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In vivo mutagenicity and initiation following oxidative DNA lesion in the kidneys of rats given potassium bromate

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To clarify the role of 8-OHdG formation as a starting point for carcinogenesis, we examined the dose-dependence and time-course of changes of *OGG1* mRNA expression, 8-OHdG levels and *in vivo* mutations in the kidneys of *gpt* delta rats given $KBrO_3$ in their drinking water for 13 weeks. There were no remarkable changes in *OGG1* mRNA in spite of some increments being statistically significant. Increases of 8-OHdG occurred after 1 week at 500 p.p.m. and after 13 weeks at 250 p.p.m. Elevation of Spi$^-$ mutant frequency, suggestive of deletion mutations, occurred after 9 weeks at 500 p.p.m. In a two-stage experiment, F344 rats were given $KBrO_3$ for 13 weeks then, after a 2-week recovery, treated with 1% NTA in the diet for 39 weeks. The incidence and multiplicity of renal preneoplastic lesions in rats given $KBrO_3$ at 500 p.p.m. followed by NTA treatment were significantly higher than in rats treated with NTA alone. Results suggest that a certain period of time might be required for 8-OHdG to cause permanent mutations. The two-step experiment shows that cells exposed to the alteration of the intranuclear status by oxidative stress including 8-OHdG formation might be able to form tumors with appropriate promotion. (*Cancer Sci* 2006; 97: 829–835)

Oxidative DNA damage is caused by reactive oxygen species derived from various processes of cellular metabolism, especially metabolism of exogenous mutagens and carcinogens. 8-OHdG, a form of guanine oxidized at the C-8 position, is believed to be fairly stable and the most abundant oxidative lesion[1] among the many oxidized nucleosides known. It is established that 8-OHdG lesions are repaired mainly by the so-called GO system.[2] In this system: OGG1 DNA glycosylase and apurinic/apyrimidic lyase act to correct 8-OH-G:C pairs;[3] MYH glycosylase removes an A base mispaired with 8-OHdG;[4] and MTH 8-OH-dGTpase hydrolyzes 8-OH-dGTP in the nucleotide pool for prevention of its incorporation into DNA.[5] Thus, the existence of these three genes for repair of 8-OHdG in DNA points to 8-OHdG as a biologically deleterious base lesion. Induction of *OGG1* mRNA expression and an increase of OGG1 activity following application of exogenous oxidative stimuli have been demonstrated.[6–9]

$KBrO_3$, which induces renal cell tumors in F344 rats after oral administration at concentrations of 250 and 500 p.p.m,[10] has been classified as a genotoxic carcinogen based on positive mutagenicity in the Ames,[11] chromosome aberration,[12] and micronucleus[13] tests. Effective prevention of $KBrO_3$ clastogenicity by antioxidants,[14,15] and induction of 8-OHdG by $KBrO_3$ *in vitro* and *in vivo* strongly suggest that 8-OHdG plays a key role in $KBrO_3$ mutagenesis and carcinogenesis.[16–19] $KBrO_3$ has therefore received much attention as a suitable agent for research into 8-OHdG-related carcinogenesis. With a single dose of $KBrO_3$ by i.p. injection, 8-OHdG glycosylase activity in the rat kidney is increased in association with 8-OHdG formation.[20] A recent study using *OGG1*-deficient *gpt* delta mice found high amounts of 8-OHdG in the genome DNA and GC:TA transversion mutations following $KBrO_3$ exposure at a concentration of 2000 p.p.m. for 12 weeks.[21] However, a single high dose of $KBrO_3$ (300 mg/kg) did not induce tumors in rats,[22] and its carcinogenicity in mice is equivocal.[23] Therefore, interpretation of data for 8-OHdG and consequent mutations with reference to their significance for carcinogenesis is difficult.

Our aim is to determine conditions required for cells with 8-OHdG that survive specific repair systems to develop mutations and have tumor-initiating potential. For this purpose, carcinogenic doses of $KBrO_3$ were administered in drinking water and the dose-dependence and time-course of changes in *OGG1* mRNA expression, 8-OHdG levels and *in vivo* MFs in the kidneys of *gpt* delta rats were measured. In a second experiment, F344 rats were given NTA as a kidney tumor-promoter,[24] in a two-stage rat renal carcinogenesis experiment to assess tumor-initiation activity of $KBrO_3$ given at the same doses as in the first experiment.

Materials and methods

Chemicals

$KBrO_3$ and NTA were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Tokyo Kasei (Tokyo, Japan),

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Abbreviations: 6-TG, 6-thioguanine; 8-OHdG, 8-hydroxydeoxyguanosine; AHs, atypical hyperplasias; ATs, atypical tubules; BD, basal diet; H–E, hematoxylin–eosin; $KBrO_3$, potassium bromate; dG, deoxyguanosine; DW, distilled water; MFs, mutant frequencies; NTA, nitrilotriacetic acid trisodium salt; PCR, polymerase chain reaction; p.p.m., parts per million; RCTs, renal cell tumors; SD, standard deviation.

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respectively. Alkaline phosphatase was obtained from Sigma Chemical (St. Louis, MO, USA) and nuclease P1 was from Yamasa Shoyu (Chiba, Japan).

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 (Tokyo, Japan). Five-week-old male *gpt* delta rats carrying approximately 10 tandem copies of the transgene lambda EG10 per haploid genome and F344 rats were obtained from Japan SLC (Shizuoka, Japan) and from Charles River Japan (Kanagawa, Japan), respectively. They were housed in polycarbonate cages (five 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 BD (Charles River Japan) and tap water.

Animal treatment

Experiment I. Groups of five male gpt delta rats were given $KBrO_3$ solution at concentrations of 0, 60, 125, 250 and 500 p.p.m. in the drinking water for 13 weeks. Additional subgroups of five male *gpt* delta rats were given $KBrO_3$ solution at a dose of 500 p.p.m. in the drinking water for 1, 5 or 9 weeks. At the end of each period, the animals were killed under ethyl ether anesthesia and a part of one kidney was homogenized in Isogen (Nippon Gene, Tokyo, Japan) and stored at −80°C until used for isolation of total RNA. The remaining kidney was also stored at −80°C for 8-OHdG measurement and *in vivo* mutation assays.

Experiment II. F344 rats were used in the tumor initiation study rather than Sprague–Dawley rats, a back strain of *gpt* delta rats, because of the accumulated data on the effects of $KBrO_3$ on the former strain. Ninety F344 male rats were randomly divided into seven groups. Fifteen animals each in groups 2–5 were given $KBrO_3$ at concentrations of 60, 125, 250 and 500 p.p.m. for 13 weeks. After a 2-week recovery period, rats received NTA as a promoter at a concentration of 1% in the diet for 37 weeks. Ten animals each in groups 1 and 6 were given DW in place of $KBrO_3$, followed by the NTA regimen at doses of 0 and 1%, respectively. Additionally, 10 animals in group 7 were maintained untreated following $KBrO_3$ treatment at a concentration of 500 p.p.m. for 13 weeks until the end of the experiment. Rats were killed at week 52 under ethyl ether anesthesia and the kidneys were removed immediately and fixed in 10% buffered formalin.

