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Regular article

In Vivo Mutagenesis Caused by Diesel Exhaust in the Testis of *gpt* delta Transgenic Mice

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Diesel exhaust (DE) is a major airborne pollutant in urban areas. In this study, we estimated the systemic effect of diesel exhaust inhalation by investigating mutations in extraplumonary organs such as the testis and liver. *gpt* delta Transgenic mice carrying the guanine phosphoribosyltransferase (*gpt*) transgene for the detection of mutations in genomic DNA were exposed to inhalation of 3 mg m^{-3} diesel exhaust (as suspended particulate matter) for 12 or 24 weeks. Compared to the control mice, DE resulted in a 2.0-fold increase in mutant frequency in the testis of mice that were exposed to DE for 24 weeks (inhaled group, 1.17×10^{-5}; control group, 0.57×10^{-5}), but not in the testis of mice exposed for 12 weeks (0.61×10^{-5}). The mutant frequency in the lungs was 2.6-fold higher in mice exposed to DE for 24 weeks than the control group, but it was not elevated in the liver (0.67×10^{-5}). In the testis, the major mutations on the *gpt* gene were G:C→T:A transversions, 1 base deletions and G:C→A:T transitions, while the major mutation in the lung was G:C→A:T transitions. The mutations on nucleotide nos. 402, 406, 409 and 416–418 in the *gpt* gene in testis seemed to be characteristic of DE inhalation in the testis. Our results suggest that inhalation of diesel exhaust is genotoxic to the testis as well as respiratory organs.

Key words: diesel exhaust emission, testis, *gpt* delta transgenic mouse, 6-thioguanine selection

Introduction

Diesel exhaust (DE) emission is a major source of air pollutant in urban areas, and has been implicated in causing allergic respiratory disease and lung cancer (1,2). Diesel exhaust particles (DEP) have been known to contain potent carcinogens and mutagens, such as polyaromatic hydrocarbons (PAH; e.g., benzo[*a*]pyrene (B[*a*]P)) and nitrated PAH (e.g., 1,6-dinitropyrene (1,6-DNP)), of which mutagenicity has been evaluated *in vitro* using a *Salmonella typhimurium* TA98 assay (3,4). Exposure to DEP through inhalation or intratracheal instillation have been shown to cause oxidative DNA damage (5,6) and DNA adduct formation (7,8) in rat and mouse lungs, and long periods of inhalation of DE resulted in respiratory tract tumors in rats (9–12). These observations suggest that mutagens in DE induce mutations in the lung, a primary target organ of inhalation, and are responsible for inducing lung cancer. Furthermore, we have previously demonstrated that typical mutagens such as B[*a*]P (13) and 1,6-DNP (14), as well as inhalation of DE (15,16), caused mutations in the lungs using transgenic rodents for analyzing *in vivo* mutagenesis (Big Blue® rat and *gpt* delta mouse). Metabolites of PAH contained in suspended particulate matter in ambient air have been detected in human urine (17), suggesting that mutagenic PAH in DE are absorbed in the lungs and transported to extrapulmonary organs, such as the testis and liver, where they could exert possible genotoxicity. Watanabe *et al.* showed that the number of daily sperm and Sertoli cells in fetuses and male rats was decreased by DE exposure (18,19). However, the mutagenic effect of DE on the extrapulmonary organs has remained unclear.

We intended to evaluate the *in vivo* mutagenicity of DE in testis and liver to obtain fundamental data for assessing the health risks of air pollution. In order to evaluate *in vivo* mutagenicity, we used the *gpt* delta transgenic mice carrying the lambda phage EG10 as a transgene for detecting mutations on genomic DNA (20,21). When the rescued phage is infected into *E. coli* expressing Cre recombinase, the phage DNA is converted into plasmids harboring the chloramphenicol (Cm)-resistance gene and guanine phosphoribosyltransferase (*gpt*) gene. The *gpt* mutants can be positively detected as colonies arising on plates containing Cm and 6-thioguanine (6-TG). Our study revealed an elevated mutant frequency and alterations in the mutation spectrum in the testis of DE-inhaled *gpt* delta transgenic mice in which

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the mutant frequency in the lung has already been reported to increase (16).

In this study, we show that inhalation of 3 mg m^{-3} DE (as suspended particulate matter (SPM)) for 24 weeks resulted in a 2.0-fold increase in mutant frequency in the testis of *gpt* delta mice compared to the controls, but the inhalation for 12 weeks did not elevate the mutant frequency in the testis. The mutant frequency in the liver was not increased by inhalation of DE under conditions where the mutant frequency in the testis and lungs were significantly increased. The predominant mutation spectrum in the testis in response to DE inhalation included G:C→T:A transversions, 1-base deletions and G:C→A:T transitions, while the major mutations in the lungs were G:C→A:T transitions (16). These data suggest that DE inhalation exerts genotoxicity on testis systemically.

Materials and Methods

Treatment of mice: *gpt* delta Mice carry ca. 80 copies of lambda EG10 DNA on each chromosome 17 in a C57BL/6J background (22). Exposure to DE (12 h d^{-1}, 7 d $week^{-1}$) was performed in chambers equipped by the National Institute for Environmental Studies (16,23) under the same conditions as those in our previous report on *in vivo* mutations in the lung (16). Three to five 7-week-old mice were exposed to 3 mg m^{-3} DE (as SPM) for 12 or 24 weeks. Seven mice were maintained in filtered clean air (control group). The animals were sacrificed 3 days after the last exposure and their testis and liver were removed, frozen in liquid nitrogen and stored at −80°C for this study.

***Gpt* mutation assay:** The *gpt* assay was performed as described previously (20). Genomic DNA was extracted from the testis and liver tissue using the RecoverEase DNA Isolation Kit (Stratagene Co., La Jolla, CA) and Lambda EG10 phages were rescued using the Transpack® Packaging Extract (Stratagene). *E. coli* YG6020 was infected with the phage, spread on M9 salt plates containing Cm and 6-TG (19), and then incubated for 72 h at 37°C for selection of the colonies harboring a plasmid carrying the chloramphenicol acetyltransferase (CAT) gene and a mutated *gpt* gene. Isolates from the 6-TG-resistant phenotype were cultured in LB broth containing 25 μg/mL^{-1} Cm at 37°C overnight, harvested by centrifugation (7,000 rpm, 10 min) and stored at −80°C.

PCR and DNA sequencing of the 6-TG-resistant mutants: A 739 bp DNA fragment containing the *gpt* gene was amplified by PCR and sequenced as described previously (13,20). Sequencing was performed using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an Applied Biosystems model 3730xl DNA analyzer.

Statistical analysis: All of the data are expressed as the mean ± SD. The statistical significance of the DE treatment was analyzed using the Student's *t*-test. $p<0.05$ was considered to be statistically significant. Mutational spectra were compared using the Adams-Skopek test (24,25).

Results

***Gpt* mutations in the testis, lung and liver of DE inhaled *gpt* delta mice:** In order to estimate the mutagenicity of DE, *gpt* delta mice inhaled DE (3 mg m^{-3} as SPM) for 12 or 24 weeks and mutations in the testis and liver were analyzed (Table 1). While the mutant frequencies in the testis of the control mice for 12 and 24 weeks inhalation were $0.57 \pm 0.04 \times 10^{-5}$ and $0.58 \pm 0.07 \times 10^{-5}$, respectively, inhalation of DE for 12 and 24 weeks resulted in 1.1 and 2.0-fold increases in mutant frequency ($0.61 \pm 0.08 \times 10^{-5}$ and $1.17 \pm 0.45 \times 10^{-5}$, respectively) compared with the controls (Table 1). Significant increases in the mutant frequency in the testis were observed in the group that inhaled DE for 24 weeks compared with the control group and the group that inhaled DE for 12 weeks. Our previous report demonstrated that inhalation of 3 mg m^{-3} DE for 24 weeks resulted in a 2.6-fold increase in the mutant frequency in the lung (Table 1) (16); however, the mutant frequency in the liver ($0.67 \pm 0.23 \times 10^{-5}$) was not elevated even after inhalation for 24 weeks compared with the control ($0.56 \pm 0.14 \times 10^{-5}$).

Alterations in the mutation spectrum in testis are induced by DE inhalation: In order to determine the mutation spectrum induced by DE inhalation, 170 6-TG-resistant mutants in a total were sequenced. As shown in Table 2, mutations of the *gpt* gene were detected in 149 mutants obtained from the testis of DE-inhaled and control mice (Table 1). The mutation type analysis indicated that the percentages of G:C→T:A transversions and 1-base deletions were increased in DE-inhaled mice (DE all) comparing to control mice (Control all). To characterize DE-induced mutagenesis precisely, the frequency of each mutation was calculated from data in Table 2 (Fig. 1). In the groups that inhaled DE for 24 weeks, the mutant frequency of G:C→T:A transversions, 1-base deletions and G:C→A:T transitions was 3.8×10^{-6}, 2.9×10^{-6} and 2.4×10^{-6}, whereas that of the control mice was, 1.0×10^{-6}, 0.6×10^{-6} and 1.9×10^{-6}, respectively. DE inhalation for 24 weeks caused a significant difference in the types of mutation in the control and DE inhalation groups ($p=0.04$, Adams-Skopek test).

