Carcinogenesis vol.32 no.10 pp.1512–1517, 2011
doi:10.1093/carcin/bgr130
Advance Access publication July 28, 2011

Chemopreventive effects of silymarin against 1,2-dimethylhydrazine plus dextran sodium sulfate-induced inflammation-associated carcinogenicity and genotoxicity in the colon of *gpt* delta rats

Naomi Toyoda-Hokaiwado, Yumiko Yasui[1,3], Mina Muramatsu[2], Kenichi Masumura, Makiko Takamune, Masami Yamada, Toshihiro Ohta[2], Takuji Tanaka[1,4] and Takehiko Nohmi*

Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo 158-8501, Japan, [1]Department of Oncologic Pathology, Kanazawa Medical University, Ishikawa 920-0293, Japan, [2]School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, Tokyo 192-0392, Japan, [3]Department of Physiology and Nutrition, School of Veterinary Medicine, Rakuno Gakuen University, Hokkaido 069-8501, Japan and [4]The Tokai Cytopathology Institute: Cancer Research and Prevention, Gifu 500-8285, Japan

*To whom correspondence should be addressed. Tel: +81 3 3700 9872; Fax: +81 3 3700 2348;
Email: nohmi@nihs.go.jp

Silymarin, a natural flavonoid from the seeds of milk thistle, is used for chemoprevention against various cancers in clinical settings and in experimental models. To examine the chemopreventive mechanisms of silymarin against colon cancer, we investigated suppressive effects of silymarin against carcinogenicity and genotoxicity induced by 1,2-dimethylhydrazine (DMH) plus dextran sodium sulfate (DSS) in the colon of F344 *gpt* delta transgenic rats. Male *gpt* delta rats were given a single subcutaneous injection of 40 mg/kg DMH and followed by 1.5% DSS in drinking water for a week. They were fed diets containing silymarin for 4 weeks, starting 1 week before DMH injection and samples were collected at 4, 20 and 32 weeks after the DMH treatment. Silymarin at doses of 100 and 500 p.p.m. suppressed the tumor formation in a dose-dependent manner and the reduction was statistically significant. In the mutation assays, DMH plus DSS enhanced the *gpt* mutant frequency (MF) in the colon, and the silymarin treatments reduced the MFs by 20%. Silymarin also reduced the genotoxicity of DMH in a dose-dependent manner in bacterial mutation assay with *Salmonella typhimurium* YG7108, a sensitive strain to alkylating agents, and the maximum reduction was >80%. These results suggest that silymarin is chemopreventive against DMH/DSS-induced inflammation-associated colon carcinogenesis and silymarin might act as an antigenotoxic agent, in part.

Introduction

Silymarin, an extract from the milk thistle fruit (*Silybum marianum*, Family Asteraceae), has been utilized for remedy of liver diseases such as sirrhosis or hepatitis for many years (1). Silymarin is actually the collective name of the extract and composed of at least seven flavonolignans and one flavonoid, and silybinin is the major active constituent (2,3). Silymarin inhibits proliferation of various cancer cells and reduces carcinogenesis in various animal models (4,5). Therefore, it has been used in the experimental therapy of cancer and chemoprevention and even in human clinical trials. Because silymarin possesses a variety of biological properties, such as antioxidant and anti-inflammatory activities, induction of phase II enzymes and apoptosis (1), it may suppress cancer development via multiple mechanisms. However, few studies that evaluate antigenotoxic properties of silymarin are available and the contribution to the chemopreventive effects remains elusive.

In this study, we explored the antigenotoxic and chemopreventive effects of silymarin in the colon of rats. We chose colon because silymarin is highly distributed in colon mucosa when it is administered to humans orally (6) and the colon cancer is one of the most frequent human cancers worldwide (7). In fact, silymarin inhibits growth of colorectal carcinoma cells *in vitro* (8,9) and suppresses colon carcinogenesis induced by methylating agents *in vivo* (10,11). The anti-inflammatory and anticancer effects in chemically induced and spontaneous intestinal carcinogenesis in mice are also reported (12,13). To evaluate the antigenotoxic and anticarcinogenic properties, we employed F344 *gpt* delta transgenic rats treated with 1,2-dimethylhydrazine (DMH) plus dextran sodium sulfate (DSS). DMH and its metabolite azoxymethane (AOM) are potent genotoxic agents and the following treatment with a non-genotoxic agent, i.e. DSS, strongly induces inflammation in the colon, thereby enhancing colon carcinogenesis in mice (14,15) and rats (16,17). *gpt* delta transgenic rats carry approximately five copies of λEG10 DNA at a single site in the chromosome 4 (18). The λDNA carries reporter genes for *in vivo* mutagenesis, and thus point mutations and deletions can be identified in any organs of rats at the sequence levels (19,20). Because the transgene is not expressed *in vivo*, the transgenic rats are expected to display very similar sensitivity to chemical carcinogens to non-transgenic F344 rats. We also conducted bacterial mutation assay with *Salmonella typhimurium* YG7108, a sensitive strain to alkylating agents (21,22), to examine whether silymarin inhibits genotoxicity of DMH and its metabolite AOM *in vitro*. From the results, we conclude that silymarin suppresses the inflammation-associated colon carcinogenesis and suggest that the antigenotoxic property contributes to the chemopreventive effects at least partly.

Materials and methods

Materials

DMH and silymarin (silymarin group, a mixture of isomers, molecular weight = 482.44) were purchased from Sigma–Aldrich Co. (St Louis, MO). AOM, *N*-methyl-*N*-nitrosourea (MNU) and dimethyl sulfoxide were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Animals, diet and housing conditions

Male 6-week-old F344 *gpt* delta transgenic rats (20) were obtained from Japan SLC and housed three or four animals per polycarbonate cage under specific pathogen-free standard laboratory conditions: room temperature, 23 ± 2°C; relative humidity, 60 ± 5%, with a 12:12 h light–dark cycle and free access to Charles River formula-1 basal diet (Oriental Yeast Company, Tokyo, Japan) and tap water.

Treatments of animals

The protocol for this study was approved by the Animal Care and Utilization Committee of Kanazawa Medical University. One-hundred rats were randomly divided into seven groups (Figure 1). Groups 1–4 received single subcutaneous injection of DMH (40 mg/kg body wt). Groups 5–7 received no injections. One week after the carcinogen treatment, Groups 1–3 and 5 were treated with 1.5% DSS in drinking water for a week. Groups 4, 6 and 7 had just drinking water instead of 1.5% DSS solution. Groups 2, 3 and 6 were fed diets containing 100 or 500 p.p.m. silymarin for 4 weeks, starting 1 week before DMH injection. Group 7 served as an untreated control. All rats were carefully observed for clinical welfare and weighed weekly and experimental diet consumptions were recorded. Animals were killed at 4 weeks (short), 20 weeks (medium) and the experiment was terminated at 32 weeks (long).

Histological analysis

At autopsy, liver, kidneys, spleen and intestine were macroscopically examined for the presence of pathologic lesions and then isolated. The intestine was

Abbreviations: AOM, azoxymethane; DMH, 1,2-dimethylhydrazine; DSS, dextran sodium sulfate; MAM, methylazoxymethanol; MF, mutant frequency; MNU, *N*-methyl-*N*-nitrosourea.

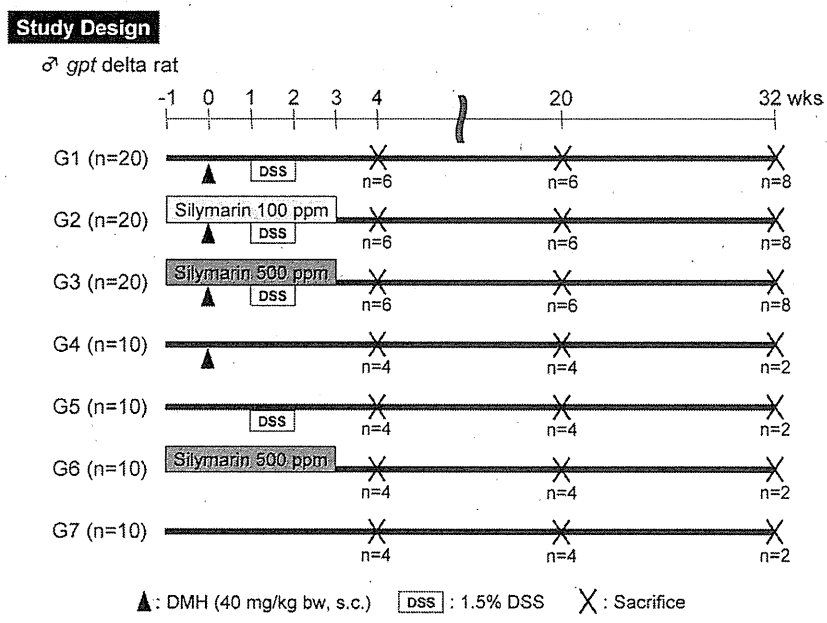

Fig. 1. Experimental protocol. Arrow heads, DMH 40 mg/kg body wt, subcutaneous injection; X, sacrifice.

excised, opened longitudinally, flushed clean with saline and examined for the presence of tumors. Colons were fixed in 10% buffered formalin and processed to hematoxylin- and eosin-stained sections. Neoplastic lesions of colorectal mucosa were histopathologically classified into dysplastic foci, adenomas and adenocarcinomas. At 4 weeks periods, 5 cm long colorectal tissues from distal segment were excised and frozen in liquid nitrogen for mutation assay. Then, colons were fixed in 10% buffered formalin and then processed for aberrant crypt foci analysis by conventional methods (23). One centimeter-long slice from stump was processed into serial paraffin sections by *en face* preparation and stained with hematoxylin and eosin and immunohistochemistry. Remained tissues were routinely embedded in paraffin and hematoxylin and eosin stained and histopathologically examined by light microscopy. The histological analysis of β-catenin-accumulated crypts and ulcer was performed based on the criteria described previously (24–26). Tumor incidence (%) means number of rat with colon tumors per total number in the experimental group and tumor multiplicity indicates number of colon tumors per rat in the experimental group.

Immunohistochemical procedures

Paraffin sections of colon were immunostained with a polyclonal anti-β-catenin antibody. Antigen retrieval was carried out by autoclaving for 15 min in 10 mmol/L citrate buffer (pH 6.0). Immunohistochemical staining was done by the avidin–biotin complex method (ABC) using the Vectastain Elite ABC system (Vector Laboratories, Burlingame, CA). Biotinylated goat anti-rabbit IgG (Vector Laboratories) was used as a secondary antibody at a dilution of 1:200. Sections were lightly counterstained with hematoxylin for microscopic examination.

DNA isolation, in vitro packaging and *gpt* mutation assay

High-molecular weight genomic DNA was extracted from the colon using the RecoverEase DNA Isolation Kit (Stratagene by Agilent Technologies, Santa Clara, CA). λEG10 phages were rescued using Transpack Packaging Extract (Stratagene). The *gpt* assay was conducted according to previously published methods (27,28). The mutant frequencies (MFs) of the *gpt* gene (*gpt* MFs) in the colon were calculated by dividing the number of confirmed 6-thioguanine (6-TG)-resistant colonies by the number of rescued plasmids. DNA sequencing of the *gpt* gene was performed with the BigDye Terminater Cycle Sequencing Ready Reaction (Applied Biosystems by Life Technologies, Carlsbad, CA) on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). All of the confirmed *gpt* mutants recovered from the carcinogen-untreated colons and selected confirmed *gpt* mutants recovered from the carcinogen-treated colons (basically, 10 mutants per animal were analyzed) were sequenced; identical mutations from the same rat were counted as one mutant.