Real-time reverse transcription–PCR for mRNA of *OGG1*

Total mRNA was isolated using the Isogen total mRNA isolation reagent (Nippon Gene) according to the manufacturer's instructions. After reverse-transcription with random hexamers using an SYBR RT-PCR Kit (Takara Bio, Shiga, Japan), PCR was carried out with specific primers for rat *OGG1* (5′-CAACATTGCTCGCATCACTGG-3′ and 5′-ATGGCTTTAGCACTGGCACATACA-3′) (Smart Cycler; Cepheid, Sunnyvale, CA) and r*GAPDH* (5′-GACAACTTTG-GCATCGTGGA-3′ and 5′-ATGCAGGGATGATGTTCTGG-3′) (Ex Taq, RT-PCR version; Takara Bio). Real-time monitoring of PCR products was achieved with fluorescence of SYBR green I (Takara Bio), and expression levels of *OGG1* were determined as ratios to *GAPDH* levels obtained with the same master reaction.[25] All of the procedures after isolation of total RNA were carried out at the Dragon Genomics Center of Takara Bio (Mie, Japan).

Measurement of nuclear 8-OHdG

In order to prevent 8-OHdG formation as a by-product during DNA isolation,[26] kidney DNA was extracted using a slight modification of the method of Nakae *et al.*[27] Briefly, nuclear DNA was extracted with a commercially available DNA Extracter WB Kit (Wako Pure Chemical Industries) containing an antioxidant NaI solution to dissolve cellular components. For further prevention of auto-oxidation in the cell lysis step, deferoxamine mesylate (Sigma Chemical) was added to the lysis buffer.[28] The DNA was digested to deoxynucleotides with nuclease P1 and alkaline phosphatase and levels of 8-OHdG (8-OHdG/10^5 dG) were measured by high-performance liquid chromatography with an electrochemical detection system (Coulochem II; ESA, Bedford, MA).

In vivo mutation assays

6-TG and Spi$^-$ (insensitive P2 interference) selection was carried out as previously described.[29] Briefly, genomic DNA was extracted from the kidneys, and lambda EG10 DNA (48 kb) was rescued as the lambda phage by *in vitro* packaging. For 6-TG selection, the packaged phage was incubated with *Escherichia coli* YG6020, expressing Cre recombinase, and converted to a plasmid 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, infected cells were also poured on plates containing chloramphenicol without 6-TG. The plates were incubated at 37°C for the selection of 6-TG-resistant colonies, and the *gpt* MF was calculated by dividing the number of *gpt* mutants after clonal correction by the number of rescued phages.

For Spi$^-$ selection, the packaged phage was 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 soft agar and poured onto lambda-trypticase agar plates. The plaques (Spi$^-$ candidates) detected on the plates were suspended in SM buffer. In order to confirm the Spi$^-$ phenotype of candidates, the suspensions were spotted on three types of plates containing XL-1 Blue MRA, XL-1 Blue MRA P2, or WL95 P2 strains and were spread with soft agar. The numbers of mutants that made clear plaques on each plate were counted as confirmed Spi$^-$ mutants. The Spi$^-$ MF was calculated by dividing the number of Spi$^-$ mutants by the number of rescued phages.

Histopathology for the initiation bioassay

Formalin-fixed kidneys were processed for embedding in paraffin and sectioned at 2 μm. Sections were routinely stained with H–E for histopathological assessment. All sections were coded and read without knowledge of the treatment for counting of ATs, AHs and RCTs. The

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diagnostic criteria for preneoplastic and neoplastic lesions of the kidney proposed by Dietrich and Swenberg,[30] were used to distinguish ATs, AHs and RCTs.

Statistics

The significance of differences in the results for mRNA levels of *OGG1*, 8-OH-dG levels and MFs was evaluated with ANOVA, followed by Dunnett's multiple comparison test. The significance of differences in the multiplicity of lesions in the initiation bioassay was evaluated using Tukey's test, and that for incidences with Fisher's exact probability test.

Results

Experiment I

OGG1 *mRNA expression.* Figure 1(a) illustrates changes of *OGG1* mRNA expression in kidneys of *gpt* delta rats given $KBrO_3$ solution at concentrations of 0, 60, 125, 250 and 500 p.p.m. in the drinking water for 13 weeks. Significant ($P < 0.05$) elevation of expression occurred at 250 p.p.m. At 500 p.p.m., a significant increase of expression ($P < 0.01$) was evident 5 weeks after the start of the exposure (Fig. 1b).

8-OHdG levels. Figure 2(a) shows data for 8-OHdG levels in kidneys of *gpt* delta rats given $KBrO_3$ in the drinking water for 13 weeks. 8-OHdG levels were elevated compared to the control value (0.28 ± 0.06 8-OHdG/10^5 dG) at $KBrO_3$ concentrations of 250 and 500 p.p.m. in a clearly dose-dependent manner (250 p.p.m., 0.45 ± 0.19 8-OHdG/10^5 dG, $P < 0.05$; 500 p.p.m., 0.59 ± 0.16 8-OHdG/10^5 dG, $P < 0.01$). Figure 2(b) summarizes the data from kidneys of *gpt* delta rats given $KBrO_3$ solution at a concentration of 500 p.p.m. for 1, 5, 9 and 13 weeks. 8-OHdG levels increased with time, with a peak at week 5 and a gradual decrease thereafter. All of the 8-OHdG levels for the treated rats were statistically significant ($P < 0.01$) compared to the control value (1 week, 0.54 ± 0.10 8-OHdG/10^5 dG; 5 weeks, 0.88 ± 0.10 8-OHdG/10^5 dG; 9 weeks, 0.75 ± 0.02 8-OHdG/10^5 dG).