The spectrum of *gpt* mutations in the testis that were induced by DE inhalation for 12 weeks and 24 weeks (Table 3) indicated a prevalence of G:C→T:A transversions with three mutation sites (nucleotide nos. 402, 406 and 409) being identified as hotspots in three or more mice, as well as G:C→A:T transition hotspots on

Table 1. Summary of mutant frequencies in the testis, lung and liver of *gpt* delta mice after inhalation of DE

Organ	DE concentration (mg m^{-3})	Exposure time (weeks)	ID of animals	Number of colonies		Mutant frequency ($\times 10^{-5}$)	Average mutant frequency ± SD ($\times 10^{-5}$)
				Mutant	Total		
Testis	0	12	1	7	1,265,600	0.55	
			2	9	1,428,800	0.63	
			3	10	1,792,000	0.56	
			4	10	1,820,800	0.55	
			Total	36	6,307,200		0.57 ± 0.04
	3	12	1	12	1,996,800	0.60	
			2	12	1,984,800	0.61	
			3	14	1,881,600	0.74	
			4	14	2,318,400	0.60	
			5	7	1,374,400	0.51	
			Total	59	9,556,000		0.61 ± 0.08
	0	24	1	9	1,676,800	0.54	
			2	5	756,800	0.66	
			3	7	1,291,200	0.54	
			Total	21	3,724,800		0.58 ± 0.07
	3	24	1	9	1,409,600	0.64	
			2	29	1,910,400	1.52	
			3	16	1,176,000	1.36	
			Total	54	4,496,000		1.17 ± 0.45*
Lung†	0	24	1	13	1,551,000	0.84	
			2	8	1,074,000	0.74	
			3	8	903,000	0.89	
			Total	29	3,528,000		0.82 ± 0.07
	3	24	1	10	462,500	2.16	
			2	11	546,000	2.01	
			3	16	745,600	2.15	
			Total	37	1,754,100		2.11 ± 0.08**
Liver	0	24	1	4	952,000	0.42	
			2	8	1,148,800	0.70	
			3	4	724,800	0.55	
			Total	16	2,825,600		0.56 ± 0.14
	3	24	1	2	275,200	0.73	
			2	2	483,200	0.41	
			3	8	937,600	0.85	
			Total	12	1,696,000		0.67 ± 0.23

Significant differences were detected between the control and DE-treated group (*: $p < 0.05$, **: $p < 0.001$).
†: data from our previous study (16).

another three sites (nucleotide nos. 64, 110 and 115). The predominant frameshift mutations induced by DE were single-base pair deletions in run sequences (22/29 = 76%); in this case the hotspot was located at nucleotide nos. 416–418. Therefore, the mutations on nucleotide nos. 402, 406, 409 and 416–418 seem to be characteristic of DE inhalation in testis, but were not hotspots in the lungs of DE-inhaled mice, while nucleotide no. 402 was a hotspot of G:C→A:T transitions in the lung.

Discussion

In this study we demonstrate that, as a result of inhalation of 3 mg m^{-3} DE, the mutant frequency in the testis of *gpt* delta mice increased with the duration of treatment (Table 1), but the mutant frequency in the liver was not elevated, indicating that DE inhalation exerts genotoxicity systemically on testis as well as on respiratory organs. This article is the first report on an increase in the mutant frequency in testis in response to DE inhalation (Table 1). Indeed, DE inhalation has also been

Table 2. Classification of *gpt* mutations from the testis of control and DE-inhaled mice

Type of mutation in the *gpt* gene	Control (weeks) 12	24	all	DE (weeks) 12	24	all
	%			%		
Base substitution						
Transition						
G:C→A:T	42	31	39	24	24	24
A:T→G:C	6	6	6	2	4	3
Transversion						
G:C→T:A	12	38	20	24	37	30
G:C→C:G	12	6	10	17	0	9
A:T→T:A	3	0	2	2	2	2
A:T→C:G	0	0	0	0	2	1
Deletion						
−1	12	13	12	30	28	29
>2	6	6	6	0	2	1
Insertion	6	0	4	2	0	1
Other	0	0	0	0	0	0
Total	100	100	100	100	100	100
Total number of mutants*	33	16	49	54	46	100

*: 149 of 170 6-TG-resitant mutants have mutation in the *gpt* gene.

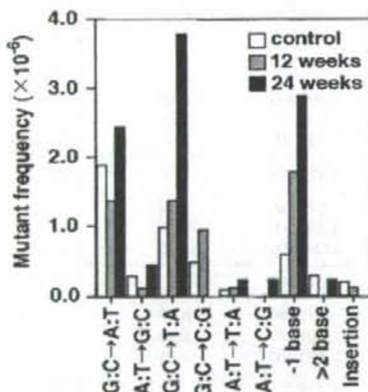

Fig. 1. Comparison of the 6-TG-resistant mutation spectra in control and DE inhaled *gpt* delta mice. The mutant frequencies of control mice and those exposed to DE for 12 weeks and 24 weeks were calculated by dividing the number of each type of *gpt* mutations in the Control all, DE 12 weeks and DE 24 weeks, respectively, by the corresponding total number of colonies (shown in Table 1).

shown to cause a decrease in the number of daily sperm and Sertoli cells in fetuses and male rats (18,19). Daily sperm production in the testis decreased dose-dependently in response to DE exposure for 6 months; a 53% reduction in sperm production was observed in rats exposed to DE (26) at the same concentration (3 mg m^{-3}) used in this study. These observations indicate that DE inhalation induces an increase in mutant frequency in the testis under the same conditions in which the reproduction of sperm was suppressed.

A significant increase in mutant frequency was observed in the testis after DE inhalation for 24 weeks but not for 12 weeks, while the mutant frequency in the lungs was elevated after inhalation for 24 weeks as well as 12 weeks (16). Delayed mutagenesis in germ cells has been observed in *lacZ* transgenic mice after 35 days treatment with ethyl nitrosourea (ENU) (27). Mutagens contained in DE were absorbed in the lung, systemically transported to the testis and possibly caused DNA adduct formation in spermatogonial stem cells and spermatogonia. These DNA adducts may be fixed as delayed mutations in germ cells through errors in DNA replication in continuous cell division during germ-cell development from spermatogenic cells to sperm. On the other hand, DNA adducts may be formed in the liver, but might not be fixed as mutations because of the low rate of cell division and/or high degree of DNA repair. However, Masumura *et al.* (28) showed that a heterocyclic amine, PhIP, was metabolically activated and induced point mutations in the liver but not the testis of *gpt* delta mice, suggesting that any factors governing the distribution and metabolism of mutagens in the body may determine the tissue specificity of mutagenesis.

The predominant mutation spectrum in the testis in response to DE included G:C→T:A transversions, 1-base deletions and G:C→A:T transitions (Table 2 and Fig. 1) as well as mutation hotspots on nucleotide nos. 402, 406 and 409, nos. 416–418 and nos. 64, 110 and 115, respectively (Table 3), while mutations in the lung were predominantly only G:C→A:T transitions (16). Mutation hotspots on nucleotide no. 406 and nos. 416–418 were identified in the testis of mice that inhaled DE for 12 weeks, in which the mutant frequency did not significantly increase, suggesting that DE acts as mutagenic agent even after inhalation for 12 weeks. G:C→T:A transversions have been known to be induced in *gpt* delta mice by B[*a*]P treatment (13) and 8-hydroxy-deoxyguanine (8-OHdG) generated by reactive oxygen species (ROS) (29). We have shown that the G:C→T:A transversion was a predominant mutation in Nrf2 deficient mice (30), in which the levels of the phase II detoxification enzymes and ROS-scavenging enzymes were suppressed (31,32) and DNA adduct formation was accelerated in the lung (33). These observations suggest some contribution of ROS to inducing mutation hotspots of G:C→T:A transversions (nucleotide nos. 402, 406 and 409) in the testis of mice subjected to DE inhalation. Nucleotide nos. 64, 110 and 115 were mutation hotspots of G:C→A:T transitions in the testis of DE-inhaled mice as well as in 1,6-dinitropyrene (DNP)-instilled lungs of *gpt* delta mice (14), and were also mutation hotspots in non-treated mice (34). The compo-

Table 3. DNA sequence analysis of *gpt* mutations obtained from the testis of DE-treated and control mice

