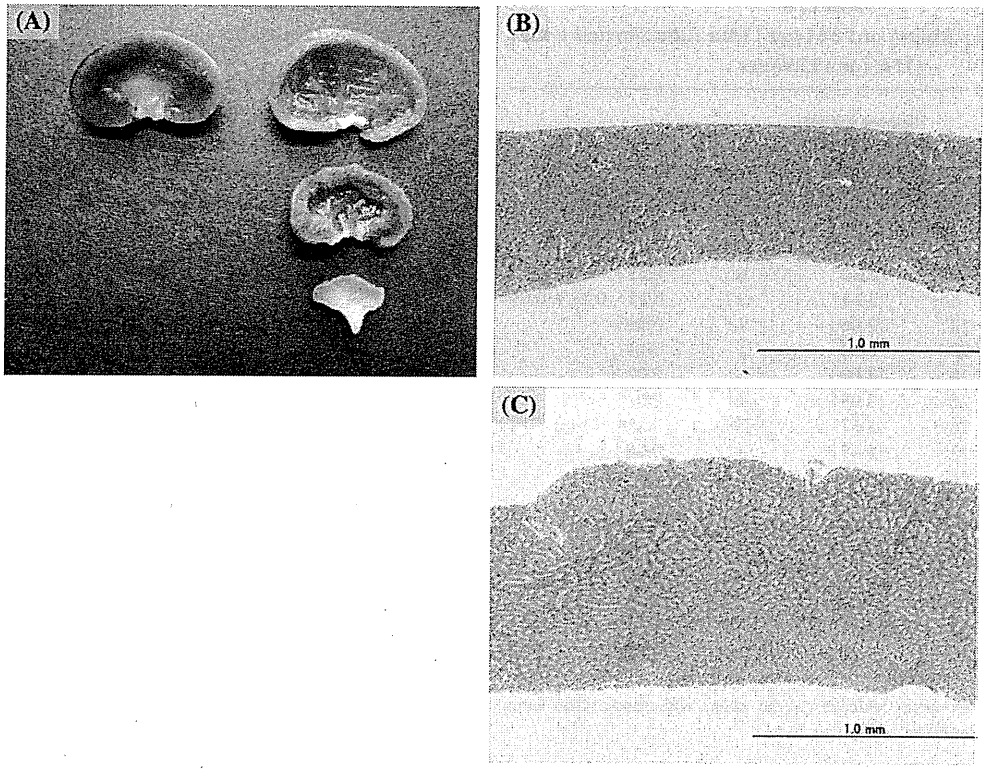

FIG. 2. (A) Kidney cut on the long axis (left). The kidney is divided into three parts, COR (upper right), OM (mid right), and the inner medulla (lower right). (B) Microscopic examination of the S1 and S2 segments of the proximal tubules and the glomeruli, but not the medullar components of the COR. (C) Microscopic examination showed primarily the S3 segment of the proximal tubules in the OM. HE stain.

stimuli (Adler *et al.*, 2009). Thus, the overall data indicate that the distribution of OTA in the kidney is not uniform but is very eccentrically located at the OSOM. Therefore, the observation that even a 13-week exposure to OTA, which is much longer than the 4 weeks recommended in the standard protocol for reporter gene mutation assays (WHO, 2006), did not affect the MFs of *gpt* and *red/gam* in DNA extracted from whole kidneys does not imply that genotoxic mechanisms are not involved in OTA-induced renal carcinogenesis.

Until now, in cases where chemical carcinogens target epithelial cells of hollow viscera, DNA was extracted after scraping the mucosal surface to be examined in reporter gene mutation assays (Okamura *et al.*, 2010). In the present study, the kidney was cut into three parts based on morphological characteristics: the COR, OM, and the inner medulla. Histopathological analyses confirmed the identities of each section. In particular, it was recognized that the OM was composed chiefly of the S3 segment of the proximal tubules, an OTA target site. The reporter gene mutation assay was then performed using DNA extracted from the COR and OM. The MFs of the *red/gam* genes in the OM were significantly increased despite a lack of mutations in the *gpt* genes. MFs of the *gpt* and *red/gam* genes were not changed in the COR. Thus, OTA was able to induce reporter gene mutations at the target site, strongly suggesting involvement of genotoxic mechanisms in OTA-induced renal carcinogenesis.

Because inactivation of the red/gam protein requires a disruption spanning the two genes, a positive result in the Spi^- assay is indicative of an increase in deletion mutations (~10,000 bp) (Nohmi *et al.*, 2000; WHO, 2006). Therefore, the present data suggest that OTA exposure is capable of inducing deletion mutations in the DNA of the target site. We demonstrated that potassium bromate, a renal carcinogen, is positive in Spi^- assay along with 8-OHdG formation in kidney DNA (Umemura *et al.*, 2006). Yang *et al.* (2006) found that deletion mutations occurred following 8-OHdG formation during base excision repair by *OGG1* encoding a specific repair enzyme for 8-OHdG adduct. It has also been reported that OTA suppresses some antioxidant enzymatic activities and generates lipid peroxidation (Ozçelik *et al.*, 2004) and that the toxin inhibits the activity of the transcription factor Nrf2 induced by oxidative stress (Cavin *et al.*, 2007). Accordingly, it has been proposed that oxidative stress might be involved in OTA-induced tumorigenesis (JECFA, 2008; Pfohl-Leszkowicz and Manderville, 2007). However, OTA exposure to rats failed to elevate 8-OHdG

TABLE 6
gpt MFs in the COR or OM of Male F344 *gpt* Delta Rats Treated with OTA for 4 Weeks

Sites	Treatment	Animal no.	Cm^R colonies ($\times 10^5$)	Cm^R and 6-TG^R colonies	MF ($\times 10^{-5}$)	Mean ± SD
COR	Control	1	6.03	1	0.17	
		2	4.28	1	0.23	
		3	6.98	3	0.43	
		4	10.67	4	0.38	
		5	5.31	1	0.19	0.28 ± 0.12
	OTA	11	5.85	3	0.51	
		12	10.98	2	0.18	
		13	7.92	0[a]	—	
		14	4.10	1	0.24	
		15	8.33	1	0.12	0.26 ± 0.17
OM	Control	1	13.14	1	0.08	
		2	11.61	2	0.17	
		3	6.39	2	0.31	
		4	9.18	1	0.11	
		5	2.84	1	0.35	0.20 ± 0.12
	OTA	11	3.92	1	0.26	
		12	10.26	4	0.39	
		13	8.42	4	0.48	
		14	3.96	2	0.51	
		15	3.78	1	0.26	0.38 ± 0.12

[a]No mutant colonies were detected on the plate, with those data being excluded from the calculation of MF.

TABLE 7
Spi^- MFs in the COR or OM of Male F344 *gpt* Delta Rats Treated with OTA for 4 Weeks

Sites	Treatment	Animal no.	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaque within WL95 (P2)	MF ($\times 10^{-5}$)	Mean ± SD
COR	Control	1	13.32	6	0.45	
		2	13.68	2	0.15	
		3	14.31	5	0.35	
		4	5.67	1	0.18	
		5	7.83	1	0.13	0.25 ± 0.14
	OTA	11	6.03	2	0.33	
		12	13.32	4	0.30	
		13	10.44	3	0.29	
		14	10.8	1	0.09	
		15	11.52	4	0.35	0.27 ± 0.10
OM	Control	1	12.51	6	0.48	
		2	20.43	1	0.05	
		3	11.52	0[a]	—	
		4	16.2	1	0.06	
		5	14.76	1	0.07	0.16 ± 0.21
	OTA	11	17.28	9	0.52	
		12	15.75	6	0.38	
		13	15.12	4	0.26	
		14	10.53	7	0.66	
		15	13.68	6	0.44	0.45 ± 0.15*

[a]No mutant colonies were detected on the plate, with those data being excluded from the calculation of MF.
*Significantly different from the control group at $p < 0.05$.

levels in whole-kidney DNA (Mally *et al.*, 2005), in line with our present data. Also, DNA extracted from the OM showed no changes in 8-OHdG levels. Along with deletion mutations, it is well known that 8-OHdG causes GC:TA transversion mutations due to the mispair with adenine. However, OTA exposure did not significantly increase frequencies of GC:TA transversion mutations in the *gpt* mutant colonies. Thus,

TABLE 8
Mutant Spectra of *gpt* Mutant Colonies in the COR and OM of Male F344 *gpt* Delta Rats Treated with OTA for 4 Weeks

	COR				Females			
	Control		OTA		Control		OTA	
	Number (%)	MF ($\times 10^{-5}$)	Number (%)	MF ($\times 10^{-5}$)	Number (%)	MF ($\times 10^{-5}$)	Number (%)	MF ($\times 10^{-5}$)
Base substitution								
Transversions								
GC:TA	5[a] (50.0)	0.14 ± 0.13	0	0	0	0	3 (25.0)	0.15 ± 0.14
GC:CG	1 (10.0)	0.04 ± 0.08	0	0	0	0	1 (8.3)	0.02 ± 0.05
AT:TA	0	0	0	0	0	0	1 (8.3)	0.02 ± 0.04
AT:CG	0	0	0	0	0	0	1 (8.3)	0.05 ± 0.11
Transitions								
GC:AT	2 (20.0)	0.05 ± 0.08	4 (57.1)	0.16 ± 0.07	6 (80.6)	0.17 ± 0.11	4 (33.3)	0.09 ± 0.12
AT:GC	0	0	1 (14.0)	0.02 ± 0.05	0	0	0	0
Deletions								
Single bp	0	0	0	0	0	0	0	0
Over 2 bp	0	0	0	0	0	0	0	0
Insertions	0	0	1 (14.0)	0.04 ± 0.09	1 (14.3)	0.03 ± 0.07	0	0
Complexes	2 (20.0)	0.05 ± 0.07	1 (14.3)	0.04 ± 0.09	0	0	2 (16.7)	0.04 ± 0.06
Total	10	0.28 ± 0.12	7	0.26 ± 0.17	7	0.20 ± 0.12	12	0.38 ± 0.12

[a]The number of colonies with independent mutations.

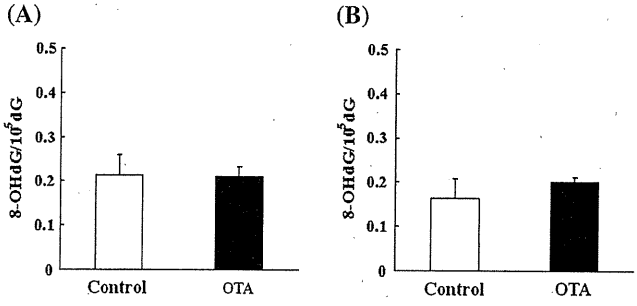

FIG. 3. Changes in 8-OHdG levels of DNA extracted from the COR (A) or OM (B) of *gpt* delta rats fed 5 ppm OTA for 4 weeks. Values are means ± SD of data for five rats.

although the participation of any other oxidized bases strictly remains unclear, it is highly probable that oxidative DNA damage contributes to neither induction of deletion mutations nor renal carcinogenesis following OTA exposure of rats. Some exogenous and endogenous agents that can induce deletion mutations cause double-stranded DNA breaks through a direct interaction with DNA, resulting in pyrimidine dimers, interstrand cross-links, apurinic sites, or modified bases (Nohmi and Masumura, 2005). Because OTA exposure of rats is subject to the formation of apurinic sites (Cavin *et al.*, 2007), it has been assumed that OTA-induced deletion mutations might result in DNA strand breaks, which might be supported by the positive results of the alkaline comet assay (Zeljezić *et al.*, 2006).