Bacterial reverse mutation test (Ames test)

The mutagenic activities of DMH and silymarin were assayed in a bacterial reverse mutation assay using *S. typhimurium* tester strains YG7108, as TA1535 but is $\Delta ada_{st}\Delta ogt_{st}$ (21,22). The test was conducted by the preincubation method with modification (29). Briefly, silymarin was dissolved in dimethyl sulfoxide and mixed with DMH or AOM, dissolved in distilled water. In the case of MNU, it was dissolved in dimethyl sulfoxide. The chemicals were mixed with overnight culture of YG7108 in the presence or the absence of S9 mix and incubated for 20 min at 37°C. The reaction mixture containing bacteria, an alkylating agent and silymarin was poured onto agar plates with soft agar and incubated for 2 days at 37°C. Assays were performed on triplicate.

Statistical analysis

The statistical significance of the difference in the value of MFs between treated groups and negative controls was analyzed by the Student's *t*-test. A P value <0.05 denoted the presence of a statistically significant difference. Variances in values for body weight, organ weight and pathological data were examined by Tukey multiple comparison post-test using GraghPad InStat (GraphPad Software, La Jolla, CA) to compare the differences. The tumor incidence was examined by Yates m × n χ^2-test.

Results

General conditions of animals

No marked clinical symptoms were observed during experimental periods. Body weight gain and food consumption were similar between each group. Final body weights at killing were not significantly different among groups (supplementary Tables I–III are available at *Carcinogenesis* Online).

Silymarin suppressed preneoplastic lesions

At 4 weeks necropsy, no obvious macroscopic changes were detected. Pathological findings are shown in Table I. Aberrant crypt foci developed in rats treated with DMH and DSS. The frequency of aberrant crypt foci/colon in Group 2 (DMH/DSS/S100) and Group 3 (DMH/DSS/S500), which received DMH + DSS and silymarin at 100 and 500 p.p.m., respectively (Figure 1), was significantly lower than that of Group 1 (DMH/DSS), which received DMH + DSS alone ($P < 0.001$). The frequency of β-catenin-accumulated crypt was also reduced by dietary silymarin treatments (Group 2, $P < 0.05$; Group 3,

$P < 0.01$). In addition, number of colon mucosal ulcer was significantly reduced in these groups (Group 2, Group 3, $P < 0.001$). On microscopic observation, major changes were observed in the distal colon.

Gene mutation assay in vivo

DMH treatments enhanced *gpt* MF in the colon 100 times over the control levels (Table II). Silymarin itself was non-genotoxic [Group 6 (S500) in Figure 1]. DSS treatments did not show marked effects on the MFs. The dietary administration of silymarin at 100 and 500 p.p.m. [Group 2 (DMH/DSS/S100) and Group 3 (DMH/DSS/S500) in Figure 1] reduced the DMH-induced MF by 20%. Because of the large standard of deviation, however, the difference was not statistically significant. Dominant base substitution induced by DMH treatment was G:C to A:T transitions and silymarin treatments did not change the mutation spectra substantially (supplementary Table IV is available at *Carcinogenesis* Online).

The colon neoplasms were reduced by silymarin treatment

The incidence and multiplicity of tumors at 20 and 32 weeks are shown in Tables III and IV, respectively. Most of adenomas and adenocarcinomas were observed in the distal colon. Although 20 week observation did not show any statistical significance between groups, the number of tumors per rat was significantly reduced by dietary silymarin administration in a dose-dependent manner at 32 weeks. In the silymarin-treated groups [Group 2 (DMH/DSS/S100) and Group 3 (DMH/DSS/S500) in Figure 1], incidence and multiplicity of proliferative lesions were markedly reduced compared with Group 1 (DMH/DSS).

Silymarin inhibited genotoxicity of DMH in S. typhimurium *YG7108*

To further characterize the suppressive effects of silymarin against DMH-induced genotoxicity, bacterial mutation assay was performed. Silymarin itself was non-genotoxic either with or without S9 activation (Figure 2 and supplementary Figure S1 is available at *Carcinogenesis* Online). DMH at a dose of 400 μg/plate, AOM at a dose of 4000 μg/plate and MNU at a dose of 10 μg/plate induced 2800, 800 and 1400 His^+ revertants per plate, respectively (Figure 2). Silymarin reduced the genotoxicity of these alkylating agents in a dose-dependent manner and the number of His^+ revertants per plate decreased by >50% at the highest dose of silymarin (Figure 2). Furthermore, silymarin showed antigenotoxic efficacy with or without S9 activation under the DMH treatments (Figure 2 and supplementary Figure S1 is available at *Carcinogenesis* Online).

Discussion

In this study, silymarin inhibited colon cancer development significantly, suggesting that it might be a quite efficient chemopreventive agent. Previously, the efficacy of silymarin against colon carcinogenesis was reported in several animal models (10–13). Kohno *et al.* (10) report that oral administration of silymarin enhances glutathione *S*-transferase activity in liver. In addition, cell proliferation in colonic mucosa is reduced and apoptosis is significantly increased by silymarin administration. Here, we reported that tumor number and incidences were greatly reduced and the MF induced by DMH was reduced by the silymarin treatments (Tables II–IV). It suggests that the antigenotoxic efficiency might contribute to the tumor reduction at least partly *in vivo*.

In *in vivo* situation, DMH is first oxidized to azomethane, which appears in the exhaled air of DMH-treated animals (30). Azomethane is oxidized to AOM, which is hydroxylated to methylazoxymethanol (MAM). AOM and MAM are also detected in the urine. MAM is unstable and decomposes to methyldiazonium, which is a highly reactive methylating intermediate (30). DMH and its metabolites, i.e. AOM and MAM, are potent carcinogens that induced colorectal carcinomas in rodent. Here, we revealed that silymarin was clearly antigenotoxic against potent alkylating carcinogens DMH, AOM and MNU *in vitro* (Figure 2). Silymarin showed similar inhibitory effects against DMH-induced genotoxicity with or without S9 mix

Table I. Pathological findings in colon (4 weeks)

Group	No. of rats	No. of mucosal ulcer/rat	No. of BCAC/rat	No. of ACF/rat	No. of foci containing	
					<4 crypts	≥4 crypts
1	6	4.50 ± 1.38[a]	3.33 ± 1.21	34.83 ± 9.20	27.17 ± 6.46	7.67 ± 3.67
2	6	1.33 ± 1.21***	1.67 ± 1.03*	17.50 ± 2.51***	16.17 ± 2.64**	1.33 ± 1.37***
3	6	0.83 ± 0.75***	0.67 ± 0.82**	13.83 ± 3.82***	13.50 ± 4.04***	0.33 ± 0.52***
4	4	0	0	0	0	0
5	4	0	0	0	0	0
6	4	0	0	0	0	0
7	4	0	0	0	0	0

BCAC, β-catenin-accumulated crypt; ACF, aberrant crypt foci.
[a]Mean ± SD.
*, **, ***: Significantly different from group 1 at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, by Tukey multiple comparison post-test.

Table II. *gpt* MF in colon (4 weeks)

Group	Treatment			No. of rats	MF ($\times 10^{-6}$) (mean ± SD)	*P* value[a] (*t*-test)
	DMH	DSS	Test chemical			
1	+	+		6	557.7 ± 213.4 (100%)[b]	
2	+	+	Silymarin 100 p.p.m.	6	423.2 ± 246.3 (75.9%)	0.1679
3	+	+	Silymarin 500 p.p.m.	6	457.7 ± 186.5 (82.1%)	0.2039
4	+	−		4	646.8 ± 231.1 (116%)	0.2741
5	−	+		4	9.8 ± 11.0 (1.8%)	
6	−	−	Silymarin 500 p.p.m.	4	5.3 ± 5.2 (1.0%)	
7	−	−		4	5.6 ± 6.4 (1.0%)	

[a]*P* values were calculated by the Student's *t*-test.
[b]Percentage of the MF of each Group against that in Group 1.

Table III. Incidence of colon tumors in each group

Group	Treatment			Medium term (20 weeks)				Long term (32 weeks)			
	DMH	DSS	Test chemical	No. of rats	No. of rats with tumors			No. of rats	No. of rats with tumors		
					Total	AD	ADC		Total	AD	ADC
1	+	+		6	0 (0%)	0 (0%)	0 (0%)	8	8 (100%)	6 (75%)	8 (100%)
2	+	+	Silymarin 100 p.p.m.	6	1 (17%)	1 (17%)	0 (0%)	8	6 (75%)	2 (25%)	6 (75%)
3	+	+	Silymarin 500 p.p.m.	6	2 (33%)	0 (0%)	2 (33%)	8	2*(25%)	0*(0%)	2*(25%)
4	+	−		4	0	0	0	2	0	0	0
5	−	+		4	0	0	0	2	0	0	0
6	−	−	Silymarin 500 p.p.m.	4	0	0	0	2	0	0	0
7	−	−		4	0	0	0	2	0	0	0

AD, adenomas; ADC, adenocarcinomas.
*Significantly different from group 1 at $P < 0.05$ by Yates m × n χ^2-test multiple comparison post-test.

Table IV. Multiplicity of colon tumors in each group

Group	Treatment			Medium term (20 weeks)			Long term (32 weeks)		
	DMH	DSS	Test chemical	No. of tumors/rat			No. of tumors/rat		
				Total	AD	ADC	Total	AD	ADC
1	+	+		0	0	0	6.3 ± 4.8	1.6 ± 1.7	4.6 ± 3.6
2	+	+	Silymarin 100 p.p.m.	0.2 ± 0.4[a]	0.2 ± 0.4	0	1.5 ± 1.9*	0.3 ± 0.5*	1.3 ± 1.6*
3	+	+	Silymarin 500 p.p.m.	0.7 ± 1.0	0	0.7 ± 1.0	0.4 ± 0.7**	0*	0.4 ± 0.7**
4	+	−		0	0	0	0	0	0
5	−	+		0	0	0	0	0	0
6	−	−	Silymarin 500 p.p.m.	0	0	0	0	0	0
7	−	−		0	0	0	0	0	0

AD, adenomas; ADC, adenocarcinomas. Mean ± SD.
*, **: Significantly different from group 1 at $P < 0.05$ and $P < 0.01$, respectively, by Tukey multiple comparison post-test.