Type of mutation	Mutation			Amino acid change	Number			
	Nucleotide number	Sequence Change			Control		DE	
					12 weeks	24 weeks	12 weeks	24 weeks
Base substitution								
Transition								
G:C→A:T	3	atG → atA		Met → Ile	1			
	64	Cga → Tga	CpG	Arg → Stop	1		$4^{\dagger}$	
	82	Caa → Taa		Gln → Stop	1			
	86	tGg → tAg		Trp → Stop	1			
	107	aGc → aAc		Ser → Asn		1		
	110	cGt → cAt	CpG	Arg → His	2^{*}		2	2^{*}
	113	gGc → gAc		Gly → Asp		1	1	
	115	Ggt → Agt	CpG	Gly → Ser	1		3^{*}	3^{*}
	116	gGt → gAt		Gly → Asp	2^{*}	1	1	1
	145	Gaa → Aaa		Glu → Lys			1	
	176	tGt → tAt		Cys → Tyr	1			
	202	Cag → Tag		Gln → Stop				1
	401	tGg → tAg		Trp → Stop	1			
	402	tgG → tgA		Trp → Stop			1	1
	418	Gat → Aat		Asp → Asn	$3^{\dagger}$	1		3^{*}
	451	Ggt → Agt	CpG	Gly → Ser		1		
A:T→G:C	56	cTc → cCc		Leu → Pro	1			1
	410	cAg → cGg		Gln → Arg				1
	415	Tgg → Cgg		Trp → Arg	1			
	419	gAt → gGt		Asp → Gly		1	1	
Transversion								
G:C→T:A	7	Gaa → Taa	CpG	Glu → Stop				1
	59	gCa → gAa		Ala → Glu				1
	110	cGt → cTt	CpG	Arg → Leu				1
	127	Ggt → Tgt		Gly → Cys				1
	140	gCg → gAg	CpG	Ala → Glu		2	1	1
	145	Gaa → Taa		Glu → Stop				1
	189	taC → taA	CpG	Tyr → Stop		1	1	
	208	Gag → Tag	CpG	Glu → Stop				1
	287	aCt → aAt		Thr → Asn			1	
	304	Gaa → Taa		Glu → Stop	1		1	1
	401	tGg → tTg		Trp → Leu	1			1
	402	tgG → tgT		Trp → Cys	1		2^{*}	1
	406	Gaa → Taa		Glu → Stop			$4^{\dagger}$	4^{*}
	409	Cag → Aag		Gln → Lys			1	2^{*}
	413	cCg → cAg	CpG	Pro → Gln	1	2^{*}		1
	418	Gat → Tat		Asp → Tyr		1	2^{*}	
G:C→C:G	3	atG → atC		Met → Ile			1	
	6	agC → agG	CpG	Ser → Arg	1			
	109	Cgt → Ggt	CpG	Arg → Gly			1	
	143	cGt → cCt	CpG	Arg → Pro	1			
	145	Gaa → Caa		Glu → Gln			1	
	262	Gat → Cat		Asp → His	1			
	289	Gcg → Ccg		Ala → Pro			1	
	340	Gca → Cca	CpG	Ala → Pro			2^{*}	
	401	tGg → tCg		Trp → Ser			1	
	402	tgG → tgC		Trp → Cys		1		
	413	cCg → cGg	CpG	Pro → Arg			2^{*}	
	418	Gat → Cat		Asp → His	1			
A:T→T:A	35	tTg → tAg		Leu → Stop				1
	146	gAa → gTa		Glu → Val			1	
	179	aTt → aAt		Ile → Asn	1			
A:T→C:G	106	Agc → Cgc		Ser → Arg				1

Table 3. cont.

Type of mutation	Nucleotide	Sequence Change	Number			
			Control		DE	
			12 weeks	24 weeks	12 weeks	24 weeks
Deletion	8–12	gAAAAAt → gAAAAt	1		2	1
−1 base	126–128	cGGGt → cGGt			1	
	133–134	gTTa → gTa			1	
	155–156	aTTc → aTc	1			
	179–181	aTTTc → aTTc				2
	230	gCa → ga				1
	237	gCg → gg	1			1
	244	cGa → ca			1	
	249	gCt → gt	1			
	277	tAc → tc			1	
	387–389	tCCCg → tCCg			1	
	416–418	tGGGa → tGGa		1	7[†]	5[†]
	420	aTa → aa				1
	426	gCg → gg			1	
	431	gTa → ga		1		
	442–443	gCCa → gCa				1
	451–452	cGGt → cGt				1
	454	tCg → tg			1	
>2	26–34	tGGGACATGTTg → tg		1		
	170–171	aCCg → ag	1			
	238–249	cGATGGCGAAGGCt → ct	1			1
Insertion	75	ct → cAt	1			
	107	ag → aTg	1			
	214–216	taaag → tAaaag			1	
Total			33	16	54	46

* and †: Mutations found in 2 and 3 different mice, respectively.

nents in DE, such as 1,6-DNP and related compounds, may also contribute to enhance spontaneous mutations *via* the generation of ROS in the lung and also in the testis in response to DE inhalation.

Potent mutagens such as B[*a*]P and 1,6-DNP in DE are suspected to cause tumors in the lung, but their effect on the germline remains to be investigated. Previously, B[*a*]P was shown to induce a dominant-lethal mutation in the germ cells of male mice (35). We show that inhalation of DE, a major air pollutant in urban air, induces mutations in the testis, suggesting that mutagenic PAH and other mutagenic compounds in DE cause germline mutations. Previously, a germline mutation has been reported to occur in herring gulls living in an urban area (36). Recently, heritable DNA mutations in micro-satellite DNA were identified in mice that inhaled polluted ambient air in an industrial area (37,38); exposure to polluted ambient air for 10 weeks, followed by 6 weeks in the laboratory, was required for a significant increase in the sperm mutant frequency in these mice (38). This observation (38) corresponds to the delayed induction of point mutations in the testis in our study. Mutagenic compounds in ambient air may contribute to the induction of germline mutations. However, further studies are required to confirm that DE and other air pollutants cause mutations in germline cells, which are good markers for assessing the health risk of air pollution.

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Possible participation of oxidative stress in causation of cell proliferation and *in vivo* mutagenicity in kidneys of *gpt* delta rats treated with potassium bromate

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ABSTRACT

Clarifying the participation of oxidative stress among possible contributing factors in potassium bromate ($KBrO_3$)-induced carcinogenesis is of importance from the perspective of human health protection. In the present study, utilizing the antioxidative effects of α-tocopherol (α-TP) or sodium ascorbic acid (SAA) to attenuate oxidative stress, alterations in bromodeoxyuridine labeling indices (BrdU-LIs) and reporter gene mutations in kidneys of male and female *gpt* delta rats given $KBrO_3$ were examined. Five male and female *gpt* delta rats in each group were given $KBrO_3$ at a concentration of 500 ppm in the drinking water for 9 weeks, with 1% of α-TP or SAA administered in the diet from 1 week prior to the $KBrO_3$ treatment until the end of the experiment. Increases in 8-hydroxydeoxyguanosine levels in kidney DNA of both sexes of rats given $KBrO_3$ were significantly inhibited by SAA, but not α-TP. While BrdU-LIs in the proximal tubules of female rats were also significantly reduced by SAA, those in the males and *gpt* mutant frequencies in kidney DNA of both sexes were not affected by SAA or α-TP. Immunohistochemical and Western blot analyses for α_{2u}-globulin strongly suggested that induction of cell proliferation observed in the males might primarily result from accumulation of this protein, independent of oxidative stress. The overall data indicated that while oxidative stress well correlates with induction of cell proliferation in females, its role in males and in generation of *in vivo* mutagenicity by $KBrO_3$ in both sexes is limited.

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1. Introduction

During the bread making process, bromate is considered to be converted to bromide (Kurokawa et al., 1990), so that use of potassium bromate ($KBrO_3$) has been permitted as a flour improver for bread making in Japan and the USA in spite of its carcinogenicity (Kurokawa et al., 1986; DeAngelo et al., 1998). However, since ozonation of surface water for disinfection yields $KBrO_3$ as a by-product (Cavanagh et al., 1992), there is still concern regarding the human hazard presented by its renal carcinogenicity. As is clear from the specific use as a food additive, $KBrO_3$ is a potent oxidizing agent. This property is responsible for changes in DNA bases as well as lipid peroxidation (LPO), in the kidneys of treated rats (Chipman et al., 1998; Umemura et al., 1998). Since 8-hydroxydeoxyguanosine (8-OHdG), a form of guanine oxidized at C-8 position, is known to be fairly stable (Kasai and Nishimura, 1991), elevation of this oxidized base following $KBrO_3$ exposure implies involvement of oxidative stress in $KBrO_3$-induced carcinogenesis (Umemura and Kurokawa, 2006; Delker et al., 2006). Simultaneous treatment with antioxidants is known to prevent elevation of 8-OHdG and LPO induced by $KBrO_3$ (Cadenas and Barja, 1999; El-Sokkary, 2000), but it remains unclear how oxidative stress contributes to $KBrO_3$-carcinogenesis.

In two-stage model using *N*-ethyl-*N*-hydroxyethyl-nitrosamine as an initiator, $KBrO_3$ enhances renal tumorigenesis in both male and female rats (Kurokawa et al., 1985; Umemura et al., 1995). Also, short-term exposure to $KBrO_3$ in males was found to significantly elevate bromodeoxyuridine-labeling indices (BrdU-LIs) in proximal convoluted tubules (PCTs) in the same dose-dependent manner as evident in the promotion assay (Umemura et al., 1993). As a possible mode of action, we have proposed involvement of α_{2u}-globulin accumulation in $KBrO_3$-induced cell proliferation in males (Umemura et al., 2004). However, the fact that PCT BrdU-LIs in females exposed to $KBrO_3$ were also increased, albeit at higher doses than in males, implies the existence of other causal factors.

