Figure 3. Histopathological findings of SD *gpt* delta rats treated with 2-MF for 13 weeks

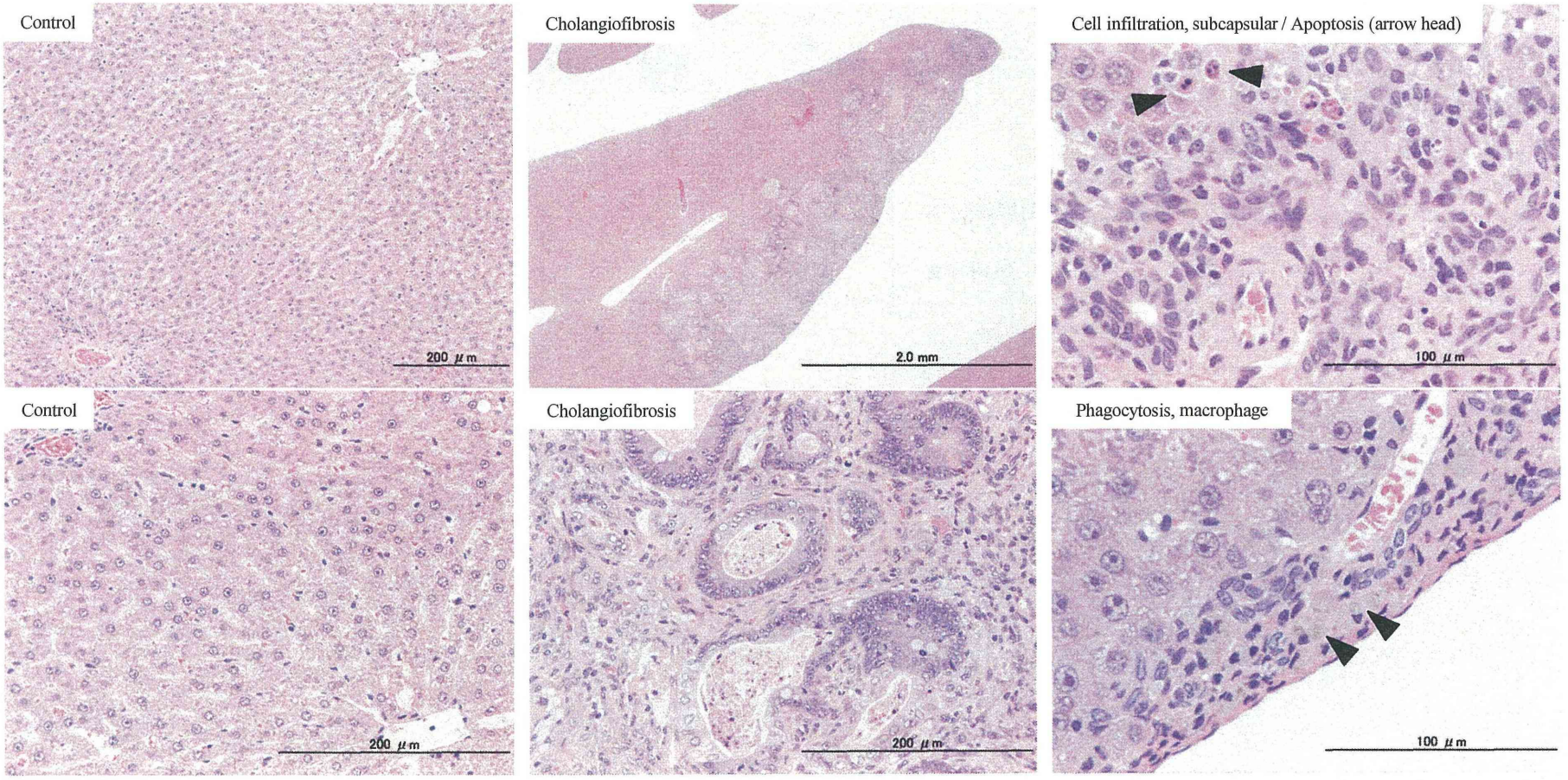
