0.5 mM DTT, 1 mM $MgCl_2$, 1.5% glycerol, 2.5 nM labeled oligonucleotide, and 50 μg/mL BSA. Three hundred femtomoles of TDG protein was reacted in 20 μL of a mixture containing 20 mM sodium phosphate (pH 7.6), 1 mM DTT, 0.5 mM EDTA, 1.5% glycerol, 2.5 nM labeled oligonucleotide, and 50 μg/mL BSA. Three hundred femtomoles of MUTYH protein was reacted in 20 μL of a mixture containing 20 mM Hepes-KOH (pH 7.5), 50 mM NaCl, 0.5 mM DTT, 0.5 mM $ZnCl_2$, 1.5% glycerol, 2.5 nM labeled oligonucleotide, and 50 μg/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 ^{32}P-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 μM CAA (Wako, Tokyo, Japan), 0–50 μM 4-ONE, or 0–50 μM 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 (pH 7.5) 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 sodium cacodylate (pH 6.5) 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 was treated with buffer containing 20 mM sodium phosphate (pH 7.0), 17% ethanol, and 5 mM 4-ONE at 37 °C for 3 h and with buffer containing 20 mM sodium phosphate (pH 7.0), 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 (pH 7.4), 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 (pH 5.3), 2 μL of 1 M $ZnCl_2$, 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 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: εdC [252.1→136.05, 35 V, 14 eV], $^{15}N_3$-εdC [255.1→139.05, 35 V, 14 eV], heptanone-ethenodeoxycytidine (HεdC) [364.2→248.15, 35 V, 14 eV], $^{15}N_3$-HεdC [367.2→251.15, 35 V, 14 eV], butanone-ethenodeoxycytidine (BεdC) [322.13→206.08, 35 V, 14 eV], $^{15}N_3$-BεdC [325.13→209.08, 35 V, 14 eV], butanone-ethenodeoxyguanisine (BεdG) [362.13→246.08, 35 V, 14 eV], and $^{15}N_5$-BεdG [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 was 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 εA, εC, BεC, BεG, HεC, and HεG, 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 toward 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 εA, εC, BεC, BεG, HεC, or HεG 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 toward the etheno-DNA adduct itself and an unmodified base paired with an etheno adduct. When an oligonucleotide containing an εA 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 εA:A, εA:T, εA:C, or εA:G, which agreed with previous findings [37] (Fig. 1A). When an oligonucleotide containing an εC adduct was reacted, the cleavage product was detected in the lanes of the reaction of the SMUG1 protein and an oligonucleotide containing εC:A, εC:T, εC:C, or εC:G and in the lane of the reaction of the TDG protein and an oligonucleotide containing εC:G (Fig. 1B). Although the cleavage activity of SMUG1 and TDG to the εC:G substrate has been previously reported [24,25], the effects of SMUG1 on the εC:A, εC:T, and εC:C substrates are novel findings. Since the DNA cleavage assay against εC and HεC showed signal background at the cleavage product position to some extent, there was some difficulty in judging the cleavage activity of some proteins (Figs. 1B and 3A). So, we extended a reaction time to 1 h, and examined DNA glycosylase activity toward an oligonucleotide containing εC and HεC 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 εC-containing oligonucleotide and those in the lanes of the reaction of the all the

