Table 9
Mutation spectra of *gpt* mutant colonies in the livers of F344 *gpt* delta rats given safrole for 13 weeks.

Sex	Base substitution	Control		0.1% safrole		0.5% safrole	
		Number (%)	Mutation frequency (10^{-5})	Number (%)	Mutation frequency (10^{-5})	Number (%)	Mutation frequency (10^{-5})
Male	Transversions						
	GC-TA	2[a] (25.0)	0.06 ± 0.12	4 (20.0)	0.15 ± 0.21	6 (17.1)	0.30 ± 0.30
	GC-CG	1 (12.5)	0.05 ± 0.09	2 (10.0)	0.09 ± 0.20	4 (11.4)	0.26 ± 0.31
	AT-TA	0	0	2 (10.0)	0.07 ± 0.10	5 (14.3)	0.29 ± 0.33
	AT-CG	0	0	0	0	1 (2.9)	0.08 ± 0.17
	Transitions						
	GC-AT	4 (50.0)	0.11 ± 0.12	8 (40.0)	0.30 ± 0.25	5 (14.3)	0.19 ± 0.18
	AT-GC	0	0	3 (15.0)	0.12 ± 0.17	10 (28.6)	0.54 ± 0.40**
	Deletion						
	Single bp	1 (12.5)	0.05 ± 0.10	1 (5.0)	0.04 ± 0.10	4 (11.4)	0.19 ± 0.21
	Over 2 bp	0	0	0	0	0	0
	Insertion	0	0	0	0	0	0
	Complex	0	0	0	0	0	0
	Total	8	0.26 ± 0.21	20	0.77 ± 0.63	35	1.89 ± 0.67
Female	Transversions						
	GC-TA	4 (19.0)	0.11 ± 0.19	10 (26.3)	0.27 ± 0.17	5 (25.0)	0.33 ± 0.45
	GC-CG	3 (14.3)	0.08 ± 0.12	2 (5.7)	0.04 ± 0.06	1 (5.0)	0.06 ± 0.13
	AT-TA	1 (4.8)	0.04 ± 0.09	6 (15.8)	0.18 ± 0.19	2 (10.0)	0.12 ± 0.28
	AT-CG	1 (4.8)	0.04 ± 0.08	3 (7.9)	0.08 ± 0.14	0	0
	Transitions						
	GC-AT	12 (57.1)	0.38 ± 0.22	8 (21.1)	0.18 ± 0.15	6 (15.0)	0.37 ± 0.34
	AT-GC	0	0	8 (21.1)	0.21 ± 0.05	5 (25.0)	0.31 ± 0.31
	Deletion						
	Single bp	0	0	1 (2.6)	0.03 ± 0.06	1 (5.0)	0.07 ± 0.15
	Over 2 bp	0	0	0	0	0	0
	Insertion	0	0	0	0	0	0
	Complex	0	0	0	0	0	0
	Total	21	0.65 ± 0.39	38	0.98 ± 0.39	20	1.26 ± 1.04

[a] Number of colonies with independent mutations.
** Significantly different from the control group at $p < 0.01$.

calcification and interstitial cell infiltration in the kidney were significantly increased in males of the treated groups. The overall data indicated that safrole is a nephrotoxicant as well as a hepatotoxicant. In previous studies, the suppression of body weight gain and liver enlargement were also observed in safrole-treated rats (Homburger et al., 1962; Hagan et al., 1965). These results show that the *gpt* delta rat has a similar sensitivity to safrole in comparison to non-transgenic wild rats. This implies that the *gpt* delta rat model can be used to investigate general toxicities of agents.

Safrole forms safrole-specific DNA adducts through hepatic cytochrome P450 biotransformation and subsequent conjugation by sulfotransferase (Miller and Miller, 1983). Alternatively, safrole can be biotransformed though the methylenedioxy ring-opening to hydroxychavicol. Hydroxychavicol could be biotransformed to o-quinone through 2-electron oxidation, and this redox-active quinone is considered to induce oxidative damages (Klungsoyr and Scheline, 1983; O'Brien, 1991). In fact, the levels of 8-OHdG were significantly increased in both sexes of the safrole-treated groups as compared to those of the control group. To the best of our knowledge, there are no reports demonstrating the significant increase of 8-OHdG levels in livers of rats treated with a low dose (half of a carcinogenic dose) for 13 weeks. However, the genotoxicity of safrole remained unknown in conventional genotoxicity tests such as the Ames test, sister chromatid exchanges (SCE) test and micronucleus test in spite of its hepatocarcinogenicity being clear (Green and Savage, 1978; Swanson et al., 1979; Baker and Bonin, 1985; Bradley, 1985; Gocke et al., 1981). The present study demonstrated that an increase or increasing tendency of the *gpt* MFs was observed in both sexes in the 0.5% group, a carcinogenic dose, despite the Spi$^-$ MFs being unchanged. These results suggested that safrole has a potential to be genotoxic *in vivo* in the livers of rats. In the mutation spectra, the AT:GC transitions were significantly induced by safrole in males of the 0.5% group. If has been reported that 8-OHdG is capable to form a base pair with adenine and subsequently produce a GC:AT transversion mutation. In addition, recent studies suggest that 8-OHdG can cause large deletion mutations associated with double strand break during base excision repair by *OGG1* (Umemura

Table 10
8-OHdG, PCNA and GST-P levels in the livers of F344 *gpt* delta rats given safrole for 13 weeks.

Sex	Treatment	Control	0.1% safrole	0.5% safrole
Male	8-OHdG	0.27 ± 0.02[a]	0.35 ± 0.05*	0.52 ± 0.06**
	PCNA-positive ratio	0.27 ± 0.12	0.54 ± 0.17*	0.51 ± 0.14*
	GST-P (number/cm^2)	0.00 ± 0.00	0.88 ± 0.55	9.42 ± 3.51**
	GST-P (mm^2/cm^2)	0.00 ± 0.00	0.005 ± 0.004	0.134 ± 0.070**
Female	8-OHdG	0.36 ± 0.04	0.51 ± 0.06**	0.62 ± 0.06**
	PCNA-positive ratio	0.20 ± 0.07	0.66 ± 0.09	1.06 ± 0.55**
	GST-P (number/cm^2)	0.00 ± 0.00	0.44 ± 0.46*	2.78 ± 1.22**
	GST-P (mm^2/cm^2)	0.00 ± 0.00	0.004 ± 0.005	0.026 ± 0.016**

[a] Mean ± SD.
* Significantly different from the controls at the levels of $p < 0.05$ (Dunnett's test).
** Significantly different from the controls at the levels of $p < 0.01$ (Dunnett's test).

et al., 2007). Thus, in the light of the type of mutations induced by safrole, it is unlikely that 8-OHdG formation contributes to safrole-induced genotoxicity, although there is a possibility of any other oxidized DNA damages being involved. The present data showing a significant increase in PCNA-positive hepatocytes might suggest the possible participation of oxidative stress in cell proliferation.