A two-stage model using nitrilotriacetate as a promoter has further shown that $KBrO_3$ possesses initiating activity for renal carcinogenesis in male rats (Umemura et al., 2006). In addition to previous positive results in several mutagenicity tests (Ishidate et al., 1984; Ishidate and Yoshioka, 1980; Hayashi et al., 1988), recent findings using isolated rat kidney cells (Nesslany et al., 2007) and

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human peripheral lymphocytes (Kaya and Topaktas, 2007) point to genotoxic potential. Also, in an *in vivo* mutation assay using reporter gene transgenic rats, $KBrO_3$ proved capable of elevating the transgene mutation frequency (Umemura et al., 2006; Yamaguchi et al., 2008). Although induction of micronuclei in rat peripheral blood reticulocytes by $KBrO_3$ was inhibited by antioxidants (Sai et al., 1992), there have been few reports demonstrating clear relationships between oxidative stress and its genotoxicity.

Assessment of the participation of oxidative stress in $KBrO_3$ carcinogenesis is clearly necessary for accurate estimation of its hazard risk to humans. In the present study, taking advantage of the inhibitory effects of two different types of antioxidants, α-tocopherol (α-TP) and sodium ascorbic acid (SAA), changes in BrdU-LIs and α_{2u}-globulin accumulation in PCT, and transgene mutations in kidney DNA of male and female *gpt* delta rats given $KBrO_3$ were investigated.

2. Materials and methods

2.1. Chemicals

$KBrO_3$, α-TP and SAA were purchased from Wako Pure Chemical Industries (Osaka, Japan). Alkaline phosphatase was obtained from Sigma Chemical (St. Loius, MO, USA) and nuclease P1 was from Yamasa Shoyu (Chiba, Japan). Anti-BrdU monoclonal and anti-α_{2u}-globulin polyclonal antibodies were from DakoCytomation (Glostrup, Denmark) and R&D Systems, Ltd. (Minneapolis, MN, USA), respectively.

2.2. Animals, diet and housing conditions

The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Five-week-old male and female *gpt* delta F344 rats carrying about five tandem copies of the transgene lambda EG10 per haploid genome were obtained from Japan SLC (Shizuoka, Japan). They were housed in polycarbonate cages (5 rats per cage) with hardwood chips for bedding in a conventional animal facility, maintained under conditions of controlled temperature (23 ± 2 °C), humidity (55 ± 5%), air change (12 times per hour), and lighting (12 h light/dark cycle) and were given free access to CRF-1 basal diet (BD: Charles River Japan) and tap water.

2.3. Animal treatments

Groups of 5 male and female *gpt* delta rats were administered $KBrO_3$ solution at a concentration of 500 ppm in the drinking water for 9 weeks. Additional subgroups of 5 male and female *gpt* delta rats were fed α-TP or SAA at a dose of 1% in the diet from 1 week prior to the $KBrO_3$ treatment until the end of the experiment. Further groups of 5 male and female rats each were given basal diet and distilled water (DW) throughout the experimental period as controls. All animals were injected with BrdU (100 mg/kg) i.p. twice a day for the final 2 days of the exposure and once on the day of termination, 2 h before killing. At the end of each period, the animals were killed under ethyl ether anesthesia and a part of left kidney was homogenized in Isogen (Nippon Gene, Tokyo, Japan) and stored at −80 °C until use for isolation of total RNA. The remaining left kidney was also stored at −80 °C for 8-OHdG measurement, Western blot analysis and *in vivo* mutation assays. Portions of right kidneys were fixed in ice-cold acetone for 3 days and processed for embedding in paraffin, sectioning (4 μm), and immunostaining for BrdU after histochemical demonstration of γ-glutamyltranspeptidase (γ-GT) activity. The remaining kidney tissue was fixed in buffered formalin and then routinely processed for embedding in paraffin, sectioning and immunostaining for α_{2u}-globulin.

2.4. Measurement of nuclear 8-OHdG

To prevent 8-OHdG formation as a byproduct during DNA isolation (Kasai, 2002), kidney DNA was extracted by a slight modification of the method of Nakae et al. (1995). Briefly, nuclear DNA was extracted with a commercially available DNA Extractor WB Kit (Wako Pure Chemical Industries, Ltd.) containing an antioxidant NaI solution to dissolve cellular components. For further prevention of autooxidation in the cell lysis step, deferoxamine mesylate (Sigma Chemical Co.) was added to the lysis buffer (Helbock et al., 1998). The DNA was digested to deoxynucleotides with nuclease P1 and alkaline phosphatase, and levels of 8-OHdG (8-OHdG/10^5 deoxyguanosine) were assessed by high-performance liquid chromatography (HPLC) with an electrochemical detection system (Coulochem II, ESA, Bedford, MA, USA).

2.5. Immunohistochemical procedures

For immunohistochemical staining of BrdU, sections were treated sequentially with normal horse serum, monoclonal mouse anti-BrdU (1:100), biotin-labeled horse anti-mouse IgG (1:400), and avidin-biotin-peroxidase complex (ABC) after denaturation of DNA with 4N HCl. Before the denaturation step, sections were processed histochemically for demonstration of γ-GT activity by the method of Rutenburg et al. (1969) using L-glutamyl-4-methoxy-β-naphtylamide (Polysciences Ltd., Warrington, PA, USA) as the substrate in order to assist in distinguishing the three kinds of tubules, as previously described (Umemura et al., 1992). The sites of peroxidase binding were demonstrated by incubation with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.). For immunohistochemical staining of α_{2u}-globulin, sections were treated sequentially with normal goat serum, polyclonal rabbit anti-α_{2u}-globulin (1:100), biotin-labeled goat anti-rabbit IgG (1:400), and ABC after denaturation of DNA with 4N HCl. The immunostained sections were lightly counterstained with hematoxylin for microscopic examination.

2.6. Cell proliferation quantification

At least 3000 tubule cells in each kidney were counted and BrdU-LIs were calculated as the percentages of cells positive for BrdU incorporation.

2.7. Western blotting for α_{2u}-globulin

Kidney samples were homogenized with a Teflon homogenizer in ice-cold 50 mM Tris–HCl, pH 7.4 containing 0.25 M sucrose and a 1% protease inhibitor cocktail (Sigma Chemical Co.). The homogenate was centrifuged for 10 min at 10,000 × g, 4 °C, and the resulting supernatant was collected. Protein concentrations were determined with a BCA Protein Assay kit (Pierce Biotechnology Ltd., Rockford, IL, USA). The samples containing 20 μg protein were resolved by SDS-PAGE, transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA, USA) and analyzed with anti-α_{2u}-globulin (1:200), as well as anti-β-actin as a loading control (1:8000, Sigma Chemical Co.). Appropriate peroxidase-conjugated secondary antibodies (1:2000, Dako Cytomation) were used to detect proteins with ECL Plus (Amersham Bioscience Corp., Piscataway, NJ, USA) reagents.

2.8. In vivo mutation assays

6-TG and Spi⁻ selections were performed as previously described (Umemura et al., 2007) using the first three animals each group. Briefly, genomic DNA was extracted from the kidneys of the first 3 animals in each group, and lambda EG10 DNA (48 kb) was rescued as phages by *in vitro* packaging.

For 6-TG selection, packaged phages were incubated with *Escherichia coli* YG6020, which expresses Cre recombinase, and converted to plasmids carrying *gpt* and chloramphenicol acetyltransferase. Infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol and 6-TG. In order to determine the total number of rescued plasmids, 3000-fold diluted phages were used to infect YG6020, and poured on plates containing chloramphenicol without 6-TG. The plates were then incubated at 37 °C for selection of 6-TG-resistant colonies. Positively selected colonies were counted on day 3 and collected on day 4. The mutant frequency (MF) was calculated by dividing the number of *gpt* mutants by the number of rescued phages.

For Spi⁻ selection, packaged phages were incubated with *E. coli* XL-1 Blue MRA for survival titration and *E. coli* XL-1 Blue MRA P2 for mutant selection. Infected cells were mixed with molten lambda-trypticase agar plates. Next day, plaques (Spi⁻ candidates) were punched out with sterilized glass pipetters and the agar plugs were suspended in SM buffer. In order to confirm the Spi⁻ phenotype of candidates, the suspensions were spotted on three types of plates where XL-1 Blue MRA, XL-1 Blue MRA P2, or WL95 P2 strains were spread with soft agar. Real Spi⁻ mutants, which made clear plaques on every plate, were counted.

For characterizing the mutation spectra of *gpt* mutants, a 739 bp DNA fragment containing the 456 bp coding region of the *gpt* gene was amplified by PCR as described previously (Nohmi et al., 2000). DNA sequencing was performed with the Big Dye™ Terminater Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM™ 310 Genetic Analayzer (Applied Biosystems).