In vivo *mutations.* Changes in *gpt* and Spi$^-$ MFs in *gpt* delta rats given $KBrO_3$ solution for 13 weeks are shown in Figs 3 and 4. There was no statistically significant increment in *gpt* MFs among the treated animals in spite of the dose-dependent increase of 8-OHdG observed in rats treated with 250 p.p.m. $KBrO_3$ (Fig. 3a,b). In contrast, Spi$^-$ MF in rats

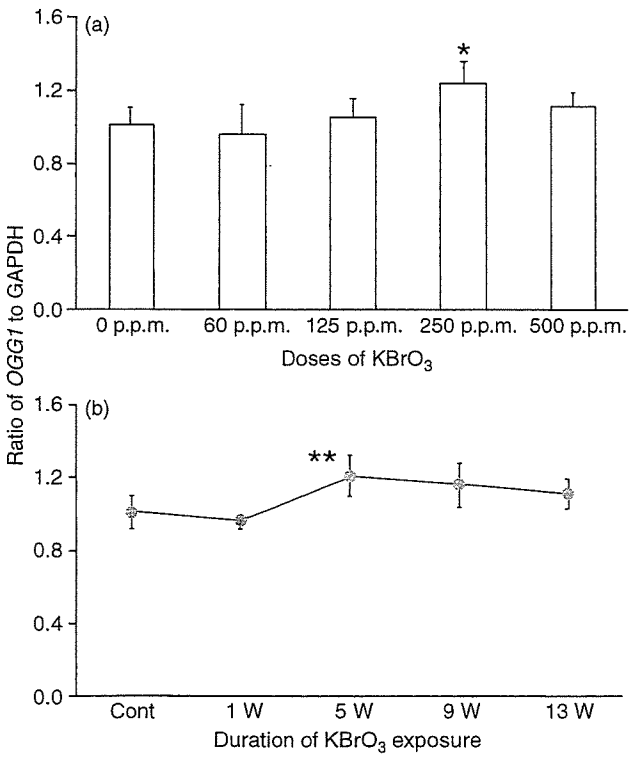

Fig. 1. (a) Dose-dependent expression of *OGG1* mRNA in kidneys of *gpt* delta rats given $KBrO_3$ at concentrations of 0, 60, 125, 250 and 500 p.p.m. in their drinking water for 13 weeks. Values are means ± SD of data for five rats. $*P < 0.05$, significantly different from the controls (0 p.p.m.). (b) Time-course of changes in expression of *OGG1* mRNA in kidneys of *gpt* delta rats given $KBrO_3$ at a concentration of 500 p.p.m. in their drinking water for 1, 5, 9 and 13 weeks. The values at 0 and 500 p.p.m. in the dose–response study were used as the control and 13-week values, respectively. Means ± SD of data for five rats are given. $**P < 0.01$, significantly different from the controls.

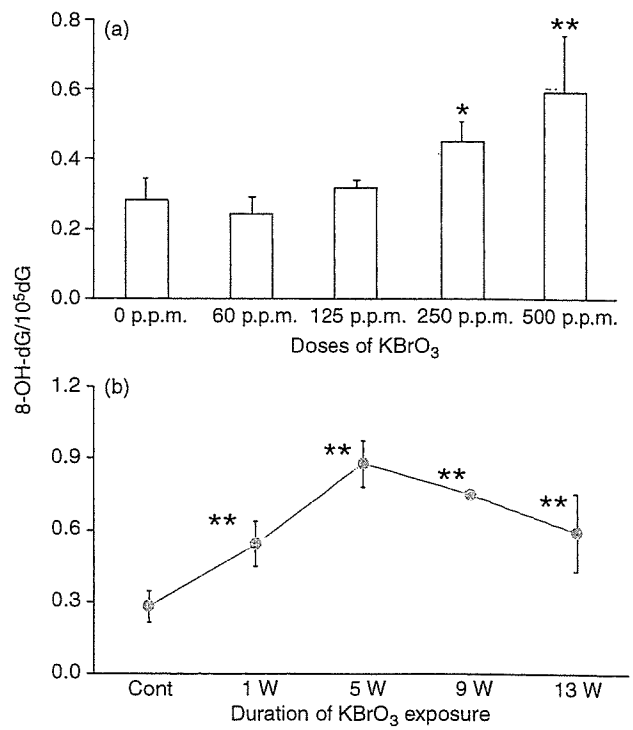

Fig. 2. (a) Dose-dependent induction of 8-OHdG in kidney DNA of *gpt* delta rats given $KBrO_3$ at concentrations of 0, 60, 125, 250 and 500 p.p.m. in their drinking water for 13 weeks. Values are means ± SD of data for five rats. $*P < 0.01$, $**P < 0.05$, significantly different from the controls (0 p.p.m.). (b) Time course of changes in levels of 8-OHdG in kidneys of *gpt* delta rats given $KBrO_3$ at a concentration of 500 p.p.m. in their drinking water for 1, 5, 9 and 13 weeks. The values at 0 and 500 p.p.m. in the dose–response study were used as the control and 13-week values, respectively. Means ± SD of data for five rats are given. $**P < 0.01$, significantly different from the controls.

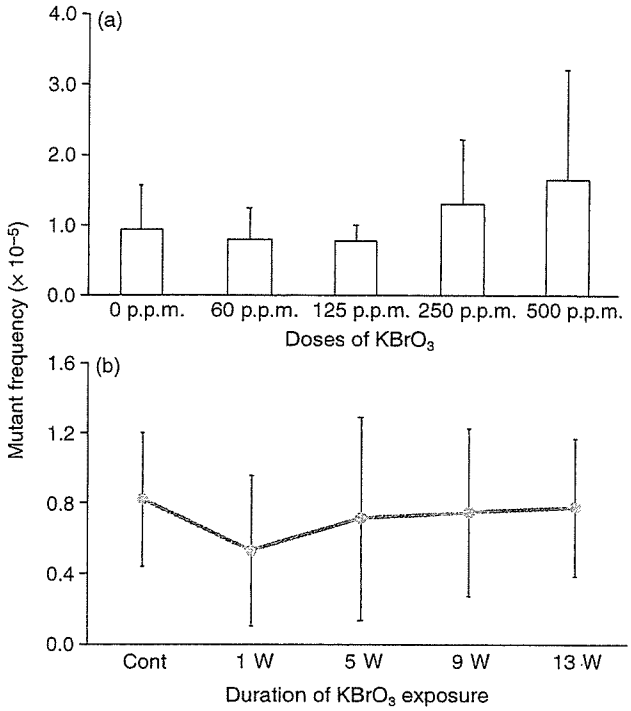

Fig. 3. (a) Dose–response data for *gpt* MFs in kidneys of *gpt* delta rats given $KBrO_3$ at concentrations of 0, 60, 125, 250 and 500 p.p.m. in their drinking water for 13 weeks. Values are means ± SD of data for five rats. (b) Time-course of changes in *gpt* MFs in kidneys of *gpt* delta rats given $KBrO_3$ at a concentration of 500 p.p.m. in their drinking water for 1, 5, 9 and 13 weeks. The values for controls and 13 weeks were obtained from re-analysis of the samples at 0 and 500 p.p.m., respectively, in the dose–response study. Values are means ± SD of data for five rats.