Epidemiological data for humans indicate a relationship between exposure to OTA and urothelial tumors, but not renal tumors. OTA can be a substrate for a human organic anion transporter taking part in the uptake of OTA into proximal tubule cells (Jung *et al.*, 2001). In addition, karyomegaly in the renal tubules of patients with high OTA blood concentration (Hassen *et al.*, 2004) and OTA-specific DNA adducts in the kidneys of patients in the endemic areas of the Balkan endemic nephropathy (Pfohl-Leszkowicz *et al.*, 1993) enable us to extrapolate the present data to humans.

In conclusion, genotoxic mechanisms, except oxidative DNA damage, might be involved in OTA-induced renal carcinogenesis in rats. Site-specific reporter gene mutation assays based on histopathological analyses could be a powerful tool for the investigation of chemical carcinogenesis.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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Involvement of mismatch repair proteins in adaptive responses induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine against γ-induced genotoxicity in human cells

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ABSTRACT

As humans are exposed to a variety of chemical agents as well as radiation, health effects of radiation should be evaluated in combination with chemicals. To explore combined genotoxic effects of radiation and chemicals, we examined modulating effects of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), a direct-acting methylating agent, against genotoxicity of γ-radiation. Human lymphoblastoid TK6 cells and its mismatch-deficient derivative, i.e., MT1 cells, were treated with MNNG for 24 h before they were exposed to γ-irradiation at a dose of 1.0 Gy, and the resulting genotoxicity was examined. In TK6 cells, the pretreatments with MNNG at low doses suppressed frequencies of the thymidine kinase (TK) gene mutation and micronucleus (MN) formation induced by γ-irradiation and thus the dose responses of TK and MN assays were U-shaped along with the pretreatment doses of MNNG. In contrast, the genotoxic effects of MNNG and γ-irradiation were additive in MT1 cells and the frequencies of TK mutations and MN induction increased along with the doses of MNNG. Apoptosis induced by γ-radiation was suppressed by the pretreatments in TK6 cells, but not in MT1 cells. The expression of p53 was induced and cell cycle was delayed at G2/M phase in TK6, but not in MT1 cells, by the treatments with MNNG. These results suggest that pretreatments of MNNG at low doses suppress genotoxicity of γ-radiation in human cells and also that mismatch repair proteins are involved in the apparent adaptive responses.

1. Introduction

Humans are consistently exposed to a variety of environmental hazardous chemicals and physical factors, which may play important roles in etiology of cancer. Among the chemicals, cigarette smoke may be the most causative one associated with increase in cancer risk [1]. In fact, tobacco smoking plays critical roles in the etiology in lung, oral cavity and esophageal cancers and various chronic degenerative diseases. Although cigarette smoke is a mixture of 4000 chemicals, including more than 60 known human carcinogens, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (nicotine-derived nitrosamino ketone, NNK) is the most mutagenic and carcinogenic tobacco-specific nitrosamine [2,3]. NNK induces O^6-methylguanine in DNA when it is metabolically activated by cytochrome P450 in the lung cells. This modified base induces G:C to A:T transitions in Ki-ras proto-oncogene, which is an initiation event of tumor development [4,5]. On the other hand, radiation is one of the most causative physical factors that induce human cancer. Radiation induces double-stranded beaks (DSBs) in DNA, which lead to chromosome aberrations and cell deaths, and generates a variety of oxidative bases in DNA, which induce gene mutations [6]. Even at low doses, residential exposure to radioactive radon and its decay products may account for about 10% of all lung cancer deaths in the United States and about 20% of the lung cancer cases in Sweden [7,8].

Since humans are exposed to a number of chemical and physical agents that may induce cancer, these factors may interact with each other and the action of one agent may be influenced by exposure to other agents. The risk from combined exposure to more than one agent may be higher or lower than predicted from the sum of individual agents. To explore the combined effects of radiation and chemicals, we have examined genotoxic effects of NNK and γ-irradiation using *gpt* delta transgenic mice [9]. In the mouse model, mutations can be detected in any organ of mice and sequence alterations can also be identified at the molecular level. Under the conditions used, treatments with NNK significantly suppressed the induction of mutations caused by γ-radiation in the lung of mice [9]. Therefore, we hypothesized that alkylating agents might suppress

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genotoxicity of γ-irradiation in vivo. In accordance with the finding, it is reported that pretreatments with *N*-ethyl-*N*-nitrosourea exhibited an adaptive response and suppressed the mutation induced by X-rays in mouse [10].

To explore the possible mechanisms underlying the adaptive responses induced by alkylating agents against genotoxicity of radiation, we took advantage of human lymphoblastoid TK6 cells and the isogenic derivative, i.e., MT1 cells, which is deficient in mismatch repair (MMR) protein MSH6 [11,12] and examined the combined genotoxicity of a methylating agent and γ-irradiation. MT1 cells were employed because we speculated that MMR proteins might act as a sensor of mismatched base pairs in DNA induced by alkylating agents, thereby inducing signals to suppress genotoxicity of γ-radiation. We treated TK6 and MT1 cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) followed by γ-irradiation to examine the combined genotoxicity. MNNG was chosen because it is a methylating agent as NNK, but does not need metabolic activation by CYP (P450) to generate the methylated DNA. We analyzed the combined genotoxicity with multiple endpoints, i.e., cell survival, gene mutation, micronucleus (MN) induction, apoptosis, delay of cell cycle and activation of p53 and Chk1 proteins. The results suggest that pretreatments with MNNG at low doses may induce adaptive responses against γ-radiation in TK6 cells, but not in MT1 cells. Possible roles of mismatch repair proteins in the apparent adaptive responses are discussed.

2. Materials and methods

2.1. Cell culture

The human lymphoblastoid cell lines TK6 and its isogenic MMR deficient derivative MT1 were grown in RPMI1640 medium (Nakalai Tesque, Kyoto, Japan) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated horse serum and 200 μg/ml sodium pyruvate [13–15]. TK6 and MT1 were incubated at 37 °C in a 5% CO_2 atmosphere with 100% humidity.

2.2. Chemicals

MNNG (CAS No.70-25-7) and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan, and trifluorothymidine (TFT) was purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.3. Irradiation

γ-Ray irradiation was performed using a Pantak HF-320 machine (PANTAK Ltd., East Haven, CT, USA) at 200 kV, 20 mA, and a dose rate of 1.0 Gy/min.

2.4. Combined MNNG treatment and γ-ray exposure

MNNG was dissolved in DMSO. TK6 and MT1 were treated with MNNG for 24 h at concentrations that gave similar surviving rates, i.e., 1.5, 3.0 and 4.5 ng/mL for TK6 and 150, 300 and 450 ng/mL for MT1. After the treatments, the cells were washed with 10 mM phosphate buffer saline pH 7.4 (PBS), and then immediately exposed to γ-ray irradiation at a dose of 1.0 Gy. The dose rate was 1.0 Gy/min.

2.5. Genotoxicity assays

Cells pretreated with MNNG followed by γ-irradiation were incubated for 3 days at 37 °C (Fig. 1). As controls, we prepared both cells pretreated with MNNG without γ-irradiation and cells without pretreatment with MNNG but with γ-irradiation. As the third negative control, cells treated neither MNNG nor γ-irradiation were also prepared. The cells were collected by centrifugation. *TK* gene mutation assay and cytotoxicity assay were conducted according to the published methods [13,14]. In the *TK* gene mutation assay, TK6 and MT1 were plated 40,000 cells/well and 4000 cells/well, respectively, with TFT at a concentration of 3 μg/ml. In the cytotoxicity assay, the cells were seeded 1.6 cells/well in 96-well plates. All plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2. We counted the number of colonies on the cytotoxicity assay plates after incubation for two weeks. We counted the number of normally growing-colonies after incubation for two weeks and that of slowly growing-colonies after incubation for four weeks on the mutation assay plates containing TFT.

MN assay was conducted as described [16]. Briefly, approximately 10^6 cells were suspended in 0.075 M KCl and then incubated for 10 min at room temperature. The suspended cells were fixed with ice-cold methanol containing 25% acetic acid. Then the fixed cells were centrifuged and re-suspended with ice-cold methanol containing 25% acetic acid. In the final fixation, the fixed cells were suspended in ice-cold methanol containing 1.0% acetic acid. A drop of fixing cell solution was spotted on each slide glass, and then the glass was air dried. The fixed cells were stained

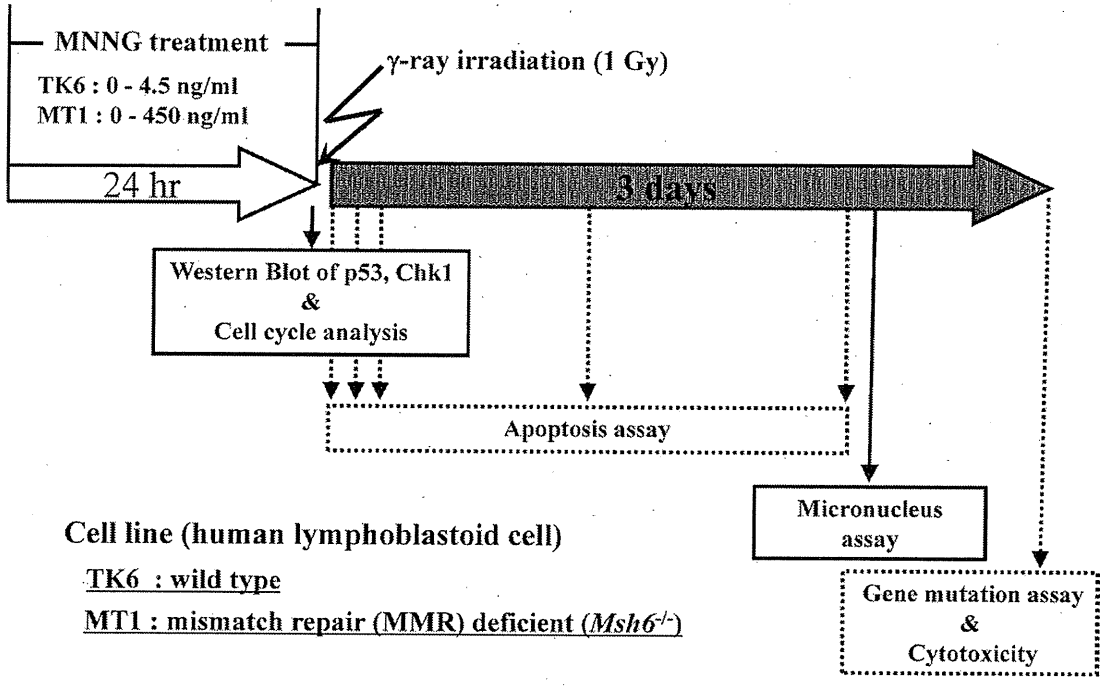

Fig. 1. An experimental design to elucidate the combined effects of γ-irradiation and MNNG treatments in TK6 and MT1 cells. The cells were pre-treated with MNNG at a concentration of 0, 1.5, 3.0 or 4.5 ng/ml in TK6 and 0, 150, 300 or 450 ng/ml in MT1, respectively. After the treatments with MNNG, both cell lines were immediately irradiated with γ-ray at a dose of 1.0 Gy. For gene mutation assay and cytotoxicity test, cells were collected three days after the irradiation. For micronucleus assay, cells were collected two days after the irradiation. Apoptosis assay was performed at 0, 2, 4, 24 and 48 h after the irradiation. Western blotting and cell cycle analyses were conducted 24 h after the MNNG treatments.

with acridine orange and analyzed with a fluorescence microscope (Olympus, Tokyo, Japan).