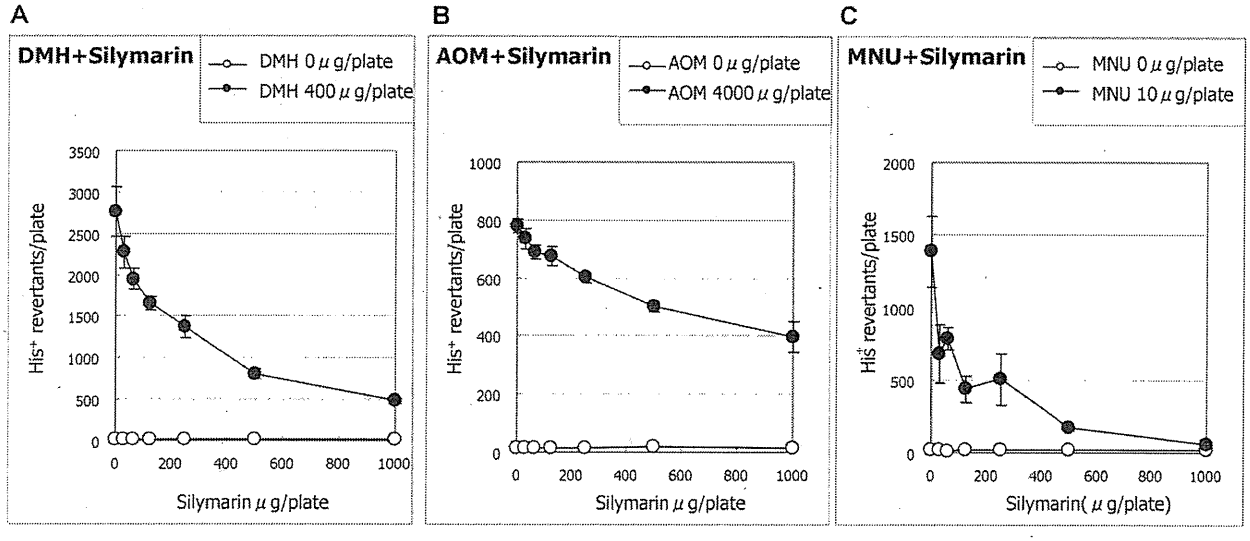

Fig. 2. Antigenotoxic activity of silymarin without S9 mix in *S. typhimurium* strain YG7108 induced by DMH (**A**), AOM (**B**) and MNU (**C**). Filled circle assayed with chemicals, open circle assayed without chemicals.

(supplementary Figure S1 is available at *Carcinogenesis* Online). It suggests that the antigenotoxic efficacy is not reduced by the metabolism *in vivo*. Nevertheless, the efficacy of antigenotoxic activity of silymarin was less pronounced *in vivo* than *in vitro*. For the bacterial mutation assays, each chemical and silymarin were directly mixed in the medium. Therefore, we speculate that the route of exposure, i.e. oral administration in rats, and the effective concentration of silymarin in the colon might account for the different efficacy between *in vivo* and *in vitro*.

When DMH is administrated by single subcutaneous injection, *N*7-methylguanine and O^6-methylguanine are detected in colon, kidney and liver in mice (31). O^6-Methylguanine DNA adduct is a potent

detrimental lesion for colorectal cancer and induces G:C to A:T transitions. The levels of O^6-methylguanine are highly distributed in the distal colon by DMH treatment (32) and histologically altered crypts often have β-catenin gene mutations (33). In the present study, the dominant base substitution in the *gpt* gene induced by DMH treatment was G:C to A:T transitions (supplementary Table IV is available at *Carcinogenesis* Online) and most of the tumors developed in distal area. To induce DNA mutations, DNA replication is required. Silymarin is known to inhibit cancer cell proliferation and induce apoptosis (8). Hence, the inhibitory effects on cell proliferation might play roles in the reduction of genotoxicity and carcinogenicity in the colon of silymarin-treated rats (Tables II–IV).

Silymarin is also reported to possess the anti-inflammatory activity (1). DMH initiation followed by DSS modification model is an established medium-term colorectal bioassay for mice (14,15) and rats (16,17). DSS induced massive inflammation on colonic mucosa by drinking administration (14,34). Under the inflammatory environment, infiltrating mast cells produced genotoxic superoxide anions. In this study, genotoxicity was not induced by DSS treatments (Table II). However, in the silymarin-treated groups, the number of colorectal mucosal tumors was reduced in a dose-dependent manner of silymarin (Tables III and IV). Oral administrated silymarin might prevent inflammation via inhibition of cytokine induction. In colon tissues from AOM-treated rats, inducible nitric oxide synthase and cyclooxygenase-2 expression levels are inhibited by dietary treatment of silibinin (35). These findings suggest that silymarin has an effective influence against promotion by DSS-induced inflammation and it might be a cause for the anticarcinogenic effect of dietary administration of silymarin.

In summary, the current study revealed the antigenotoxic potency of silymarin against alkylating agents, and suggests that the antigenotoxic efficiency along with its inhibitory effects on cell proliferation and inflammation might contribute to the effective tumor reduction *in vivo*. Our results also indicate that F344 *gpt* delta rats are useful for screening cancer chemopreventive compounds as well as environmental genotoxic carcinogens (20).

Supplementary material

Supplementary Tables I–IV and Figure S1 can be found at http://carcin.oxfordjournals.org/

Funding

This work was supported by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT, 18201010; MEXT, 22241016), the Ministry of Health, Labour and Welfare, Japan (MHLW, H21-Food-General-009) and the Japan Health Sciences Foundation (KHB1007) and for cancer research from MHLW (20 designated-8).

Conflict of Interest Statement: None declared.

References

1. Kidd,P.M. (2009) Bioavailability and activity of phytosome complexes from botanical polyphenols: the silymarin, curcumin, green tea, and grape seed extracts. *Altern. Med. Rev.*, **14**, 226–246.
2. Comelli,M.C. *et al.* (2007) Toward the definition of the mechanism of action of silymarin: activities related to cellular protection from toxic damage induced by chemotherapy. *Integr. Cancer Ther.*, **6**, 120–129.
3. Kroll,D.J. *et al.* (2007) Milk thistle nomenclature: why it matters in cancer research and pharmacokinetic studies. *Integr. Cancer Ther.*, **6**, 110–119.
4. Kaur,M. *et al.* (2007) Silymarin and epithelial cancer chemoprevention: how close we are to bedside? *Toxicol. Appl. Pharmacol.*, **224**, 350–359.
5. Ramasamy,K. *et al.* (2008) Multitargeted therapy of cancer by silymarin. *Cancer Lett.*, **269**, 352–362.
6. Hoh,C.S. *et al.* (2007) Quantitation of silibinin, a putative cancer chemopreventive agent derived from milk thistle (Silybum marianum), in human plasma by high-performance liquid chromatography and identification of possible metabolites. *J. Agric. Food Chem.*, **55**, 2532–2535.
7. Tanaka,T. (2009) Colorectal carcinogenesis: review of human and experimental animal studies. *J. Carcinog.*, **8**, 5.
8. Kaur,M. *et al.* (2009) Silibinin suppresses growth and induces apoptotic death of human colorectal carcinoma LoVo cells in culture and tumor xenograft. *Mol. Cancer Ther.*, **8**, 2366–2374.
9. Singh,R.P. *et al.* (2008) Silibinin inhibits colorectal cancer growth by inhibiting tumor cell proliferation and angiogenesis. *Cancer Res.*, **68**, 2043–2050.
10. Kohno,H. *et al.* (2002) Silymarin, a naturally occurring polyphenolic antioxidant flavonoid, inhibits azoxymethane-induced colon carcinogenesis in male F344 rats. *Int. J. Cancer*, **101**, 461–468.
11. Sangeetha,N. *et al.* (2010) Oral supplementation of silibinin prevents colon carcinogenesis in a long term preclinical model. *Eur. J. Pharmacol.*, **643**, 93–100.
12. Ravichandran,K. *et al.* (2010) Inhibitory effect of silibinin against azoxymethane-induced colon tumorigenesis in A/J mice. *Clin. Cancer Res.*, **16**, 4595–4606.
13. Rajamanickam,S. *et al.* (2010) Chemoprevention of intestinal tumorigenesis in APCmin/+ mice by silibinin. *Cancer Res.*, **70**, 2368–2378.
14. Tanaka,T. *et al.* (2003) A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. *Cancer Sci.*, **94**, 965–973.
15. Kohno,H. *et al.* (2005) Beta-catenin mutations in a mouse model of inflammation-related colon carcinogenesis induced by 1,2-dimethylhydrazine and dextran sodium sulfate. *Cancer Sci.*, **96**, 69–76.
16. Tanaka,T. *et al.* (2009) Melatonin suppresses AOM/DSS-induced large bowel oncogenesis in rats. *Chem. Biol. Interact.*, **177**, 128–136.
17. Cho,Y.M. *et al.* (2008) A new medium-term rat colorectal bioassay applying neoplastic lesions as end points for detection of carcinogenesis modifiers effects with weak or controversial modifiers. *Toxicol. Pathol.*, **36**, 459–464.
18. Hayashi,H. *et al.* (2003) Novel transgenic rat for *in vivo* genotoxicity assays using 6-thioguanine and Spi$^-$ selection. *Environ. Mol. Mutagen.*, **41**, 253–259.
19. Nohmi,T. *et al.* (2005) Molecular nature of intrachromosomal deletions and base substitutions induced by environmental mutagens. *Environ.Mol. Mutagen.*, **45**, 150–161.
20. Toyoda-Hokaiwado,N. *et al.* (2010) Integration of *in vivo* genotoxicity and short-term carcinogenicity assays using F344 *gpt* delta transgenic rats: *in vivo* mutagenicity of 2,4-diaminotoluene and 2,6-diaminotoluene structural isomers. *Toxicol Sci.*, **114**, 71–78.
21. Yamada,M. *et al.* (1997) New tester strains of *Salmonella typhimurium* lacking O^6-methylguanine DNA methyltransferases and highly sensitive to mutagenic alkylating agents. *Mutat. Res.*, **381**, 15–24.
22. Yamada,M. *et al.* (1995) Construction and characterization of mutants of *Salmonella typhimurium* deficient in DNA repair of O^6-methylguanine. *J. Bacteriol.*, **177**, 1511–1519.
23. Bird,R.P. (1987) Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. *Cancer Lett.*, **37**, 147–151.
24. Yamada,Y. *et al.* (2001) Sequential analysis of morphological and biological properties of beta-catenin-accumulated crypts, provable premalignant lesions independent of aberrant crypt foci in rat colon carcinogenesis. *Cancer Res.*, **61**, 1874–1878.
25. Cooper,H.S. *et al.* (1993) Clinicopathologic study of dextran sulfate sodium experimental murine colitis. *Lab Invest*, **69**, 238–249.
26. Suzuki,R. *et al.* (2004) Sequential observations on the occurrence of preneoplastic and neoplastic lesions in mouse colon treated with azoxymethane and dextran sodium sulfate. *Cancer Sci.*, **95**, 721–727.
27. Nohmi,T. *et al.* (1996) A new transgenic mouse mutagenesis test system using Spi$^-$ and 6-thioguanine selections. *Environ. Mol. Mutagen.*, **28**, 465–470.
28. Nohmi,T. *et al.* (2000) Recent advances in the protocols of transgenic mouse mutation assays. *Mutat. Res.*, **455**, 191–215.
29. Maron,D.M. *et al.* (1983) Revised methods for the Salmonella mutagenicity test. *Mutat. Res.*, **113**, 173–215.
30. Fiala,E.S. (1977) Investigations into the metabolism and mode of action of the colon carcinogens 1,2-dimethylhydrazine and azoxymethane. *Cancer*, **40**, 2436–2445.
31. Cooper,H.K. *et al.* (1978) DNA alkylation in mice with genetically different susceptibility to 1,2-dimethylhydrazine-induced colon carcinogenesis. *Cancer Res.*, **38**, 3063–3065.

