tion of large deletions at a dose rate of 1.5 mGy/h of γ-irradiation.

To further characterize the large deletions induced by the irradiation, we identified the size and junctions of all the 51 deletion mutants (Fig. 4). The size of deletions distributed from 1035 to 9265 bp. About half of the mutants had short homologous sequences up to 11 bp in the junctions while another half had no such short homologous sequences. Two mutants had 1 or 4 bp insertions in the junctions. There was no hot spot of the junctions so that only 2 out of 51 deletions were identified in two mice. There were no obvious differences between large deletions induced by radiation alone and those induced by radiation plus NNK treatments. These results suggest that radiation-induced DSBs in DNA caused large deletions either in the absence or the presence of NNK treatments.

4. Discussion

Humans are exposed to a variety of exogenous and endogenous genotoxic agents. Thus, biological effects of radiation at low doses or low-dose-rate should be evaluated in combination with chemical exposure [12]. In fact, survey of chromosome aberrations in habitats in high-background radiation area in China indicates that cigarette smoking has stronger effects on induction of chromosome aberrations than has the elevated level of natural radiation [23]. Epidemiological studies on underground mineworkers exposed to high levels of radon or plutonium suggest the complexity of interactions between radiation and cigarette smoke in induction of lung tumors [24,25]. Hence, it is important to understand the fundamental mechanisms underlying the interactive genotoxicity and carcinogenicity of cigarette smoking and radiation for the risk assessment on human health.

To elucidate the mechanisms involved, we examined the combined genotoxicity of low-dose-rate γ-irradiation and a tobacco-specific nitrosamine NNK in the lung of *gpt* delta mice. In this study, we focused on whether γ-irradiation would modulate NNK-induced base substitutions and whether NNK treatments would modulate radiation-induced deletions. The mice were irradiated at dose rates of 0.5, 1.0 and 1.5 mGy/h for 22 h for 2 weeks and treated with NNK, i.e., 2 mg/mouse/day for four consecutive days, with irradiation (Fig. 1). The mice were irradiated at the same dose rates for another 2 weeks before sacrifice. Base substitutions and deletions in the lung detected by *gpt* and Spi$^-$ selection, respectively, were analyzed at the molecular levels. We chose the dose rates, i.e., 0.5, 1.0 and 1.5 mGy/h of γ-ray, since Sakai et al. [26] report the suppression of carcinogenicity of 3-methylchoranthrene in ICR female mice by chronic low-dose-rate irradiation of γ-ray at 0.95 mGy/h. According to the report, there is an optimum dose rate of about 1 mGy/h to observe the suppressive effects, and the higher or lower dose rates fail to suppress the tumor induction.

In the present study, NNK treatments significantly enhanced the *gpt* MF (Fig. 2). We observed, however, no modulating effects, i.e., enhancement or suppression, of γ-irradiation at any given dose rate, on the NNK-induced mutations (Fig. 2). This conclusion holds true even when we analyzed the detailed mutation spectra (Table 1). NNK treatments induced similar pattern of base substitutions, i.e., G:C to A:T, G:C to T:A, A:T to T:A and A:T to C:G regardless of the dose rates of combined radiation. In contrast, we observed a suppressive effect of NNK treatments on the radiation-induced deletions. γ-Irradiation enhanced the MF of large deletions in the size of more than 1 kb in a dose-dependent manner (Fig. 3A and Table 2). When combined with NNK treatments, however, the dose–response curve became bell-shaped and the MF at the highest dose rate, i.e., 1.5 mGy/h, was reduced by more than 50% (Fig. 3B and Table 2). The total radiation dose at the highest dose rate was 1.02 Gy. The size of the large deletions was between about 1 and 9 kb, and about half of the large deletions had short homologous sequences in the junctions while other did not (Fig. 4). These features are similar to those of large deletions induced by high dose irradiation with heavy ion, X-ray and γ-ray [20]. Thus, we suggest that NNK induced an adaptive response that eliminated the cells bearing radiation-induced DSBs in DNA.

Previous studies show that low-dose radiation can induce an adaptive response, which causes cells to become resistant to damage by subsequent high doses of radiation [13,27]. Although the exact mechanisms of the adaptive response are not well understood, it is assumed that some proteins are induced by low-dose radiation and they recognize and remove the cells bearing DSB in DNA. Tucker et al. [28] report that the frequency of *Dlb-1* mutations in the small intestine in female F1 mice obtained by crossing SWR/J and C57BL/6 increases along with the total radiation doses of γ-ray, but it saturates and slightly decreases at high doses, i.e., 2–3 Gy (55 mGy/day × 42 or 63 days). Interestingly, our results also suggest that the MFs of the large deletions saturated slightly at the highest dose of 1.02 Gy (Fig. 3A). Thus the adaptive response might be induced slightly at the highest radiation dose even without NNK treatments. Nevertheless, concomitant NNK treatments much clearly suppressed the occurrence of large dele-