3. Results

As shown in Fig. 1, 8-OHdG levels in kidney DNA of male and female *gpt* delta rats given $KBrO_3$ were significantly increased as compared to the controls. Although the levels in *gpt* delta rats of both sexes co-treated with α-TP or SAA were still significantly higher than the controls, significant decreases in either sex of rats were evident as compared to $KBrO_3$-treated animals.

PCT BrdU-LIs in male and female gpt delta rats exposed to $KBrO_3$, with or without antioxidants, are shown in Fig. 2. In the males, $KBrO_3$ exposure induced prominent rise of BrdU-LIs with statistical significance, which was not affected by α-TP or SAA treatment. In the females, $KBrO_3$ significantly increased BrdU-LIs as in the males,

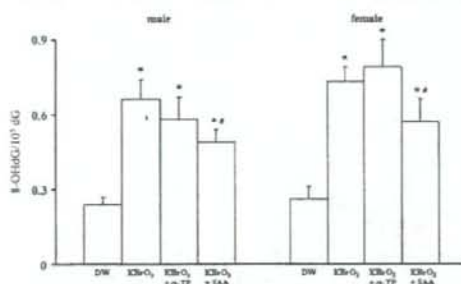

Fig. 1. 8-OHdG levels in kidneys of male and female *gpt* delta rats co-treated with $KBrO_3$ and α-TP or SAA. Values are means ± SDs of data for 5 rats. *$p < 0.01$, significantly different from the controls (DW). #$p < 0.01$, significantly different from the $KBrO_3$ alone group.

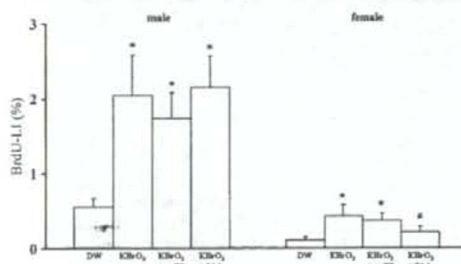

Fig. 2. BrdU-LIs for proximal convoluted tubules (PCT) of male and female *gpt* delta rats co-treated with $KBrO_3$ and α-TP or SAA. Values are means ± SDs of data for 5 rats. *$p < 0.01$, significantly different from the controls (DW). #$p < 0.01$, significantly different from the $KBrO_3$ alone group.

but in this case, co-treatment with SAA, but not α-TP, was associated with suppression of the elevation.

Immunohistochemical data for α_{2u}-globulin are shown in Fig. 3(A–E). Because α_{2u}-globulin is a male rat-specific urinary protein, in controls scattered accumulation was limited to males (Fig. 3 A). $KBrO_3$ caused accumulation of the protein only in males (Fig. 3 B), which was not inhibited by any antioxidant treatments (Fig. 3 C and D), no binding being evident even in the $KBrO_3$-treated female rats (Fig. 3 E). These findings were directly in line with Western blot results for α_{2u}-globulin (Fig. 3 F).

Data for *gpt* MFs analyzed by 6-TG selection are summarized in Tables 1 and 2. In the males, although statistical analysis could not be performed because no *gpt* mutant colonies were detected in one control rat, MFs in all the treated groups showed a tendency for increase (Table 1). Likewise, in the females, elevation of MFs in all the treated groups were found, the increase in the $KBrO_3$ alone group being statistically significant (Table 2). To characterize *gpt* mutations DNA sequencing was performed (Table 3). Among the groups in which the MFs were significantly increased, there were no common types of mutations. GC:AT transitions in α-TP treated males, GC:TA and AT:TA transversions in SAA treated males and single base deletions in $KBrO_3$ alone treated females showed the highest mutation frequencies. As shown in Table 4, there were no changes in Spi^- MFs in males. In all the treated females, a tendency for elevation of Spi^- MFs was apparent, with statistical significance in the α-TP treatment case (Table 5). However, co-treatment with the antioxidants did not appear to exert any effects on MFs for the *gpt* gene in the kidneys of rats given $KBrO_3$.

4. Discussion

In the present study, increases of 8-OHdG levels in kidney DNA of male and female rats following $KBrO_3$ exposure were significantly suppressed by SAA, but not α-TP. Although precise mechanisms responsible for the differences in efficacy between the two antioxidants remain to be determined, it has been reported that dietary ascorbic acid is capable of accumulating more effectively in renal cortical tissue of rats than is the case with dietary α-TP (Craven et al., 1997). In consideration of the fact that $KBrO_3$ is efficiently reduced by GSH at brush borders on the luminal surfaces of PCT cells (Murata et al., 2001), eventually yielding oxidative stress (Ballmaier and Epe, 1995, 2006), it is plausible that an aqueous antioxidant would exert preventive effects. Previous study demonstrated that dietary vitamin E inhibited 8-OHdG levels in kidney DNA induced by $KBrO_3$ at higher dose (Cadenas and Barja, 1999). The incompatible results might involve differences in the nature of damage to DNA produced by low vs. high doses of $KBrO_3$. In the present study, simultaneous treatment with SAA was in fact able to attenuate oxidative damage caused by $KBrO_3$.

$KBrO_3$ at a concentration of 500 ppm has been reported to promote tumor development in the rat kidney of both sexes (Kurokawa et al., 1985; Umemura et al., 1995). Induction of cell proliferation, regarded as a contributing factor, was observed even at 30 ppm of $KBrO_3$ in males, in contrast to the lowest effective dose in females being 250 ppm (Umemura et al., 2004). Interestingly, α_{2u}-globulin accumulation in the kidneys of male rats also occurred in a

Table 1
Effects of antioxidants on *gpt* mutant frequencies in the kidneys of male *gpt* delta rats given $KBrO_3$.

Treatment		Animal no.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies	Mutant frequency ($\times 10^{-5}$)	Mean ± SD
Water	Diet					
DW	BD	1	6.0	0[a]	0	0.12
		2	9.3	1	0.11	
		3	7.7	2	0.26	
$KBrO_3$	BD	6	9.2	3	0.33	0.43 ± 0.29
		7	98.8	2	0.20	
		8	6.6	5	0.76	
$KBrO_3$	α-TP	11	8.7	8	0.92	1.14 ± 0.30
		12	5.4	8	1.48	
		13	9.7	10	1.03	
$KBrO_3$	SAA	16	10.1	3	0.30	0.53 ± 0.24
		17	7.8	6	0.77	
		18	9.3	5	0.53	

DW: Distilled water, BD: basal diet.
[a] Two colonies were found on the plate, but neither harbored any *gpt* mutations.

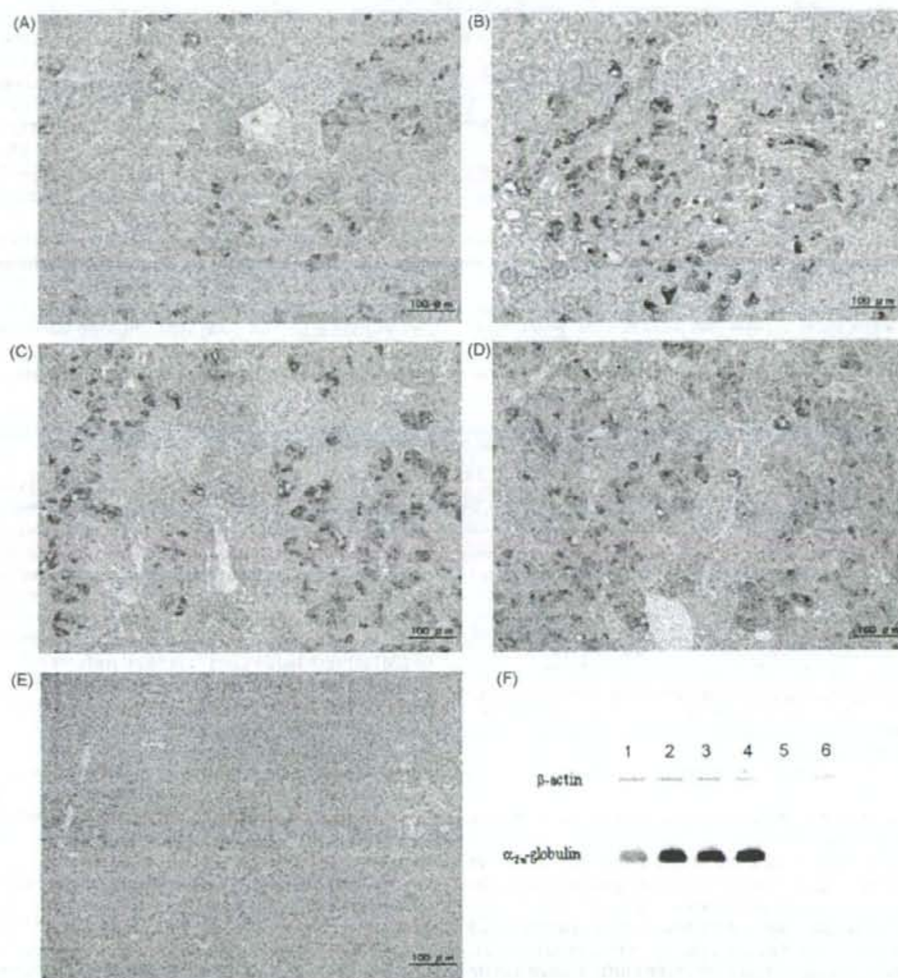

Fig. 3. Photomicrographs of immunohistochemical staining for α_{2u}-globulin in the kidneys of male (A–D) and female (E) *gpt* delta rats given DW (A), $KBrO_3$ (B), $KBrO_3$ and α-TP (C), $KBrO_3$ and SAA (D), or $KBrO_3$ (E). Western blot analysis of α_{2u}-globulin (F) from kidneys of male (lanes 1–4) and female (lanes 5 and 6) *gpt* delta rats given DW (lane 1), $KBrO_3$ (lane 2), $KBrO_3$ and α-TP (lane 3), $KBrO_3$ and SAA (lane 4), DW (lane 5), and $KBrO_3$ (lane 6). α_{2u}-globulin accumulation is more prominent in $KBrO_3$-treated male rats (B) as compared to the controls (A), and is not affected by α-TP (C) or SAA (D) treatment. Note the lack of accumulation in $KBrO_3$-treated females (E), in line with the Western blot analysis (F).