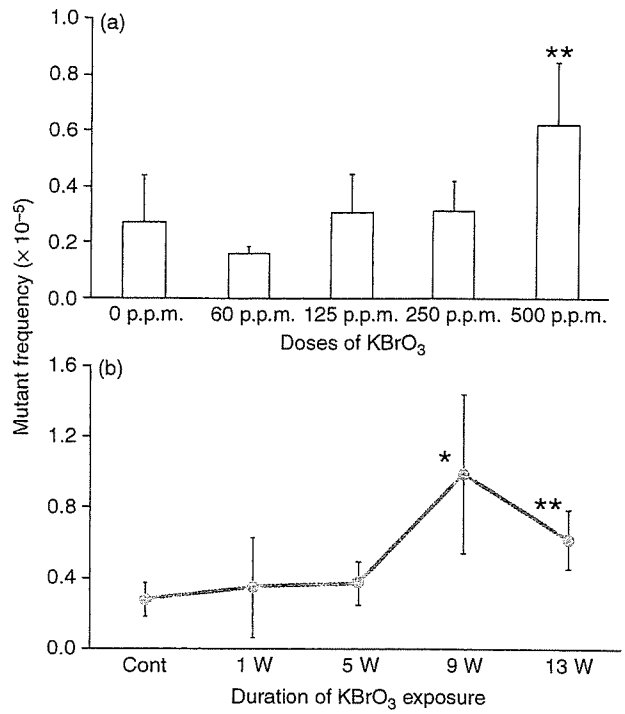

Fig. 4. (a) Dose–response data for Spi^- MFs in kidneys of *gpt* delta rats given $KBrO_3$ at concentrations of 0, 60, 125, 250 and 500 p.p.m. in their drinking water for 13 weeks. Values are means ± SD of data for five rats. $^{**}P < 0.01$, significantly different from the controls (0 p.p.m.). (b) Time-course of changes in Spi^- MFs in kidneys of *gpt* delta rats given $KBrO_3$ at a concentration of 500 p.p.m. in their drinking water for 1, 5, 9 and 13 weeks. The values for the controls and at 13 weeks were obtained from re-analysis of the samples at 0 and 500 p.p.m., respectively, in the dose–response study. Values are means ± SD of data for five rats. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from the controls.

treated with 500 p.p.m. $KBrO_3$ was significantly higher ($P < 0.01$) than in untreated control rats (Fig. 4a). As shown in Fig. 4(b), a significant ($P < 0.05$) elevation of Spi^- MF appeared 9 weeks after the start of the exposure.

Experiment II

One animal given $KBrO_3$ at a dose of 250 p.p.m. followed by NTA treatment died of a malignant pheochromocytoma 49 weeks after the start of the experiment and was eliminated from the dataset. Final body weights were 391.3 ± 19.2 g (DW/BD), 3705 ± 23.2 g ($KBrO_3$ 60 p.p.m./NTA), 365.4 ± 18.5 g ($KBrO_3$ 125 p.p.m./NTA), 367.7 ± 26.6 g ($KBrO_3$ 250 p.p.m./NTA), 348.7 ± 20.1 g ($KBrO_3$ 500 p.p.m./NTA, $P < 0.01$ *versus* DW/BD), 360.2 ± 17.0 g (DW/NTA, $P < 0.05$ *versus* DW/BD) and 384.2 ± 18.4 g ($KBrO_3$ 500 p.p.m./BD). The incidences and multiplicities of renal preneoplastic lesions in rats given $KBrO_3$ at various doses for 13 weeks followed by NTA treatment are shown in Table 1. In all of the groups except the no-treatment control group, preneoplastic lesions were found (Fig. 5a,b). The multiplicity ($P < 0.01$) of ATs, and the incidence ($P < 0.05$) and multiplicity ($P < 0.05$) of AHs in rats given $KBrO_3$ at a dose of 500 p.p.m. followed by NTA were significantly elevated as compared with the values for rats given NTA only. A cystic adenoma was observed in a rat given $KBrO_3$ at the dose of 500 p.p.m. for 13 weeks followed by no-treatment for 39 weeks (Fig. 5c). However, there were no neoplastic lesions in any of the other groups.

Discussion

OGG1 mRNA levels have been found to be elevated in rat lungs after exposure to diesel exhaust particles,[6] in rat kidney after ischemic-reperfusion injury,[7] and in human lung alveolar epithelial cells following crocidolite asbestos treatment.[31] Because overexpression in these cases occurred concomitantly with an increment in OGG1 activity, it has been considered that the elevation in *OGG1* gene expression is linked with the increase of OGG1 activity,[31] and formation of 8-OHdG is a trigger for induction of OGG1 activity.[32–34] However, in the present study, overexpression of *OGG1* mRNA in the kidneys of rats given $KBrO_3$ was not demonstrated. It has been reported that OGG1 activities in the kidneys of rats given $KBrO_3$ by single i.p. injection at a dose of 80 mg/kg were increased in a time- and dose-dependent manner.[20] Also, a recent report showed that $KBrO_3$ exposure at a dose of 400 p.p.m. in drinking water for 52 weeks was able to induce an approximately fourfold increase in *OGG1* mRNA expression.[35] Therefore, chronic exposure to $KBrO_3$

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Table 1. Incidence and multiplicity data for preneoplastic lesions in the kidneys of rats given $KBrO_3$ at various doses followed by NTA treatment

Exposure	Number of rats at risk	Atypical tubules		Atypical hyperplasias	
		Incidence (%)	Multiplicity (number/rat)	Incidence (%)	Multiplicity (number/rat)
DW/BD	10	0	0.0	0	0.0
$KBrO_3$ (60 p.p.m)/NTA	15	93	3.1 ± 1.8	27	0.3 ± 0.6
$KBrO_3$ (125 p.p.m)/NTA	15	87	3.0 ± 2.9	27	0.3 ± 0.6
$KBrO_3$ (250 p.p.m)/NTA	14	100	3.7 ± 1.6	36	0.4 ± 0.6
$KBrO_3$ (500 p.p.m)/NTA	15	100	10.1 ± 4.5**	80*	1.3 ± 1.1*
DW/NTA	10	80	2.2 ± 1.5	30	0.4 ± 0.7
$KBrO_3$ (500 p.p.m)/BD	10	50	0.9 ± 1.1	20	0.2 ± 0.4

$KBrO_3$ was given in the drinking water for 13 weeks. NTA was given at a dose of 1% in the diet for 37 weeks. *$P < 0.05$, ** $P < 0.01$ *versus* DW/NTA.