tions at the highest radiation dose. We speculate that NNK treatments plus radiation at the highest dose may induce p53-dependent apoptosis, which eliminates the cells bearing radiation-induced DSBs in DNA [29]. The involvement of p53 in the maintenance of genome stability is associated with several pathways such as cell cycle arrest, apoptosis and DNA repair. Low levels of DNA damage appear to enhance p53-dependent DNA repair while high levels induce apoptosis [30]. We envisage that NNK treatments plus radiation at the highest dose introduce genotoxic damage to the cells, the levels of which are enough to trigger the apoptosis. Zhou et al. [31] examined the combined effects of NNK and α-particle irradiation with human–hamster hybrid cultured cells and concluded that the induction of chromosome deletions were additive when the NNK dose was low but a suppressive effect was observed at a higher NNK concentration. In vivo studies also suggest that exposure to high levels of cigarette smoke decrease the risk of lung cancer induced by radon in dogs [32]. However, a multiplicative effect of smoking and radon is observed in rats [33]. In humans, the definitive interaction models have not been established between smoking and radiation exposure [24,25]. Thus, further work is needed to clearly establish the interactive genotoxic mechanisms between radiation and cigarette smoking in vivo.

NNK, a tobacco-specific nitrosamine, is metabolically activated by α-hydroxylation of the methyl and methylene groups [34]. Methylene hydroxylation leads to DNA methylation while methyl hydroxylation leads to pyridyloxobutylated DNA [35]. DNA methylation occurs at N7 and O^6 of guanine and O^4 and O^2 of thymine. It is suggested that O^6-methylguanine (O^6-mG) and pyridyloxobutylated DNA are responsible for G:C to A:T and G:C to T:A mutations, respectively, which activate Ki-*ras* oncogene in the mouse lung tumors induced by NNK [6]. In the present study, G:C to A:T and G:C to T:A mutations were induced by NNK treatments significantly (Table 1). Tiano et al. [36] examined the genotoxicity of NNK in AS52 hamster cells expressing human CYP2A6 and analyzed the induced mutations with the *gpt* gene as a reporter gene for mutations. Because of the lack of O^6-mG methyltransferase in the cell line, about 80% of mutations were G:C to A:T transitions. Interestingly, most of the G:C to A:T transition hotspots occur at the second G of the GGT sequence motif, which is the motif of codon 12 in the Ki-*ras* oncogene [37]. When we define the hotspot as the site where more than four G:C to A:T mutations were identified, we identified 18 hotspots in the *gpt* gene among 155 G:C to A:T mutants recovered from NNK-treated mice. They are nucleotide 27, 64, 86, 87, 92, 107, 110, 113, 115, 116, 128, 274, 281, 287, 402, 409, 417 and 418 when A of ATG of the start codon of the *gpt* gene is set as nucleotide 1. Tiano et al. [36] identified four hotspots of the second G of GGT in nucleotide 23, 116, 128 and 281 in the *gpt* gene, three of which are included in the hotspots identified by us. However, we identified other hotspots such as the second G of GGA at nucleotide 87, 274, 402 and 418, the second G of GGG at nucleotide 27, 64, 92 and 417 and the second G of GGC at nucleotide 113. Thus, we conclude that NNK preferentially induces G:C to A:T mutations at the second G of GGX where X represents any of A, T, G and C. In addition to G:C to A:T and G:C to T:A mutations, we observed an increase in the MFs of A:T to T:A and A:T to C:G in the NNK treated mice (Table 1). Substantial increases in the MFs of A:T to T:A and A:T to C:G are also reported by Hashimoto et al. [38], who examined the genotoxicity of NNK with *lacZ* transgenic mice (Muta^{TM} Mouse). Since reporter genes for mutations, such as *gpt*, *cII* or *lacZ*, are not expressed in vivo and are not imposed by any selection bias, they can reflect any genotoxic events occurring in the genomic DNA. In contrast, oncogenes such as the *ras* gene can only detect mutations that can activate the oncogenic activity of the gene products. Thus, we assume that NNK induces modifications in DNA such as O^4-methyl or O^2-methyl thymine in the lung, which may account for the induction of A:T to C:G and A:T to T:A mutations, respectively [8]. Although the toxicological significance of these mutations is currently unknown, these mutations may contribute to the carcinogenicity of cigarette smoke as well.

γ-Irradiation at dose rate of 1.0 and 1.5 mGy/h clearly enhanced the MFs of large deletions when no NNK treatments were combined (Fig. 3A). The total estimated doses were 0.68 and 1.02 Gy, which may be the lowest radiation doses that gave positive results in transgenic mice mutation assays [16]. In contrast, we could detect no significant increase in the MF of large deletions induced by NNK treatments (Table 2 and Fig. 3A and B). Thus, we suggest that NNK induces mostly base substitutions but not deletions in vivo. Interestingly, NNK treatments induce deletions in cultured mammalian cells. Tiano et al. [36] report that about 20% of mutations induced by NNK treatments are deletions in AS52 hamster cells expressing human CYP2A6. Zhou et al. [31] report that about 80% of NNK-induced mutations are large deletions in the human–hamster hybrid (A_L) cell assay. We speculate that NNK may have a potential to induce both base substitutions and large deletions in vitro but the latter can be eliminated in vivo by the p53-dependent mechanism. Chinese hamster cell lines such as CHO and V79 harbor missense mutation in the *p53*

gene [39,40]. Large deletions might have been detected in the cultured cells because of inefficient p53 functions.