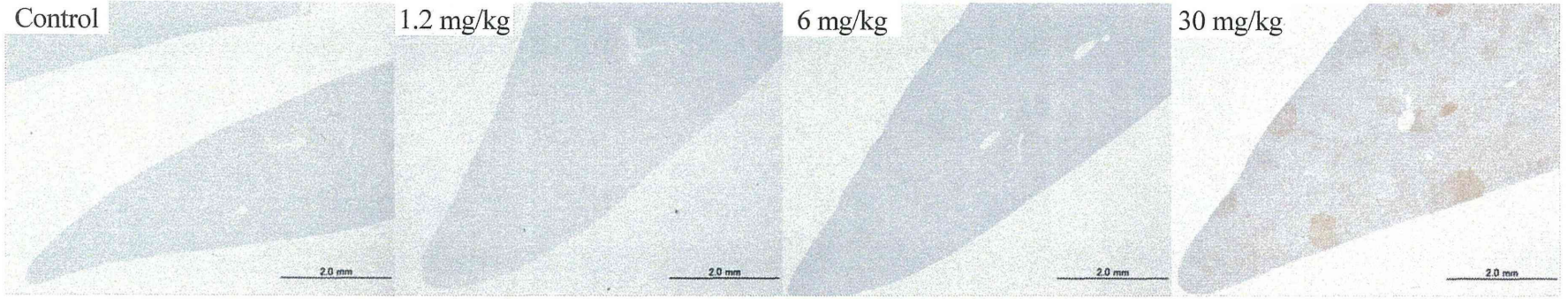
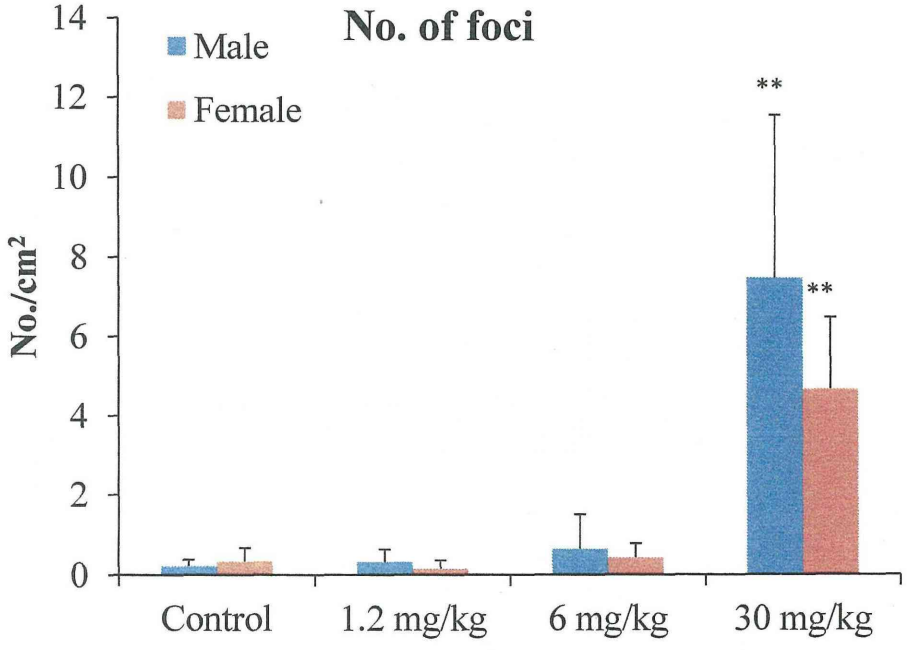
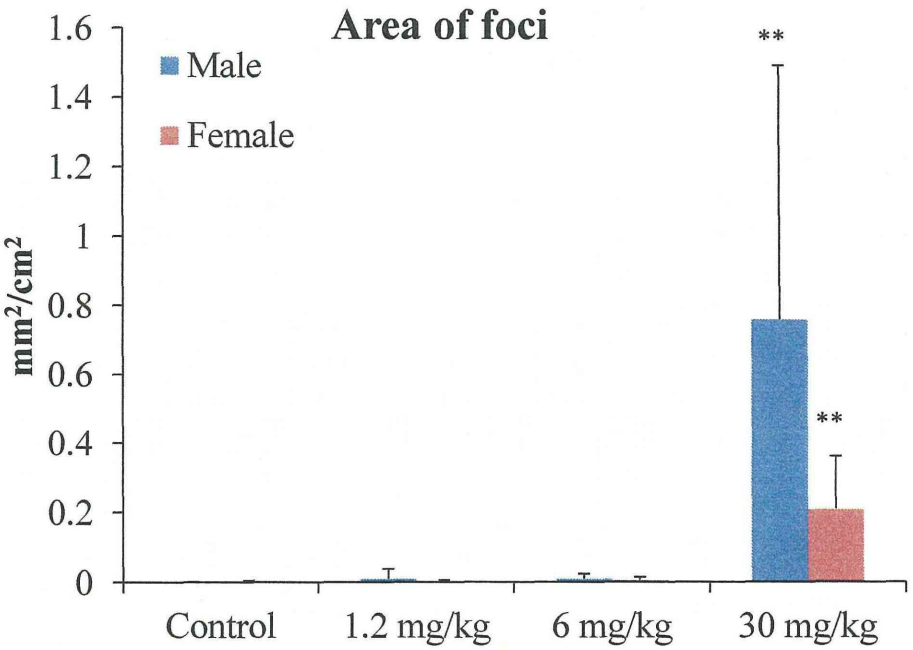