2.6. Apoptosis assay

We used APOPercentage Apoptosis Assay (Biocolor, Belfast, Northern Ireland) to assess the induction of apoptosis [17]. The cells with or without pretreatments with MNNG were harvested 0, 2, 4, 24 or 48 h after γ-irradiation and stained with APOPercentage dye. Then we measured the apoptotic cells using the fluorescence microscope (KEYENCE, Osaka, Japan).

2.7. Cell cycle analysis

TK6 and MT1 cells pretreated with MNNG were washed with PBS, and fixed in ethanol overnight at 4 °C. The cells were collected and re-suspended with PBS, and then the suspension was incubated with RNase for 30 min at 37 °C. Cells were re-suspended with 10 μl/ml propidium iodide solution overnight at 4 °C, and analyzed by flow-cytometry (Beckman Coulter) [18,19].

2.8. Western blot analysis

Treated cells were harvested by centrifugation, and the pellets were lysed in protein extraction solution (iNtRON Biotechnology, Korea). The supernatant containing proteins was obtained by centrifugation, mixed with loading buffer (0.125 M Tris–HCl, 10% 2-mercaptoethanol, 4% sodium dodecyl sulfate (SDS), 10% sucrose, and 0.004% bromophenol blue), and incubated for 5 min at 95 °C. The protein samples were resolved on 8% or 10% SDS-polyacrylamide gels and transferred to ECL membranes (GE Healthcare UK Ltd., London, UK) [20]. To detect p53, the membranes were probed with mouse monoclonal antibody against p53 protein as a primary antibody (DO-1) (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) and horse radish peroxidase (HRP)-conjugated anti-mouse IgG as a secondary antibody (GE Healthcare UK Ltd., London, UK) [13,21–23]. To detect Chk1, the membranes were probed with mouse monoclonal antibody against Chk1 protein as a primary antibody (G-4) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and HRP-conjugated anti-mouse IgG as a secondary antibody. To detect phospho-Chk1, the membranes were probed with rabbit polyclonal anti-phospho-Chk1 (Ser317) antibody as a primary antibody (Ser317) (Cell Signaling Technology Inc., Danvers, MA, USA) and HRP-conjugated anti-rabbit IgG as a secondary antibody [24,25]. To detect actin as a loading control, the membranes were probed with goat polyclonal antibody against actin protein as primary antibody (I-19) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and HRP-conjugated anti-goat IgG as a secondary antibody. The antibody-treated membranes were incubated with ECL Plus Western blotting detection reagent (GE Healthcare UK Ltd., London, UK) and the detected proteins were visualized using an image analyzer LAS-1000 (Fuji film, Co., Ltd., Tokyo, Japan).

2.9. Statistical analyses

For all the assays, statistical analyses were performed with Student's *t*-test. Data analyses were performed with Excel software (Microsoft).

3. Results

3.1. Suppression of cytotoxicity and mutagenicity of γ-irradiation by pretreatments with MNNG in TK6 cells

Since MT1 cells exhibit about 100-times resistance to the killing effects of MNNG than TK6 cells [11], we treated TK6 and MT1 cells with different concentrations of MNNG. In fact, treatments of TK6 with MNNG at concentrations of 1.5, 3.0 and 4.5 ng/mL and those of MT1 with MNNG at concentrations of 150, 300 and 450 ng/mL gave similar cell survival (Fig. 2a). Treatments of TK6 and MT1, respectively, with MNNG at concentrations of 4.5 and 450 ng/mL gave about 70% cell survival. The cell survival decreased along with the concentrations of MNNG in both TK6 and MT1 cells. The γ-irradiation alone at 1 Gy reduced the cell survival to 20% in both TK6 and MT1 cells. When combined the treatments with MNNG and γ-irradiation, we found that the survival of TK6 cells pretreated with MNNG at a concentration of 1.5 ng/mL was significantly higher than that of cells without pretreatments with MNNG ($p = 0.004$). The survival of cells pretreated with MNNG at a concentration of 1.5 ng/mL followed by γ-irradiation was higher than that of cells pretreated with MNNG at concentrations of 3.0 or 4.5 ng/mL too. These results suggest that the pretreatment with MNNG at a concentration of 1.5 ng/mL exhibits suppressive effects on the cell killing effects of γ-irradiation. In contrast, the survival of MT1 cells after γ-irradiation decreased along with the concentrations of MNNG. It can be said from the results that killing effects of MNNG and γ-irradiation are additive in MT1 cells.

In the mutation assay, MNNG treatments without γ-irradiation enhanced TK mutation frequency of both TK6 and MT1 cells in a dose-dependent manner (Fig. 2b). The spontaneous TK mutation frequency was about 10 times higher in MT1 cells compared to TK6 cells. The γ-irradiation alone induced the mutations at

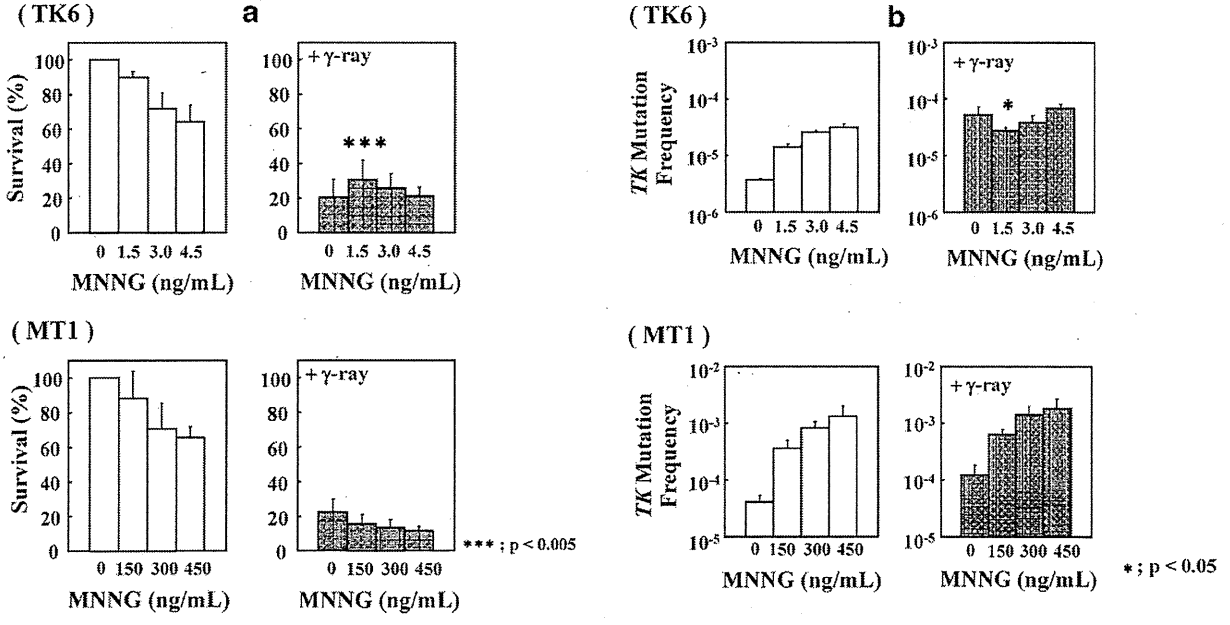

Fig. 2. Cell survival and *TK* gene mutation assays in TK6 and MT1 cells. The effects on cell survival (a) and gene mutations (b) are separately presented. An asterisk (*) and asterisks (***) denote $p < 0.05$ and $p < 0.005$, respectively, in the *t*-test of comparison between MNNG-treated and untreated cells.

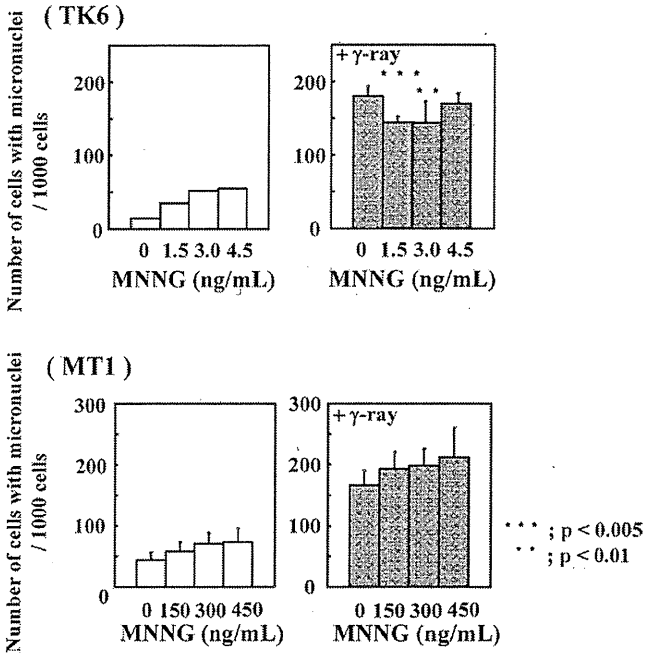

Fig. 3. Micronucleus assay in TK6 and MT1 cells. An asterisk (*) and asterisks (***) denote $p < 0.01$ and $p < 0.005$, respectively, in the *t*-test of comparison between MNNG-treated and untreated cells.