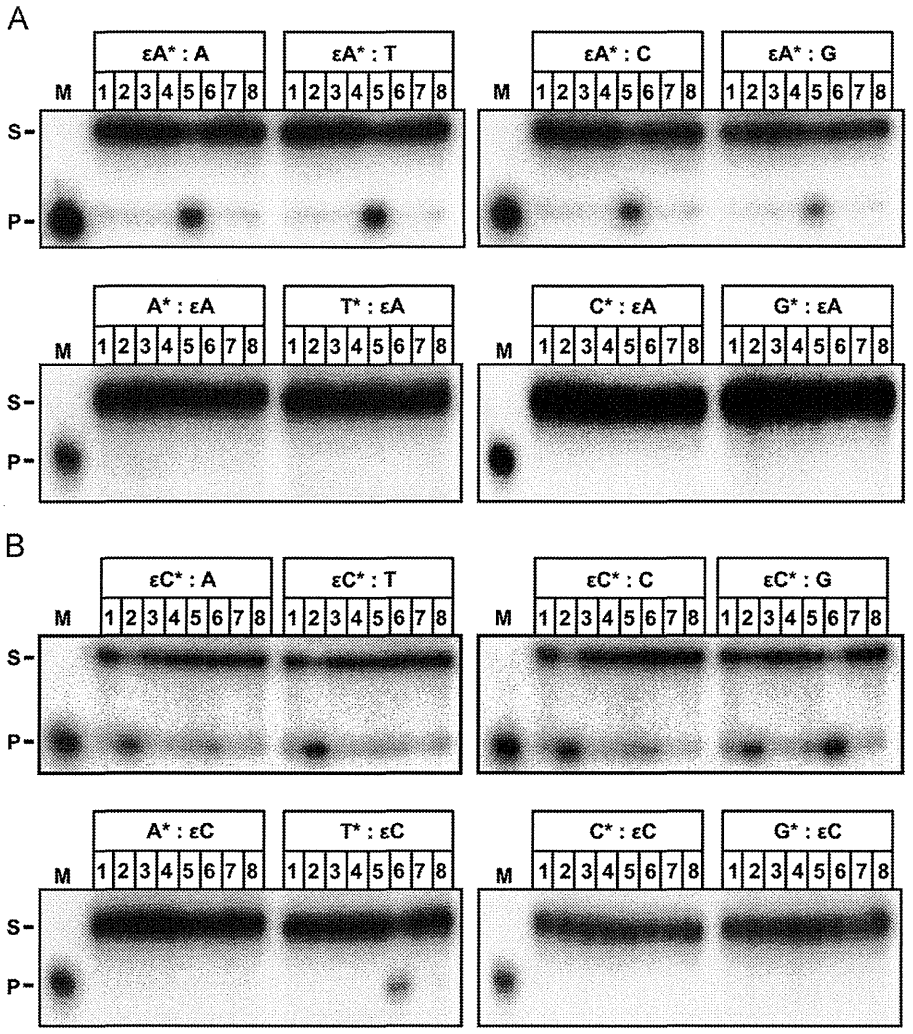

Fig. 1. Evaluation of repair activities of 8 DNA glycosylase proteins against 1,N^6-ethenoadenine (εA) and 3,N^4-ethenocytosine (εC). The abilities of the DNA glycosylase proteins OGG1, SMUG1, NTH1, UNG2, MPG, TDG, MUTYH, and NEIL1 to repair double-stranded DNA containing εA (A) or εC (B) were examined using a DNA cleavage assay. Each BER protein (300 fmol) 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 ^{32}P-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 ^{32}P-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.

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 eight DNA glycosylase proteins do not have 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 toward 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 percentage 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 was 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 εC, BεC, BεG, HεC, or HεG as well as εC in the εC: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 εC) 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 μM CAA (Fig. 5A).