dose-dependent manner from 30 ppm, the protein levels being statistically significant at 125 ppm and above (Umemura et al., 2004). Therefore, it is very likely that this protein accumulation is involved in the cell proliferation observed in the males. In the present study, immunohistochemical and Western blot analysis of α_{2u}-globulin clearly demonstrated accumulation due to $KBrO_3$ exposure, which was not affected by either of antioxidants. This might account for the finding that simultaneous administration of SAA failed to block the rise in BrdU-LIs in males exposed to $KBrO_3$. In general, non-covalently binding of chemicals to the α_{2u}-globulin binding site, a highly aromatic region of the α_{2u}-globulin binding pocket (Huwe et al., 1996) seems to be an initial step, followed by accumulation of the protein in lysosomes of PCT because of resultant resistance to proteolysis. Alternatively, since activities of cystein proteases in lysosomes are prerequisite for degradation of α_{2u}-globulin (Saito et al., 1992), there might be the possibility of primary decrease of protease activity in lysosomes due to interaction of $KBrO_3$ with their thiols acting as a trigger for accumulation (Read, 1991). Although it remains uncertain whether $KBrO_3$ has affinity for the pocket or detrimental effects on lysomal functions, our data imply that induction of cell proliferation following accumulation of the protein in males occurs independently of oxidative stress. On the other hand, in the females lacking α_{2u}-globulin, the BrdU-LI elevation in PCT of rats given $KBrO_3$ was alleviated by SAA. Since mRNA levels of oxidative stress-related genes such as *c-fos*, *c-jun* and *NF-κB* were not elevated in kidneys of $KBrO_3$-treated female rats (data not shown), further studies appear warranted to determine links at the molecular level between oxidation and cell proliferation.

Exposure of female *gpt* delta rats with a genetic background of F344 to $KBrO_3$ at 500 ppm for 9 weeks induced significant elevation of *gpt* MFs along with Spi$^-$ MFs. However, the antioxidants were unable to prevent any type of mutation. 8-OHdG is not only a

Table 2
Effects of antioxidants on *gpt* mutant frequencies in the kidneys of female *gpt* delta rats given $KBrO_3$.

Treatment		Animal no.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies	Mutant frequency ($\times 10^{-5}$)	Mean ± SD
Water	Diet					
DW	BD	51	9.9	3	0.30	0.24 ± 0.07
		52	6.0	1	0.17	
		53	11.8	3	0.25	
$KBrO_3$	BD	56	11.0	7	0.64	0.53 ± 0.11*
		57	9.6	4	0.42	
		58	11.4	6	0.53	
$KBrO_3$	α-TP	61	8.6	6	0.70	0.48 ± 0.20
		62	9.5	4	0.42	
		63	13.0	4	0.31	
$KBrO_3$	SAA	66	6.8	2	0.29	0.44 ± 0.34
		67	10.2	2	0.20	
		68	8.4	7	0.83	

DW: Distilled water, BD: basal diet.
* $p < 0.01$ vs. DW/BD.

Table 3
Mutation spectra of *gpt* mutant colonies.

Sex	Male				Female			
Treatment (water/diet)	DW/BD	$KBrO_3$/BD	$KBrO_3$/α-TP	$KBrO_3$/SAA	DW/BD	$KBrO_3$/BD	$KBrO_3$/α-TP	$KBrO_3$/SAA
Base substitution								
Transversions								
GC:TA	0[a]	1(0.04)	4(0.17)	3(0.11)	2(0.07)	1(0.03)	0	2(0.08)
GC:CG	0	0	0	1(0.04)	0	1(0.03)	1(0.03)	1(0.04)
AT:TA	0	2(0.08)	4(0.17)	3(0.11)	0	3(0.09)	4(0.13)	2(0.08)
AT:CG	0	1(0.04)	2(0.08)	0	0	3(0.09)	0	0
Transitions								
GC:AT	2(0.09)	1(0.04)	8(0.34)	2(0.07)	2(0.07)	4(0.13)	6(0.19)	3(0.12)
AT:GC	0	1(0.04)	3(0.13)	1(0.04)	0	1(0.03)	1(0.03)	0
Deletion								
Single bp	0	1(0.04)	3(0.13)	1(0.04)	2(0.07)	6(0.19)	2(0.06)	1(0.04)
Over 2 bp	0	1(0.04)	2(0.08)	2(0.07)	1(0.04)	1(0.03)	0	1(0.04)
Insertion	0	1(0.04)	0	0	0	0	1(0.03)	1(0.04)
Complex	1(0.04)	1(0.04)	0	0	0	0	1(0.03)	0
Total	3(0.13)	10(0.39)	26(1.09)	14(0.51)	7(0.25)	20(0.63)*	14(0.51)	11(0.63)

DW: Distilled water, BD: basal diet. Values appearing in parenthesis indicates mutation frequency, $\times 10^{-5}$. * $p < 0.01$ vs. DW/BD.
[a] The number of colonies with independent mutations.

representative marker for oxidative stress but also a primary cause of GC:TA transversions due to mispairing with A (Cheng et al., 1992; Shibutani et al., 1991). Nevertheless, spectrum analysis of the *gpt* mutants induced by $KBrO_3$ did not indicate a majority of GC:TA transversions. Instead, deletions were most common in concord with the results of Spi^- mutation assays. In our previous study using male *gpt* delta rats with a Sprague–Dawley genetic background, significant elevation of Spi^- MFs was rather apparent (Umemura et al., 2006). Furthermore, in an *in vitro* genotoxicity assay for $KBrO_3$ using human lymphoblastoid TK6 cells (Luan et al., 2007) or mouse lymphoma cells (Harrington-Brock et al., 2003), $KBrO_3$ induced large deletions, including loss of heterozygosity at

Table 4
Effects of antioxidants on *red/gam* mutant frequencies in the kidneys of male *gpt* delta rats given $KBrO_3$.

Treatment		Animal no.	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaques within XL-1 Blue MRA (P2) (Spi^-)	Mutant frequency ($\times 10^{-5}$)	Mean ± SD
Water	Diet					
DW	BD	1	19.2	8	0.42	0.82 ± 0.52
		2	20.3	13	0.64	
		3	17.8	25	1.40	
$KBrO_3$	BD	6	15.3	36	2.36	1.04 ± 1.15
		7	21.8	8	0.37	
		8	15.7	6	0.38	
$KBrO_3$	α-TP	11	23.4	9	0.39	0.43 ± 0.05
		12	26.0	11	0.42	
		13	14.3	7	0.49	
$KBrO_3$	SAA	16	17.8	9	0.51	0.57 ± 0.08
		17	14.5	8	0.55	
		18	19.5	13	0.67	

DW: Distilled water, BD: basal diet.

Table 5
Effects of antioxidants on *red/gam* mutant frequencies in the kidneys of female *gpt* delta rats given $KBrO_3$.

Treatment: Water	Treatment: Diet	Animal no.	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaques within XL-1 Blue MRA (P2) (Spi$^-$)	Mutant frequency ($\times 10^{-5}$)	Mean ± SD
DW	BD	51	15.2	2	0.13	0.45 ± 0.30
		52	6.8	5	0.73	
		53	10.5	5	0.48	
$KBOrO_3$	BD	56	5.2	6	1.16	0.89 ± 0.42
		57	4.9	2	0.41	
		58	6.4	7	1.09	
$KBrO_3$	α-TP	61	5.6	7	1.24	1.02 ± 0.20*
		62	8.4	8	0.96	
		63	3.5	3	0.85	
$KBrO_3$	SAA	66	5.2	8	1.55	0.97 ± 0.51
		67	7.0	5	0.71	
		68	7.8	5	0.64	

DW: Distilled water, BD: basal diet.
* $p < 0.05$ vs. DW/BD.