It is well known that the results of bioassay using GST-P-positive foci show good correlation with those of the 2-year cancer bioassay (Ito et al., 2000; Ogiso et al., 1985). Therefore, it has been widely accepted that the analysis of GST-P positive foci may be a useful indicator to predict carcinogenicity of agents. In the present study, the number and the area of GST-P positive foci were significantly increased in both sexes in the 0.5% group. The data on quantitative analysis for GST-P foci using *gpt* delta rats are also in agreement with the carcinogenicity data previously reported by Long et al. (1963).

In conclusion, the present medium-term animal model using F344 *gpt* delta rats confirmed previous reports of the hepatotoxicity and hepatocarcinogenicity of safrole. The genotoxicity of safrole, which remained unknown, so far, was clearly demonstrated in the target organ by this *in vivo* model. Thus, this animal model might be a promising tool for investigating comprehensive toxicities of agents. The acquisition of additional data on key events in chemical carcinogenesis, such as base modification and cell proliferation, could assist in understanding the modes of action. Applications of this model should be further expanded in future studies.

Conflict of interest statement

None.

Acknowledgements

We thank Ms. Ayako Kaneko and Ms. Yoshimi Komatsu for their expert technical assistance. This work was supported by a Grant-in-Aid for Research on Food Sanitation from the Ministry of Health, Labor and Welfare of Japan (H21-shokuhin-ippan-010).

References

Baker, R.S.U., Bonin, A.M., 1985. Tests with *Salmonella* plate incorporation assay. In: Ashby, J., De Serres, F.J. (Eds.), Evaluation of Short-term Tests for Carcinogens. Elsevier, NY, pp. 177–180.

Borchert, P., Wislocki, P.G., Miller, J.A., Miller, E.C., 1973. The metabolism of the naturally occurring hepatocarcinogen safrole to 1′-hydroxysafrole and the electrophilic reactivity of 1′-acetoxysafrole. Cancer Res. 33, 575–589.

Bradley, M.O., 1985. Measurement of DNA single-strand breaks by alkaline elution in rat hepatocytes. In: Ashby, J., De Serres, F.J. (Eds.), Evaluation of Short-term Tests for Carcinogens. Elsevier, NY, pp. 353–357.

Daimon, H., Sawada, S., Asakura, S., Sagami, F., 1998. In vivo genotoxicity and DNA adduct levels in the liver of rats treated with safrole. Carcinogenesis 19, 141–146.

Dorange, J.L., Janiaud, P., Delaforge, M., et al., 1978. Comparative survey of microsomal activation system for mutagenic studies of safrole. Mutat. Res. 52, 179–180.

Dunnet, C.W., 1955. A multiple comparison procedure for comparing several treatments with a control. Am. Stat. Assoc. J. 50, 1096–1121.

Furia, T.E., Bellanca, N., 1975. Fenaroli's Handbook of Flavor Ingredients, vol. 2., 2nd ed. CRC press, Cleveland, OH.

Gocke, E., King, M.T., Eckardt, K., Wild, D., 1981. Mutagenicity of cosmetics ingredients licensed by the European Community. Mutat. Res. 90, 91–109.

Green, N.R., Savage, J.R., 1978. Screening of safrole, eugenol, their ninhydrin positive metabolites and selected secondary amines for potential mutagenicity. Mutat. Res. 57, 115–121.

Hagan, E.C., Jenner, P.M., Jones, W.I., et al., 1965. Toxic properties of compounds related to safrole. Toxicol. Appl. Pharmacol. 7, 18–24.

Hayashi, H., Kondo, H., Masumura, K., et al., 2003. Novel transgenic rat for *in vivo* genotoxicity assays using 6-thioguanine and Spi$^-$ selection. Environ. Mol. Mutagen. 41, 253–259.

Homburger, F., Kelley Jr., T., Baker, T.R., Russfield, A.B., 1962. Sex effect on hepatic pathology from deficient diet and safrole in rats. Arch. Pathol. 73, 118–125.

IARC, 1976. Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man. Safrole, Isosafrole and Dihydrosafrole, vol. 10. International Agency for Research on Cancer, Lyon, France, pp. 231–244.

Ioannides, C., Delaforge, M., Parke, D.V., 1981. Safrole: its metabolism, carcinogenicity and interactions with cytochrome P450. Food Cosmet. Toxicol. 19, 657–666.

Ito, N., Imaida, K., Asamoto, M., Shirai, T., 2000. Early detection of carcinogenic substances and modifiers in rats. Mutat. Res. 462, 209–217.

Kasai, H., 2002. Chemistry-based studies on oxidative DNA damage: formation, repair, and mutagenesis. Free Radic. Biol. Med. 33, 450–456.

Klungsoyr, J., Scheline, R.R., 1983. Metabolism of safrole in the rat. Acta Pharmacol. Toxicol. 52, 211–216.

Leung, A.Y., 1980. Encyclopedia of Common Natural Ingredients Used in Food, Drugs, and Cosmetics. John Wiley, New York.

Liu, T.Y., Chen, C.C., Chen, C.L., Chi, C.W., 1999. Safrole-induced oxidative damage in the liver of Sprague-Dawley rats. Food Chem. Toxicol. 37, 697–702.

Long, E.L., et al., 1963. Liver tumors produced in rats by feeding safrole. Arch. Pathol. 75, 595–604.

Masumura, K., Horiguchi, M., Nishikawa, A., et al., 2003. Low dose genotoxicity of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinolone (MeIQx) in *gpt* delta transgenic mice. Mutat. Res. 541, 91–102.

Miller, J.A., Miller, E.C., 1983. The metabolic activation and nucleic acid adducts of naturally-occurring carcinogens. Recent results with ethyl carbamate and the spice flavors safrole and estragole. Br. J. Cancer 48, 1–15.

Nakae, D., Mizumoto, Y., Kobayashi, E., et al., 1995. Improved fenomic/nuclear DNA extraction for 8-hydroxydeoxyguanosine analysis of small amounts of rat liver tissue. Cancer Lett. 97, 233–239.

Natarajan, A.T., Darroudi, F., 1991. Use of human hepatoma cells for *in vitro* metabolic activation of chemical mutagens/carcinogens. Mutagenesis 6, 399–403.

Nohmi, T., Katoh, M., Suzuki, H., et al., 1996. A new transgenic mouse mutagenesis test system using Spi$^-$ and 6-thioguanine selections. Environ. Mol. Mutagen. 28, 465–470.

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

O'Brien, P.J., 1991. Molecular mechanisms of quinine cytotoxicity. Chem. Biol. Interact. 80, 1–11.

Ogiso, T., Tatematsu, M., Tamano, S., et al., 1985. Comparative effects of carcinogens on the induction of placental glutathione S-transferase-positive liver nodules in a short-term assay and of hepatocellular carcinomas in a long-term assay. Toxicol. Pathol. 13, 257–265.

Swanson, A.B., Chambliss, D.D., Blanquist, J.C., et al., 1979. The mutagenicities of safrole, estragole, trans-anethole and some of their known or possible metabolites for *Salmonella thyphimurium* mutants. Mutat. Res. 60, 143–153.

To, L.P., Hunt, T.P., Andersen, M.E., 1982. Mutagenicity of trans-anethole, estragole, eugenol, and safrole in the Ames *Salmonella typhimurium* assay. Bull. Environ. Contam. Toxicol. 28, 647–654.