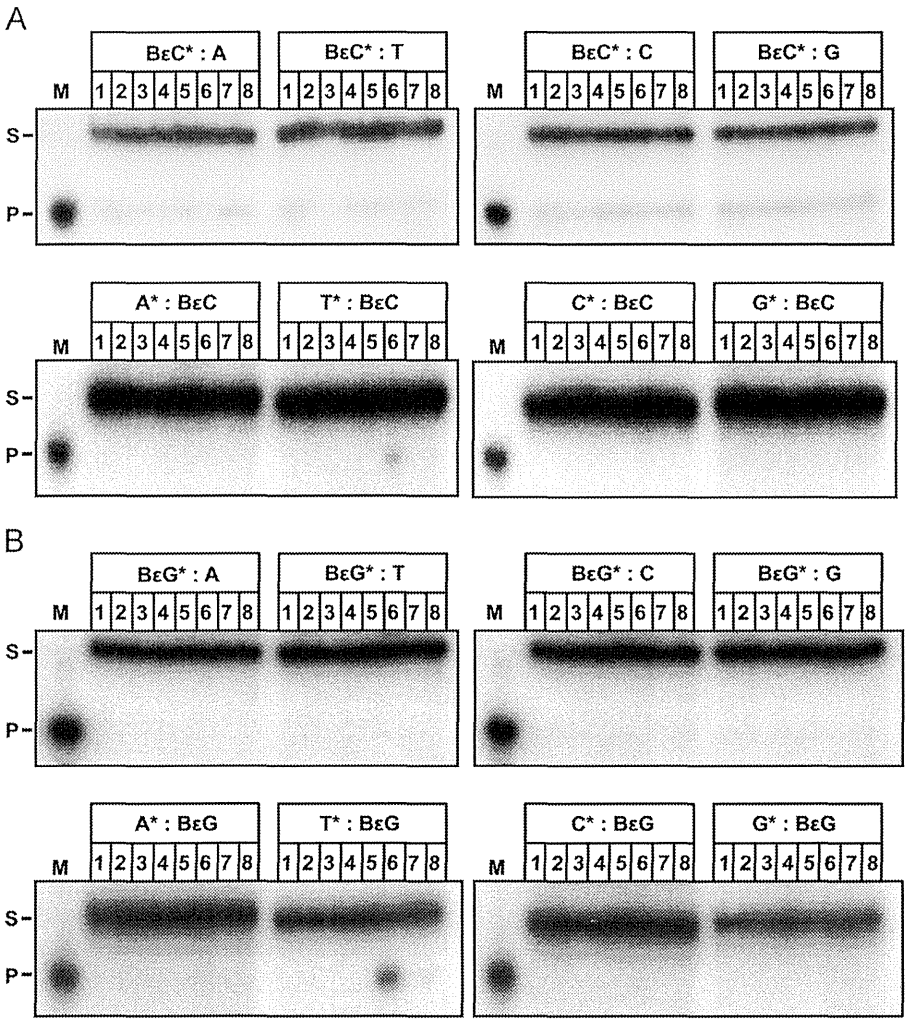

Fig. 2. Evaluation of repair activities of 8 DNA glycosylase proteins against 4-oxo-hexenal adducts. The abilities of the DNA glycosylase proteins OGG1, SMUG1, NTH1, UNG2, MPG, TDG, MUTYH, and NEIL1 to repair double-stranded DNA containing butanone-ethenocytosine (BεC) (A) or butanone-ethenoguanine (BεG) (B) were examined using a DNA cleavage assay. Each BER protein (300 fmol) 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 ^{32}P-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 ^{32}P-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 survival of cells exposed to 5–30 μM 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 μM 4-ONE or 4-OHE (Fig. 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 εdC, HεdC or butanone-etheno adducts, respectively, using an LC-MS/MS analysis. A dramatic increase was observed in the levels of εdC, HεdC, 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 εdC, HεdC, BεdC, and BεdG 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 HεdC, BεdC and BεdG were seen between the TDG-knockdown cells and the control cells (Fig. 6B–D); however, remarkably, the εdC 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 εC 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 CAA; the mutation frequency was then compared between the 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 the TDG-knockdown cells (Figs. 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