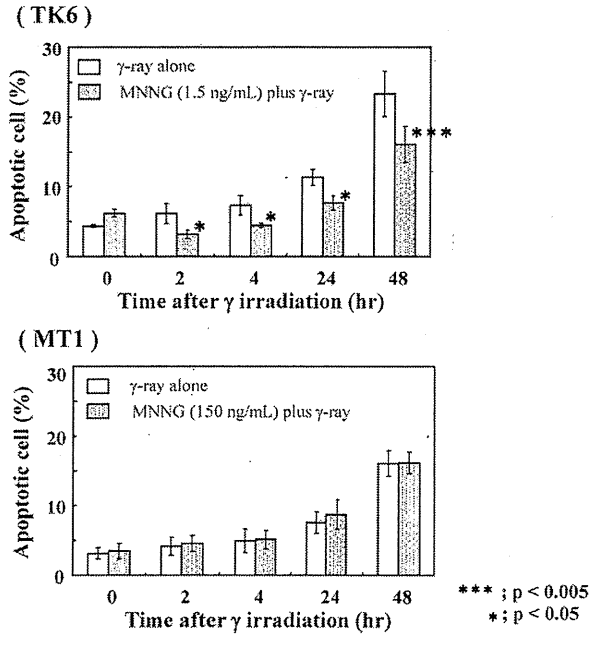

Fig. 4. Apoptosis assay in TK6 and MT1 cells. An asterisk (*) and asterisks (***) denote $p < 0.05$ and $p < 0.005$, respectively, in the *t*-test of comparison between MNNG-treated and untreated cells.

about 10^{-4} in both cells. In combination of pretreatments with MNNG and γ-irradiation, the dose response was U-shaped in TK6 cells. The mutation frequency significantly decreased when the cells were pretreated with MNNG at a concentration of 1.5 ng/mL ($p < 0.05$), and then increased along with the pretreatment doses of MNNG, i.e., 3.0 and 4.5 ng/mL. In contrast, the dose response curve increased along with the pretreatment concentrations of MNNG in MT1 cells. The examination implies that the involvement of MMR proteins, i.e., MSH6, in the apparent adaptive responses against γ-irradiation by pretreatments with MNNG.

3.2. Suppression of γ-induced MN formation by pretreatment of MNNG in TK6 cells

Treatments with MNNG alone enhanced frequencies of MN formation, i.e., numbers of cells with MN per 1000 cells, in a dose-dependent manner in both TK6 and MT1 cells (Fig. 3). MT1 cells did not exhibit significantly higher MN frequencies compared to TK6 cells. The γ-irradiation at a dose of 1 Gy alone induced about 18% of MN frequencies in both cells. In the combined treatments, the MN frequencies significantly decreased when the cells were pretreated with MNNG at concentrations of 1.5 and 3.0 ng/mL in TK6 cells ($p < 0.005$ and $p < 0.01$, respectively). Thus, MNNG pretreatments apparently inhibit MN formation induced by γ-irradiation in TK6 cells. In contrast, the MN frequencies were almost the sum of those of γ-irradiation and pretreatments with MNNG in MT1 cells. The results suggest the pretreatments with MNNG at 1.5 and 3.0 ng/mL exhibit suppressive effects on γ-induced MN formation in TK6 cells and the involvement of MMR proteins in the adaptive responses.

3.3. Pre-treatments with MNNG reduce the apoptosis induced by irradiation in TK6 cells

To examine whether the suppressive effects are due to induction of apoptosis, which kills damaged cells, we investigated the induction of apoptotic cells after the γ-irradiation with or without pretreatments with MNNG (Fig. 4). γ-Irradiation alone enhanced frequencies of apoptotic cells and the frequencies increased along with the incubation time after γ-irradiation in both cell lines. The frequency of apoptosis in TK6 cells at 48 h upon γ-irradiation was significantly higher ($p < 0.05$) than that of apoptosis in MT1 cells. Furthermore, combined treatments exhibited different patterns of apoptosis in TK6 and MT1 cells. In TK6 cells, pretreatments with MNNG at a concentration of 1.5 ng/mL significantly suppressed the frequencies of apoptotic cells induced by γ-irradiation ($p < 0.05$ for 2, 4 and 24 h pretreatments and $p < 0.005$ for 48 h pretreatments). The frequencies and the standard deviations of the cells treated with γ-irradiation alone versus γ-irradiation combined with pretreatments with MNNG were 4.33 ± 0.25 versus 6.20 ± 0.56, 6.17 ± 1.45 versus 3.17 ± 0.59, 7.30 ± 1.39 versus 4.43 ± 0.32, 11.33 ± 1.16 versus 7.67 ± 1.03, 23.30 ± 3.24 versus 16.10 ± 2.60, respectively, for induction times of 0, 2, 4, 24 and 48 h after γ-irradiation. In contrast, the pretreatments did not affect the frequencies of apoptotic cells in MT1 cells. These results suggest that the suppressive effects of pretreatments with MNNG in TK6 cells are not due to induction of apoptosis. Rather, the pretreatments appeared to alleviate the induction of apoptosis in the cells.

3.4. G2/M delay in TK6 cells by treatments with MNNG

Next, to examine whether the adaptive responses are due to delay of progression of cell cycle, we analyzed distribution of G1, S and G2/M phases of cells treated with MNNG (Table 1). In TK6 cells, the distributions were 34.0 ± 1.0 (%), 57.8 ± 1.4 and 8.2 ± 1.5 in G1, S and G2/M, respectively, without the treatments. After treatments with MNNG at a concentration of 1.5 ng/mL, the percentage of cells in G2/M significantly increased compared to the control cells (13.2% for the treated cells versus 8.1% for untreated cells, $p < 0.005$). On the other hand, there were no significant changes of distribution of cells in G1, S and G2/M phases in MT1 cells associated with MNNG treatments. Thus, the results indicate that treatments with MNNG induce cell cycle delay at the G2/M phase in TK6 cells and also that

Table 1
Cell cycle distribution in TK6 and MT1 cells treated with MNNG for 24 h.

	TK6			MT1		
MNNG	0 (ng/ml)	1.5 (ng/ml)	150 (ng/ml)	0 (ng/ml)	1.5 (ng/ml)	150 (ng/ml)
G1	34.2	32.8	26.4	39.6	41.6	39.4
S	57.8	53.0	64.1	47.0	43.7	47.2
G2/M	8.1	13.2***	9.5	13.4	14.7	14.7

Asterisks (***) denote $p < 0.005$ in the *t*-test of comparison between MNNG-treated (1.5 ng/ml MNNG) and untreated TK6 cells.

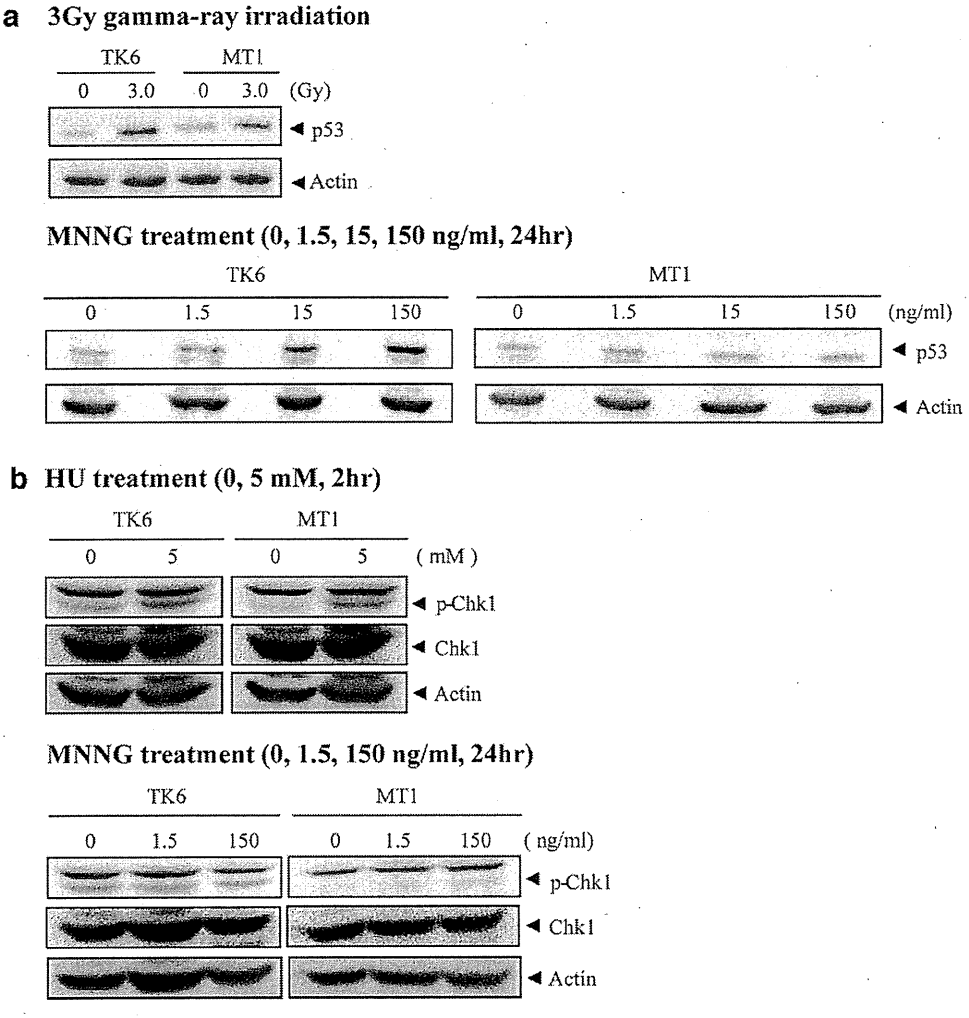

Fig. 5. Western blotting analysis in TK6 and MT1 cells. (a) Activation of p53. The cells were irradiated with γ-ray at a dose of 3 Gy as a positive control. Alternatively, the cells were treated with MNNG at a dose of 1.5, 15 or 150 ng/ml for 24 h and subjected to the analysis. (b) Phosphorylation of Chk1. The cells were treated with hydroxyurea (HU) for 2 h as a positive control or MNNG at a dose of 1.5 or 150 ng/ml for 24 h. In these experiments, actin was used as a loading control.

the delay may cause the mismatch-dependent adaptive responses against γ-irradiation.

3.5. MNNG treatments induce the p53 activation

To further elucidate the mechanisms underlying the adaptive response, we examined the activation of p53 and Chk1, which are the key proteins of DNA repair and cell cycle checkpoint. As the positive control, we irradiated both cells with γ-ray at a dose of 3.0 Gy (Fig. 5a). The γ-irradiation induced p53 expression approximately 2.5-fold and 1.5-fold, respectively, higher compared with non-irradiated cells in TK6 and MT1 cells. When the cells were treated with MNNG at concentrations of 1.5, 15 and 150 ng/mL, p53 expression level increased in a dose dependent manner in TK6 cells. However, virtually no increases were observed in MT1 cells. For the phosphorylation of Chk1 protein, we treated both cells with hydroxyurea (HU) at a concentration of 5 mM as the positive control (Fig. 5b). As expected, the phosphorylated form of Chk1 was observed in both cells after HU treatments. However, no phosphorylation of Chk1 was observed after treatments with MNNG at concentrations of 1.5 and 150 ng/mL in both cells.