Toyoda-Hokaiwado, N., Inoue, T., Masumura, K., 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.

Umemura, T., Kuroiwa, Y., Tasaki, M., et al., 2007. Detection of oxidative DNA damage, cell proliferation and *in vivo* mutagenicity induced by dicyclanil, a non-genotoxic carcinogen, using *gpt* delta mice. Mutat. Res. 633, 46–54.

Umemura, T., Tasaki, M., Kijima, A., et al., 2009. Possible participation of oxidative stress in causation of cell proliferation and *in vivo* mutagenicity in kidneys of *gpt* delta rats treated with potassium bromate. Toxicology 257, 46–52.

Watanabe, T., Katsura, Y., Yoshitake, A., et al., 1994. IPAP: image processor for analytical pathology. J. Toxicol. Pathol. 7, 353–361.

Wislocki, P.G., Miller, E.C., Miller, J.A., et al., 1977. Carcinogenic and mutagenic activities of safrole. 1′-Hydroxysafrole, and same known or possible metabolites. Cancer Res. 37, 1883–1891.

Genes and Environment, Vol. 33, No. 4 pp. 160–166 (2011)

Regular article

Modulatory Effects of Capsaicin on N-diethylnitrosamine (DEN)-induced Mutagenesis in Salmonella typhimurium YG7108 and DEN-induced Hepatocarcinogenesis in gpt Delta Transgenic Rats

Naomi Toyoda-Hokaiwado[1], Yumiko Yasui[2,3], Makiko Takamune[1], Masami Yamada[1], Mina Muramatsu[1,4], Kenichi Masumura[1], Toshihiro Ohta[4], Takuji Tanaka[2,5] and Takehiko Nohmi[1,6]

[1]Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo, Japan
[2]Department of Oncologic Pathology, Kanazawa Medical University, Ishikawa, Japan
[3]Department of Physiology and Nutrition, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido, Japan
[4]School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan
[5]The Tokai Cytopathology Institute: Cancer Research and Prevention, Gifu, Japan

(Received July 12, 2011; Revised August 21, 2011; Accepted August 24, 2011)

Capsaicin from the red chili pepper is a prospective chemopreventive agent. To explore the possible antigenotoxic effects of capsaicin on N-diethylnitrosamine (DEN)-induced mutagenesis in vitro, we conducted bacterial mutation assays with Salmonella typhimurium YG7108, a sensitive strain to mutagenic alkylating agents. Capsaicin was not mutagenic either with or without S9 activation. Unexpectedly,it enhanced the mutagenicity of DEN in the presence of S9 activation significantly. Capsaicin also enhanced the mutagenicity of 2-aminoanthracene and benzo[a]pyrene in the presence of S9 activation and benzo[a]pyrene diolepoxide in the absence of S9 activation. However, it reduced the mutagenicity of ethylnitrosourea in the absence of S9 activation. To examine whether capsaicin modulates DEN-induced mutagenesis and hepatocarcinogenesis in vivo, we took advantage of gpt delta rats, transgenic rodents that carry reporter genes for mutations. Female gpt delta rats were given drinking water containing 40 ppm DEN for five weeks. They were fed diets containing capsaicin at doses of 0, 100 or 500 ppm for seven weeks, starting one week before the DEN treatment. Samples were collected at weeks 7 and 32, respectively, for mutagenicity and carcinogenicity assays. DEN enhanced gpt mutant frequency more than 200 fold in the liver. However, capsaicin displayed no modulating effects on the mutagenesis. Rather, it reduced the number of liver neoplasms, especially liver cell adenomas, in a dose-dependent manner although the reduction in hepatocellular carcinoma was statistically insignificant. These results suggest that chemopreventive effect of capsaicin against DEN-induced hepatocarcinogenesis is slight and that the effect is not due to antimutagenesis. The results also caution that chemopreventive effects of chemicals should be examined not only in vitro but also in vivo with multiple indexes, e.g., in vitro and in vivo mutations and pathological examinations.

Key words: capsaicin, chemoprevention, N-diethylnitrosamine, hepatocarcinogenicity, gpt delta transgenic rats

Introduction

Capsaicin is the principal pungent constituent of hot red chili peppers, which are the most frequently consumed spices in the world (1). In addition to a spicy dietary ingredient, capsaicine is known to exhibit various biological activities, such as inhibition of CYP-depenent xenobiotic metabolism, inhibition of cellular signal transduction and induction of apoptosis (1–6). Thus, it is expected that capsaicin can be chemopreventive against tumors via antigenotoxic mechanisms. In fact, it is reproted that dietary exposure to capsaicin suppresses azoxymethane-induced colon tumors in rats (7). Capsaicin also inhibits DNA binding of aflatoxin B1 in the presence of *in vitro* metabolic activation by S9 enzymes (8). Several reports suggest, however, that capsaicin itself is mutagenic in Ames bacterial mutation assays (9–11), V79 mammalian gene mutation assays (12) and micronucleus assays *in vivo* (13), and one report suspects the carcinogenicity (14). It remains elusive, there-

[6]Correspondence to: Takehiko Nohmi, Division of Genetics and Mutagenesis, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel: +81-3-3700-9872, Fax: +81-3-3700-2348, E-mail: nohmi@nihs.go.jp

© The Japanese Environmental Mutagen Society

fore, whether capsaicin is chempreventive and if so whether antigenotoxic mechanisms are involved in the chemopreventive action.

To examine the possible chemopreventive effects of capsaicin against *N*-diethylnitrosamine (DEN), we conducted *in vitro* and *in vivo* mutation assays. We chose DEN as a target carcinogen because it induces hepatocarcinoma effectively in a genotoxic manner (15). In addition, liver cancer is one of the most prevalent cancer diseases world wide (16). We employed *Salmonella typhimurium* YG7108 (17,18), a sensitive strain to mutagenicity of various alkylating agents, for *in vitro* mutation assays and Fischer 344 *gpt* delta transgenic rats for *in vivo* assays (19). The strain *S. typhimurium* YG7108 is sensitive to mutagenicity of alkylating agents because it lacks DNA repair enzymes of O^6-alkylguanine alkyltransferases encoded by the ada_{ST} and ogt_{ST} genes (17,18). *gpt* delta transgenic rats carry reporter genes for *in vivo* mutations (20). Point mutations and deletions can be identified in any organs or tissues of F344 rats and the mutations are analyzable at the sequence level (19). Chemopreventive effects of capsaicin against tumor induction was histopathologically evaluated in the liver of DEN-treated F344 *gpt* delta rats. Glutathione *S*-transferase placenta form (GST-P) positive foci are frequently used as an indicator of pre-neoplastic lesions of liver of rats because this bioassay shows good correlations with long-term carcinogenicity results (21). The results suggest that capcaisin suppresses DEN-induced heptatocarcinogenesis slightly. However, the chemopreventive effect is not due to antigenotoxic mechanims because capsaicin displayed no antimutgenic activity against DEN-induced mutations *in vivo*. Capsaicin is not mutagenic *in vitro* and *in vivo*. Because of the complex properties, chemopreventive effects of capsaicin should be further evaluated via multiple indexes such as mutations and proliferating lesions (preneoplasms and neoplasms) induced by other genotoxic carcinogens.