TK locus, but not GC:TA transversions. On the other hand, in the present study, Spi$^-$ MFs in males were not increased, in contrast to the previous study demonstrating significant increment. Although certain differences between genetic backgrounds should not be ignored, seemingly inconsistent results might reflect smaller increase of MFs following $KBrO_3$ exposure (2–3 fold) as compared to the case (10–30 fold) with potent genotoxic carcinogens (Kanki et al., 2005). In other words, as shown in microbial and the *Hprt* mutation assays in mammalian cells (Speit et al., 1999), the potential of $KBrO_3$ to induce mutations may be very weak (Harrington-Brock et al., 2003). Actually, we obtained negative data for transgene mutations at 250 ppm for 13 weeks (Umemura et al., 2006) and another group similarly reported negative findings with 125 ppm for 16 weeks (Yamaguchi et al., 2008). The hypothesis of weak mutagenicity is strongly supported by a bioassay showing $KBrO_3$ at 500 ppm for 13 weeks to be incapable of effecting tumor development with appropriate promotion, despite preneoplastic lesions being enhanced (Umemura et al., 2006).

In conclusion, oxidative stress generated by $KBrO_3$ might take part in induction of cell proliferation in PCT of female rats, leading to tumor promoting potential. In males, in contrast, α_{2u}-globulin accumulation independent of oxidative stress plays a major role in cell proliferation, which implies that the tumor promotion observed in males is not directly comparable to the human situation. Likewise, induction of reporter gene mutations by $KBrO_3$ is unlikely to be due to oxidative stress, the extent of which being much lower as compared to that of potent genotoxic carcinogens. The data overall allow us to speculate that the predominant contributing factor for $KBrO_3$-induced renal carcinogenesis is tumor promoting potential, which is only to a limited extent associated with oxidative stress.

Conflict of interest

None.

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— Minireview —

Spontaneous Mutagenesis in Rodents: Spontaneous Gene Mutations Identified by Neutral Reporter Genes in *gpt* Delta Transgenic Mice and Rats

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Transgenic rodents are valuable models for investigation of genotoxicity of chemicals *in vivo*. We have developed *gpt* delta transgenic mice (C57BL/6J background) and rats (Sprague-Dawley, SD), which have the ability to identify both point mutations by the *gpt* assay [6-thioguanine (6-TG) selection] and certain types of deletions by the Spi⁻ (Spi, sensitive to P2 interference) assay. Recently, the *gpt* delta SD rat was backcrossed with the Fisher 344 (F344) rat to establish an *gpt* delta F344 rat. The average spontaneous *gpt* mutation frequencies (MFs) are about 4.5×10^{-6} in both SD and F344 *gpt* delta rats as well as in *gpt* delta mice. The G:C to A:T transitions at 5′-CpG-3′sites and G:C to T:A transversions are the predominant spontaneous *gpt* mutations in rats and mice. However, there is one false mutation (*e.g.* A:T to T:A at position 299) in the rats. The base substitution may have arisen when the lambda EG10 transgene was introduced into the genome of the SD rat during transgenesis. In the Spi⁻ assay, 1-bp deletions in repetitive sequences are predominantly observed in both mice and rats. Possible mechanisms underlying the spontaneous mutations in *gpt* delta rodents are discussed.

Key words —— *gpt* delta transgenic rodent, spontaneous mutation, mutation spectrum, *gpt* assay, Spi⁻ assay

OVERVIEW OF *gpt* DELTA TRANSGENIC RODENTS

Gene mutations play an important role in the etiology of many human diseases including cancer. Since humans are exposed to a variety of endogenous and exogenous mutagens, there has been considerable interest in the relationship between exposure, genotoxic effects, and cancer incidence. To assess the risk of mutagens to the human genome, genotoxicity tests have been developed, including *in vivo* mutation assays using experimental animals, which play a crucial role in risk assessment. To investigate *in vivo* genotoxicity, a number of transgenic rodent mutation assays have been developed by introducing reporter transgenes into the chromosome of every cell of the animal.[1,2] Using these systems, mutagenic events induced in a rodent can be detected by recovering the transgene and analyzing the phenotype of the reporter gene in a bacterial host. These models permit quantitation of mutations and identification at the sequence level in any tissue or organ in the body. *lacZ*, *lacI* or *cII* have been employed as reporter genes in transgenic rodents, such as the Muta™ mouse, and the Big Blue® mouse and rat.[3–7] Despite differences in size and sequence context, spontaneous mutation frequencies of these reporter genes are in the mid-10^{-5} range and those are predominantly base substitutions in most tissues. This high background of base substitutions may make it difficult to detect rare mutations such as deletions induced by ionizing radiation.[8,9] To overcome this limitation, a transgenic "*gpt* delta" assay system has been developed for the efficient detection of both point mutations and deletions.[1,10] A unique feature of the lambda EG10 phage vector constructed for this system is the incorporation of two different positive selection methods: the *gpt* assay [6-thioguanine (6-TG) selection] using the *gpt* gene of *Escherichia coli* (*E. coli*) that detects mainly point mutations such as base substitutions and frameshifts, and the Spi⁻ (Spi, sensitive

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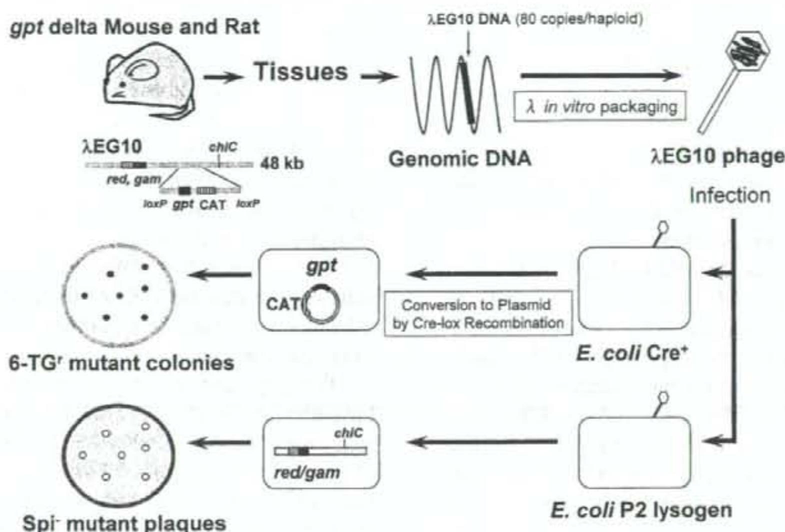

Fig. 1. Principle of *gpt* Delta Transgenic Rodent Mutation Assays[1,10,13,14]

Genomic DNA is extracted from tissue of a transgenic rodent. Lambda EG10 DNA is recovered by *in vitro* packaging and rescued as phage particles. Two distinct *E. coli* host strains are infected with the rescued lambda EG10 phages: one is *E. coli* YG6020 expressing Cre recombinase for the *gpt* assay and the other is a P2 lysogen for the Spi⁻ assay. In the *gpt* assay, lambda EG10 DNA is converted to plasmid carrying *gpt* and CAT by cre-lox recombination. Bacteria harboring the plasmids carrying mutant *gpt* are positively selected as colonies on plates containing chloramphenicol and 6-thioguanine. In the Spi⁻ assay, mutant lambda EG10 phages lacking *red/gam* gene functions are positively selected as Spi⁻ plaques on lawns of P2 lysogens. The same DNA prepared from identical tissue sample is applied to both *gpt* and Spi⁻ assays.

to P2 interference) assay (Spi⁻ selection) using the *red/gam* genes of lambda phage that detects deletions including frameshifts (Fig. 1).

To accomplish the *gpt* and Spi⁻ assay, the *gpt* delta transgenic mouse has been developed.[1,10) This mouse was established by microinjection of lambda EG10 phage DNA into the fertilized eggs of C57BL/6J mice. It carries about 80 copies of the transgene in a head to tail fashion at a single site in chromosome 17 and is maintained as a homozygote (carrying 160 copies of transgene per diploid).[11] The same lambda EG10 transgene used in the *gpt* delta mouse was integrated into the genome of Sprague-Dawley (SD) rat to establish the *gpt* delta rat.[12] The *gpt* delta rat harbors about 5–10 copies of the transgene in chromosome 4 and is maintained as a heterozygote. The *gpt* and Spi⁻ assay systems have been validated primarily in mice with many chemical mutagens/carcinogens, UV and ionizing radiation, for which mutagenicity, organ specificity and mutation spectrum have been thoroughly characterized.[1,10–28] Recently, the outbred *gpt* delta SD rat was backcrossed with Fisher 344 (F344) rat, to establish an inbred *gpt* delta rat (F344). In this review, we focus on the spontaneous mutations detected by the *gpt* and Spi⁻ assays in *gpt* delta mice and rats and discuss possible mechanisms underlying these *in vivo* mutations.

gpt ASSAY (6-TG SELECTION) FOR POINT MUTATIONS

The principles and method of the *gpt* assay have been described previously (Fig. 1).[1,10,15] Briefly, the *gpt* gene encodes guanine phosphoribosyltransferase that is involved in the purine salvage pathway of *E. coli*. This enzyme phosphoribosylates not only guanine, but also 6-TG, which is toxic to cells when it is incorporated into DNA. Thus, *E. coli* cells expressing wild-type *gpt* cannot survive on the plates containing 6-TG. Only *gpt* mutants can form colonies on plates containing 6-TG. The coding region of *gpt* is 456 bp, which is convenient for sequence analysis of the mutants. When *E. coli* strain YG6020 expressing Cre recombinase is infected with lambda EG10 rescued from *gpt* delta rodents, the plasmid region is efficiently excised, circularized and propagated as multi-copy number plasmid carrying *gpt* and chloramphenicol acetyltransferase

(CAT). Bacteria harboring mutated *gpt* genes can be positively selected as colonies on plates containing 6-TG and chloramphenicol (Cm). The number of rescued phages can be determined by plating the cells on the plates containing Cm alone. The *gpt* mutant frequency (MF) is calculated by dividing the number of the *gpt* mutant colonies by the number of rescued Cm-resistant colonies.