Fig. 4 Number and area of GST-P-positive foci for SD *gpt* delta rats treated with 2-MF for 13 weeks







**: $p < 0.01$ vs. Control

Table 1. Hematology of SD *gpt* delta rats treated with 2-MF for 13 weeks

Male

Item	Unit	Control	1.2 mg/kg	6 mg/kg	30 mg/kg
RBC	/μL	850.4 ± 51.5	846.3 ± 45.1	844.6 ± 50.3	845.4 ± 33.1
Hb	g/dL	15.0 ± 0.6	14.9 ± 0.8	14.7 ± 0.5	14.1 ± 0.6*
Ht	%	47.2 ± 2.1	47.3 ± 2.5	47.0 ± 2.4	45.9 ± 2.1
MCV	Fl	55.5 ± 1.2	55.9 ± 1.1	55.7 ± 0.8	54.2 ± 1.2*
MCH	pg	17.6 ± 0.7	17.6 ± 0.4	17.4 ± 0.8	16.6 ± 0.6**
MCHC	%	31.7 ± 0.6	31.6 ± 0.4	31.3 ± 1.0	30.7 ± 0.8*

Female

Item	Unit	Control	1.2 mg/kg	6 mg/kg	30 mg/kg
RBC	/μL	683.0 ± 42.4	705.0 ± 42.3	685.9 ± 28.4	693.9 ± 94.2
Hb	g/dL	14.1 ± 0.6	14.6 ± 0.3*	14.2 ± 0.5	13.4 ± 0.5**
Ht	%	40.7 ± 2.1	41.9 ± 1.7	40.2 ± 1.7	39.3 ± 1.8
MCV	Fl	59.7 ± 1.8	56.6 ± 8.7	58.6 ± 1.1	54.4 ± 1.3**
MCH	pg	20.7 ± 0.9	20.8 ± 1.0	20.8 ± 0.7	18.6 ± 0.7**
MCHC	%	34.6 ± 1.0	34.9 ± 0.9	35.4 ± 1.0	34.2 ± 0.9

*,**: $p < 0.05$, 0.01 vs. Control

Table 2. Serum biochemistry of SD *gpt* delta rats treated with 2-MF for 13 weeks