32. Jackson,P.E. *et al.* (2003) Associations between tissue-specific DNA alkylation, DNA repair and cell proliferation in the colon and colon tumour yield in mice treated with 1,2-dimethylhydrazine. *Carcinogenesis*, **24**, 527–533.
33. Yamada,Y. *et al.* (2000) Frequent beta-catenin gene mutations and accumulations of the protein in the putative preneoplastic lesions lacking macroscopic aberrant crypt foci appearance, in rat colon carcinogenesis. *Cancer Res.*, **60**, 3323–3327.
34. Suzuki,R. *et al.* (2005) Dose-dependent promoting effect of dextran sodium sulfate on mouse colon carcinogenesis initiated with azoxymethane. *Histol. Histopathol.*, **20**, 483–492.
35. Velmurugan,B. *et al.* (2008) Inhibition of azoxymethane-induced colonic aberrant crypt foci formation by silibinin in male Fisher 344 rats. *Cancer Prev. Res. (Phila)*, **1**, 376–384.

Received February 13, 2011; revised June 9, 2011; accepted July 29, 2011

TOXICOLOGICAL SCIENCES 122(2), 406–414 (2011)
doi:10.1093/toxsci/kfr139
Advance Access publication May 27, 2011

Site-Specific *In Vivo* Mutagenicity in the Kidney of *gpt* Delta Rats Given a Carcinogenic Dose of Ochratoxin A

Daisuke Hibi,* Yuta Suzuki,* Yuji Ishii,* Meilan Jin,* Maiko Watanabe,† Yoshiko Sugita-Konishi,† Tokuma Yanai,‡ Takehiko Nohmi,§ Akiyoshi Nishikawa,¶ and Takashi Umemura*,[1]

*Division of Pathology, and †Division of Microbiology, National Institute of Health Sciences, Tokyo 158-8501, Japan; ‡Laboratory of Veterinary Pathology, Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University, Gifu 501-1193, Japan; §Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo 158-8501, Japan; and ¶Biological Safety Research Center, National Institute of Health Sciences, Tokyo 158-8501, Japan

[1]To whom correspondence should be addressed at Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Fax: +81-3-3700-1425. E-mail: umemura@nihs.go.jp.

Received February 18, 2011; accepted April 26, 2011

Ochratoxin A (OTA) can induce renal tumors that originate from the S3 segment of the proximal tubules in rodents, but the results of conventional mutagenicity tests have caused controversy regarding the role of genotoxic mechanisms in the carcinogenesis. Human exposure to OTA from various foods is unavoidable. Therefore, an understanding of OTA-induced renal carcinogenesis is necessary for accurate estimates of the human risk hazard. In the present study, a 13-week exposure of *gpt* delta rats to OTA at a carcinogenic dose induced karyomegaly and apoptosis at the outer stripe of the outer medulla (OM) of the kidney but failed to affect the reporter gene mutations in DNA extracted from whole kidneys. This site specificity resulting from the kinetics of specific transporters might be responsible for the negative outcome of *in vivo* mutagenicity. The kidney was then macroscopically divided, based on anatomical characteristics, into the cortex, the OM, and the inner medulla, each of which was histopathologically confirmed. Spi$^-$ mutant frequencies (MFs) but not *gpt* MFs in the OM after a 4-week exposure to OTA were significantly higher than in controls despite the absence of cortical changes. There were also no changes in 8-hydroxydeoxyguanosine levels in kidney DNA. These results strongly suggest the involvement of a genotoxic mechanism, with the exception of oxidative DNA damage in OTA-induced renal carcinogenesis. In addition, the reporter gene mutation assay using DNA from target sites could be a more powerful tool to investigate *in vivo* genotoxicities.

Key Words: ***gpt*** **delta rat; mutagenicity; ochratoxin A; site specificity.**

Ochratoxin A (OTA) is a mycotoxin produced by fungal species, some of which belong to the *Penicillium* and *Aspergillus* genera and is often found as a contaminant in cereals and agricultural products (EFSA, 2006). In rodents, OTA induces a high incidence of renal adenomas/carcinomas (Bendele *et al.*, 1985; Mantle *et al.*, 2005; NTP, 1989). OTA is also a well-known nephrotoxicant and might be associated with Balkan endemic nephropathy and urinary tract tumors in humans (JECFA, 2001). Accordingly, OTA is classified by the International Agency for Research on Cancer as a 2B group compound. Nevertheless, human exposure to OTA from various foods is still considerable (EFSA, 2006), and currently, there is no way of preventing exposure to the contaminant. Therefore, an understanding of the modes of action underlying OTA-induced renal carcinogenesis is critical for assessments of the hazard to human health.

Although the formation of OTA-specific DNA adducts has been demonstrated in the kidney of rats treated with OTA (Mantle *et al.*, 2010), contradictory data of no OTA-specific DNA adducts detected have also been reported (Delatour *et al.*, 2008). An *in vitro* study using rat proximal tubular cells revealed that OTA induced oxidative DNA damage, possibly by depletion of cellular glutathione (Schaaf *et al.*, 2002), whereas an *in vivo* study found that exposure of rats to OTA increased lipid peroxidation levels in the kidneys (Ozçelik *et al.*, 2004). However, antioxidants combined with OTA attenuated the OTA-induced nephrotoxicity in rats but did not affect the incidence of renal tumors (Pfohl-Leszkowicz *et al.*, 2002). In tests for reverse gene mutations, exposure to OTA had no effect on mutagenicity in most Ames tests (JECFA, 2001); the exceptions were tests conducted on mouse kidney microsomes (Obrecht-Pflumio *et al.*, 1999) and rat hepatocytes exposed to OTA (Hennig *et al.*, 1991). The results of other genotoxicity assays such as unscheduled DNA synthesis, *in vitro* sister chromatid exchange, and *in vitro* chromosomal aberration have not yielded consistent results (JECFA, 2001).

Reporter gene mutation assays in which the absorption, distribution, metabolism, and excretion of the test chemicals can be taken into consideration are promising tools for investigation of the genotoxic mechanisms of carcinogenic chemicals that

TABLE 1
Body and Kidney Weights in F344 *gpt* Delta Rats Treated with OTA

	4 Weeks				13 Weeks			
	Males		Females		Males		Females	
	Control	OTA	Control	OTA	Control	OTA	Control	OTA
Body weight (g)	241 ± 8[a]	229 ± 14	156 ± 4	142 ± 3	356 ± 7	343 ± 18	196 ± 13	185 ± 8
Kidney weight								
Absolute weight (g)	1.63 ± 0.07	1.31 ± 0.11**	1.09 ± 0.04	0.92 ± 0.05**	2.10 ± 0.08	1.60 ± 0.11**	1.18 ± 0.09	0.94 ± 0.03**
Relative weight (g/100 g b.w.)	0.68 ± 0.03	0.57 ± 0.03**	0.70 ± 0.05	0.65 ± 0.03	0.59 ± 0.03	0.47 ± 0.02**	0.60 ± 0.04	0.51 ± 0.11*

[a]Mean ± SD.
*,**Significantly different from the control group at $p < 0.05$, 0.01, respectively.

show equivocal outcomes in conventional mutagenicity tests (WHO, 2006). An additional advantage of this assay is the ability to investigate *in vivo* mutagenicity at the target organs (Tasaki *et al.*, 2010; Umemura *et al.*, 2006). However, some chemicals exert their carcinogenic effects at specific sites in these target organs, possibly due to the kinetics of chemical transporters (Launay-Vacher *et al.*, 2006) and the location of various carcinogenic modifying factors such as activating (Lohr *et al.*, 1998) and DNA repair enzymes (Hong *et al.*, 2001). We have demonstrated reporter gene mutations in mucosal epithelium of the colon (Okamura *et al.*, 2010) and transitional epithelium of the urinary bladder (Suzuki *et al.*, 1996). Although mutant frequency (MF) of *lacI* gene in different parts of the kidney was demonstrated by means of aspirating the samples with a pipette (de Boer *et al.*, 2000), the use might be limited in terms of a small amount of the corrected tissue. Thus, there have been few reports of site-specific *in vivo* mutations in parenchymatous viscera.

In the present study, OTA-specific target sites were confirmed in the kidneys of *gpt* delta rats, and the reporter gene mutation assay was performed using DNA extracted from whole kidneys. In order to clarify involvement of genotoxic mechanisms in OTA-induced renal carcinogenesis, the kidney was divided into the cortex (COR) and medulla and DNA was subsequently extracted to examine oxidative DNA damage and reporter gene mutation frequencies.

MATERIALS AND METHODS

Experimental Animals and Housing Conditions

The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Specific pathogen-free, 5-week-old male and female F344/NSlc-Tg (*gpt* delta) rats carrying about five tandem copies of the transgene lambda EG10 per haploid genome were obtained from Japan SLC (Shizuoka, Japan) and acclimated for 1 week prior to commencement of testing. Animals were housed in a room with a barrier system and maintained under constant temperature (23 ± 2°C), relative humidity (55 ± 5%), air change (12 times/h), and lighting (12 h light-dark cycle) with free access to an Oriental CRF-1-basal diet (Oriental Yeast Co., Ltd, Tokyo, Japan) and tap water.

Test Compound

OTA was extracted from a culture of *Aspergillus ochraceus* (BD-5). Fermentative production of OTA was performed based on the culture and extraction methods of Kumata *et al.* (1980) with minor modifications. Several 500-ml Erlenmeyer flasks containing 100 g of polished rice were autoclaved before the addition of 20 ml of sterilized water. The rice was inoculated with spores of *A. ochraceus* and incubated for 2 weeks at 25°C. After incubation, 200 ml of chloroform-acetic acid (99:1) was added to the moldy rice in each flask. The extracts collected from the flasks were concentrated and precipitated with 4 l of hexane on a stirrer. The precipitate was dissolved in 500 ml of chloroform and subjected to chromatography using a silica gel column and mobile phases of benzene-acetic acid (100:0 to 88:12) with a linear gradient. The benzene-acetic acid (95:5 and 92.5:7.5) eluates were evaporated to dryness. Benzene was added to the extract, and the solution was heated followed by gentle cooling for crystallization of OTA. The purification of crystals was confirmed by high-performance liquid chromatography according to the method of Sugita-Konishi *et al.* (2006). The purity of OTA was estimated to be > 95% from the area percentage of the chromatogram (data not shown).

Animal Treatment

Experiment I. Groups of four to five male and female *gpt* delta rats were administered OTA at a concentration of 5 ppm in the basal diet or basal diet without supplement (control) for 4 or 13 weeks. The dietary dose level of 5 ppm was selected based on the carcinogenic dose reported in a 2-year carcinogenicity study of rats (Mantle *et al.*, 2005). Based on the preliminary

TABLE 2
Food Consumption and Intake of OTA in F344 *gpt* Delta Rats Treated for 13 Weeks

			Intake of OTA			
	Food consumption (g/rat/day)		Daily (mg/kg b.w./day)		Total (mg/kg b.w.)	
Group	Males	Females	Males	Females	Males	Females
Control	20.0	15.2	—	—	—	—
OTA	19.3	12.3	0.36	0.38	32.7	34.0

study for OTA stability, the diet was prepared once every week and was stored in the dark at 4°C prior to use. At necropsy, the kidneys were weighed, and a portion of the harvested kidneys were fixed in 10% neutral buffered formalin. Fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE). The remaining kidneys were stored at −80°C for *in vivo* mutation assays and the measurement of 8-hydroxydeoxyguanosine (8-OHdG) levels.