The spontaneous *gpt* MFs of *gpt* delta mice (C57BL/6J background) have been previously reported. In most experiments, the values of spontaneous *gpt* MFs are in the range of 5×10^{-6}. This is less than that of other transgenic rodent mutation assay systems, such as Muta mice and Big Blue mice, in which spontaneous MFs are usually greater than 1×10^{-5}. Different selection method and sequence context of the *gpt* gene might account for the lower MF of the *gpt* assay. Even if some types of mutation occurred in the *gpt* gene, residual enzymatic activity may still catalyze enough amount 6-TG to kill the cells. Because the *gpt* mutant cells are grown on M9 minimal medium agar plate containing 6-TG for 3–4 days, phenotypic selection of 6-TG resistance could require complete loss of enzymatic activity of the mutated *gpt* gene product as a selectable phenotype. There are no clearly observed tissue differences in the spontaneous MFs in *gpt* delta mice. Although the number of studies in which multiple tissues types have been analyzed is limited, similar spontaneous MFs were observed in six tissues (liver, spleen, colon, testis, brain and bone marrow) collected from the same animals.[11] No significant differences between male and female were observed in the spontaneous and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-induced MFs in liver and colon.[11] In *gpt* delta mice, spontaneous MF in the liver at 85 weeks of age increased by a factor of 2 over that at 19 weeks of age.[22] It is also reported that spontaneous MFs increase with age in most somatic cells in Muta mice and Big Blue mice.[29–32] However, the trend could be different in brain and germ tissue, where MFs don't increase with age in adult mice.[33–35] Interestingly, it has been reported that some gene knockout mice show different spontaneous MFs. Spontaneous *gpt* MF in the liver is significantly higher in *ogg1* gene knockout mice, which lack 8-oxo-guanine DNA glycosylase activity.[36] Interleukin-10 (IL-10)-deficient mice, which spontaneously develop intestinal inflammation, have a *gpt* mutation frequency in the colon about five times higher than that in wild type mice.[37] In transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2)-null mice, the spontaneous *gpt* mutation frequency in the lung was approximately three times higher in *nrf2*-null (*nrf2*−/−) mice than in *nrf2* heterozygous (*nrf2*+/−) or wild-type (*nrf2*+/+) mice; whereas in the liver, the mutation frequency was higher in *nrf2*−/− and *nrf2*+/− mice than in *nrf2*+/+ mice. In contrast, no difference in mutation frequency was observed in testis between the three genotypes.[38] These results suggest that the intracellular environment contributes to spontaneous mutations, perhaps through oxidative stress and/or detoxification systems.

In the *gpt* delta rat, the spontaneous *gpt* MFs we have obtained from more than 40 samples from various organs were between 0.9×10^{-6} and 8.5×10^{-6} (4.5×10^{-6} in average). No difference was observed between outbred SD rats (4.5×10^{-6}, calculated from 31 samples) and inbred F344 rats (4.4×10^{-6}, calculated from 10 samples). Although the data represent males and females, different organs (liver, kidney and mammary gland), and different ages (between 10 and 52 weeks old), these values are comparable to those in mice. On the other hand, Hayashi *et al.* reported that the spontaneous *gpt* MF in the liver of the rats was lower than that of mice.[12] The similar observation was also reported in Big Blue mice and rats.[7] Additional studies are needed to investigate the effect of genetic strain background, tissue type, and age in *gpt* delta rat in order to validate these findings.

Spontaneous *gpt* mutation spectra of *gpt* delta rodents are shown in Table 1. Regardless of species or strain background, the proportions of mutations are remarkably similar, although the sample size for F344 rats is small. The most frequent mutations are G:C to A:T transitions. More than half of these occur at 5′-CpG-3′ sequences in mice and SD rats. This suggests that deamination of methylated cytosines at CpG sites contributes to spontaneous mutations in the *gpt* gene *in vivo*.[39] Beside transitions, G:C to T:A transversions are frequently observed base substitutions. This type of mutations may reflect oxidative damage in DNA, such as 8-oxoguanine lesions[40] or abasic sites.[41] Other mutations were frameshifts, short deletions and insertions. These findings were largely consistent between different tissues. Although lower spontaneous *gpt* MFs than Muta mouse and Big Blue mouse are observed, the characteristic mutation spectra are similar, and the similarity of spontaneous mutation spectra in different tissues and different rodent strains are also observed with the

Table 1. Spontaneous *gpt* Mutation Spectra of *gpt* Delta Mice and Rats

	C57BL/6J mouse			SD rat			F344 rat		
	No.	CpG	%	No.	CpG	%	No.	CpG	%
Base substitutions									
Transitions									
G:C → A:T	59	(32)	33.3	39	(22)	43.8	12	(3)	40.0
A:T → G:C	19		10.7	6		6.7	1		3.3
Transversions									
G:C → T:A	31		17.5	18		20.2	5		16.7
G:C → C:G	4		2.3	1		1.1	1		3.3
A:T → T:A	13		7.3	1		1.1	1		3.3
A:T → C:G	11		6.2	2		2.2	1		3.3
Deletions	24		13.6	15		16.9	9		30.0
1 bp		18			12			5	
> 2 bps		6			3			4	
Insertions	10		5.6	7		7.9	0		0.0
Others[a]	6		3.4	0		0.0	0		0.0
Total No. of Mutations	177		100.0	89		100.0	30		100.0
No. of animal	23			31			10		
Sex	male			male and female			male		
Tissues	liver			liver, kidney, mammary gland			liver		
Age	10–20 weeks old			10–52 weeks old			20–24 weeks old		

a) Multiple base substitutions, bese substitutions coupled with deletion or insertion.

lacI transgene.[42,43] De Boer *et al.* reported the spontaneous *lacI* mutations for liver, spleen, bladder, stomach, kidney, bone marrow, lung and skin of Big Blue mice. They showed the similarity of the *lacI* mutational spectra in all tissues.[42] Basically, the predominant class of spontaneous mutations was G:C to A:T transitions, most of which occurred at CpG sites. The second most common class was G:C to T:A transversions. All other base substitution classes contributed less than 10% each. Of the non-base substitution events, the loss of a single base pair was the most frequently occurring event. Zhang *et al.* compared *lacI* spontaneous mutation spectra in the liver of C57BL/6, B6C3F1 and BC-1 mice and F344 rats and concluded that spontaneous mutations appear to be similar, regardless of genetic location, rodent strain, or species.[43]

In sequence analysis of the *gpt* gene recovered from *gpt* delta rats, we should note that an unexpected A:T to T:A transversions at position 299 (*e.g.* nucleotide 299 from the first codon of <u>A</u>TG, in the *gpt* sequence) was frequently observed. We conclude that this base substitution must have arisen in the lambda EG10 DNA during SD rats transgenesis, and is not induced by spontaneous or induced somatic mutagenesis. Evidence for this includes: (1) base substation is observed in untreated rats as well as mutagen-treated rats at a similar frequency; (2) it typically occurs along with another mutation in any given *gpt* mutant; (3) it was detected in gpt^+ (6-TG sensitive) colonies rescued from both *gpt* delta SD and F344 rats; (4) we observed that 96/473 (20%) *gpt* mutants recovered from *gpt* delta rats contain this base substitution [the frequency should be about 20% (2×10^{-1}) if one of five copies of integrated lambda EG10 has A:T to T:A change at position 299]; and (5) it was never found among 1680 *gpt* mutants we have analyzed in *gpt* delta mice. Thus, an A:T to T:A transversion at position 299 observed only in *gpt* delta transgenic rats is a "false" mutation. In the development of transgenic rodents, multiple copy transgenes are usually integrated at a single site of chromosome in a head-to-tail fashion. We suppose that an unintended point mutation might have been arisen in one copy of the transgenes during the first round of DNA replication when they integrated into the chromosome of *gpt* delta SD rat. Although this *gpt* mutation results in an amino acid substitution from isoleucine (Ile) to asparagine (Asn), it doesn't cause a mutated *gpt* phenotype (*e.g.* is a silent mutation) and therefore doesn't affect the *gpt* MF. Because of this, A:T to T:A mutations at 299 were excluded from the mutation spectra of *gpt* delta rats in Table 1.