4. Discussion

Humans are continuously exposed to various environmental hazardous genotoxic chemicals and physical agents. Thus, the evaluation of combined genotoxic effects of environmental mutagens and radiation is an important issue for human health. In a pre-

vious study, we observed an adaptive response in the lung of *gpt* delta mice treated with NNK, a tobacco specific nitrosamine, combined with γ-irradiation [9]. The methylating agent, i.e., NNK, significantly suppresses mutations induced by γ-irradiation in the lungs of mice. To explore the mechanisms underlying the apparent adaptive response, we examined combined effects of MNNG, a directly acting methylating agent, and γ-irradiation in the human lymphoblastoid cell lines TK6 and MT1 *in vitro* (Fig. 1). Single treatments of MNNG or γ-irradiation increased the frequencies of mutations, MN induction and cell death in both TK6 and MT1 (Figs. 2 and 3). In the combined treatments, however, induction of TK mutation, MN and cell death by γ-irradiation were significantly suppressed by pretreatments with MNNG not in MT1 but in TK6.

At first, we hypothesized that γ-induced apoptosis might be enhanced by the pretreatments with MNNG, thereby suppressing the mutations and MN induction in TK6 cells. However, the apoptosis was rather suppressed by the pretreatments with MNNG in TK6 cells (Fig. 4). No significant changes were observed in apoptosis in MT1 cells by the pretreatments. Suppression of radiation-induced apoptosis is known to be elicited by low-dose ionizing irradiation, which is called radioadaptive response [26–30]. Although the exact mechanism underlying the radioadaptive response is largely unknown, it is generally thought that cellular defense mechanisms against genotoxic effects of radiation are induced by prior exposure to low-dose radiation. Many of genes including p53, ATM and Chk1 are up- and down-regulated in the adapting cells [31]. Interestingly, we observed enhanced expression of p53 in MNNG-pretreated cells in the presence of MMR proteins (TK6) but not in the absence of them (MT1) (Fig. 5a). In fact, p53 is reported to play an important role in both apoptosis and DNA repair pathways depending on the level of DNA damage [22,23,32]. When cells are exposed to low or moderate levels of DNA damage, p53 does not induce apoptosis but activates error-free DNA repair, e.g., homologous recombination, thereby suppressing cell death. Therefore, we suggested that pretreatments with MNNG at low doses induce low or moderate levels of DNA damage and activate p53 in a way that it alleviates radiation-induced apoptosis. In this activation process, MMR proteins play crucial roles to mediate the signal from DNA damage to p53 (see below).

Because increased apoptosis was not the case, we next examined another possibility of whether MNNG pretreatments might delay cell-cycle progression, thereby acquiring resistance to genotoxicity of radiation. Intriguingly, when TK6 cells were treated with MNNG at a low dose, i.e., 1.5 ng/mL, the percentage of cells at G2/M was significantly increased (Table 1). No such increases were observed in TK6 cells treated with MNNG at a high dose, i.e., 150 ng/mL, or in MT1 cells treated with low or high doses of MNNG. Thus, we suggested that the activation of p53 by exposure to the low dose of MNNG suppresses cell cycle progression at G2/M phase, which in turn leads to enhanced error-free repair of DSBs in DNA. The enhancement of DNA repair at prolonged G2/M phase may account for the apparent adaptive response observed in TK6 cells (Fig. 6). This notion is supported by previous studies that indicate that treatments of cells with MNNG induce cell cycle delay at G2/M phase [18,33–35]. It is suggested that the MMR proteins interact with Ataxia telangiectasia mutated kinase (ATM) and ATM-and-Rad3-related kinase (ATR), thereby activating p53, Chk1 and cell cycle checkpoint proteins [21,36–38]. p38 activation is also reported to be important for methylating agent-induced G2 arrest in glioma and colon cancer cell lines [39]. In our case, however, Chk1 was not phosphorylated in both cell lines after MNNG pretreatments. This raises the possibility that Chk1 phosphorylation may not be necessary for cell cycle delay in TK6 cells. Alternatively, it is because we examined the phosphorylation of Chk1 by the Western blotting analysis earlier before it is detectably activated. Recently, Quiros et al. demonstrated that Chk1 was activated more effectively in the post-treatment cell cycle following O^6-methylguanine (O^6-MeG) induction treated by low concentration MNNG and suggested that

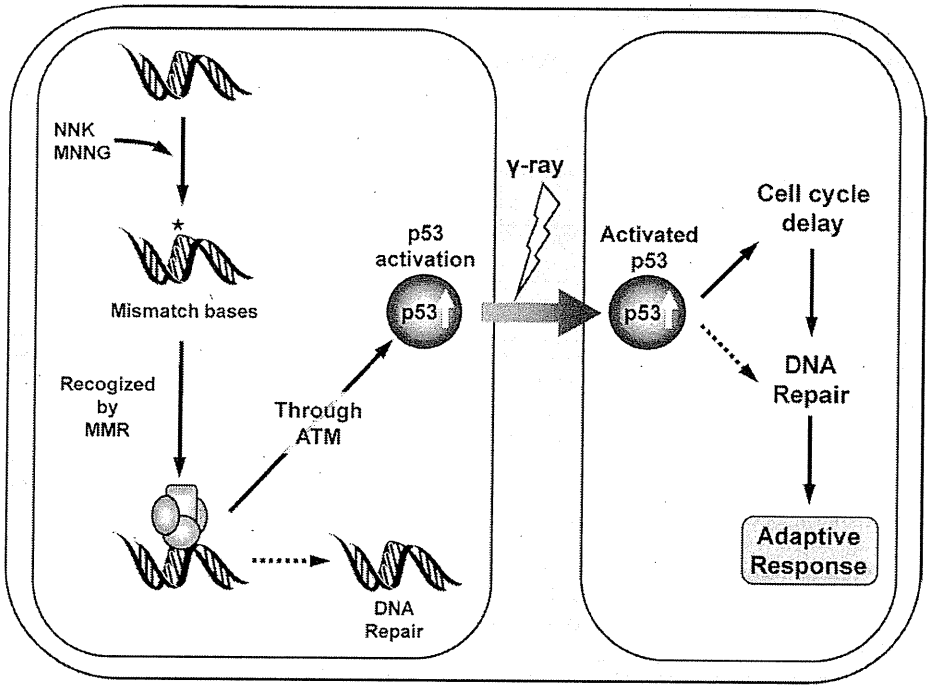

Fig. 6. A possible mechanism underlying the adaptive response of MNNG-pretreated TK6 cells against genotoxicity of γ-irradiation. Alkylating agents, i.e., NNK and MNNG, induce mismatch bases such as O^6-methylguanine paired with thymine in DNA, which are recognized by mismatch repair (MMR) proteins. After the recognition, the mismatch bases may be repaired by MMR in one pathway. In the other pathway, however, the mismatch-bound MMR recruits ATM and activates p53. The activation leads to cell cycle delay at G2/M and enhances error-free DNA repair, which results in induction of the adaptive response.

Chk1 activation requires at least two rounds of replication after the treatment of MNNG [40]. It is reported that MMR-dependent cell cycle arrest fully occurs after the second S-phase following DNA damage. We examined the Chk1 phosphorylation 24 h after the initiation of MNNG pretreatments when not all the cells might have finished the second round of DNA replication (Fig. 1). Unlike the checkpoint analysis, however, MN and gene mutations were analyzed 3 days after the termination of MNNG pretreatments. Hence, the cells replicated chromosome DNA in the presence of methylated damage, e.g., O^6-MeG, in DNA more than two times, which may be a reason why we observed clear suppressive effects against genotoxicity of γ-irradiation (Figs. 2 and 3). The results that we observed the suppressive effects 3 days after the MNNG pretreatments also raise a possibility that the adaptive response induced by MNNG may continue at least for 3 days.

The results in this study suggest that functional interactions of MMR proteins with p53 play critical roles in the adaptive response elicited by MNNG pretreatments (Fig. 6). Then, how does methylated DNA damage such as O^6-MeG induce activation of p53 via MMR proteins? One possibility is that a mismatch base-pair of O^6-MeG with thymine is recognized by MMR proteins, which in turn recruit other proteins, e.g., ATM, that stabilize and activate p53 [38]. Alternatively, the recognition of the mismatch base-pair by MMR proteins may lead to futile DNA synthesis, which results in generation of single-strand gaps and/or DSBs in DNA. These aberrant structures of DNA induce ATM, which can activate p53 and other proteins related to DNA damage response. Further work using mutant MMR proteins that can recognize the mismatch base-pairs but not interact with other proteins may give us insight into the roles of MMR proteins in induction of signals to p53.

It is evident that p53 activation leads to cell death via apoptosis when the DNA damage is severe. However, as described above, it is suggested that p53 activation leads to onset of DNA repair and enhancement of cell survival. Therefore, it is expected that there is a threshold below which p53 acts as a suppressor of cell death (or genotoxicity) and upon which p53 acts as an enhancer of cell death. Interestingly, suppression against radiation-induced genotoxicity was only observed at a specific dose of MNNG, i.e., 1.5 ng/mL for cell survival and TK gene mutations, and 1.5 and 3.0 ng/mL for MN induction (Figs. 2 and 3). Therefore, the dose responses of TK and MN assays were U-shaped along with the pretreatment doses of MNNG. These results may reflect the biphasic roles of p53 in cell survival (relief of genotoxicity) and cell death depending on the doses of MNNG. It is reported that severe DNA damage activates p53-independent apoptosis via p73, a protein homologous to p53 [41]. Because p73 is stabilized by MNNG treatments and silencing of p73 results in significant decreases in apoptosis and increases in survival, p73 appears to play a role in MMR-dependent apoptosis induced by MNNG treatment [42]. In this study, however, we could not observe enhanced apoptosis in TK6 cells pretreated with MNNG after γ-irradiation (Fig. 4). Thus, we suggest that apoptosis via p73 is not involved in the adaptive response in TK6 cells pretreated with MNNG against γ-irradiation (Figs. 2 and 3). It remains an open question, however, whether p73 may play a role in the adaptive response via other pathways, e.g., activation of DNA repair.

In summary, our results suggest that exposure to MNNG at low-doses induces adaptive response to radiation-induced genotoxicity in human cells and also that MMR proteins and p53 play critical roles in the adaptation. The results also suggest that the adaptive response depends on the priming dose of MNNG and perhaps the timing when the genotoxicity is analyzed. Obviously, further work is needed to thoroughly understand the mechanisms underlying the MNNG-induced adaptive response against radiation and how the mechanisms can be generalized to other combined genotoxicity of chemicals and radiation.

Conflict of interest

No conflict of interest.