Male

Item	Unit	Control	1.2 mg/kg	6 mg/kg	30 mg/kg
ALP	IU/L	428.6 ± 40.8	485.6 ± 64.8	486.8 ± 89.8*	566.2 ± 38.9**
T-Bil	mg/dL	0.0 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.0*
γ-GTP	IU/L	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	4.2 ± 0.8
Glucose	mg/dL	154.0 ± 26.5	144.8 ± 21.0	124.2 ± 27.2	101.8 ± 16.5**
T-Cho	mg/dL	61.8 ± 6.7	64.0 ± 12.5	67.8 ± 12.0	84.0 ± 7.1**
Na	mEq/L	142.0 ± 0.7	143.2 ± 1.1	142.4 ± 0.9	143.8 ± 0.4**
Ca	mg/dL	9.6 ± 0.2	9.7 ± 0.2	9.8 ± 0.2	9.7 ± 0.2
IP	mg/dL	6.0 ± 0.7	6.5 ± 0.3	7.0 ± 0.4*	7.6 ± 0.2**

Female

Item	Unit	Control	1.2 mg/kg	6 mg/kg	30 mg/kg
ALP	IU/L	232.7 ± 60.1	256.5 ± 92.6	198.6 ± 63.9	303.9 ± 85.8
T-Bil	mg/dL	0.1 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.1 ± 0.0
γ-GTP	IU/L	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	2.7 ± 0.9
Glucose	mg/dL	140.3 ± 20.9	147.8 ± 17.1	124.8 ± 25.7	114.9 ± 12.2*
T-Cho	mg/dL	118.0 ± 24.6	101.3 ± 13.9	105.3 ± 12.8	110.2 ± 20.6
Na	mEq/L	140.6 ± 1.2	140.9 ± 1.0	140.0 ± 1.2	141.0 ± 1.2
Ca	mg/dL	10.1 ± 0.4	10.0 ± 0.4	10.3 ± 0.2	10.6 ± 0.2**
IP	mg/dL	4.9 ± 1.2	5.1 ± 1.2	5.8 ± 0.7	6.7 ± 0.3**

*,**: $p<0.05$, 0.01 vs Control

Table 3. Final body and organ weight of SD *gpt* delta rats treated with 2-MF for 13 weeks

Male

Absolute weight (g)				
Item	0 mg/kg	1.2 mg/kg	6 mg/kg	30 mg/kg
Body weight	526.9 ± 40.0	547.6 ± 52.0	512.2 ± 35.5	456.3 ± 21.2**
Liver	14.41 ± 1.80	15.20 ± 2.41	15.02 ± 1.57	19.00 ± 1.49**
Kidneys	2.59 ± 0.24	2.61 ± 0.20	2.62 ± 0.25	2.70 ± 0.23
Relative weight (g%)				
Liver	2.73 ± 0.21	2.76 ± 0.23	2.93 ± 0.14	4.16 ± 0.23**
Kidneys	0.49 ± 0.03	0.48 ± 0.03	0.51 ± 0.03	0.59 ± 0.05**

Female

Absolute weight (g)				
Item	0 mg/kg	1.2 mg/kg	6 mg/kg	30 mg/kg
Body weight	302.9 ± 26.1	303.6 ± 22.0	286.5 ± 33.2	255.3 ± 18.5**
Liver	7.76 ± 1.19	7.56 ± 0.67	8.46 ± 1.07	10.80 ± 0.89**
Kidneys	1.60 ± 0.15	1.60 ± 0.19	1.53 ± 0.15	1.70 ± 0.15
Relative weight (g%)				
Liver	2.56 ± 0.32	2.49 ± 0.18	2.96 ± 0.21**	4.24 ± 0.24**
Kidneys	0.53 ± 0.04	0.53 ± 0.05	0.54 ± 0.04	0.67 ± 0.05**

*,**: $p<0.05$, 0.01 vs Control

Table 4 Histopathological findings of SD *gpt* delta rats treated with 2-MF for 13 weeks