Experiment II. Groups of five male *gpt* delta rats were fed 5 ppm OTA or basal diet without supplement for 4 weeks. At necropsy, the harvested kidneys were divided into the COR, the outer medulla (OM), and the inner medulla. After the kidneys were cut along the long axis, the COR was macroscopically separated with curving scissors using the arcuate arteries at the boundary of the COR and medulla as a landmark. The OM was divided on the basis of the distinguishable color of each part (i.e., OM: pale brown; inner medulla: white). The COR and OM were stored at −80°C for *in vivo* mutation assays and the measurement of 8-OHdG levels. A portion of each part was fixed in 10% neutral buffered formalin and routinely processed for histopathological examinations.

Measurement of Nuclear 8-OHdG

DNA extraction and digestion was performed according to the method of Nakae *et al.* (1995) and our previous report (Umemura *et al.*, 2006). The samples were homogenized with lysis buffer including commercial DNA isolation kit. The mixture was centrifuged at 10,000 × g for 20 s at 4°C. The deposit was dissolved in 200 µl of enzyme reaction buffer. After treatment with RNase and protease K, the DNA pellet was obtained by washing with 2-propanol and ethanol and centrifugation. The dried DNA pellet was dissolved in 20µM sodium acetate buffer, pH 4.8, and was incubated with 4 µl of nuclease P1 (2000 U/ml) at 70°C for 15 min. Then, 20 µl of 1.0M Tris-HCl buffer, pH 8.2, was added, and the sample was incubated with 4 µl of alkaline phosphatase (2500 U/ml) at 37°C for 60 min. After the addition of 20 µl of 3.0M sodium acetate buffer, pH 5.1, the digested DNA samples were passed through 100,000 NMWL filter (Millipore, Bedford, MA) and injected into the liquid chromatography with electrochemical detection.

In Vivo *Mutation Assays*

6-Thioguanine (6-TG) and Spi⁻ selections were performed using the method of Nohmi *et al.* (2000). Briefly, genomic DNA was extracted from the kidneys of male or female 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 genes encoding *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 then incubated at 37°C for selection of 6-TG-resistant colonies, and *gpt* MF was calculated by dividing the number of *gpt* mutants after clonal correction by the number of rescued phages. *gpt* Mutations were characterized by amplifying a 739-bp DNA fragment containing the 456-bp coding region of the *gpt* gene (Nohmi *et al.*, 2000) and sequencing the PCR products with an Applied Biosystems 3730*xl* DNA Analyzer (Applied Biosystems Japan Ltd). 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. The next day, plaques (Spi⁻ candidates) were punched out with sterilized glass pipettes and the agar plugs were suspended in SM buffer. The Spi⁻ phenotype

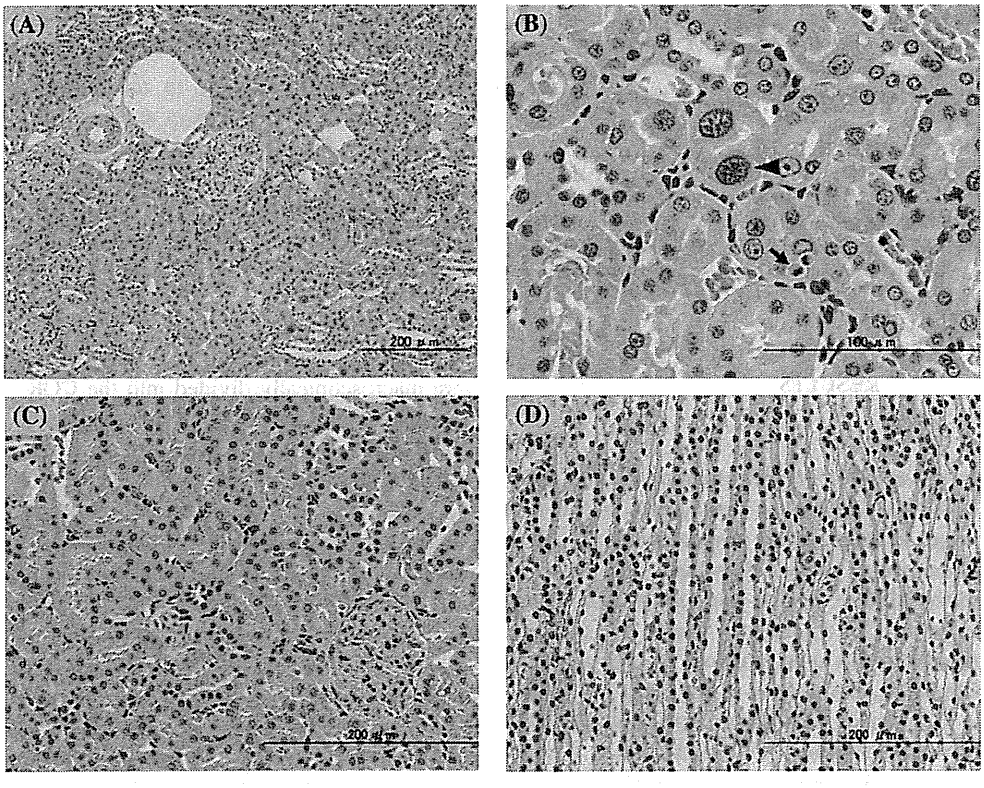

FIG. 1. Boundary of the renal COR and medulla (A) and OSOM (B) of a male F344 *gpt* delta rat treated with OTA. Vacuolation, apoptosis (arrow), and karyomegaly (arrow head) were apparent at the S3 segment of the proximal tubules of the OSOM. There were no remarkable changes in the COR (C) and the inner stripe of the OM (D). HE stain.

TABLE 3
gpt MFs in the Kidneys of F344 *gpt* Delta Rats Treated with OTA for 13 Weeks

Sexes	Treatment	Animal no.	Cm^R colonies ($\times 10^5$)	Cm^R and 6-TG^R colonies	MF ($\times 10^{-5}$)	Mean ± SD
Males	Control	5	3.78	2	0.53	
		6	3.47	0[a]	—	
		7	5.00	5	1.00	
		8	3.42	2	0.58	0.70 ± 0.26
	OTA	22	2.75	3	1.09	
		23	3.96	4	1.01	
		24	4.41	2	0.45	
		25	2.61	2	0.77	
		26	2.66	3	1.13	0.89 ± 0.28
Females	Control	35	2.66	1	0.38	
		36	5.49	3	0.55	
		37	6.84	1	0.15	
		38	4.32	0[a]	—	0.36 ± 0.20
	OTA	49	5.54	2	0.36	
		50	5.63	1	0.18	
		51	3.78	0[a]	—	
		52	3.06	1	0.33	
		53	1.85	2	1.08	0.49 ± 0.41

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

was confirmed by spotting the suspensions on three types of plates where XL-1 Blue MRA, XL-1 Blue MRA P2, or WL95 P2 strain was spread with soft agar. Spi^- mutants, which made clear plaques on every plate, were counted. Positive DNA samples (controls) were simultaneously applied in all the *in vivo* mutation assays.

Statistics

The data for body and kidney weights, 8-OHdG levels, and *gpt* and Spi^- MFs were analyzed statistically as follows. Variance in the data was checked for homogeneity with the *F*-test. When the data were homogeneous, the Student's *t*-test was applied. In heterogeneous cases, Welch's *t*-test was used instead.

RESULTS

Experiment I

Body and kidney weights, food consumption, and OTA intake. Body and kidney weights are summarized in Table 1. No significant changes in body weight were observed in either sex during the experimental period. Significant decreases in absolute and/or relative kidney weights were observed in males and females treated with OTA at 4 and 13 weeks. The average food consumption per day was approximately 20.0 g for males and 15.2 g for females in the control group and 19.3 g for males and 12.3 g for females in OTA group (Table 2). The average daily OTA intakes were 0.36 mg/kg body weight (b.w.)/day for males and 0.38 mg/kg b.w./day for females. The total intakes of OTA over the 13 weeks were 32.7 mg/kg for males and 34.0 mg/kg b.w. for females.

Histopathological findings in the kidneys. Histopathological features in the kidneys of the control and treatment groups are shown in Figure 1. In both sexes treated with OTA, apoptosis denoted by pyknotic and fragmented nuclei, karyomegaly characterized by prominent nuclear enlargement, and vacuolation in proximal tubule epithelial cells in the outer stripe of the OM (OSOM) were found (Figs. 1A and 1B). The incidence of apoptosis and karyomegaly reached 100% in all treated rats from 4 weeks. The incidence of vacuolation was 40% at 4 weeks and 100% at 13 weeks in the treated males and 100% from 4 weeks in the treated females. These changes were not found in the COR, the inner stripe of the OM, or the inner medulla (Figs. 1C and 1D).

In vivo *mutation assays in the kidneys.* Data for *gpt* and Spi^- MFs in the kidney of male and female *gpt* delta rats treated with OTA for 13 weeks are summarized in Tables 3 and 4. There were no significant differences in the *gpt* MFs in either sex between the groups (males: 0.89 ± 0.28 in the OTA group, 0.70 ± 0.26 in the control group; females: 0.49 ± 0.41 in the OTA group, 0.36 ± 0.20 × 10^{-5} in the control group). To characterize the types of *gpt* mutations caused by OTA exposure, we determined the *gpt* mutation spectra in the *gpt* mutant colonies. As a result, OTA-specific *gpt* mutation spectra were not found (Table 5). Spi^- MFs in the treated males and females (males: 0.14 ± 0.12; females: 0.32 ± 0.23 × 10^{-5}) were also not significantly different from those in the relevant control groups (males: 0.18 ± 0.04; females: 0.14 ± 0.08 × 10^{-5}).

Oxidative DNA damage in the kidneys. The results for 8-OHdG levels in the kidneys at 13 weeks of OTA exposure are shown in Supplementary figure 1. The 8-OHdG levels in both sexes treated with OTA (males: 0.31 ± 0.05; females: 0.42 ± 0.15 8-OHdG/10^5 dG) had no significant differences as compared with the control values (males: 0.39 ± 0.08 at 13 weeks; females: 0.32 ± 0.03 8-OHdG/10^5 dG).