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Acrylamide genotoxicity in young versus adult *gpt* delta male rats

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The recent discovery that the potent carcinogen acrylamide (AA) is present in a variety of fried and baked foods raises health concerns, particularly for children, because AA is relatively high in child-favoured foods such as potato chips and French fries. To compare the susceptibility to AA-induced genotoxicity of young versus adult animals, we treated 3- and 11-week-old male gpt delta transgenic F344 rats with 0, 20, 40 or 80 p.p.m. AA via drinking water for 4 weeks and then examined genotoxicity in the bone marrow, liver and testis. We also analysed the level of *N*7-(2-carbamoyl-2-hydroxyethyl)-guanine (*N*7-GA-Gua), the major DNA adduct induced by AA, in the liver, testis and mammary gland. At 40 and 80 p.p.m., both age groups yield similar results in the comet assay in liver; but at 80 p.p.m., the bone marrow micronucleus frequency and the *gpt*-mutant frequency in testis increased significantly only in the young rats, and *N*7-GA-Gua adducts in the testis was significantly higher in the young rats. These results imply that young rats are more susceptible than adult rats to AA-induced testicular genotoxicity.

Introduction

Acrylamide (AA) is a low molecular weight vinyl compound commonly used in industries and laboratories. Because individuals are exposed to AA in the workplace, health concerns originally centred on occupational exposure (1). A recent study, however, reported that low levels of AA are formed in many heat-processed foods, especially starchy ones such as potato chips, crackers and French fries (2,3), as a result of asparagine reacting with sugars (Maillard reaction) (4,5). This finding raises concerns that AA poses health risks for the general population (6).

Many animal studies have demonstrated that AA induces neurotoxicity, testicular toxicity and reproductive toxicity (7–9). AA also causes cancers such as mammary fibroadenomas, thyroid follicular cell adenomas and testicular mesotheliomas in rats (10–12). In mice, it induces gene mutations in liver, micronuclei in haematopoietic cells (13,14) and chromosome aberrations in spermatids and spermatocytes (15,16). Thus, AA is clearly genotoxic *in vivo*, although its *in vitro* genotoxicity remains unclear because it is not metabolically activated in standard *in vitro* systems (17,18). AA is metabolised to glycidamide (GA), presumably by cytochrome P450 2E1 (CYP2E1), which quickly reacts with cellular DNA and protein (6,19,20). Two major GA–DNA adducts—*N*7-(2-carbamoyl-2-hydroxyethyl)-guanine (*N*7-GA-Gua) and *N*3-(2-carbamoyl-2-hydroxyethyl)-adenine (*N*3-GA-Ade)—have been identified in mice and rats treated with AA or GA (21–23), with the level of *N*7-GA-Gua being 100 times as high as the level of *N*3-GA-Ade in the organs (22). Individual GA to AA ratios, which can be used as an indicator of the extent of AA metabolism, are highly variable, suggesting that some individuals or populations may be more susceptible than others to AA-induced genotoxicity (24). Other issues are AA intake and metabolism in children compared with adults. Children generally consume larger amounts of food relative to their body mass than adults and favour foods such as French fries and potato chips that have relatively high AA concentrations (25). These issues should be considered when evaluating the susceptibility of the paediatric population in genotoxic and carcinogenic risk assessments (26).

In the present study, to compare the susceptibility to AA-induced genotoxicity of young versus, adult age groups, we treated 3- and 11-week-old male *gpt* delta transgenic F344 rats with 0, 20, 40 or 80 p.p.m. of AA via drinking water for 4 weeks and examined genotoxicity in the bone marrow, liver and testis. We also analysed the level of *N*7-GA-Gua in the liver, testis and mammary gland.

Materials and methods

Animals, diet and housing

We purchased 20 male with 10-week-old and 15 pregnant female F344 *gpt* delta transgenic rats from Japan SLC (Shizuoka, Japan). The pregnant animals were time-mated at 10 weeks of age and arrived on gestational Day 12 or 13 to our facility. After delivery, we obtained >14 male pups from the pregnant rats. All animals were housed *three* to *five* rats in polycarbonate cage with sterilised wood chip bedding and maintained under specific pathogen-free standard laboratory conditions: room temperature, 24 ± 1°C; relative humidity, 55 ± 5%; 12-h light–dark cycle; basal diet (CRF-1; Oriental Yeast Company, Tokyo, Japan) and tap water *ad libitum* until parturition.

Treatments of animals

The protocol for this study was approved by the Animal Care and Utilisation Committee of the National Institute of Health Sciences. We randomly divided 14 and 20 of the 3- and 11-week-old rats into four groups of 3–5 animals, treated them for 4 weeks with AA (Wako Pure Chemical Co., Tokyo, Japan) at 0, 20, 40 or 80 p.p.m. in drinking water and monitored clinical signs, body weight and food and water consumption. At the end of the treatment period, we anaesthetized and killed the animals, and we excised organs for the *gpt* mutation assay (liver, testis), comet assay (liver), DNA adducts analysis (liver, testis, mammary gland and thyroid) and micronucleus (MN) test (bone marrow).

MN test

We removed bone marrow from the femur, mixed it with foetal calf serum, placed it on an acridine orange-coated glass slide, covered it with a coverslip and stained it supravitally (27). We analysed 2000 polychromatic erythrocytes per animal with a fluorescence microscope and recorded the number of micronucleated polychromatic erythrocytes, which fluoresced greenish yellow.

Alkaline comet assay

We performed the comet assay using the procedure recommended by the comet assay working group of the International Workshop on Genotoxicity Testing (IWGT) (28,29), except that we used a MAS-coat type slide glass (Matsunami Glass *Ind.* Ltd, Tokyo, Japan) instead of a conventional agarose bottom layer (30). We prepared cell suspensions from the livers, mixed them with 0.5% w/v low-melting agarose, and spotted an aliquot of the mixture onto the slide. After electrophoresis, we stained the cells with SYBR-Gold (cat. # S-11494; Molecular Probes, Invitrogen, Tokyo, Japan), and examined at least 100 cells per animal using a fluorescence microscope (BX50 and BX51; Olympus Corporation, Tokyo, Japan) connected to the comet assay scoring system (Comet IV; Perceptive Instruments Ltd, Suffolk, UK), which quantified the result as %tail intensity.

gpt mutation assay

We extracted high molecular weight genomic DNA from the liver and testis using a Recover Ease DNA Isolation Kit (Stratagene, La Jolla, CA, USA), rescued lambda EG10 phages using Transpack Packaging Extract (Stratagene) and conducted the *gpt* mutation assay as previously published (31). We calculated the *gpt*-mutant frequency (*gpt*-MF) by dividing the number of 6-thioguanine-resistant colonies by the number of colonies with rescued plasmids.

DNA adduct assay

As a standard for liquid chromatography tandem mass spectrometry analysis, *N*7-GA-Gua and [$^{15}N_5$]-labelled *N*7-GA-Gua were synthesised as described previously (18,22). We extracted DNA from the liver, testis, mammary gland and thyroid using a DNeasy 96 Blood & Tissue Kit (QIAGEN, Düsseldorf, Germany), incubated it at 37°C for 48 h for deprination. We added an aliquot of the labelled standard to each sample and filtered through an ultrafiltration membrane to remove DNA. The eluted solution was evaporated thoroughly and dissolved in water and then the solutions were subsequently quantified by a Quattro Ultima Pt triple stage quadrupole mass spectrometer (Waters-Micromass, Milford, MA, USA) equipped with a Shimadzu LC system (Shimadzu, Japan). We analysed the liver and testis for each individual rat but pooled the mammary and thyroid glands for each treatment group because the tissue yields were too small to be examined individually.

Statistical analysis

We used the Student's *t*-test to determine the statistical significance of the difference in the results of the *gpt* mutation assay and the DNA adduct assay between the treated and negative control groups and between the young and adult groups. We examined variances in body weight and results of the MN and comet assays by one-way analysis of variance using the Dunnett's test to compare the differences between the control and treated groups.

Results

Clinical signs, body weight and AA intake

We observed no clinical abnormality in either the young or adult rats during the 28-day treatment period. We found no significant differences in body weight or food and water consumption between the adult treatment groups, although we did observe a slight but statistically insignificant suppression of body weight in the young, 80-p.p.m. treatment group (Table I).

The table shows average daily food, water and AA intake of the young and adult treatment groups and their mean body weights. The average daily intakes of AA are calculated as 3.01, 5.95 and 12.19 mg/kg body weight for 20, 40 and 80 p.p.m. group, respectively, in young rats and as 1.83, 3.54 and 7.05 mg/kg body weight for 20, 40 and 80 p.p.m. group, respectively, in adult rats.

MN test

While no AA dose induced MN in adult rat bone marrow, the highest dose (80 p.p.m.) significantly increased the MN frequency in young rat bone marrow (Figure 1a). Because of the large standard deviation, however, the difference between young and adult rats was not significant (Figure 1a).

Alkaline comet assay

DNA damage induced by AA in liver was evaluated by the comet assay under alkaline conditions (Figure 1b). The comet tail intensities increased in a dose-dependent manner in both young and adult rats with no statistically significant differences between the two groups. AA significantly induced DNA damage at 40 and 80 p.p.m. in the adult rat liver and at 80 p.p.m. in the young rat liver.

gpt mutation assay

Figure 2 shows the *gpt* mutation assay results. The *gpt*-MF of control (0 p.p.m.) young and adult rat livers was 1.57 ± 0.72 ($\times 10^{-6}$) and 3.66 ± 2.14 ($\times 10^{-6}$), respectively. The control *gpt*-MF of the young rat liver was lower than that of the adult rat liver, but not significantly. AA did not increase the *gpt*-MF in the liver of either age group at any dose; but at 80 p.p.m., it approximately doubled the *gpt*-MF in the testis of both young and adult rats, but the increase in adult rats was not statistically significant.

DNA adduct formation

Figure 3 shows *N*7-GA-Gua DNA adduct levels in the liver, testis and mammary glands and thyroid of the young and adult rats. The adduct level increased in a dose-dependent manner in all the tissues. In the mammary glands and thyroid, adduct levels did not differ significantly between young and adult rats. In the liver and testis, on the other hand, the level was higher in the young rats than in the adult rats. In the testis, the DNA adduct level of young rats was approximately six times that of adult rats at all treatment doses.