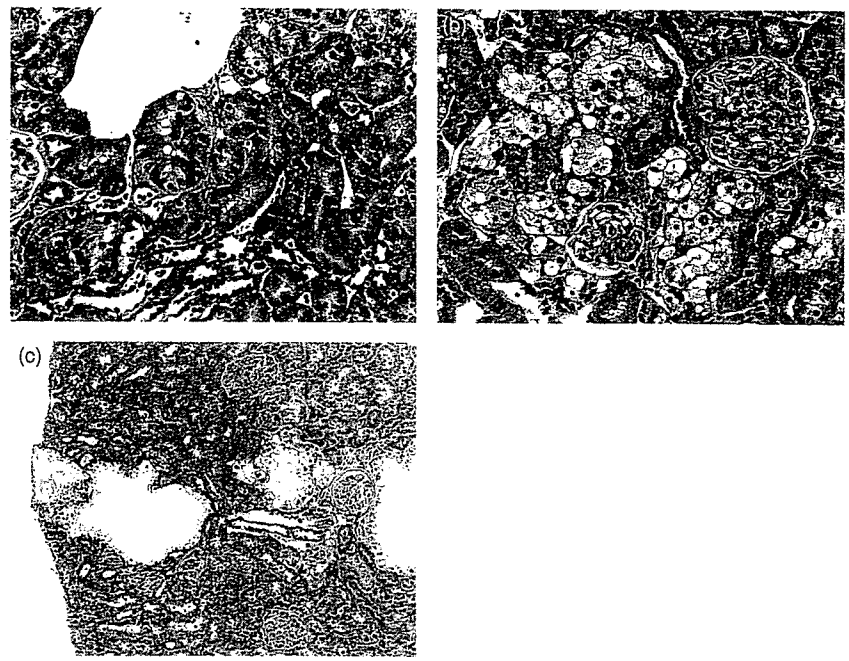

Fig. 5. (a) A single atypical tubule of basophilic cells in a rat treated with KBrO3 at a concentration of 500 p.p.m. in the drinking water for 13 weeks then, after a 2-week rest period, NTA at a concentration of 1% in the diet for 37 weeks. H–E stain. Original magnification × 80. (b) A focus of atypical hyperplasia composed of several atypical tubules, showing a solid structure and clear cell morphology in a rat treated with KBrO3 at a concentration of 500 p.p.m. in the drinking water for 13 weeks then, after a 2-week rest period, NTA at a concentration of 1% in the diet for 37 weeks. H–E stain. Original magnification × 80. (c) Cystic adenoma in a rat treated with $KBrO_3$ at a concentration of 500 pm in the drinking water for 13 weeks followed by no further treatment for 39 weeks. H–E stain. Original magnification × 33.

at carcinogenic levels for 9 weeks might be insufficient for affecting *OGG1* mRNA level.

Even though sustained increases in 8-OHdG formation were apparent through the experimental period, 9 weeks was needed to induce a significant increase of MF. In addition, the MF in the kidneys of rats given $KBrO_3$ at a dose of 250 p.p.m. did not change, despite statistically significant elevated 8-OHdG levels. Accordingly, the present data suggest that a period of time might be necessary for cells having high amounts of 8-OHdG to harbor mutations. This time factor might account for a previous bioassay finding of no initiating effects in rats given $KBrO_3$ by gavage as a single dose of 300 mg/kg, which is sufficient to increase 8-OH-dG levels in kidney DNA, and subsequently subjected to a promoting regimen for 102 weeks.[22]

In the present study, although we failed to detect an increase of *gpt* MF, significant elevation of *red/gam* MF, mainly attributable to deletion mutations, was found. Although it remains uncertain whether the mutation observed in *gpt* delta rats exposed to $KBrO_3$ originates in 8-OHdG, there have been several reports that formation of high amounts of 8-OHdG *in vivo* resulted in several types of mutations, including deletion mutations, besides GC:TA transversions. Previous work with *OGG1* knockout mice demonstrated that surprisingly high amounts of 8-OHdG due to $KBrO_3$ exposure resulted in an increase of deletion mutations and GC:AT transitions as well as GC:TA transversions.[21,36,37] Whereas mutations in NIH3T3 cells transfected with the *ras* gene, which incorporated 8-OHdG at the first position of codon 12, showed mainly GC:TA transversions, incorporation at the second position elicited GC:AT transitions to an appreciable extent.[36,38] Thus, the overall data indicate that mutations other than GC:TA transversions induced by 8-OHdG *in vivo* are possible.[39,40] Additionally, it is highly probable that other oxidized bases, such as 5-formyluracil and 5-hydroxycytosine,[41,42] were generated concomitantly with 8-OHdG formation. The

fact that deletion mutations were predominant among *p15* or *p16* lesions found in renal cell tumors induced by ferric nitrilotriacetate, an agent causing oxidative stress,[43] allows us to speculate that there might be certain types of DNA lesions related to oxidative stress that mainly cause deletion mutations.

The initiation bioassay clearly showed that a 13-week exposure to $KBrO_3$ at 500 p.p.m. was sufficient to induce renal preneoplastic lesions with significant incidence and multiplicity when followed by a typical renal tumor-promoter. Although $KBrO_3$ promotion activity has already been demonstrated in the kidneys of F344 rats when given in their drinking water,[23,44,45] this is the first report showing $KBrO_3$ initiating activity using the two-stage rat renal carcinogenesis model. Because Sprague–Dawley are a back strain of *gpt* delta rats, it seems hard to extrapolate their data to the results obtained from F344 rats. However, in addition to the fact that exposure of F344 rats to $KBrO_3$ at concentrations of 250 and 500 p.p.m. in their drinking water, not at 125 p.p.m. and below, was able to cause increase of 8-OHdG formation,[18] the initiation activity was found in the same dose-dependent fashion as *in vivo* mutagenicity in *gpt* delta rats. Further studies using newly developed *gpt* delta rats of F344 strain are now ongoing. In any case, based on the accumulated data using F344 rats it has been accepted that there is a close link between oxidative DNA damage and $KBrO_3$ carcinogenesis.[46] Considering that the increase of 8-OHdG implies occurrence of intranuclear oxidative stress, the present data suggest that the alteration of the intranuclear circumstances by oxidative stress might have the initiating potential.

In conclusion, the overall data suggest that not only the amount of 8-OHdG but also a period of time with high 8-OHdG levels might be required to bring an oxidized base lesion to mutation status. They also showed a possibility of cells with oxidative DNA damage becoming neoplastic under the influence of an appropriate tumor-promoter.