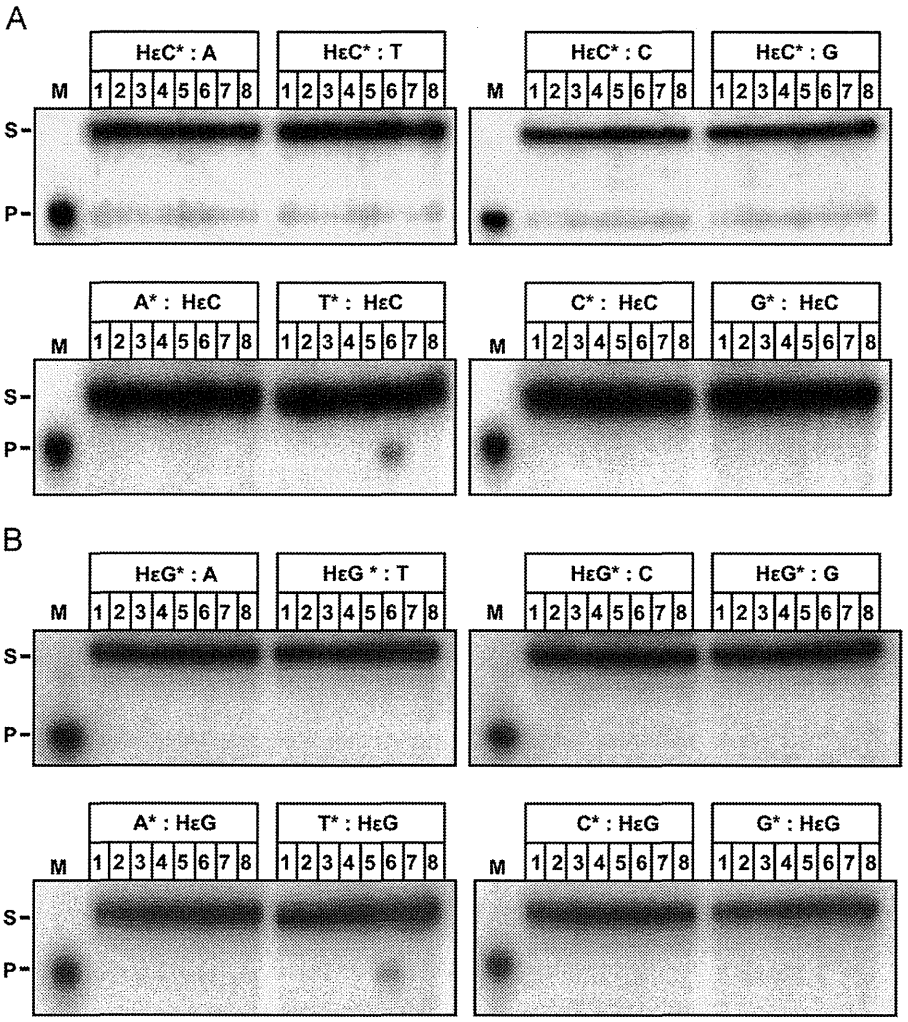

Fig. 3. Evaluation of repair activities of 8 DNA glycosylase proteins against 4-oxo-nonenal adducts. The abilities of the DNA glycosylase proteins OGG1, SMUG1, NTH1, UNG2, MPG, TDG, MUTYH, and NEIL1 to repair double-stranded DNA containing heptanone-ethenocytosine (HεC) (A) or heptanone-ethenoguanine (HεG) (B) were examined using a DNA cleavage assay. Each BER protein (300 fmol) 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 ^{32}P-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 ^{32}P-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.

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 spectra 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 spectra 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 εC in human cells may be modest.

Discussion

εA, εC, N^2,3-ethenoguanine (N^2,3-εG), and 1,N^2-ethenoguanine (1,N^2-εG) in etheno-DNA adducts are reported to be repaired in *E. coli* and human BER pathways by several groups [40]. However, the repair systems of BεC, BεG, HεC, and HεG 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 functions 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 εC, the human BER enzyme TDG is also capable of removing thymine mispaired with εC, BεC, BεG, HεC, and HεG *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 εC, 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*.