Experiment II

In vivo *mutation assay in the COR and OM.* The kidneys were macroscopically divided into the COR, the OM, and the inner medulla (Fig. 2A). The cell components of each part were confirmed using HE-stained sections. The COR included cortical components such as the S1 and S2 segments of the proximal tubules and glomeruli, but not the medullar components (Fig. 2B). The S3 segment of the proximal tubules primarily occupied the OM (Fig. 2C). Data for *gpt* and Spi^- MFs in the COR and OM in the kidney of male *gpt* delta rats treated with OTA for 4 weeks are shown in Tables 6 and 7, respectively. No significant differences in *gpt* MFs were observed in either the COR or the OM (COR: 0.28 ± 0.12 in the OTA group, 0.26 ± 0.17 × 10^{-5} in the control group; OM: 0.38 ± 0.12 in the OTA group, 0.20 ± 0.12 × 10^{-5} in the control group). Also, OTA-specific *gpt* mutation spectra were not observed (Table 8). Interestingly, in the OM, Spi^- MFs in the OTA treatment group were significantly higher ($p < 0.05$)

TABLE 4
Spi$^-$ MFs in the Kidneys of F344 *gpt* Delta Rats Treated with OTA for 13 Weeks

Sexes	Treatment	Animal no.	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaque within WL95 (P2)	MF ($\times 10^{-5}$)	Mean ± SD
Males	Control	5	14.49	0[a]	—	
		6	13.32	2	0.15	
		7	26.37	6	0.23	
		8	13.14	2	0.15	0.18 ± 0.04
	OTA	22	12.06	1	0.08	
		23	8.82	1	0.11	
		24	13.68	1	0.07	
		25	13.95	1	0.07	
		26	8.37	3	0.36	0.14 ± 0.12
Females	Control	35	16.47	1	0.06	
		36	14.4	1	0.07	
		37	27.09	5	0.18	
		38	17.28	4	0.23	0.14 ± 0.08
	OTA	49	15.12	3	0.20	
		50	13.41	4	0.30	
		51	9.45	3	0.32	
		52	10.17	7	0.69	
		53	10.89	1	0.09	0.32 ± 0.23

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

than in the control group (0.45 ± 0.15 in the OTA group, 0.16 ± 0.21 × 10^{-5} in the control group). However, there were no significant differences in Spi$^-$ MFs in the COR compared with the control group (0.27 ± 0.10 in the OTA group, 0.25 ± 0.14 × 10^{-5} in the control group).

Oxidative DNA damage in the COR and OM. The levels of 8-OHdG in the COR and OM are shown in Figure 3. There were no significant differences in 8-OHdG levels in the COR or OM of rat kidneys treated with OTA (COR: 0.21 ± 0.02, OM: 0.20 ± 0.01 8-OHdG/10^5 dG) versus the controls (COR: 0.21 ± 0.05, OM: 0.16 ± 0.04 8-OHdG/10^5 dG).

DISCUSSION

Exposure of *gpt* delta rats to OTA at the carcinogenic dose caused karyomegaly and apoptosis of tubular epithelial cells at OSOM, in line with previously reported data (Boorman *et al.*, 1992; Rached *et al.*, 2007). These histopathological changes were not observed at any other regions. Judging from their morphological features, the affected tubules were surely the S3 segment of the proximal tubules. OTA is excreted and reabsorbed in the renal tubules through several transporter proteins such as organic anion transporters, H^+-dipeptide cotransporter, and multidrug-resistant protein-2 (Pfohl-Leszkowicz and Manderville, 2007). In rats, reabsorption rates that are dependent on organic anion transporters are highest at the S3 segment (Dahlmann *et al.*, 1998). Although the pathogenesis of apoptosis remains uncertain, it is thought that OTA can potentially arrest G2/M progression in the cell cycle, consequently leading to the induction of abnormally enlarged nuclear formation under higher cell proliferating

TABLE 5
Mutant Spectra of *gpt* Mutant Colonies in the Kidneys of F344 *gpt* Delta Rats Treated with OTA for 13 Weeks

	Males				Females			
	Control		OTA		Control		OTA	
	Number (%)	MF ($\times 10^{-5}$)	Number (%)	MF ($\times 10^{-5}$)	Number (%)	MF ($\times 10^{-5}$)	Number (%)	MF ($\times 10^{-5}$)
Base substitution								
Transversions								
GC:TA	3[a] (33.3)	0.25 ± 0.05	1 (7.14)	0.05 ± 0.10	2 (40.0)	0.19 ± 0.19	2 (33.3)	0.09 ± 0.10
GC:CG	0	0	0	0	0	0	1 (16.7)	0.14 ± 0.27
AT:TA	0	0	0	0	0	0	0	0
AT:CG	0	0	1 (7.14)	0.05 ± 0.11	0	0	0	0
Transitions								
GC:AT	3 (33.3)	0.20 ± 0.35	9 (64.3)	0.60 ± 0.43	1 (20.0)	0.06 ± 0.11	1 (16.7)	0.14 ± 0.27
AT:GC	0	0	0	0	2 (40.0)	0.11 ± 0.10	1 (16.7)	0.08 ± 0.16
Deletions								
Single bp	0	0	1 (7.14)	0.07 ± 0.16	0	0	1 (16.7)	0.05 ± 0.01
Over 2 bp	0	0	0	0	0	0	0	0
Insertions	1 (11.1)	0.07 ± 0.12	0	0	0	0	0	0
Complexes	2 (22.2)	0.19 ± 0.16	2 (14.3)	0.12 ± 0.18	0	0	0	0
Total	9	0.70 ± 0.26	14	0.89 ± 0.28	5	0.36 ± 0.20	6	0.49 ± 0.41

[a]The number of colonies with independent mutations.

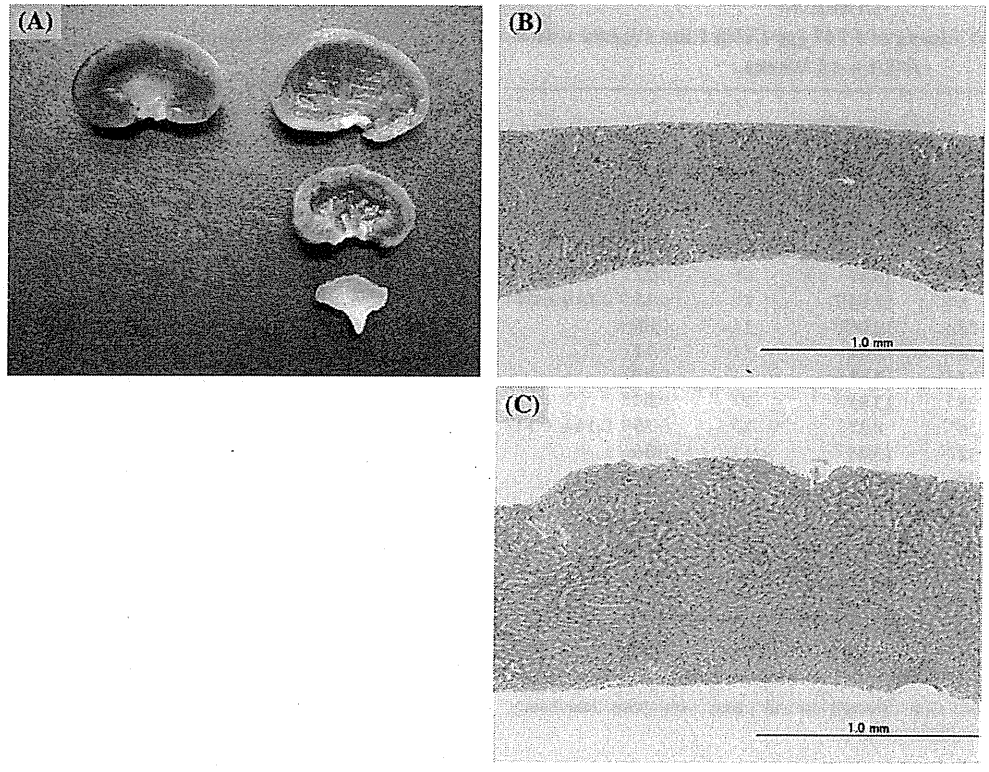

FIG. 2. (A) Kidney cut on the long axis (left). The kidney is divided into three parts, COR (upper right), OM (mid right), and the inner medulla (lower right). (B) Microscopic examination of the S1 and S2 segments of the proximal tubules and the glomeruli, but not the medullar components of the COR.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

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

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

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

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

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

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

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

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

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

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

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

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

[a]The number of colonies with independent mutations.

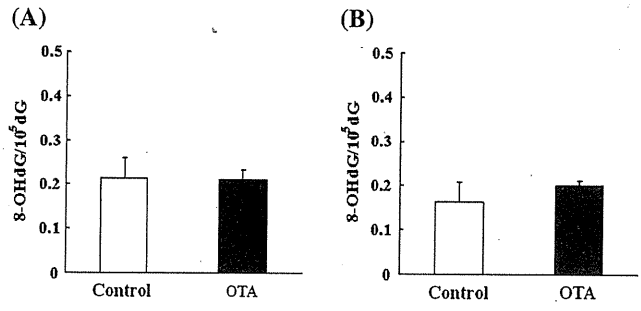

FIG. 3. Changes in 8-OHdG levels of DNA extracted from the COR (A) or OM (B) of *gpt* delta rats fed 5 ppm OTA for 4 weeks. Values are means ± SD of data for five rats.

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

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

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

SUPPLEMENTARY DATA

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

FUNDING

Grant-in-Aid for Technical Research on the Assessment of Food Effects on Health from the Food Safety Commission (#0903); Grant-in-Aid for Cancer Research from the Ministry of Labor and Welfare of Japan.

ACKNOWLEDGMENTS

We appreciate the expert technical assistance of Ms Aki Kijima, Ms Ayako Kaneko, and Ms Yoshimi Komatsu.

REFERENCES

Adler, M., Müller, K., Rached, E., Dekant, W., and Mally, A. (2009). Modulation of key regulators of mitosis linked to chromosomal instability is an early event in ochratoxin A carcinogenicity. *Carcinogenesis* **30,** 711–719.

Bendele, A. M., Carlton, W. W., Krogh, P., and Lillehoj, E. B. (1985). Ochratoxin A carcinogenesis in the (C57BL/6J X C3H) F1 mouse. *J. Natl. Cancer Inst.* **75,** 733–742.

Boorman, G. A., McDonald, M. R., Imoto, S., and Persing, R. (1992). Renal lesions induced by ochratoxin A exposure in the F344 rat. *Toxicol. Pathol.* **20,** 236–245.

Cavin, C., Delatour, T., Marin-Kuan, M., Holzhäuser, D., Higgins, L., Bezençon, C., Guignard, G., Junod, S., Richoz-Payot, J., Gremaud, E., *et al.* (2007). Reduction in antioxidant defenses may contribute to ochratoxin A toxicity and carcinogenicity. *Toxicol. Sci.* **96,** 30–39.

Dahlmann, A., Dantzler, W. H., Silbernagl, S., and Gekle, M. (1998). Detailed mapping of ochratoxin A reabsorption along the rat nephron *in vivo*: the nephrotoxin can be reabsorbed in all nephron segments by different mechanisms. *J. Pharmacol. Exp. Ther.* **286,** 157–162.

de Boer, J. G., Holcroft, J., Cunningham, M. L., and Glickman, B. W. (2000). Tris(2,3-dibromopropyl)phosphate causes a gradient of mutations in the cortex and outer and inner medullas of the kidney of *lacI* transgenic rats. *Environ. Mol. Mutagen.* **36,** 1–4.

Delatour, T., Mally, A., Richoz, J., Ozden, S., Dekant, W., Ihmels, H., Otto, D., Gasparutto, D., Marin-Kuan, M., Schilter, B., *et al.* (2008). Absence of 2'-deoxyguanosine-carbon 8-bound ochratoxin A adduct in rat kidney DNA monitored by isotope dilution LC-MS/MS. *Mol. Nutr. Food Res.* **52,** 472–482.

European Food Safety Authority (EFSA). (2006). Opinion of the Scientific Panel on Contaminants in the Food Chain on a Request from the commission related to ochratoxin A in food. *EFSA J.* **365,** 1–56.