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GENOTOXICITY AND CARCINOGENICITY

Lack of in vivo mutagenicity and oxidative DNA damage by flumequine in the livers of *gpt* delta mice

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Abstract Flumequine (FLU), an anti-bacterial quinolone agent, has been recognized as a non-genotoxic carcinogen for the mouse liver, but recent reports have suggested that some genotoxic mechanism involving oxidative DNA damage may be responsible for its hepatocarcinogenesis. In the present study, we investigated this possibility in the mouse liver using male and female B6C3F1 *gpt* delta mice fed diet containing 0.4% FLU, a carcinogenic dose, for 13 weeks. Measurements of 8-hydroxydeoxyguanosine levels in liver DNA, and *gpt* point and deletion mutations revealed no significant increases in any of these parameters in either sex. Histopathologically, centrilobular swelling of hepatocytes with vacuolation was apparent, however, together with significant increase in bromodeoxyuridine-labeling indices in the treated males and females. These results suggest that genotoxicity, including oxidative DNA damage, is not involved in mouse hepatocarcinogenesis by FLU, which might rather solely exert tumor-promoting effects in the liver.

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Keywords Flumequine · In vivo mutagenicity · Oxidative DNA damage · Cell proliferation · *gpt* delta mouse

Introduction

Flumequine (FLU) is a fluoroquinolone compound with anti-microbial activity against gram-negative organisms used in the treatment of enteric infections in domestic animals (Greenwood 1998), which has also limited application in humans for the treatment of urinary tract infections (JECFA 2004). Flumequine and its metabolites are suspected to persist in the edible tissues of domestic animals and fish (Choma et al. 1999). Toxicity and carcinogenicity studies of FLU have already been performed using rats and mice, and FLU-induced hepatocellular tumors in an 18-month carcinogenicity study in CD-1 mice (JECFA 1998). However, negative results were obtained in an in vivo chromosome aberration test, a reverse mutation test in bacteria and gene mutation tests in mammalian cells (JECFA 1998). On the basis of these data, the Food and Agriculture Organization (FAO)/World Health Organization (WHO) Joint Expert Committee on Food Additives (JECFA) concluded that FLU is a non-genotoxic hepatocarcinogen, and that hepatocellular necrosis-regeneration cycles due to hepatotoxicity are mechanistically relevant to its induction of liver tumors in mice (JECFA 1998).

Previously, Yoshida et al. (1999) reported that the administration of FLU in the diet at a concentration of 4,000 ppm for 30 weeks induced basophilic liver cell foci in CD-1 mice and also increased the number of 8-hydroxydeoxyguanosine (8-OHdG) positive hepato-

cytes immunohistochemically. In addition, heterozygous *p53*-deficient CBA mice, a strain sensitive to genotoxic carcinogens, receiving 4,000 ppm FLU for 26 weeks developed basophilic liver foci (Takizawa et al. 2001). Positive results of in vivo comet assays in ddY mice, and increases of the number of hepatocellular foci in C3H mice using a two-stage liver carcinogenesis model have also been reported (Kashida et al. 2002), strongly pointing to a necessity for determination of whether FLU has initiating potential for mouse liver. Based on the results, JECFA temporarily withdrew the acceptable daily intake values (ADI), but this was shortly re-established at 0–30 mg/kg bw based on negative results for unscheduled DNA synthesis with FLU in rat liver cells in vivo (JECFA 2003, 2004). Thus, since conclusive evidence regarding the mode of action of FLU has yet to be provided, clarification of its in vivo mutagenicity is required for accurate assessment of hazard risk for humans.

Rodents transfected with *gpt* as a reporter gene are useful tools for estimating in vivo genotoxicity and carcinogenic risk of environmental chemicals (Gorelich et al. 1996; Nohmi et al. 2000; Nishikawa et al. 2001). In this transgenic mouse mutation assay, the reporter gene is integrated into mouse chromosome as part of λ shuttle vectors, which are easily recovered as phage particles from mouse genomic DNA by in vitro packaging reactions. Transgenic mice carrying the λ vector are treated with a test compound, and the mutant phages are infected to specific *E. coli* host cells and selected. An advantage of this *gpt* delta mouse model is to be able to detect two distinct types of mutations: point mutations can be positively identified by 6-thioguanine (6-TG) selection and deletions with sizes of more than 1 K base pairs by Spi^- selection (Nohmi et al. 2000). In the present study, we therefore performed in vivo mutation assays of FLU using B6C3F1 *gpt* delta mice, along with measurement of 8-OHdG formation in liver DNA and hepatocyte bromodeoxyuridine-labeling indices (BrdU-LIs).

Materials and methods

Chemicals

Flumequine, a white crystallized powder (purity 99.3%), was kindly provided by Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). Alkaline phosphatase and BrdU were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and nuclease P1 from Yamasa Co. (Chiba, Japan).

Animals and treatments

The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Male and female B6C3F1 *gpt* delta mice carrying 80 tandem copies of the transgene lambda EG10 in haploid genome were raised from mating between C57BL/6 *gpt* delta and non-transgenic C3H/He mice, a strain of mice with high sensitivity to hepatocarcinogens (Japan SLC, Inc. Shizuoka, Japan). Twenty male and 20 female B6C3F1 *gpt* delta mice were each randomized by weight into two groups. They were housed in a room with a barrier system, and maintained under the following constant conditions: temperature of 23 ± 2°C, relative humidity of 55 ± 5%, ventilation frequency of 18 times/h, and a 12 h light–dark cycle, with free access to CRF-1 basal diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water.

Starting at 8 weeks of age the mice were fed diet containing 0.4% FLU or maintained as non-treatment controls for 13 weeks. At the end of the experiment, five males and females from each group were sacrificed and a part of left lateral lobe of the liver was preserved at –80°C for subsequent mutation assays and 8-OHdG measurement. The rest of the lobes were fixed in 10% buffered formalin solution and routinely processed to paraffin blocks for histopathological examination as well as immunohistochemistry. Hematoxylin and eosin (H–E)-stained tissue preparations cut from the blocks were examined by light microscopy. At autopsy, the body and liver weights were measured.

Quantification of hepatocyte proliferation

In order to examine the proliferative activity of hepatocytes, the remaining five animals from each group not used for other analyses were given BrdU (100 mg/kg) by i.p. injection once a day for the final 2 days and once on the day of termination at 2 h before being euthanatized at autopsy. For immunohistochemical staining of BrdU, after first denaturing DNA with 4*N* HCl, tissue sections were treated sequentially with normal horse serum, monoclonal mouse anti-BrdU (Becton, Dickinson & Co., Franklin Lakes, NJ, USA) (1:100), biotin-labeled horse anti-mouse IgG (1:400), and avidin–biotin–peroxidase complex (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA, USA). The site of peroxidase binding was demonstrated by incubation with 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Co.). The immunostained sections were lightly counterstained with hematoxylin for microscopic examination. At least

2,000 hepatocytes in each liver were counted and labeling indices (LIs) were calculated as the percentages of cells positive for BrdU incorporation.

Measurement of 8-OHdG in liver DNA

In order to prevent 8-OHdG formation as a byproduct during DNA isolation (Kasai 2002), liver 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., Osaka, Japan) containing 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).