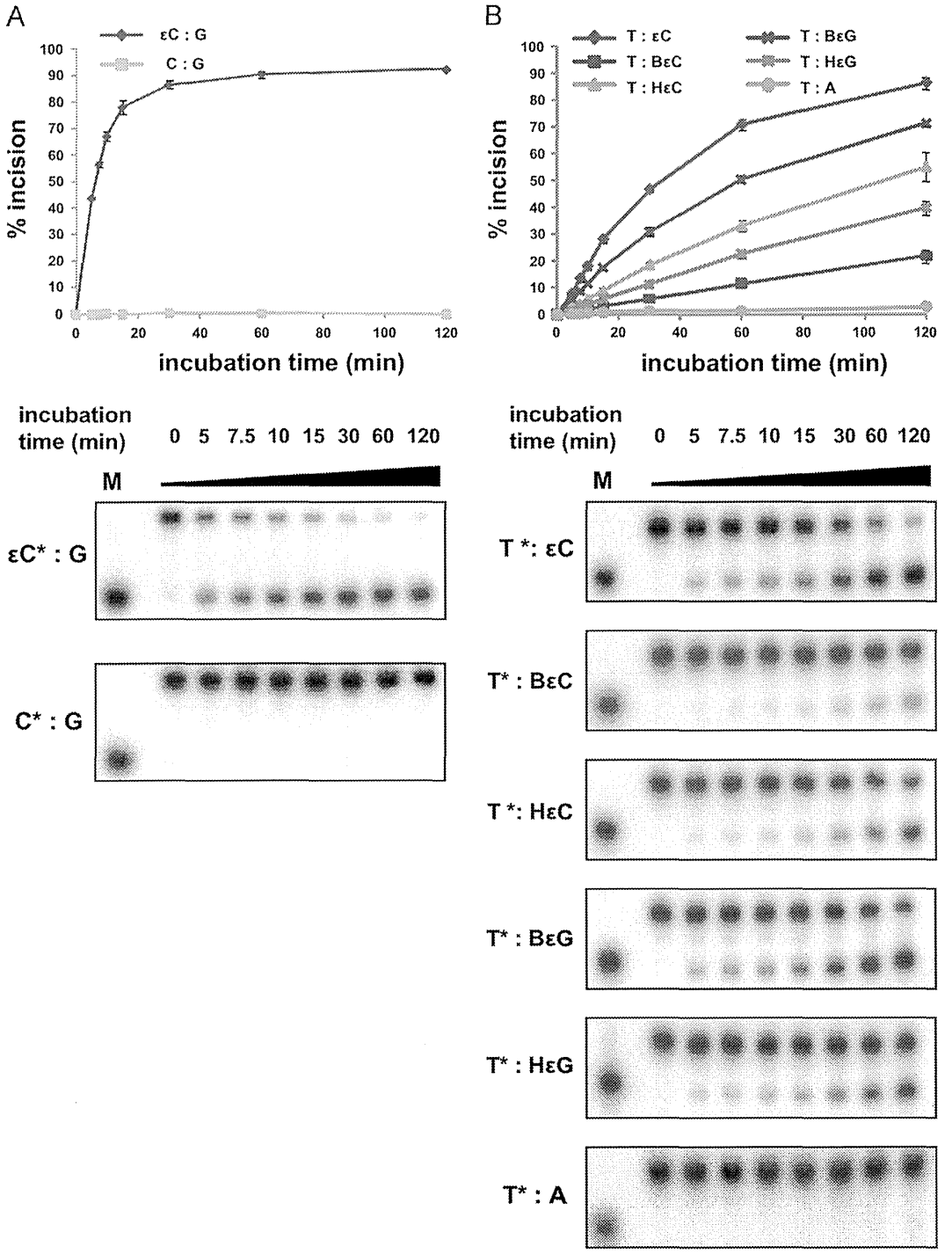

Fig. 4. Time-course assay for cleavage of DNA containing an etheno adduct by TDG protein. (A) Time-course assay for the cleavage of DNA containing εC:G and C:G base pairs by TDG protein. TDG protein (300 fmol) was incubated at 37 °C for 0–120 min with double-stranded oligonucleotides containing εC:G and C:G base pairs (50 fmol). The amount of cleavage products as a proportion of the total oligonucleotides was calculated as the % incision. The % incision valves 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 fmol) 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 fmol). The lower panels show representative results of the DNA cleavage assays for TDG protein.

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 embryonic 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 break 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

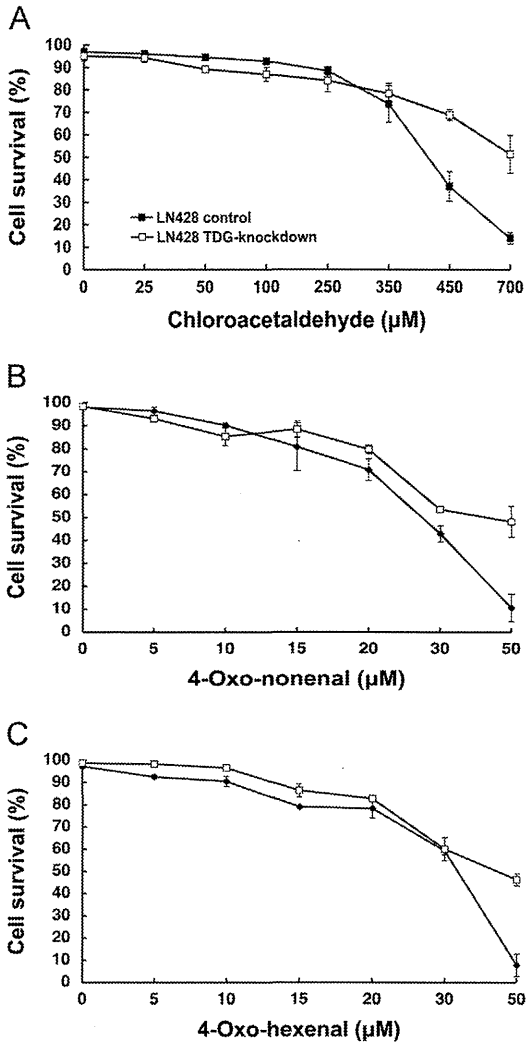

Fig. 5. Cell survival of LN428-control and LN428-TDG-knockdown cells treated with CAA, 4-ONE, or 4-OHE. Cells were treated with (A) 0–700 μM CAA, (B) 0–50 μM 4-ONE, or (C) 0–50 μM 4-OHE for 2 h; after the medium was exchanged with fresh medium, survival was measured after 12 h using a trypan blue exclusion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

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 doses 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 the 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 εC, especially C:G to A:T transversions. When the DNA polymerase inserts T, DNA containing εC falls into a futile repair cycle, and suffers from persistent single-strand break. If the polymerase inserts A and C, εC: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 HεC has been reported that HεC 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 HεC can also be prevented by TDG. The position where the bases were removed by TDG are reportedly reconstituted by BER pathway-related enzymes including DNA polymerase beta [48]. In future studies, we would like to clarify the details on how TDG and its related enzymes work after T against etheno-DNA adduct was removed.

In this paper, the *supF* forward mutation assay showed a 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 εC. 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 εC DNA adducts, but also other DNA adducts, such as εG, in DNA [49].