Materials and Methods

Materials: Capsaicin (synthetic, *N*-vanillylnonanamide, CAS: 2444-46-4) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The purity was >96%. DEN (CAS No.: 55-18-5) and ethylnitrosourea (ENU; CAS: 759-73-9) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). 2-Aminoanthracen (2-AA; CAS No.: 103404-81-5), benzo[*a*]pyrene (BP; CAS No.: 50-32-8), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1; CAS No.: 67730-11-4), and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Benzo[*a*]pyrene-dihydrodiol epoxide (BPDE; CAS No.: 60268-85-1) was purchased from Midwest Research Institute Global (Missouri, MO, USA). S9 prepared from male Sprague-Dawley rats pretreated with phenobarbital and 5,6-benzoflavone was purchased from Kikkoman Cooperation, Chiba, Japan.

Bacterial reverse mutation test (Ames test): Modulating effects of capsaicin against DEN-induced mutagenesis were assayed in a bacterial reverse mutation assay using *S. typhimurium* tester strains YG7108 (17,18), as TA1535 but is $\Delta ada_{ST} \Delta ogt_{ST}$, in the presence of S9 enzymes. The test was conducted by the pre-incubation method with modifications (22). Briefly, capsaicin dissolved in DMSO was mixed with S9 mix for 5 min on ice. Then, DEN dissolved in distilled water was added, followed by addition of overnight culture of *S. typhimurium* YG7108. The mixture was incubated for 20 min at 37°C and poured onto agar plates with soft agar. The plates were incubated for 2 days at 37°C. Assays were performed on triplicate. When the antimutagenic effects of capsaicin on other chemicals were examined, the test conditions, i.e., *S. typhimurium* strains and requirements for S9 mix, were as follows: 2-AA and Glu-P-1, TA98, +S9 mix; BP, TA100, +S9 mix; BPDE, TA100, −S9 mix; ENU, YG7108, −S9 mix.

Animals, diet and housing conditions: Female six-week-old F344 *gpt* delta transgenic rats (19) 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 CRF-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. Fifty-four rats were randomly divided into five groups (Fig. 1). Groups

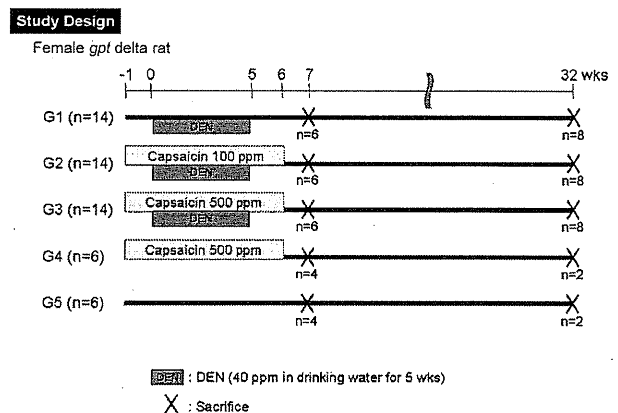

Fig. 1. Experimental protocol to examine *in vivo* modulating effects of capsaicin against DEN-induced mutagenesis and hepatocarcinogenesis using F344 *gpt* delta rats.

1 through 3 were treated with 40 ppm DEN in drinking water for five weeks. Group 2 was fed diets containing 100 ppm capsaicin, and Groups 3 and 4 were fed diets containing 500 ppm capsaicin for seven weeks, starting one week before DEN administration. Group 5 served as an untreated control. All rats were carefully observed for clinical welfare and weighed weekly, and experimental diet consumption was recorded. The experiment was terminated at 32 weeks after the start of DEN administration. During the study, animals were killed at week 7 to determine the effects of capsaicin on the mutation frequency. At autopsy, liver, kidneys and spleen were macroscopically examined for the presence of pathologic lesions, and then isolated. Tissues were fixed in 10% buffered formalin and processed to hematoxylin and eosin (HE) stained sections. Neoplastic lesions of liver were histopathologically classified into adenomas and hepatocellular carcinomas (HCC). Left lobes of the livers from rats sacrificed at week 7 were excised and frozen in liquid nitrogen for mutation assay. Then, remaining livers were fixed in 10% buffered formalin, embedded in paraffin, sectioned, stained by HE and histopathologically examined.

DNA isolation, in vitro packaging and gpt mutation assay: High-molecular-weight genomic DNA was extracted from the liver using the RecoverEase DNA Isolation Kit (Stratagene by Agilent Technologies, Santa Clara, CA, USA). λ EG10 phages were rescued using Transpack Packaging Extract (Stratagene). The *gpt* assay was conducted according to previously published methods (23). The mutant frequencies of the *gpt* gene (*gpt* MFs) in the liver were calculated by dividing the number of confirmed 6-thioguanine-resistant colonies by the number of rescued plasmids.

Immunohistochemical procedures: Liver sections of 3 μm thickness from short period groups were treated with rabbit anti-rat GST-P antibody (1:1,000; Medical & Biological Laboratories, Nagoya, Japan). Immunohistochemical staining was done by the avidin-biotin complex method (ABC) using the Vectastain Elite ABC system (Vector Laboratories, Inc., Burlingame, CA, USA). 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. Areas and numbers of GST-P-positive foci larger than 0.1 mm in diameter of the liver sections were quantitatively measured with an image processor for analytical pathology (IPAP-WIN; Sumika Technos Company, Osaka, Japan).

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

Results

Capsaicin enhanced mutagenicity of DEN in S. typhimurium YG7108: To examine modulating effects of capsaicin on DEN-induced mutagenicity, bacterial mutation assays with *S. typhimurium* YG7108 were performed (Fig. 2). Capsaicin itself was not mutagenic with or without S9 activation in *S. typhimurium* TA98, TA100 and YG7108. DEN itself at a dose of 200 μg/plate induced about 500 His$^+$ revertants per plate in the presence of S9 activation. When capsaicin was added in the reaction mixture, it enhanced the mutagenicity of DEN in a dose-dependent manner, and the number of His$^+$ revertants per plate reached about 4,000 at a dose of 50 μg capsaicin/plate. We also examined the modulating effects with 2-AA, BP and Glu-P-1 in the presence of S9 activation, and with ENU and BPDE in the absence of S9 activation. We used *S. typhimurium* strains TA98 or TA100 when the test chemicals were not alkylating agents. Capsaicin substantially enhanced mutations induced by 2-AA and BP in a dose-dependent manner as in the case of DEN. It enhanced mutations induced by BPDE dose-dependently at doses less than 100 μg of capsaicin per plate. Capsaicin slightly enhanced the mutagenicity of Glu-P-1 at lower doses and then reduced it at higher doses. Capsaicin reduced the mutagenicity of ENU.