Hassen, W., Abid-Essafi, S., Achour, A., Guezzah, N., Zakhama, A., Ellouz, F., Creppy, E. E., and Bacha, H. (2004). Karyomegaly of tubular kidney cells in human chronic interstitial nephropathy in Tunisia: respective role of ochratoxin A and possible genetic predisposition. *Hum. Exp. Toxicol.* **23,** 339–346.

Hennig, A., Fink-Gremmels, J., and Leistner, L. (1991). Mutagenicity and effects of ochratoxin A on the frequency of sister chromatid exchange after metabolic activation. *IARC Sci. Publ.* **115,** 255–260.

Hong, M. Y., Chapkin, R. S., Morris, J. S., Wang, N., Carroll, R. J., Turner, N. D., Chang, W. C., Davidson, L. A., and Lupton, J. R. (2001). Anatomical site-specific response to DNA damage is related to later tumor development in the rat azoxymethane colon carcinogenesis model. *Carcinogenesis* **22,** 1831–1835.

Joint FAO/WHO Expert Committee on Food Additives (JECFA). (2001). *Ochratoxin A*. JECFA Food Additives Series 47, WHO, Geneva, Switzerland.

Joint FAO/WHO Expert Committee on Food Additives (JECFA). (2008). *Safety Evaluation of Certain Food Additives and Contaminants*. WHO Food Additives Series 59, pp. 357–429. WHO, Geneva, Switzerland.

Jung, K. Y., Takeda, M., Kim, D. K., Tojo, A., Narikawa, S., Yoo, B. S., Hosoyamada, M., Cha, S. H., Sekine, T., and Endou, H. (2001). Characterization of ochratoxin A transport by human organic anion transporters. *Life Sci.* **69,** 2123–2135.

Kumata, K., Amano, R., Ichinoe, M., and Uchiyama, S. (1980). Culture conditions and purification method for large-scale production of ochratoxins by Aspergillus ochraceus. *Shokuhin Eiseigaku Zasshi* **21,** 171–176.

Launay-Vacher, V., Izzedine, H., Karie, S., Hulot, J. S., Baumelou, A., and Deray, G. (2006). Renal tubular drug transporters. *Nephron Physiol.* **103,** 97–106.

Lohr, J. W., Willsky, G. R., and Acara, M. A. (1998). Renal drug metabolism. *Pharmacol. Rev.* **50,** 107–141.

Mally, A., Völkel, W., Amberg, A., Kurz, M., Wanek, P., Eder, E., Hard, G., and Dekant, W. (2005). Functional, biochemical, and pathological effects of repeated oral administration of ochratoxin A to rats. *Chem. Res. Toxicol.* **18,** 1242–1252.

Mantle, P., Kulinskaya, E., and Nestler, S. (2005). Renal tumourigenesis in male rats in response to chronic dietary ochratoxin A. *Food Addit. Contam.* **22** (Suppl. 1), 58–64.

Mantle, P. G., Faucet-Marquis, V., Manderville, R. A., Squillaci, B., and Pfohl-Leszkowicz, A. (2010). Structures of covalent adducts between DNA and ochratoxin A: a new factor in debate about genotoxicity and human risk assessment. *Chem. Res. Toxicol.* **23,** 89–98.

Nakae, D., Mizumoto, Y., Kobayashi, E., Noguchi, O., and Konishi, Y. (1995). Improved genomic/nuclear DNA extraction for 8-hydroxydeoxyguanosine analysis of small amounts of rat liver tissue. *Cancer Lett.* **97,** 233–239.

National Toxicology Program (NTP). (1989). Toxicology and carcinogenesis studies of ochratoxin A (CAS No. 303-47-9) in F344/N rats (gavage studies). *Natl. Toxicol. Program Tech. Rep. Ser.* **358,** 1–142.

Nohmi, T., and Masumura, K. (2005). Molecular nature of intrachromosomal deletions and base substitutions induced by environmental mutagens. *Environ. Mol. Mutagen.* **45,** 150–161.

Nohmi, T., Suzuki, T., and Masumura, K. (2000). Recent advances in the protocols of transgenic mouse mutation assays. *Mutat. Res.* **455,** 191–215.

Obrecht-Pflumio, S., Chassat, T., Dirheimer, G., and Marzin, D. (1999). Genotoxicity of ochratoxin A by Salmonella mutagenicity test after bioactivation by mouse kidney microsomes. *Mutat. Res.* **446,** 95–102.

Okamura, T., Ishii, Y., Suzuki, Y., Inoue, T., Tasaki, M., Kodama, Y., Nohmi, T., Mitsumori, K., Umemura, T., and Nishikawa, A. (2010). Effects of co-treatment of dextran sulfate sodium and MeIQx on genotoxicity and possible carcinogenicity in the colon of p53-deficient mice. *J. Toxicol. Sci.* **35,** 731–741.

Ozçelik, N., Soyöz, M., and Kilinç, I. (2004). Effects of ochratoxin A on oxidative damage in rat kidney: protective role of melatonin. *J. Appl. Toxicol.* **24,** 211–215.

Pfohl-Leszkowicz, A., Bartsch, H., Azémar, B., Mohr, U., Estève, J., and Castegnaro, M. (2002). MESNA protects rats against nephrotoxicity but not carcinogenicity induced by ochratoxin A, implicating two separate pathways. *Facta Univ. Ser. Med. Biol.* **9,** 57–63.

Pfohl-Leszkowicz, A., Grosse, Y., Castegnaro, M., Nicolov, I. G., Chernozemsky, I. N., Bartsch, H., Betbeder, A. M., Creppy, E. E., and Dirheimer, G. (1993). Ochratoxin A-related DNA adducts in urinary tract tumours of Bulgarian subjects. *IARC Sci. Publ.* **124,** 141–148.

Pfohl-Leszkowicz, A., and Manderville, R. A. (2007). Ochratoxin A: an overview on toxicity and carcinogenicity in animals and humans. *Mol. Nutr. Food Res.* **51,** 61–99.

Rached, E., Hard, G. C., Blumbach, K., Weber, K., Draheim, R., Lutz, W. K., Ozden, S., Steger, U., Dekant, W., and Mally, A. (2007). Ochratoxin A: 13-week oral toxicity and cell proliferation in male F344/N rats. *Toxicol. Sci.* **97,** 288–298.

Schaaf, G. J., Nijmeijer, S. M., Maas, R. F., Roestenberg, P., de Groene, E. M., and Fink-Gremmels, J. (2002). The role of oxidative stress in the ochratoxin A-mediated toxicity in proximal tubular cells. *Biochim. Biophys. Acta.* **1588,** 149–158.

Sugita-Konishi, Y., Tanaka, T., Nakajima, M., Fujita, K., Norizuki, H., Mochizuki, N., and Takatori, K. (2006). The comparison of two clean-up procedures, multifunctional column and immunoaffinity colomn, for HPLC determination of ochratoxin A in cereals, raisins and green coffee beans. *Talanta* **69,** 650–655.

Suzuki, T., Itoh, T., Hayashi, M., Nishikawa, Y., Ikezaki, S., Furukawa, F., Takahashi, M., and Sofuni, T. (1996). Organ variation in the mutagenicity of dimethylnitrosamine in Big Blue mice. *Environ. Mol. Mutagen.* **28,** 348–353.

Tasaki, M., Umemura, T., Suzuki, Y., Hibi, D., Inoue, T., Okamura, T., Ishii, Y., Maruyama, S., Nohmi, T., and Nishikawa, A. (2010). Oxidative DNA damage and reporter gene mutation in the livers of *gpt* delta rats given non-genotoxic hepatocarcinogens with cytochrome P450-inducible potency. *Cancer Sci.* **101,** 2525–2530.

Umemura, T., Kanki, K., Kuroiwa, Y., Ishii, Y., Okano, K., Nohmi, T., Nishikawa, A., and Hirose, M. (2006). *In vivo* mutagenicity and initiation following oxidative DNA lesion in the kidneys of rats given potassium bromate. *Cancer Sci.* **97,** 829–835.

World Health Organization (WHO). (2006). *Transgenic Animal Mutagenicity Assays*. Environmental Health Criteria 233. WHO, Geneva, Switzerland.

Yang, N., Chaudhry, M. A., and Wallace, S. S. (2006). Base excision repair by hNTH1 and hOGG1: a two edged sword in the processing of DNA damage in gamma-irradiated human cells. *DNA Repair (Amst)* **5,** 43–51.

Zeljezić, D., Domijan, A. M., and Peraica, M. (2006). DNA damage by ochratoxin A in rat kidney assessed by the alkaline comet assay. *Braz. J. Med. Biol. Res.* **39,** 1563–1568.

Mutation Research 713 (2011) 56–63

Contents lists available at ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

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

Ayumi Yamamoto[1], Yasuteru Sakamoto[2], Kenichi Masumura, Masamitsu Honma, Takehiko Nohmi*

Division of Genetics and Mutagenesis, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

ARTICLE INFO

Article history:
Received 12 February 2011
Received in revised form 20 May 2011
Accepted 23 May 2011
Available online 15 June 2011

Keywords:
Adaptive response
Mismatch repair
Alkylating agents
Radiation

ABSTRACT

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

1. Introduction

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

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

* Corresponding author. Tel.: +81 3 3700 9872; fax: +81 3 3700 2348.
E-mail address: nohmi@nihs.go.jp (T. Nohmi).
[1] Present address: Hachinohe National College of Technology, 16-1 Uwanotai, Tamonoki, Hachinohe-shi, Aomori-ken 039-1192, Japan.
[2] Present address: Ajinomoto Pharmaceuticals Co., Ltd., 1-1 Suzuki-cho, Kawasaki-ku, Kanagawa, 210-8681, Japan.

0027-5107/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.mrfmmm.2011.05.014

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

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

2. Materials and methods

2.1. Cell culture

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

2.2. Chemicals

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

2.3. Irradiation

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

2.4. Combined MNNG treatment and γ-ray exposure

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

2.5. Genotoxicity assays

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

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

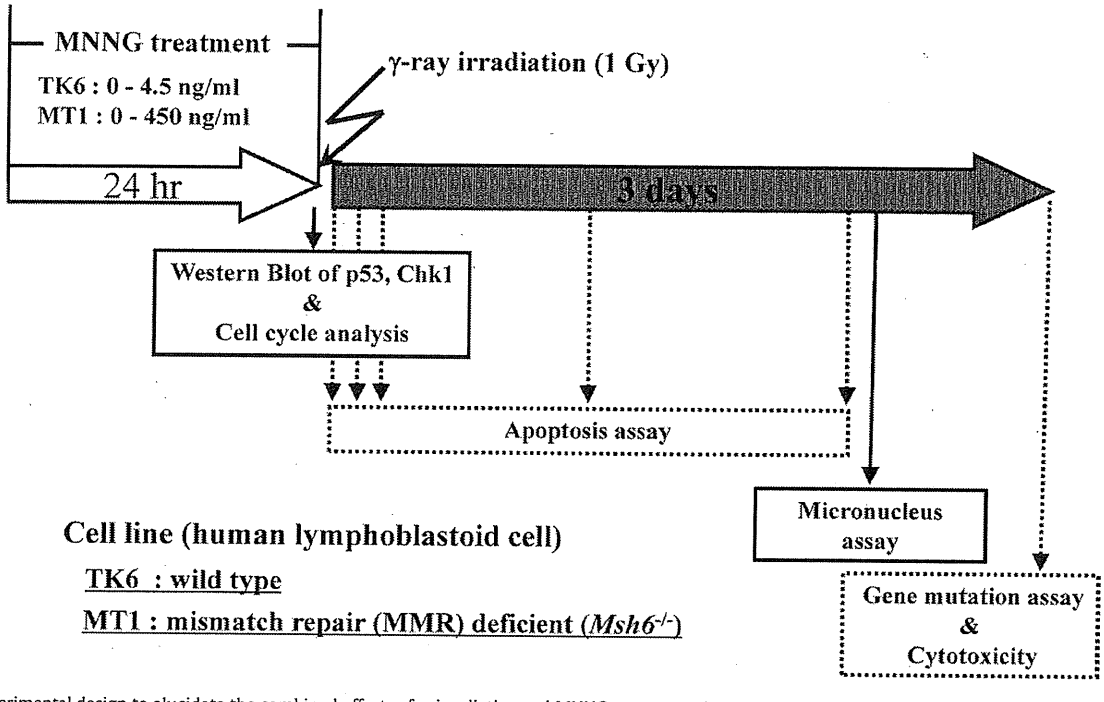

Fig. 1. An experimental design to elucidate the combined effects of γ-irradiation and MNNG treatments in TK6 and MT1 cells. The cells were pre-treated with MNNG at a concentration of 0, 1.5, 3.0 or 4.5 ng/ml in TK6 and 0, 150, 300 or 450 ng/ml in MT1, respectively. After the treatments with MNNG, both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