In our quantitative analysis of adduct level in transfected plasmids (Fig. 6A), the εdC levels of plasmids transfected into TDG-knockdown cells were higher than those of plasmids transfected into control cells. The reduced repair activity of TDG toward εC in TDG-knockdown cells may compensate the ordinary power to reduce εdC levels in the cells. Molatore et al. [50] and Turco et al. [51] have reported that the 8OHG levels in immortalized MEFs derived from Mutyh-/- mice were higher than in wild-type MEFs after exposure to $KBrO_3$, a base damage inducer. The results of both papers suggest that a difference in DNA glycosylase activity toward 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 εC:T to εC:G, the loss of this function of TDG itself may also contribute to the remaining εdC 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

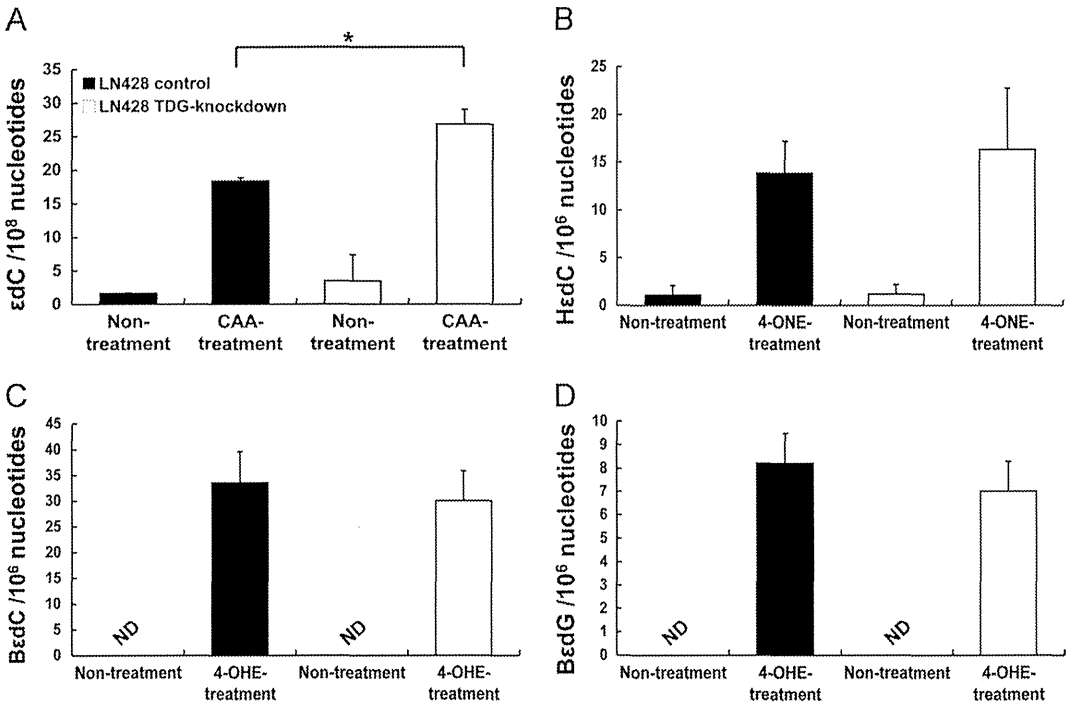

Fig. 6. Measurement of repair activity against etheno-DNA adducts in LN428-control and LN428-TDG-knockdown cells. Adduct-modified pEGFP plasmids were transfected into the cells; after incubation for 24 h, the plasmids were collected and the levels of εdC (A), HεdC (B), BεdC (C), and BεdG (D) were measured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