General observation of in vivo study: To reveal modulating effects of capsaicin *in vivo*, genotoxicity assay and carcinogenesis study were conducted with *gpt* delta rats. Consumptions of capsaicin-mixed diets were 25% lower than those of normal diet at the first experimental week (Groups 2 to 4, data not shown). It recovered, however, to the level similar to the control group at the second experimental week. During drinking administration of 40 ppm DEN, growth of body weight of DEN-treated animals was slightly reduced (Groups 1 to 3, data not shown). At week 7, their body weight except for Group 3, which received DEN plus 500 ppm capsaicin (Suppl. Table 1, available at http://www.j-ems.org/journal/) did not differ from that of the control group. This difference was, however, not observed at week 32 (Suppl. Table 2, available at http://www.j-ems.org/journal/). Organ weight did not show any differences among the groups (Suppl. Table 1 and Suppl. Table 2).

Capsaicin did not affect formation of preneoplastic hepatocellular lesions: DEN induced small GST-P positive foci in liver at week 7 (Group 1, Table 1).

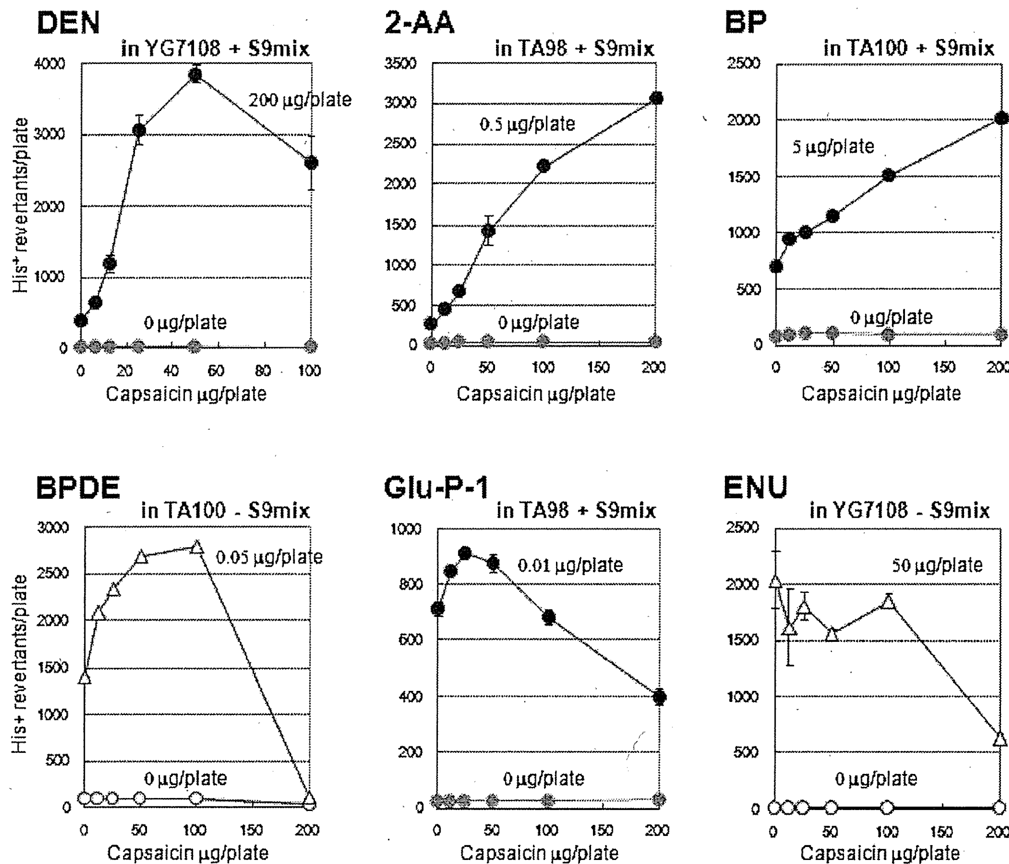

Fig. 2. Modulating effects of capsaicin on *in vitro* mutagenesis induced by DEN, 2-AA, BP, BPDE, Glu-P-1 and ENU. Doses of each chemical, 0.01–200 μg/plate, in the condition with chemical were indicated on the graph and doses of capsaicin were indicated on the X axis. Strains and S9 mix conditions are indicated on each panel.

Table 1. Quantification of GST-P positive foci at week 7

Group	Treatment		No. of rats	No. of foci [No./cm^2]	Area of foci [mm^2/cm^2]
	DEN	Test chemical			
1	+	—	6	15.425±7.233*	0.445±0.280
2	+	Capsaicin 100 ppm	6	16.553±10.543	0.538±0.429
3	+	Capsaicin 500 ppm	6	20.405±14.939	0.718±0.816
4	−	Capsaicin 500 ppm	4	0.000±0.000	0.000±0.000
5	−	—	4	0.000±0.000	0.000±0.000

*Mean±SD. Experimental period was 7 weeks. Values were compared with Group 1 by Student's *t*-test.

Quantitative analysis (number and area) of the lesions did not show any difference among the DEN-treated groups, i.e., Groups 1, 2 and 3 (Table 1). These preneoplastic lesions did not develop in the liver of rats treated without DEN (Groups 4 and 5).

Capsaicin did not affect gene mutations in vivo: DEN treatments enhanced *gpt* MF in the liver 200 times over the control levels (Table 2 and Suppl. Table 3, available at http://www.j-ems.org/journal/). Capsaicin was non-genotoxic (Group 4). Unlike the *in vitro* results, capsaicin treatments did not show any substantial effects on DEN-induced mutagenesis *in vivo*. The *gpt* MFs of Groups 2 and 3, which received DEN plus 100 and 500 ppm capsaicin, respectively, were not different from that of Group 1 that received DEN alone (Table 2).

Capsaicin slightly suppressed hepatocarcinogenesis: The incidence and multiplicity of tumors at week 32 were slightly reduced in the capsaicin treated groups (Groups 2 and 3, Table 3). The treatment with capsaicin

decreased the incidence and multiplicity of liver tumors by 20–40% and 47–64%, respectively. The number of adenomas per rat was significantly decreased by the capsaicin treatment ($p<0.05$), although the reduction of HCCs was statistically insignificant.

Discussion

In this study, we examined the modulating effects of capsaicin on DEN-induced mutagenesis *in vitro* and *in vivo*. We could not confirm previously reported mutagenicity of capsaicin in *S. typhimurium* TA98 and TA100 in the presence of S9 activation (9,11,13). Capsaicin was not mutagenic not only *in vitro* (Fig. 2) but also *in vivo* (Table 2). Purity of the samples may account for the different findings (24). Instead, we found that capsaicin effectively enhanced mutagenicity of DEN, 2-AA and BP in the presence of S9 activation *in vitro* (Fig. 2). At first, we assumed that capsaicin might modulate the activities of CYP enzymes involved in metabolic activation of the xenobiotics, thereby enhancing the mutagenesis. In fact, it is suggested that metabolites of capsaicin bind microsomal proteins, such as CYP enzymes (25–27). However, in this study capsaicin also enhanced mutations induced by BPDE without S9 activation. Therefore, we suggested that capsaicin modulated not only metabolic activation but also mutagenesis and/or DNA repair. Interestingly, capsaicin displayed opposite modulating effects on mutagenesis, i.e., reduction of mutagenesis by Glu-P-1 in the presence of S9 enzymes and by ENU in the absence of S9 (Fig. 2). These results suggest that capsaicin can suppress metabolic activation by S9 enzymes and mutagenesis/DNA repair in some cases. It is puzzling, however, why capsaicin enhanced mutations induced by DEN but reduced those induced by ENU, although both DEN and ENU induce mutagenic O^6-ethylguanine in DNA. One possible explanation for the complex modulating effects is that capsaicin might enhance membrane permeability of bacteria to chemical carcinogens, thereby displaying various modulating effects on the mutagenicity of chemicals. Complex modulating effects of capsaicin *in vitro* have been described by Huynh and Teel (28). They have reported that capsaicin at doses of 0.25 μmol (76.3 μg) and 0.5 μmol (152.7 μg) per plate reduced mutations in *S. typhimurium* TA98 induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and Glu-P-1, but enhanced those by Trp-P-2 in the presence of S9 enzymes (28). Collectively, our results illustrated in Fig. 2 along with other reports (2,29,30) suggest that capsaicin has ability to modulate multiple steps leading to mutations at least *in vitro*.