In vivo mutation assays

6-TG and Spi$^-$ selection were performed as previously described (Nohmi et al. 2000). Briefly, genomic DNA was extracted from each liver, and lambda EG10 DNA (48 kb) was rescued as the lambda phage by in vitro packaging. For 6-TG selection, the packaged phage was incubated with *E. coli* YG6020, which expresses Cre recombinase, and converted to a plasmid 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, 3,000-fold diluted phages were used to infect YG6020, and were poured on the plates containing chloramphenicol without 6-TG. The plates were 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 was calculated by dividing the number of *gpt* mutants by the number of rescued phages.

For the Spi$^-$ selection, the packaged phage was 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 soft agar and poured onto lambda-trypticase agar plates. Next day, plaques (Spi$^-$ candidates) were punched out with sterilized glass pipettes 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 on which 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.

Statistical evaluation

For statistical analysis, the Student's *t* test was used to compare liver and body weights, as well as quantitative data for BrdU-LIs, 8-OHdG levels and MFs, between groups.

Results

Body and liver weights and FLU intake

Data for final body and organ weights and intake of FLU are shown in Table 1. The final body weights were significantly ($P < 0.01$) decreased in FLU-treated males and females. Daily food consumption was also decreased in the FLU-treated animals, particularly females, as compared to the control group value. Daily FLU intake calculated from the consumption values were 590 and 763 mg/kg/day in males and females, respectively (Table 1). The doses used in a previous carcinogenicity study by gavage were 400 and 800 mg/kg/day, both of which were carcinogenic in mice (JECFA 2004). Liver/body weight ratios were significantly ($P < 0.01$) increased in the FLU-treated males and females.

Histopathology and immunohistochemical analysis of BrdU

Histopathologically, swelling of centrilobular hepatocytes with vacuolation was observed in FLU-treated males (Fig. 1b) and females. Slight infiltration of lymphocytes and neutrophils was also observed, although distinct hepatocellular necrosis was not found. There were no distinct sex differences in the degree of lesion development. The number of BrdU-positive liver cells (Fig. 1c, d) was increased in the FLU-treated group (Fig. 2), mostly appearing in the mid-zone of normal-looking cells adjacent to the damaged cells. The BrdU-LI in males given FLU was significantly ($P < 0.05$) higher than that in females (Fig. 2).

8-OHdG level in liver DNA

The data for 8-OHdG levels in the livers of FLU-treated males and females are shown in Fig. 3. No

Table 1 Body and liver weights, and food and flumequine intake data

Treatment	Number of mice	Body weight (g)[a]	Liver/body weight ratio (%)[a]	Food consumption (g/mouse/day)	Flumequine intake	
					Total (mg/mouse)	Daily (mg/kg/day)
Males						
Control	10	36.3 ± 1.0	4.56 ± 0.62	5.4	–	–
0.4% Flumequine	10	31.4 ± 1.5*	5.31 ± 0.24*	4.2	1,517	590
Females						
Control	10	25.7 ± 1.9	4.28 ± 0.25	7.0	–	–
0.4% Flumequine	10	23.3 ± 0.8*	5.42 ± 0.37*	4.4	1,610	763

[a]Data are mean ± SD values

*$P < 0.01$ (vs. control)

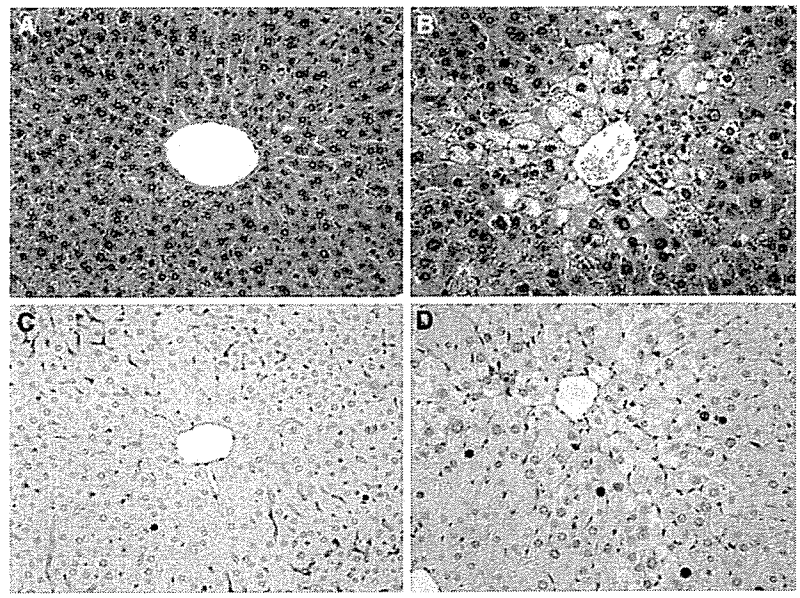

Fig. 1 Photomicrographs of livers of male gpt delta mice treated with basal diet (**a**, **c**) and 0.4% flumequine for 13 weeks (**b**, **d**). Note no obvious alterations (**a**) and centrilobular hepatocytes swelling with vacuolation (**b**). H–E staining at ×360 original magnification. Note BrdU-positive hepatocytes were few (**c**) and remarkably seen adjacent to the damaged cells (**d**). BrdU immunohistochemical staining at ×360 original magnification

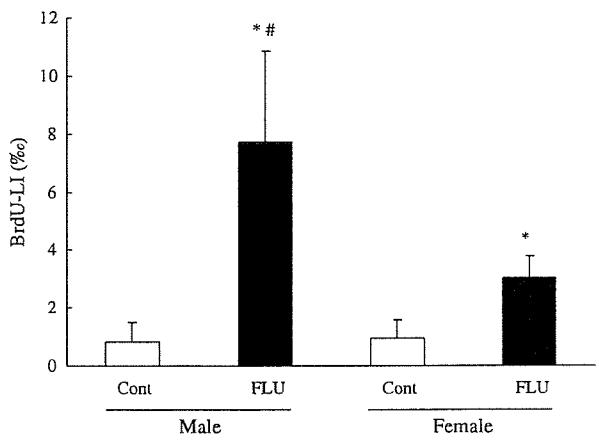

Fig. 2 BrdU-LIs for hepatocytes in male and female *gpt* delta mice fed 0.4% flumequine for 13 weeks. Values are mean ± SD of data for five mice. * Significant increase ($P < 0.05$) from the control group. # Significant difference ($P < 0.05$) between the sexes