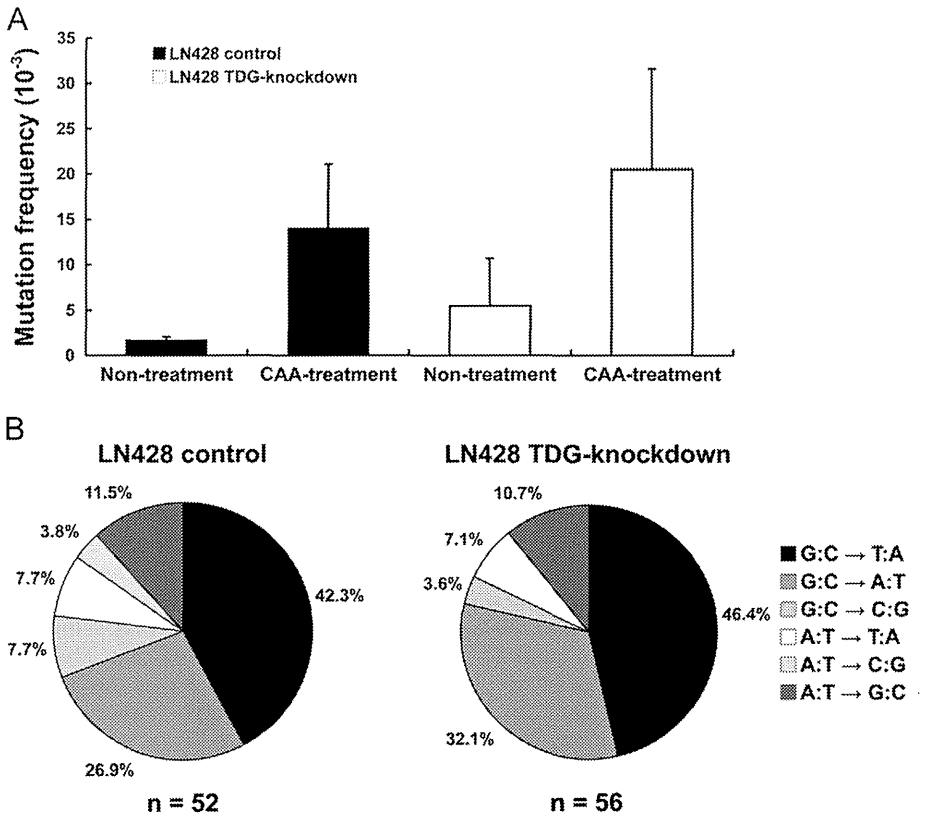

Fig. 7. *SupF* forward mutation assay using CAA-treated plasmid pMY189 in LN428-control and LN428-TDG knockdown cells. (A) The mutation frequency of the *supF* gene in pMY189 was measured using a *supF* forward mutation assay. The mutation frequency is shown as the mean ± standard deviation of data from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

that etheno-DNA adducts are decomposed under alkaline conditions and converted into ring-opened structures [46,52,53]. 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 an apurinic/apyrimidinic site. Interestingly, alkali treatment against etheno-DNA adducts seems to have a stronger influence on

the cytosine-DNA adducts, in particular on εC, than on guanine-DNA adducts.

There is nothing simple in the repair of etheno-DNA adducts. εA and εC are repaired not only by BER systems but also by *E. coli* AlkB family proteins and their human homologues ABH2 and ABH3 proteins [54–56]. AlkB enzymes directly reverse etheno-DNA adducts to normal base in DNA [54,57]. 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 [58]. TDG is known to also excise T mispaired with O^6-meG [59,60], 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 carcinomas [61,62]. In addition, the loss of heterozygosity of the *TDG* gene, which is located at chromosome 12q24.1, has been detected in gastric cancers [63]. 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 [48,64] and in the current study, as well as other TDG functions, such as DNA demethylation, may be involved in such cancer-preventing roles.

Acknowledgments

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Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2014.07.044.

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Glycemic Variability Is Associated With Quality of Life and Treatment Satisfaction in Patients With Type 1 Diabetes

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Patients with type 1 diabetes have greater glycemic variability than that in patients with type 2 diabetes. However, neither glucose variability nor hypoglycemia is detected precisely by HbA_{1c}. This study investigated whether glycemic variability assessed by continuous glucose monitoring influences quality of life (QOL) and treatment satisfaction in patients with type 1 diabetes.

The study was conducted in Kyoto University Hospital between September 2011 and June 2012. The study protocol was approved by the institutional review board (UMIN Clinical Trials Registry UMIN000005833). Twenty-eight patients with type 1 diabetes aged ≥18 years were included in analyses (age, 45.9 ± 14.5 [mean ± SD] years; diabetes duration, 15.0 ± 8.2 years; 57% female; 21% using insulin pump; HbA_{1c}, 8.1 ± 1.2% [64.5 ± 13.0 mmol/mol]). Glycemic variability in everyday life was assessed for a 72-h period using the CGMS System Gold continuous glucose monitoring system (Medtronic, Northridge, CA), and mean absolute glucose (MAG) change was calculated as a glycemic variability measure. This is a summation of all absolute changes in glucose divided by the time over which the measurements were taken (1). QOL and treatment satisfaction were evaluated by the diabetes quality-of-life measure (DQOL) and the Diabetes Treatment Satisfaction Questionnaire (DTSQ) (available from www.healthpsychologyresearch.com) (2–4). We divided the participants into two groups by HbA_{1c} level—<8% (64.0 mmol/mol), good/fair-control group (n = 14); ≥8% (64.0 mmol/mol),