In contrast, capsaicin neither affected development of DEN-induced preneoplastic lesions, GST-P positive foci (Table 1) nor mutagenesis in the liver of rats (Table 2). These results were unexpected because capsaicin substantially enhanced the mutagenicity of DEN *in vitro* (Fig. 2) and it is reported that capsaicin inhibits metabolism of carcinogens, including dimethylnitrosamine (DMN), which are preferentially activated by CYP2E1 (26,27). In the report, capsaicin at 0.25 μmol (76.3 μg) per plate reduces the mutagenicity of DMN in

Table 2. Mutant frequency in Liver at week 7

Group	Treatment		No. of rats	Mutant frequency ($\times 10^{-6}$)
	DEN	Test chemical		
1	+	—	6	225.31±52.51*
2	+	Capsaicin 100 ppm	6	254.76±83.47
3	+	Capsaicin 500 ppm	6	245.10±114.52
4	−	Capsaicin 500 ppm	4	1.10±1.09
5	−	—	4	1.49±1.90

*Mean±SD. Values were examined by Student's *t*-test. Significant differences among groups 1, 2 and 3 were not observed.

Table 3. Pathological findings in liver at week 32

Group	Treatment		No. of rats	Incidence			Multiplicity		
				No. of rats with tumors			No. of tumors/ rats		
	DEN	Test chemical		Total	AD	HCC	Total	AD	HCC
1	+	—	8	8 (100%)	7 (88%)	5 (63%)	2.75±2.12*	1.75±1.28	1.00±1.07
2	+	Capsaicin 100 ppm	8	6 (75%)	5 (63%)	4 (50%)	1.38±1.30	0.75±0.71	0.63±0.74
3	+	Capsaicin 500 ppm	8	5 (63%)	5 (63%)	3 (38%)	1.13±1.13	0.63±0.52†	0.50±0.76
4	−	Capsaicin 500 ppm	2	0 (0%)	0 (0%)	0 (0%)	0.00±0.00	0.00±0.00	0.00±0.00
5	−	—	2	0 (0%)	0 (0%)	0 (0%)	0.00±0.00	0.00±0.00	0.00±0.00

AD, adenoma; HCC, hepatocellular carcinoma. *Mean±SD. †Significantly different from group 1 by Dunnett multiple comparison test ($p<0.05$).

S. typhimurium TA100 by more than 50% (26). A possible reason for the discrepancy between *in vitro* and *in vivo* findings is that, unlike *in vitro* where capsaicin can interact with S9 enzymes at high concentrations, the local concentration (tissue distribution) in the liver of rats might not be high enough to modulate the activity of CYP enzymes. In fact, there is a report suggesting that capsaicin did not inhibit any of CYP enzyme activities at concentrations occurring after ingestion of chili peppers (31). Direct inhibition may be observed at much higher concentrations. Although exact concentrations in the liver are unavailable in this study, the negative modulating effects of capsaicin in the liver of rats caution that chemopreventive effects of chemicals should be evaluated not only *in vitro* but also *in vivo*.

Interestingly, capsaicin slightly suppressed the incidence and multiplicity of hepatocellular tumors at week 32. Although the reduction in the multiplicity of liver cell carcinomas did not reach the statistical significance, the value of liver cell adenomas was significantly reduced by feeding with capsaicin at a dose level of 500 ppm ($p<0.05$, Table 3). Capsaicin was previously demonstrated to be chemopreventive against azoxymethane-induced colon carcinogenesis (7). Capsaicin has multiple biological activities, such as block of signal transduction pathways leading to carcinogenesis, induction of apoptosis, cell-cycle delay and anti-inflammation (1,3,5,6,32). It is unclear which biological activities are involved in the slight reduction of DEN-induced hapatocarcinogenesis. However, we suggest that antimutagenesis does not play roles in the weak chemopreventive ability because of the negative modulating effects on DEN-induced mutagenesis *in vivo*.

In summary, we revealed that capsaicin was slightly chemopreventive against liver cell tumors induced by DEN in rats through mechanisms other than antigenotoxicity. Our study highlights the importance of employment of multiple biological parameters, such as mutations and pathological biomarkers, to investigate the mechanisms underlying the chemopreventive effects of chemicals. In this regard, F344 *gpt* delta rats (19) are quite useful, because mutations *in vivo* as well as pathological alterations (incidences and multiplicities of tumors and preneoplasms) can be analyzed at the same time in target tissues of the same rats that received carcinogens and/or test agents.

Acknowledgements: 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 Science Foundation (KHB1007); and for cancer research from MHLW (20 designated-8).

References

1 Surh YJ, Lee E, Lee JM. Chemoprotective properties of some pungent ingredients present in red pepper and ginger. Mutat Res. 1998; 402: 259–67.
2 Zhang Z, Hamilton SM, Stewart C, Strother A, Teel RW. Inhibition of liver microsomal cytochrome P450 activity and metabolism of the tobacco-specific nitrosamine NNK by capsaicin and ellagic acid. Anticancer Res. 1993; 13: 2341–6.
3 Aggarwal BB, Shishodia S. Molecular targets of dietary agents for prevention and therapy of cancer. Biochem Pharmacol. 2006; 71: 1397–421.
4 Kang HJ, Soh Y, Kim MS, Lee EJ, Surh YJ, Kim HR, Kim SH, Moon A. Roles of JNK-1 and p38 in selective induction of apoptosis by capsaicin in ras-transformed human breast epithelial cells. Int J Cancer. 2003; 103: 475–82.
5 Oyagbemi AA, Saba AB, Azeez OI. Capsaicin: a novel chemopreventive molecule and its underlying molecular mechanisms of action. Indian J Cancer. 2010; 47: 53–8.
6 Reilly CA, Yost GS. Metabolism of capsaicinoids by P450 enzymes: a review of recent findings on reaction mechanisms, bio-activation, and detoxification processes. Drug Metab Rev. 2006; 38: 685–706.
7 Yoshitani SI, Tanaka T, Kohno H, Takashima S. Chemoprevention of azoxymethane-induced rat colon carcinogenesis by dietary capsaicin and rotenone. Int J Oncol. 2001; 19: 929–39.
8 Teel RW. Effects of capsaicin on rat liver S9-mediated metabolism and DNA binding of aflatoxin. Nutr Cancer. 1991; 15: 27–32.
9 Azizan A, Blevins RD. Mutagenicity and antimutagenicity testing of six chemicals associated with the pungent properties of specific spices as revealed by the Ames *Salmonella*/microsomal assay. Arch Environ Contam Toxicol. 1995; 28: 248–58.
10 Nagabhushan M, Bhide SV. Nonmutagenicity of curcumin and its antimutagenic action versus chili and capsaicin. Nutr Cancer. 1986; 8: 201–10.
11 Toth B, Rogan E, Walker B. Tumorigenicity and mutagenicity studies with capsaicin of hot peppers. Anticancer Res. 1984; 4: 117–9.
12 Lawson T, Gannett P. The mutagenicity of capsaicin and dihydrocapsaicin in V79 cells. Cancer Lett. 1989; 48: 109–13.
13 Nagabhushan M, Bhide SV. Mutagenicity of chili extract and capsaicin in short-term tests. Environ Mutagen. 1985; 7: 881–8.
14 Verschoyle RD, Steward WP, Gescher AJ. Putative cancer chemopreventive agents of dietary origin-how safe are they? Nutr Cancer. 2007; 59: 152–62.
15 International Agency for Research on Cancer. *N*-Nitrosodiethylamine. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Some Inorganic Substances, Chlorinated Hydrocarbons, Aromatic Amines, *N*-Nitroso Compounds and Natural Products. 1972; 1: 107–24.