In summary, we have examined the combined genotoxicity of γ-irradiation and NNK treatments in the lung of *gpt* delta mice. Although radiation did not modulate the NNK-induced base substitutions, NNK treatments suppressed the induction of large deletions in size more than 1 kb induced by the irradiation. NNK treatments might induce an adaptive response, which eliminates the cells bearing radiation-induced DSBs in DNA. This finding may be helpful in understanding the molecular mechanisms of genotoxicity as a result of interactions of more than one genotoxic agents in vivo.

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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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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)

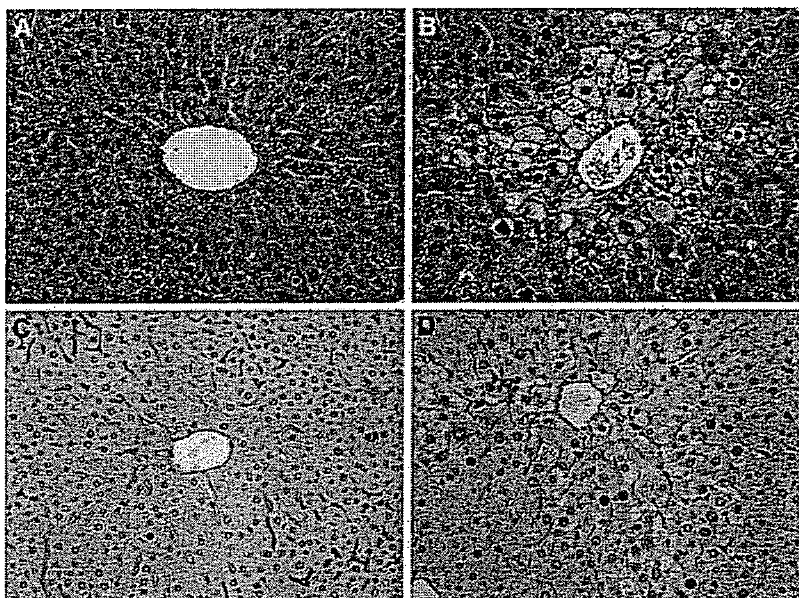

Fig. 1 Photomicrographs of livers of male gpt delta mice treated with basal diet (**a, c**) and 0.4% flumequine for 13 weeks (**b, d**). Note no obvious alterations (**a**) and centrilobular hepatocytes swelling with vacuolation (**b**). H–E staining at ×360 original magnification. Note BrdU-positive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

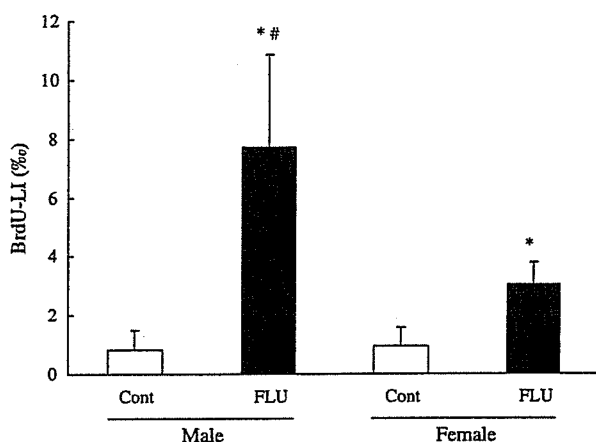

Fig. 2 BrdU-LIs for hepatocytes in male and female *gpt* delta mice fed 0.4% flumequine for 13 weeks. Values are mean ± SD of data for five mice. * Significant increase ($P < 0.05$) from the control group. # Significant difference ($P < 0.05$) between the sexes

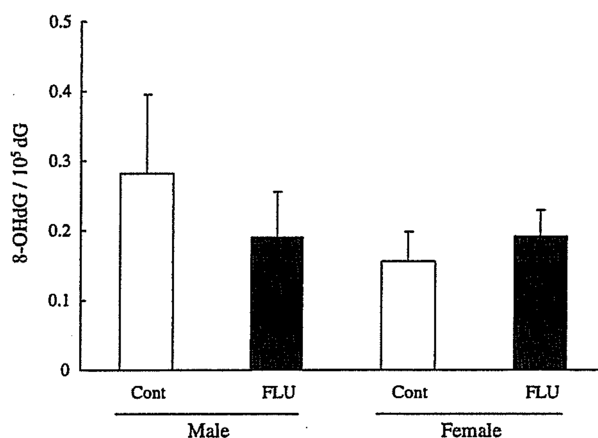

Fig. 3 8-OHdG levels in the livers of male and female *gpt* delta mice fed 0.4% flumequine for 13 weeks. Values are means ± SD of data for five mice. No significant differences were observed

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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