		Male				Female			
		Control	1.2 mg/kg	6 mg/kg	30 mg/kg	Control	1.2 mg/kg	6 mg/kg	30 mg/kg
Organs	Findings	10	10	10	10	10	10	10	10
Liver	Apoptosis, hepatocyte	0	0	0	6	0	0	1	6
	Foci of cellular alteration	0	0	0	10	0	0	0	10
	Bile duct proliferation	0	0	1	8	0	0	1	8
	Oval cell proliferation	0	0	0	2	0	0	0	0
	Cholangiofibrosis	0	0	0	9	0	0	0	9
	Cell infiltration, subcapsular	0	0	7	10	0	0	2	10
	Phagocytosis, macrophage	0	0	4	5	1	0	5	5
	Microgranuloma	0	0	0	0	1	0	0	0
Kidney	Hyalline cast	1	-	-	1	1	-	-	1
	Mineralization	1	-	-	2	2	-	-	2
	Tubular regeneration	2	-	-	2	1	-	-	2
	Focal inflammation	0	-	-	0	1	-	-	0
Heart	Focal inflammation	1	-	-	1	0	-	-	0
Thyroid	Remnant of ultimobranchial body	0	-	-	3	1	-	-	3
Pituitrary	Cyst	0	-	-	0	4	-	-	0

別紙４

研究成果の刊行に関する一覧表レイアウト

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
	該当なし						

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kohei Matsushita, Aki Kijima, Yuji Ishii, Shinji Takasu, Meilan Jin, Ken Kuroda, Hiroaki Kawaguchi, Noriaki Miyoshi, Takehiko Nohmi, Kumiko Ogawa, Takashi Umemura:	Development of a medium-term animal model using gpt delta rats to evaluate chemical carcinogenicity and genotoxicity.	*J. Toxicol. Pathol.*	26	19-27	2013
Jin M, Kijima A, Hibi D, Ishii Y, Takasu S, Matsushita K, Kuroda K, Nohmi T, Nishikawa A, Umemura T.	*In vivo* genotoxicity of methyleugenol in *gpt* delta transgenic rats following medium-term exposure.	*Toxicol. Sci.*	131	387-394	2013
Jin M, Kijima A, Suzuki Y, Hibi D, Ishii Y, Nohmi T, Nishikawa A, Ogawa K, Umemura T.	*In vivo* genotoxicity of 1-methylnaphthalene from comprehensive toxicity studies with B6C3F$_1$ *gpt* delta mice.	*J. Toxicol. Sci.*	37	711-721	2012

Ⅳ. 研究成果の刊行物・別刷り

J Toxicol Pathol 2013; 26: 19–27

Original Article

Development of a Medium-term Animal Model Using *gpt* Delta Rats to Evaluate Chemical Carcinogenicity and Genotoxicity

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Abstract: In this study, the potential for development of an animal model (GPG46) capable of rapidly detecting chemical carcinogenicity and the underlying mechanisms of action were examined in *gpt* delta rats using a reporter gene assay to detect mutations and a medium-term rat liver bioassay to detect tumor promotion. The tentative protocol for the GPG46 model was developed based on the results of dose-response exposure to diethylnitrosamine (DEN) and treatment with phenobarbital over time following DEN administration. Briefly, *gpt* delta rats were exposed to various chemicals for 4 weeks, followed by a partial hepatectomy (PH) to collect samples for an *in vivo* mutation assay. The mutant frequencies (MFs) of the reporter genes were examined as an indication of tumor initiation. A single intraperitoneal (ip) injection of 10 mg/kg DEN was administered to rats 18 h after the PH to initiate hepatocytes. Tumor-promoting activity was evaluated based on the development of glutathione S-transferase placental form (GST-P)-positive foci at week 10. The genotoxic carcinogens 2-acetylaminofluorene (2-AAF), 2-amino-3-methylimidazo [4,5-f] quinolone (IQ) and safrole (SF), the non-genotoxic carcinogens piperonyl butoxide (PBO) and phenytoin (PHE), the non-carcinogen acetaminophen (APAP) and the genotoxic non-hepatocarcinogen aristolochic acid (AA) were tested to validate the GPG46 model. The validation results indicate that the GPG46 model could be a powerful tool in understanding chemical carcinogenesis and provide valuable information regarding human risk hazards. (DOI: 10.1293/tox.26.19; J Toxicol Pathol 2013; 26: 19–27)