16 Yuen MF, Hou JL, Chutaputti A. Hepatocellular carcinoma in the Asia pacific region. J Gastroenterol Hepatol. 2009; 24: 346–53.

17 Yamada M, Matsui K, Sofuni T, Nohmi T. New tester strains of *Salmonella typhimurium* lacking O^6-methylguanine DNA methyltransferases and highly sensitive to mutagenic alkylating agents. Mutat Res. 1997; 381: 15–24.

18 Yamada M, Sedgwick B, Sofuni T, Nohmi T. Construction and characterization of mutants of *Salmonella typhimurium* deficient in DNA repair of O^6-methylguanine. J Bacteriol. 1995; 177: 1511–9.

19 Toyoda-Hokaiwado N, Inoue T, Masumura K, Hayashi H, Kawamura Y, Kurata Y, Takamune M, Yamada M, Sanada H, Umemura T, Nishikawa A, Nohmi T. 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. 2010; 114: 71–8.

20 Hayashi H, Kondo H, Masumura K, Shindo Y, Nohmi T. Novel transgenic rat for *in vivo* genotoxicity assays using 6-thioguanine and Spi^- selection. Environ Mol Mutagen. 2003; 41: 253–9.

21 Ogiso T, Tatematsu M, Tamano S, Tsuda H, Ito N. Comparative effects of carcinogens on the induction of placental glutathione S-transferase-positive liver nodules in a short-term assay and of hepatocellular carcinomas in a long-term assay. Toxicol Pathol. 1985; 13: 257–65.

22 Maron DM, Ames BN. Revised methods for the *Salmonella* mutagenicity test. Mutat Res. 1983; 113: 173–215.

23 Nohmi T, Suzuki T, Masumura K. Recent advances in the protocols of transgenic mouse mutation assays. Mutat Res. 2000; 455: 191–215.

24 Proudlock R, Thompson C, Longstaff E. Examination of the potential genotoxicity of pure capsaicin in bacterial mutation, chromosome aberration, and rodent micronucleus tests. Environ Mol Mutagen. 2004; 44: 441–7.

25 Reilly CA, Yost GS. Structural and enzymatic parameters that determine alkyl dehydrogenation/hydroxylation of capsaicinoids by cytochrome p450 enzymes. Drug Metab Dispos. 2005; 33: 530–6.

26 Surh YJ, Lee RC, Park KK, Mayne ST, Liem A, Miller JA. Chemoprotective effects of capsaicin and diallyl sulfide against mutagenesis or tumorigenesis by vinyl carbamate and *N*-nitrosodimethylamine. Carcinogenesis. 1995; 16: 2467–71.

27 Gannett P, Iversen P, Lawson T. The mechanism of inhibition of cytochrome P450IIE1 by dihydrocapsaicin. Bioorg Chem. 1990; 18: 185–98.

28 Huynh HT, Teel RW. *In vitro* antimutagenicity of capsaicin toward heterocyclic amines in *Salmonella typhimurium* strain TA98. Anticancer Res. 2005; 25: 117–20.

29 Miller CH, Zhang Z, Hamilton SM, Teel RW. Effects of capsaicin on liver microsomal metabolism of the tobacco-specific nitrosamine NNK. Cancer Lett. 1993; 75: 45–52.

30 Espinosa-Aguirre JJ, Reyes RE, Rubio J, Ostrosky-Wegman P, Martinez G. Mutagenic activity of urban air samples and its modulation by chili extracts. Mutat Res. 1993; 303: 55–61.

31 Babbar S, Chanda S, Bley K. Inhibition and induction of human cytochrome P450 enzymes *in vitro* by capsaicin. Xenobiotica. 2010; 40: 807–16.

32 Chow J, Norng M, Zhang J, Chai J. TRPV6 mediates capsaicin-induced apoptosis in gastric cancer cells—Mechanisms behind a possible new "hot" cancer treatment. Biochim Biophys Acta. 2007; 1773: 565–76.

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.

© The Author 2011. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com

Downloaded from carcin.oxfordjournals.org at National Institute of Health Sciences on October 3, 2011

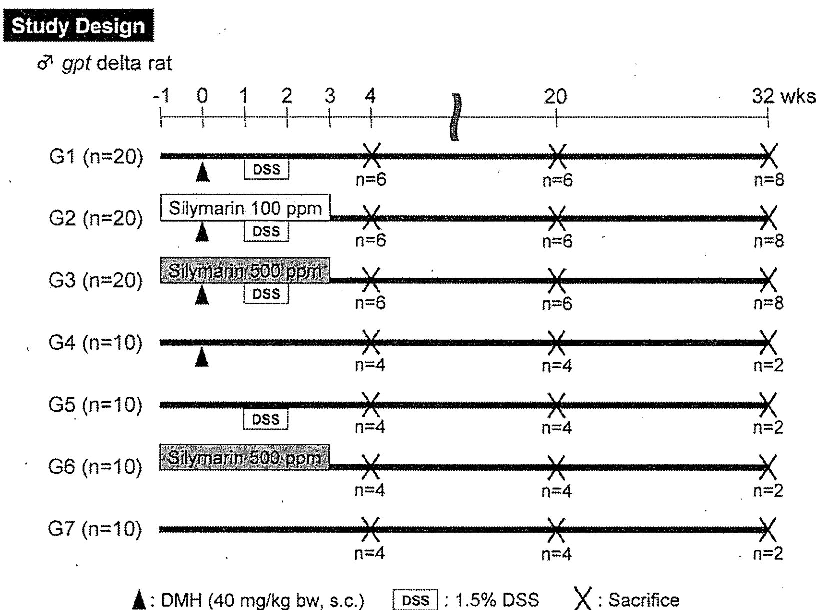

Fig. 1. Experimental protocol. Arrow heads, DMH 40 mg/kg body wt, subcutaneous injection; X, sacrifice.