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

2.6. Apoptosis assay

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

2.7. Cell cycle analysis

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

2.8. Western blot analysis

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

2.9. Statistical analyses

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

3. Results

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

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

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

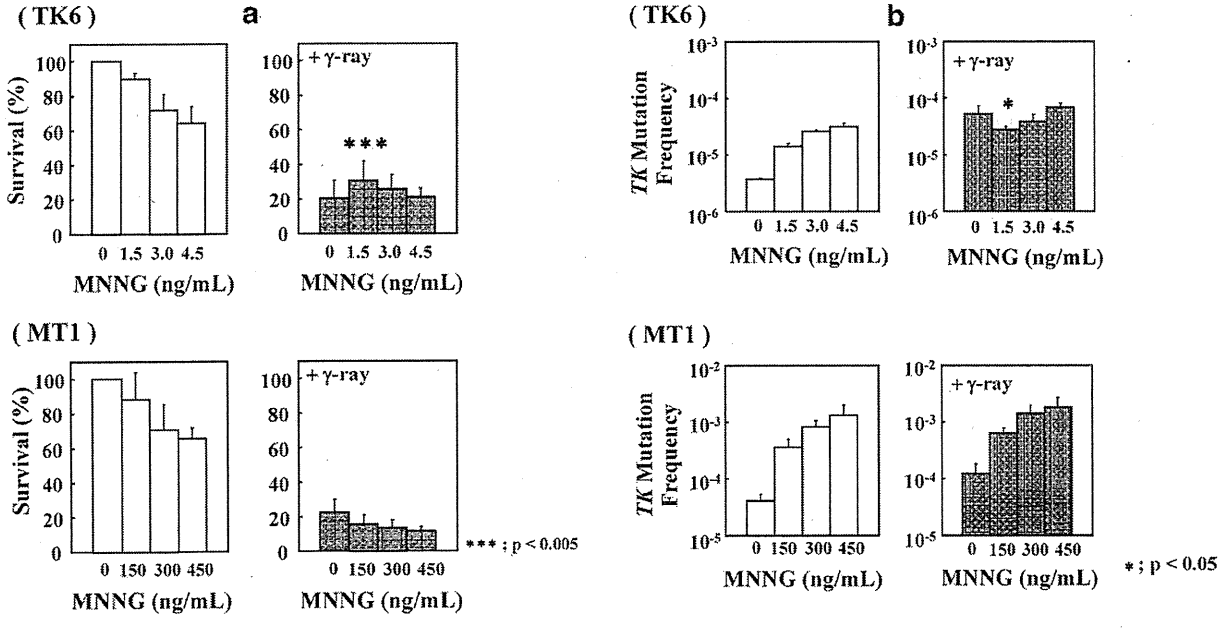

Fig. 2. Cell survival and *TK* gene mutation assays in TK6 and MT1 cells. The effects on cell survival (a) and gene mutations (b) are separately presented. An asterisk (*) and asterisks (***) denote $p < 0.05$ and $p < 0.005$, respectively, in the *t*-test of comparison between MNNG-treated and untreated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

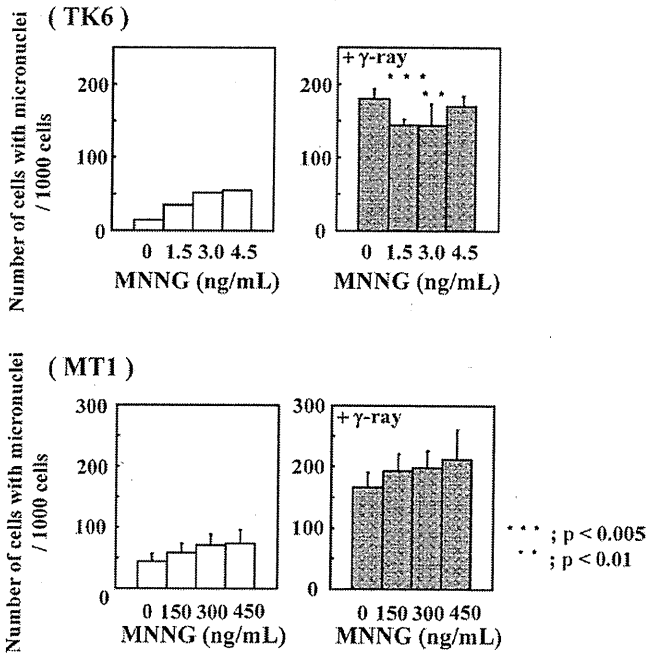

Fig. 3. Micronucleus assay in TK6 and MT1 cells. An asterisk (*) and asterisks (***) denote $p<0.01$ and $p<0.005$, respectively, in the *t*-test of comparison between MNNG-treated and untreated cells.

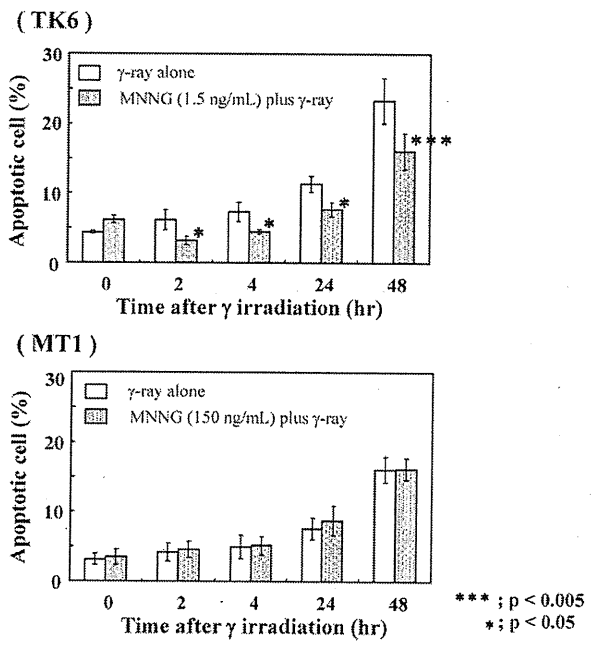

Fig. 4. Apoptosis assay in TK6 and MT1 cells. An asterisk (*) and asterisks (***) denote $p<0.05$ and $p<0.005$, respectively, in the *t*-test of comparison between MNNG-treated and untreated cells.

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

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

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

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

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

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

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

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

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

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

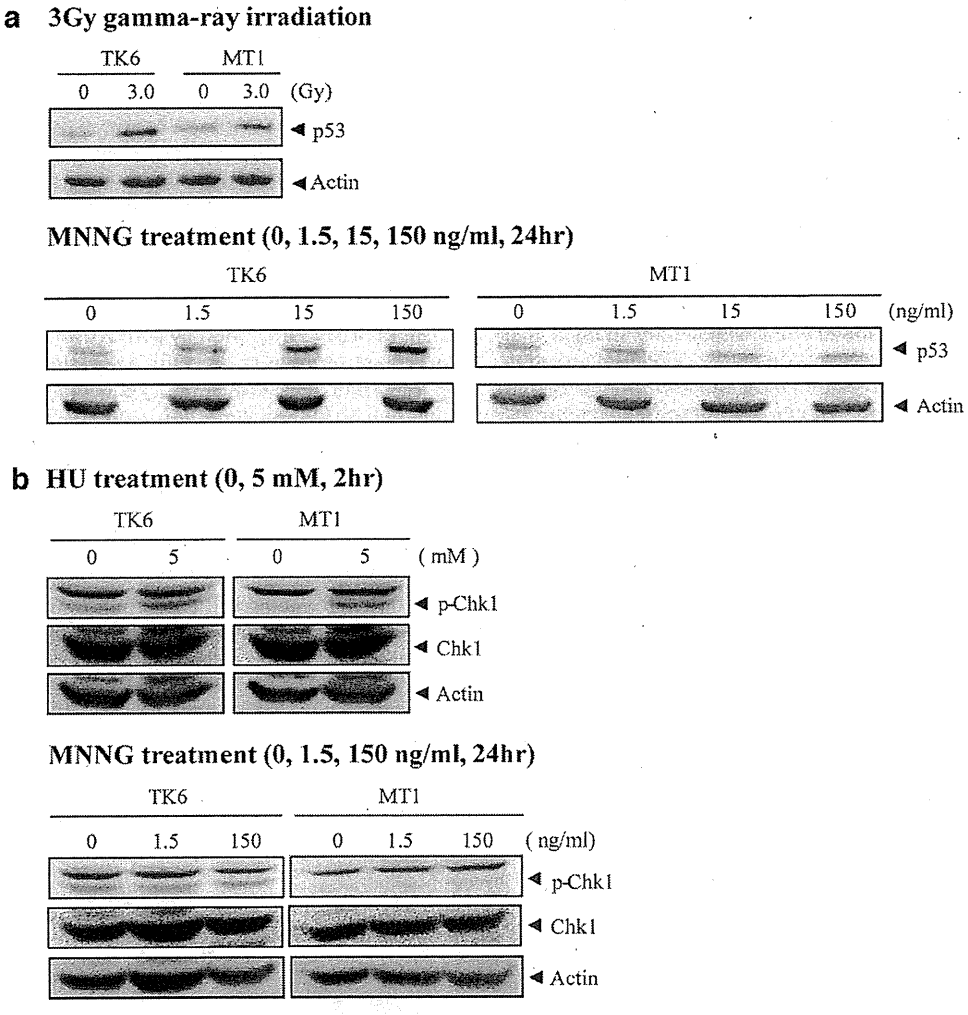

Fig. 5. Western blotting analysis in TK6 and MT1 cells. (a) Activation of p53. The cells were irradiated with γ-ray at a dose of 3 Gy as a positive control. Alternatively, the cells were treated with MNNG at a dose of 1.5, 15 or 150 ng/ml for 24 h and subjected to the analysis. (b) Phosphorylation of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

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

3.5. MNNG treatments induce the p53 activation

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

4. Discussion

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