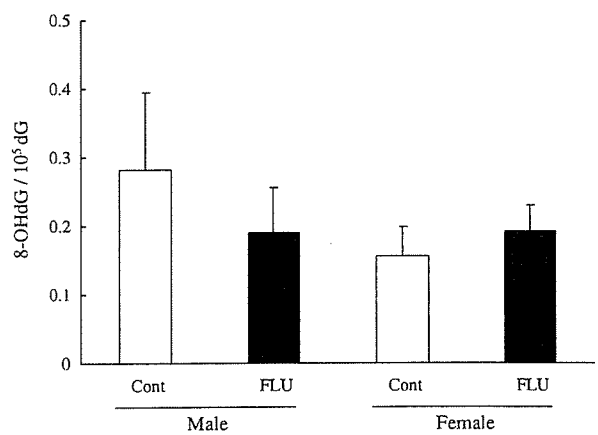

Fig. 3 8-OHdG levels in the livers of male and female *gpt* delta mice fed 0.4% flumequine for 13 weeks. Values are means ± SD of data for five mice. No significant differences were observed

Table 2 Guanine phosphoribosyltransferase *(gpt)* mutant frequencies (MFs) in the livers

Treatment	Number of mice	Total population	6-TG^r colonies	Total *gpt* mutants	MF ($\times 10^{-5}$)[a]
Male					
Control	5	3,378,000	27	22	0.80 ± 0.44
0.4% Flumequine	5	6,126,000	66	55	1.01 ± 0.52
Female					
Control	5	5,166,000	33	25	0.46 ± 0.28
0.4% Flumequine	5	6,864,000	63	43	0.65 ± 0.25

No significant difference was observed in MFs

[a]Data are mean ± SD values

significant effect of the FLU treatment was noted in either sex.

Mutation assays

Data for *gpt* MFs analyzed by 6-TG selection are summarized in Table 2. There were no significant increases of *gpt* MFs in the liver DNA of the FLU-treated males or females as compared to the non-treatment control values. Data for Spi$^-$ selection assessing deletion mutations are summarized in Table 3. Again, there was no significant variation in Spi$^-$ MFs values between FLU-treated and control mice.

Discussion

The present study did not provide support for the earlier finding from immunohistochemical analysis of increased 8-OHdG adducts in hepatocytes of mice given FLU (Yoshida et al. 1999). A marker widely used for oxidative damage to DNA (Shigenaga et al. 1991), 8-OHdG pairs with adenine as well as cytosine, generating GC-to-TA transversions upon replication by DNA polymerases (Cheng et al. 1992). Therefore, it has been postulated that this oxidized base is responsible for mutagenicity and carcinogenicity of many epigenetic carcinogens (Le Page et al. 1995; Nakae et al. 2002). In the present study, we quantitated 8-OHdG in the FLU-treated mouse livers by HPLC-ECD, but found no significant increase in either sex of treated mice. In addition to the fact that the present experimental conditions regarding animal strain and duration of exposure were different from these used previously (Yoshida et al. 1999), it is widely accepted that HPLC-ECD method is more precise and suitable for the detection of dose responses than immunohistochemistry (ESCODD 2000). There is a major body of evidence in favor of most sensitive detection of 8-OHdG elevation by HPLC-ECD in target organ DNA of animals exposed to hepatocarcinogens causing oxidative stress (Fiala et al. 1993; Umemura et al. 1996; Kasai 1997). Therefore, it is clear that FLU dose not cause oxidative DNA damage in the mouse liver at least under the present experimental conditions.

Table 3 Spi$^-$ MFs in the livers

Treatment	Number of mice	Total population	Total Spi$^-$ mutants	MF ($\times 10^{-5}$)[a]
Male				
Control	5	4,932,000	20	0.40 ± 0.14
0.4% Flumequine	5	5,350,500	20	0.38 ± 0.31
Female				
Control	5	7,587,000	25	0.33 ± 0.11
0.4% Flumequine	5	5,476,500	24	0.48 ± 0.36

No significant difference was observed in MFs

[a]Data are mean ± SD values

Similarly, in the present study, there were also no remarkable increases in *gpt* or Spi- mutation frequencies in the liver DNA of male or female *gpt* delta mice treated with FLU. We previously reported that many chemicals classified as genotoxic carcinogens increase mutation frequency with characteristic mutation spectra in target organ DNA of *gpt* delta mice (Nohmi and Masumura 2005; Kanki et al. 2005; Masumura at al. 2003). We also confirmed no increases of mutation frequency in the reporter gene in any organs of transgenic mice treated with non-genotoxic carcinogens or non-carcinogen, and in non-target organs treated with genotoxic carcinogens (Kanki et al. 2005; Nishikawa et al. 2001). Recently, we found that an increase in the mutation frequency with chemical exposure in a reported non-target organ was able to lead to tumor formation with the aid of an appropriate tumor-promoting regimen (Nishikawa et al. 2005). Thus, the data overall strongly suggest that the in vivo mutation assay using *gpt* delta mice is a reliable tool to predict the potential of a chemical for tumor-initiation. From the results of a comet assay for FLU, Kashida et al. (2002) suggested FLU cause DNA strand breaks in infant or regenerative livers of ddY mice, and sporadically in adult liver. However, the data were also in line with effects limited to cells with high mitotic activity. Although we should consider a possibility of other oxidative lesions than 8-OHdG occurring, the overall data

suggested that any lesions failed to exceed the thresholds for inducing their relevant genotoxicity. Accordingly, it can be said that FLU is not a tumor-initiating compound, genotoxicity including oxidative DNA damage not being involved in its hepatocarcinogenesis.

The present study revealed elevated cell proliferation in FLU-treatment in terms of BrdU incorporation, in agreement with a previous report of increase of proliferating cell nuclear antigen (PCNA)-positive cells in FLU-treated mice (Yoshida et al. 1999; Takizawa et al. 2001), and our data for liver weights. Together with the body weight suppression, these data imply hepatotoxicity of FLU (JECFA 1998; Yoshida et al. 1999). Focal necrosis of hepatocytes was observed in CD-1 mice at 400 and 800 mg/kg/day in an 18-month study earlier (JECFA 1998), although distinct hepatocellular necrosis was not found in the present study. The present finding that BrdU-LIs in FLU-treated males were significantly higher than in females corresponded to the previous report of a sex differentiation in FLU toxicity (JECFA 1998). Therefore, our data strongly support JECFA's conclusion that the induction of hepatocellular necrosis–regeneration cycles due to FLU hepatotoxicity is the relevant to 'promotion' of liver tumor development (JECFA 2004).

In conclusion, our data clearly demonstrate that FLU dose not cause either oxidative DNA damage or mutagenicity in the mouse liver when given even at a carcinogenic dose. Therefore, it is concluded that genotoxicity, including oxidative DNA damage, is not involved in mouse hepatocarcinogenesis by FLU and it can be classified as a mouse liver tumor promoter.

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