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,

Downloaded from carcin.oxfordjournals.org at National Institute of Health Sciences on October 3, 2011

$P < 0.01$). 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.

Downloaded from carcin.oxfordjournals.org at National Institute of Health Sciences on October 3, 2011

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.

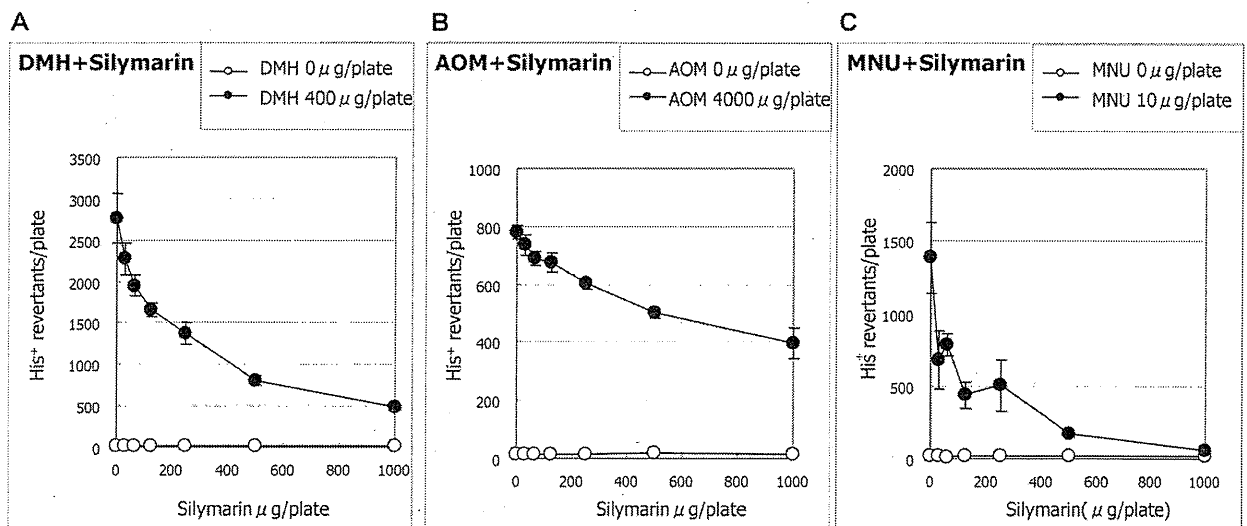

Fig. 2. Antigenotoxic activity of silymarin without S9 mix in *S. typhimurium* strain YG7108 induced by DMH (**A**), AOM (**B**) and MNU (**C**). Filled circle assayed with chemicals, open circle assayed without chemicals.

(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

Downloaded from carcin.oxfordjournals.org at National Institute of Health Sciences on October 3, 2011

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.

Downloaded from carcin.oxfordjournals.org at National Institute of Health Sciences on October 3, 2011

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

Downloaded from carcin.oxfordjournals.org at National Institute of Health Sciences on October 3, 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

© The Author 2011. Published by Oxford University Press on behalf of the Society of Toxicology. All rights reserved.
For permissions, please email: journals.permissions@oup.com

Downloaded from http://toxsci.oxfordjournals.org/ at Eishi Shikenjo Tosho on March 5, 2012

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

	Food consumption (g/rat/day)		Intake of OTA			
			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

Downloaded from http://toxsci.oxfordjournals.org/ at Eishi Shikenjo Tosho on March 5, 2012

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

Downloaded from http://toxsci.oxfordjournals.org/ at Eishi Shikenjo Tosho on March 5, 2012

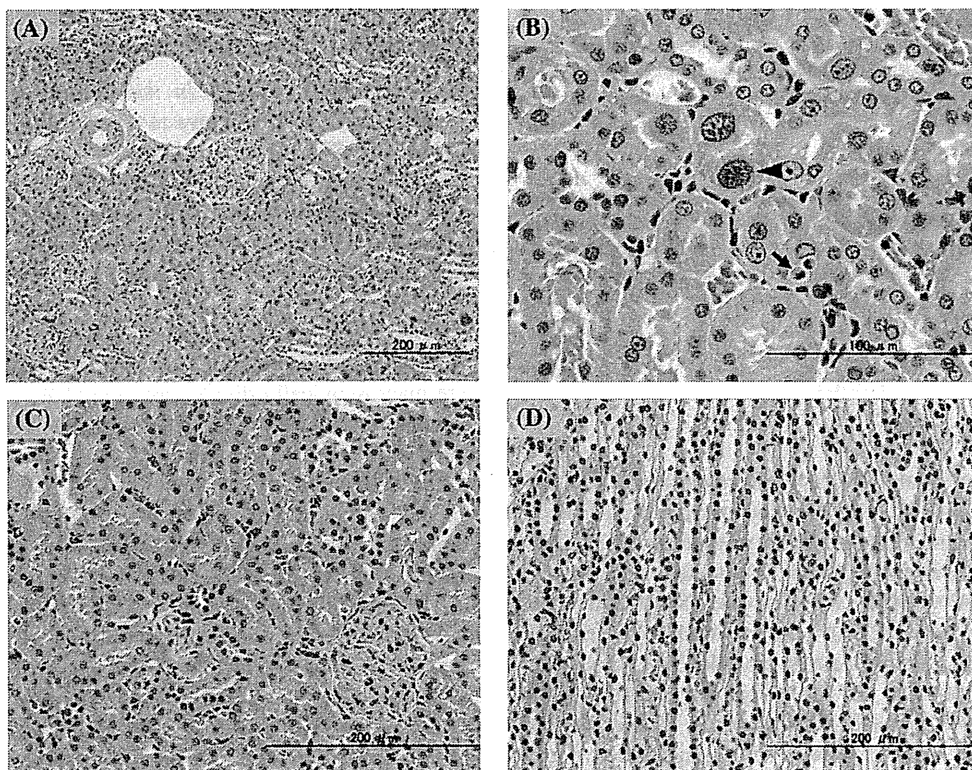

FIG. 1. Boundary of the renal COR and medulla (A) and OSOM (B) of a male F344 *gpt* delta rat treated with OTA. Vacuolation, apoptosis (arrow), and karyomegaly (arrow head) were apparent at the S3 segment of the proximal tubules of the OSOM. There were no remarkable changes in the COR (C) and the inner 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 ($\times10^5$)	Cm^R and 6-TG^R colonies	MF ($\times10^{-5}$)	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 $\times 10^{-5}$ 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 $\times 10^{-5}$) were also not significantly different from those in the relevant control groups (males: 0.18 ± 0.04; females: 0.14 ± 0.08 $\times 10^{-5}$).

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 $\times 10^{-5}$ in the control group; OM: 0.38 ± 0.12 in the OTA group, 0.20 ± 0.12 $\times 10^{-5}$ 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 ($p < 0.05$)

Downloaded from http://toxsci.oxfordjournals.org/ at Eishi Shikenjo Tosho on March 5, 2012

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 $\times 10^{-5}$ 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 $\times 10^{-5}$ 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.

Downloaded from http://toxsci.oxfordjournals.org/ at Eishi Shikenjo Tosho on March 5, 2012