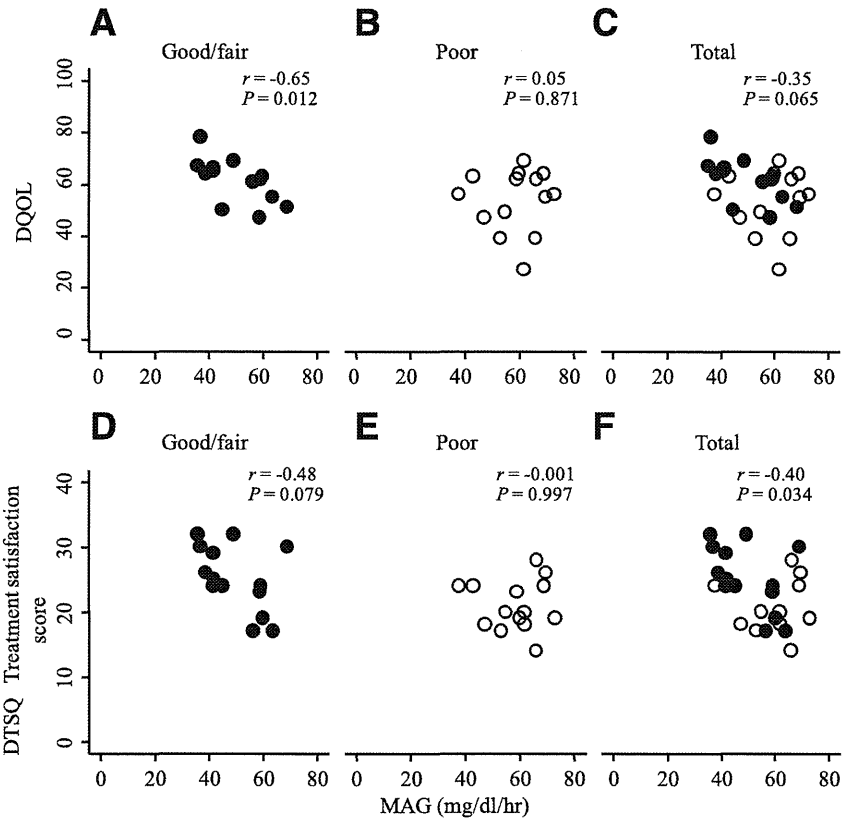

Figure 1—Correlation between glycemic variability and DQOL (*A*–*C*) and DTSQ (*D*–*F*).

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poor-control group ($n = 14$)—considering HbA_{1c} as a potential intermediate variable between glycemic variability and patient-reported outcomes (5). Potential confounding factors were use of carbohydrate-counting with insulin adjustment, age, sex, diabetes duration, and use of insulin pump and the Clarke hypoglycemic score.

Glycemic variability correlated negatively with DQOL in the good/fair-control group ($r = -0.65$, $P = 0.01$), whereas there was no correlation in the poor-control group ($r = 0.05$, $P = 0.87$) (Fig. 1). Glycemic variability correlated negatively with DTSQ across all patients ($r = 0.40$, $P = 0.03$). No significant confounding effect was identified by stepwise selection.

Our study identifies the important association of glycemic variability with diabetes-related QOL and treatment satisfaction in patients with type 1 diabetes. Interestingly, the association between glycemic variability and diabetes-related QOL, which measures satisfaction, impact, social worries, and diabetes worries, was limited to the group with good/fair glycemic control, indicating that the contribution of glycemic variability to QOL is emphasized by better glycemic control. On the other hand, the insignificant association in the poor-control group may imply other important predictors of QOL in patients with poorer glycemic control. The strong points of this study include use of a relatively new indicator, MAG change. It differs from standard deviation, a measure of how spread out data values are around the mean, in that it represents not only dispersion but also the rate of change of blood glucose. Although the limitations of our study include small sample size and exclusion of 12 of 40 participants whose continuous glucose monitoring data were less than 48 h because of disconnection of the sensor, calibration errors, or out-of-range data, this is the first report about the important association between glycemic variability and patient-reported outcomes in type 1 diabetes.

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Author Contributions. S.A.-T. designed the study, collected and researched data, analyzed data, interpreted data, and wrote the manuscript. K.I. designed the study, collected and researched data, analyzed data, interpreted data, and reviewed and edited the manuscript. S.F. designed the study, analyzed data, interpreted data, and reviewed and edited the manuscript. A.H., S.H., K.T., Y.F., K.N., and D.T. collected data and contributed to the discussion. N.I. reviewed and edited the manuscript and contributed to the discussion. N.I. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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