Discussion

The *in vivo* genotoxicity of AA has been clearly demonstrated by various rodent genotoxicity tests including MN tests in peripheral blood (13,14,32) and gene mutation and comet assays in various organs (14,33,34). However, there has been no report for the comparison of genotoxicity between young and adult animals. In this study of the genotoxicity of AA in various organs of young (3-week-old) and adult (11-week-old) male rats, we showed that the testis were more vulnerable to AA genotoxicity in the young rat than in the adult rat. Especially, *N*7-GA-Gua DNA adduct was much higher accumulated in the testis of young rats than of adult rats (Figure 3). The daily intake of AA per weight in young rats was ~1.5-fold of the adult rats because the younger animals drank more water. It can explain the higher accumulation of adduct in the young rat liver, but the level in testis was

Table I. Body weight, food and water consumption and AA intake of young and adult rats

Group	AA dose (p.p.m.)	No. of animals	Initial body weight (g) mean ± SD	Final body weight (g) mean ± SD	Food consumption (mg/rat/day)	Water consumption (ml/rat/day)	Intake of AA (mg/kg/day)
Young	0	4	40.5 ± 2.7	168.9 ± 14.3	11.2	17.9	0
	20	3	37.1 ± 3.5	164.7 ± 17.8	11.3	16.9	3.01
	40	3	38.2 ± 2.1	165.1 ± 3.6	11.1	16.7	5.95
	80	4	40.6 ± 2.5	157.4 ± 8.2	11.0	16.9	12.19
Adult	0	5	249.8 ± 10.0	301.3 ± 11.5	16.4	25.8	0
	20	5	249.8 ± 8.2	299.9 ± 7.3	16.1	25.4	1.83
	40	5	250.5 ± 8.7	302.4 ± 12.2	16.2	24.8	3.54
	80	5	249.1 ± 7.7	306.6 ± 5.4	16.8	24.6	7.05

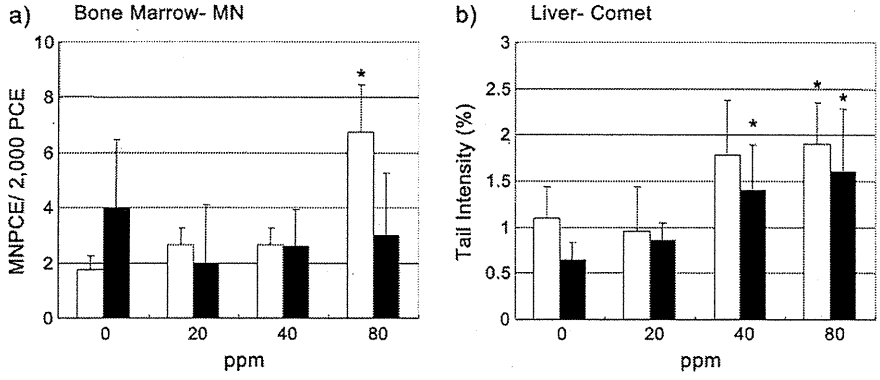

Fig. 1. (**a**) MN frequency in bone marrow of AA-treated young (open bars) and adult (closed bars) *gpt* delta rats. (**b**) Tail intensity (%) in the comet assay in liver of AA-treated young (open bars) and adult (closed bars) *gpt* delta rats. The values represent the mean of experiments ± standard deviations. *is statistically significant experiment compared with the untreated control ($P < 0.05$)discussion.

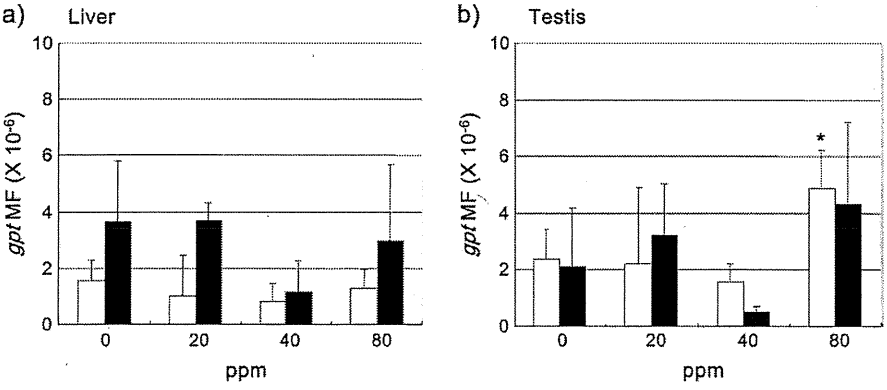

Fig. 2. *gpt* Mutation frequency in liver (**a**) and testis (**b**) of AA administered young (open bars) and adult (closed bars) *gpt* delta rats. The values represent the mean of experiments ± standard deviations. *is statistically significant experiment compared with the untreated control ($P < 0.05$).

approximately six times high in the young rats than in the adult rats, suggesting that AA metabolism in testis is different depending on animal age. Testis is one of the target organs of AA-induced genotoxicity (15,16,35–39). We believe that this is the first report of an age difference in the effect.

AA is primarily metabolised in animals via two competing pathways: oxidation by CYP2E1 to form GA (activation) and conjugation by glutathione *S*-transferase (GST) with reduced glutathione (detoxification) (19,40,41). GA may subsequently undergo conjugation or hydrolysis catalysed by epoxide hydrolase. The balance between activation and detoxification probably determines AA genotoxicity *in vivo*. Rat testis shows CYP2E1 activity (42). Wang *et al.* (43) reported that the treatment of 1.4 and 7.0 mM of AA or GA via drinking water for 4 weeks induced the testicular *cII* mutation in Big Blue mice. The *cII* mutation spectra significantly differed between testis and liver, suggesting that testis may have different pathway to metabolise AA and GA. However, the

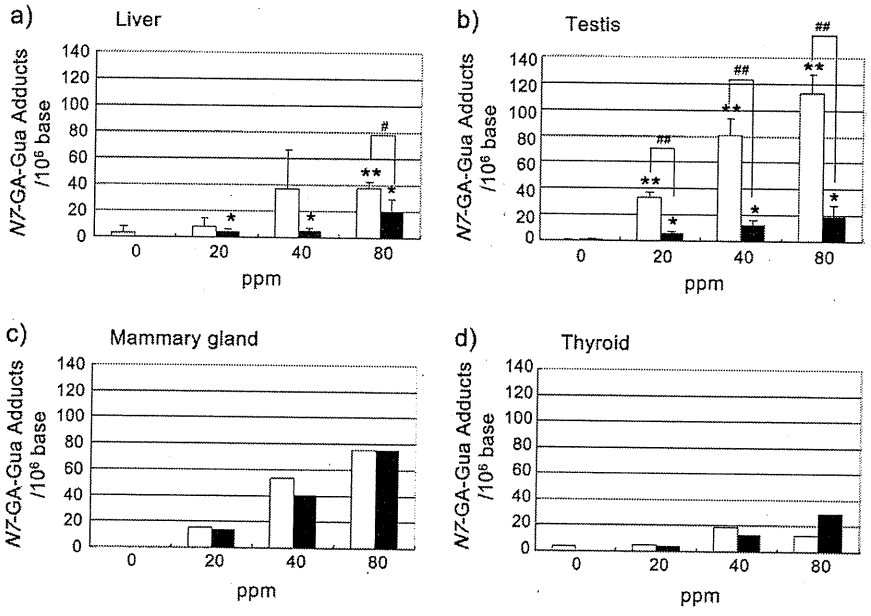

Fig. 3. Levels of *N*7-GA-Gua in the liver (**a**) testis (**b**) mammary gland (**c**) thyroid and (**d**) administered AA young (open bars) and adult (closed bars) *gpt* delta rat. The mammary gland and thyroid were pooled and analysed in the treatment group. Data are expressed as the number of adducts in 10^6 nucleotides. * and **are statistically significant experiment compared with the untreated control (*$P < 0.05$, **$P < 0.05$). # and ##are statistically significant experiment compared between young and adult *gpt* delta rat (#$P < 0.05$, ##$P < 0.05$).

developmental changes were not studied. Recently, Takahashi *et al.*. (44) showed that GST activity in the testis was significantly lower in young rats than in adult rats and that could explain the different age-related *N*7-GA-Gua adduct levels and *gpt*-MFs in the present study. The greater mutagenicity of aflatoxin B1 in liver of neonatal mice than of adult mice corresponds to liver GST levels (45). The GST level in the organs could be responsible for the expression of genotoxicity of AA and aflatoxin B1.

While the *N*7-GA-Gua adduct level in liver and testis clearly increased in a dose-dependent manner and significantly differed between young and adult rats, the *gpt* mutation results were not clear. We treated the rats with doses that were lower than those used in other studies (14,34,43), and these doses may have been insufficient to induce gene mutations in our study. Indication of the DNA adduct must be good biomarker to demonstrate genotoxic insult under low-dose exposure condition.

In conclusion, this finding that young rats were more susceptible than adult rats to AA-induced genotoxicity, especially in the testis, suggests that we should be concerned about the risk to children exposed to AA via ordinary foods.

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Mutational Specificities of Brominated DNA Adducts Catalyzed by Human DNA Polymerases

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Chronic inflammation is known to lead to an increased risk for the development of cancer. Under inflammatory condition, cellular DNA is damaged by hypobromous acid, which is generated by myeloperoxidase and eosinophil peroxidase. The reactive brominating species induced brominated DNA adducts such as 8-bromo-2′-deoxyguanosine (8-Br-dG), 8-bromo-2′-deoxyadenosine (8-Br-dA), and 5-bromo-2′-deoxycytidine (5-Br-dC). These DNA lesions may be implicated in carcinogenesis. In this study, we analyzed the miscoding properties of the brominated DNA adducts generated by human DNA polymerases (pols). Site-specifically modified oligodeoxynucleotides containing a single 8-Br-dG, 8-Br-dA, or 5-Br-dC were used as a template in primer extension reactions catalyzed by human pols α, κ, and η. When 8-Br-dG-modified template was used, pol α primarily incorporated dCMP, the correct base, opposite the lesion, along with a small amount of one-base deletion (4.8%). Pol κ also promoted one-base deletion (14.2%), accompanied by misincorporation of dGMP (9.5%), dAMP (8.0%), and dTMP (6.1%) opposite the lesion. Pol η, on the other hand, readily bypassed the 8-Br-dG lesion in an error-free manner. As for 8-Br-dA and 5-Br-dC, all the pols bypassed the lesions and no miscoding events were observed. These results indicate that only 8-Br-dG, and not 5-Br-dC and 8-Br-dA, is a mutagenic lesion; the miscoding frequency and specificity vary depending on the DNA pol used. Thus, hypobromous acid-induced 8-Br-dG adduct may increase mutagenic potential at the site of inflammation.

Introduction

Chronic parasitic infections lead to inflammation, and chronic inflammation has been recognized as a factor of carcinogenesis and linked to cancer development.[1] At sites of inflammation, neutrophils and eosinophils are activated and then increased in the blood and tissues.[2] These elements play important roles in host defense against microbial parasite using reactive oxidants such as nitric oxide and hypobromous acid (HOBr).[3] The excessive oxidants in this process, however, can produce several DNA base damages[4–6] and may lead to mutagenesis in adjacent epithelial cells.

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Abbreviations used: HOBr, hypobromous acid; MPO, myeloperoxidase; EPO, eosinophil peroxidase; 8-Br-dG, 8-bromo-2′-deoxyguanosine; 8-Br-dA, 8-bromo-2′-deoxyadenosine; 5-Br-dC, 5-bromo-2′-deoxycytidine; 8-Oxo-dG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; dNTP, 2′-deoxynucleoside triphosphate; Alexa546, AlexaFluor-546 dye; pol, human DNA polymerase; F_{ins}, frequency of insertion; F_{ext}, frequency of extension; LPS, lipopolysaccharide; 5-Br-dU, 5-bromo-2′-deoxyuridine.

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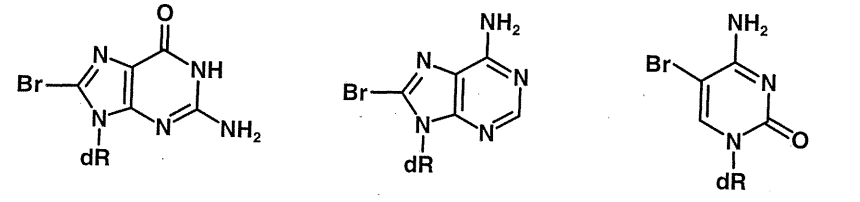

Fig. 1. Structures of brominated DNA adduct.

At the inflammation site, the NADPH oxidase complex of leukocytes is activated and assembles at the plasma membrane. The complex catalyzes the one-electron reduction of oxygen to superoxide ($O_2^{\cdot-}$). This reduced oxygen then dismutates to hydrogen peroxide (H_2O_2), which is utilized as a reactive oxidant. In the presence of plasma halides (Cl^-, 100 mM; Br^-, 20–100 μM; I^-, <1 μM),[7] myeloperoxidase (MPO) and eosinophil peroxidase (EPO), which are released by neutrophils and eosinophils, respectively, generate HOBr using H_2O_2 and Br^- as substrates (Eq. 1).[3,8–10]

$$Br^- + H_2O_2 + H^+ \rightarrow HOBr + H_2O \quad (1)$$

HOBr is a potent oxidant that oxidizes the cellular components of invading pathogens. This reactive brominating species also damages the host DNA and the cellular protein under chronic inflammatory conditions.[11,12] 5-Bromouracil (5-bromo-2′-deoxyuridine (5-Br-dU) in nucleoside form) has been detected in human inflammatory tissues.[13] 5-Br-dU is well known as a thymidine analog and causes G:C → A:T and A:T → G:C transitions.[14,15] It has been previously reported that 5-bromo-2′-deoxycytidine (5-Br-dC) is produced by EPO and can be converted to 5-Br-dU by cellular cytidine deaminases.[4,16,17] 8-Bromo-2′-deoxyguanosine (8-Br-dG) and 8-bromo-2′-deoxyadenosine (8-Br-dA) are also generated by exposure of calf thymus DNA or free deoxyribonucleoside to the EPO/H_2O_2/Br^- system[6] (Fig. 1). Moreover, 8-Br-dG was recently detected from the hepatic DNA of lipopolysaccharide (LPS)-treated rats and the urine of hepatocellular carcinoma patients.[5] Thus, 5-Br-dC, 8-Br-dA, and 8-Br-dG may contribute to the burden of carcinogenesis in inflammatory tissues.

Primer extension reactions past 8-Br-dG lesion have been performed by using only human DNA polymerase (pol) α and β in the presence of a single or two 2′-deoxynucleoside triphosphates (dNTPs) on single-stranded gap DNA templates containing a 8-Br-dG lesion.[18] The previous report showed that both pols incorporated dCMP and dGMP opposite 8-Br-dG. The miscoding properties of 5-Br-dC and 8-Br-dA have not been determined yet. No quantitative analysis has been used for exploring miscoding events generated by 8-Br-dG, 8-Br-dA, and 5-Br-dC.

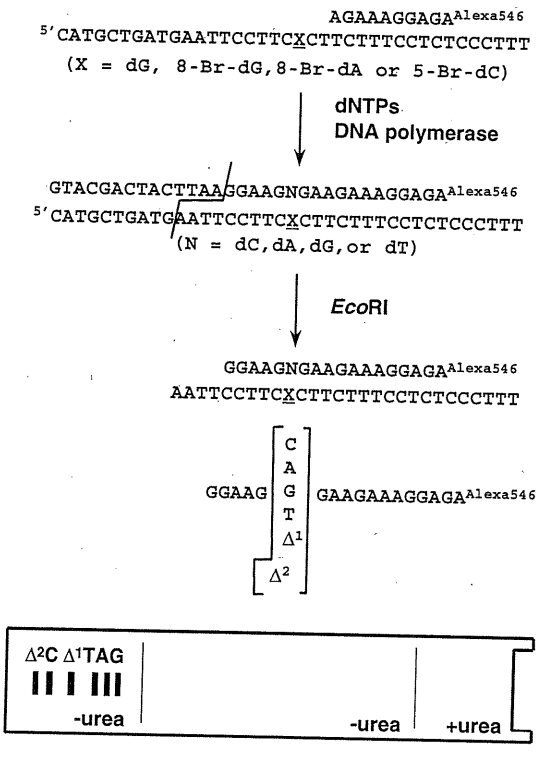

Fig. 2. Diagram of the fluorescent two-phase PAGE analysis. Unmodified and modified 38-mer templates were annealed to an Alexa546-labeled 10-mer primer. Primer extension reactions catalyzed by pols α, κ, and η were conducted in the presence of all four dNTPs. Fully extended products formed during DNA synthesis were recovered from the 20% polyacrylamide gel, annealed with the complementary 38-mer, cleaved with EcoRI, and subjected to two-phase PAGE. To determine miscoding specificities, mobilities of the reaction products were compared with those of 18-mer standards containing dC, dA, dG, or dT opposite the lesion and one-base (Δ^1) or two-base (Δ^2) deletions.

In this study, site-specifically modified oligodeoxynucleotides containing single 8-Br-dG, 8-Br-dA, and 5-Br-dC were used as a DNA template for primer extension reactions catalyzed by human DNA pols α, κ, and η in the presence of all four dNTPs. We determined the miscoding properties of the brominated DNA lesions that occurred during *in vitro* DNA replication using two-phase gel electrophoresis, which allows the quantification of base substitutions and deletions (Fig. 2).[19–21] Relative bypass frequencies past the brominated DNA adducts were also determined by steady-state kinetic studies.

Results

Primer extension reactions catalyzed by human DNA pols on 8-Br-dG-, 8-Br-dA-, and 5-Br-dC-modified DNA templates

Primer extension reactions were carried out using 8-Br-dG-, 8-Br-dA-, and 5-Br-dC-modified 38-mer templates in the presence of all four dNTPs and varying amounts of pol α, κ, or η (Fig. 3). Using the unmodified DNA template, the primer extension rapidly occurred to form fully extended products. When the 5-Br-dC-modified template was used, these pols readily bypassed the lesion, and the bypass efficiency was almost the same as that of the unmodified dC template (Fig. 3). With 8-Br-dG- and 8-Br-dA-modified templates, the primer extension reactions catalyzed by pols α and κ were slightly retarded by the lesions, showing that pols α and κ were blocked opposite (13-mer) and one base before (12-mer) both lesions, respectively (Fig. 3a and b). When the amount of pols was increased, products representing more than 32-mer bases long were produced on the 8-Br-dG- and 8-Br-dA-modified templates. Pol η easily bypassed the 8-Br-dG and 8-Br-dA lesions as efficiently as unmodified dG and dA, respectively (Fig. 3c). Blunt-end addition to the fully extended product (33- to 34-mer) was observed in all primer extension reactions, as reported earlier for *Escherichia coli* and mammalian DNA pols.[22,23]

Miscoding frequencies and specificities of brominated DNA adducts

Translesion synthesis catalyzed by pols α, κ, and η was conducted in the presence of all four dNTPs. The fully extended products (approximately 28- to 34-mer) past 8-Br-dG-, 8-Br-dA-, and 5-Br-dC-modified adducts were recovered, digested by EcoRI, and subjected to two-phase PAGE for quantitative analysis of base substitutions and deletions as described in Materials and Methods. A standard mixture of six Alexa Flour 546 dye (Alexa546)-labeled oligomers containing dC, dA, dG, or dT opposite the lesion or one- and two-base deletions can be resolved by this method (Fig. 2). The percentage of 2′-deoxynucleoside monophosphate incorporation was normalized to the amount of the starting primer.

When unmodified dG template was used, the incorporation of dCMP, the correct base, was observed opposite dG at 74.1%, 85.3%, and 65.7% of the starting primers for pols α, κ, and η, respectively (Fig. 4). Using 8-Br-dG-modified template, pol α frequently incorporated dCMP (69.9%) opposite the lesion. As indicated by the arrowhead in Fig. 4a, one-base deletion (4.8%) was also detected. With pol κ, the correct base dCMP (55.2%) opposite the lesion was incorporated as the primary product. Moreover, pol κ promoted one-base deletion (14.2%) associated with dGMP (9.5%), dAMP (8.0%), and dTMP (6.1%) misincorporation opposite the 8-Br-dG lesion (Fig. 4b). Pol η exclusively incorporated dCMP opposite both unmodified dG and 8-Br-dG (Fig. 4c). Small amounts of unknown products were also detected (arrowheads in Fig. 4c). The miscoding specificities of 8-Br-dA and 5-Br-dC catalyzed by pols α, κ, and η were also determined by using two-phase PAGE. All the pols exclusively incorporated the correct bases dTMP and dGMP opposite 8-Br-dA and 5-Br-dC lesions, respectively, indicating that no miscoding events occurred (Supplementary Fig. S1).

Steady-state kinetic studies on 8-Br-dG-modified DNA template

A steady-state kinetic analysis was performed by using pols α and κ to determine the frequency of dNTP incorporation (F_{ins}) opposite 8-Br-dG and chain extension (F_{ext}) from the primer terminus (Table 1). With pol α, the F_{ins} value for dGTP (0.22), the wrong base, was 2.4 times higher than that for dCTP (9.20×10^{-2}). The relative bypass frequency ($F_{ins} \times F_{ext}$) past dG:8-Br-dG (5.48×10^{-6}) was, however, 13.1 times lower than that for dC:8-Br-dG (7.16×10^{-5}) because the F_{ext} for the dG:8-Br-dG pair (2.49×10^{-5}) was 31.2 times lower than that for the dC:8-Br-dG pair (7.78×10^{-4}). The values of F_{ins} and F_{ext} for dA:8-Br-dG and dT:8-Br-dG were not detectable.

When pol κ was used, the F_{ins} value for dCTP (9.82×10^{-2}) opposite 8-Br-dG was only 1.4 times lower than that for dGTP (0.14) and 13.4 and 15.2 times higher than that for dATP (7.32×10^{-3}) and dTTP (6.46×10^{-3}), respectively. The F_{ext} for dC:8-Br-dG (3.05×10^{-2}) was 12.0, 26.1, and 14.0 times higher than that for dG:8-Br-dG (2.54×10^{-3}), dA:8-Br-dG (1.17×10^{-3}), and dT:8-Br-dG (2.18×10^{-3}), respectively. Therefore, the relative bypass frequency past the dC:8-Br-dG was only 8.43 times higher than that for dG:8-Br-dG.