Key words: medium-term animal model, carcinogenicity, *gpt* delta rats, *in vivo* genotoxicity, glutathione S-transferase placental form

Introduction

Environmental chemicals, including pharmaceuticals, agrochemicals and food additives, are important in various aspects of daily life. However, these chemicals may pose a risk to humans, and their toxicities have been extensively assessed in animal studies. In particular, carcinogenicity is a key component of safety assessments because the resulting lesions can be irreversible and are often fatal. The current gold standard for assessing the risk of cancer is a lifetime bioassay in rodents, but this method requires over 3 years to complete, including histopathological procedures[1]. It is estimated that only approximately 1500 chemicals have been tested over the past 30 years despite the addition of nearly 4000 new chemicals in the Chemical Abstracts Service (CAS) Registry database every day[2,3]. Although conventional lifetime bioassays can provide data regarding the potential carcinogenicity and target organs of various chemicals, these assays do not provide any information about the associated mechanisms of action that influence carcinogenesis. The development of bioassays that can rapidly detect chemical carcinogenicity and provide information about the underlying mechanisms of action is currently being pursued.

Thresholds in dose-related chemical carcinogenicity curves depend on the involvement of genotoxic mechanisms[4]. Mutagenicity and carcinogenicity are important factors when determining risk assessments[5]. Although *in vitro* genotoxic assays, such as the Ames test, the micronucleus test and the chromosomal aberration test, are considered standard tools for investigating chemical mutagenicity, the results of these methods are not necessarily indicative of carcinogenicity[5]. Reporter gene mutation assays are promising genotoxic techniques because *in vivo* metabolic processes can be evaluated at the target organs[6]. Comprehensive toxicity studies and the measurement of DNA adducts, oxidative stress and enzymatic activities have been demon-

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strated in animal models using *gpt* delta rodents[7–10]. Using the reliable preneoplastic marker glutathione S-transferase placental form (GST-P) foci, medium-term rat liver bioassays have been developed to rapidly detect tumor promoters because the liver is the most common target organ for carcinogenesis[11]. However, the conventional medium-term bioassays do not provide information regarding the involvement of genotoxic mechanisms in carcinogenesis as a result of exposure to test compounds.

In this study, we evaluated the possibility of developing a new animal model designed to rapidly detect chemical carcinogenicity and underlying molecular mechanisms using a reporter gene mutation assay and a medium-term liver bioassay. The conditions were optimized to establish a tentative experimental protocol, and validation of the model was confirmed using several carcinogens.

Materials and Methods

Chemicals

Diethylnitrosamine (DEN) and safrole (SF) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Phenobarbital (PhB), 2-acetylaminofluorene (2-AAF), piperonylbutoxide (PBO), and phenytoin (PHE) were obtained from Wako Pure Chemical Industries (Osaka, Japan), and acetaminophen (APAP) was purchased from MP Biomedicals (Irvine, CA, USA). 2-Amino-3-methylimidazo [4,5-f] quinolone (IQ) and aristolochic acid (AA) were obtained from Toronto Research Chemicals (North York, ON, Canada) and Sigma-Aldrich (St. Louis, MO, USA), respectively.

Experimental animals and housing conditions

The protocol was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Five- or nine-week-old specific pathogen-free F344/NSlc rats or five-week-old specific pathogen-free F344/NSlc-Tg (*gpt* delta) rats carrying approximately 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 testing. The rats were housed in polycarbonate cages (two or three rats per cage) with hardwood chips for bedding in a conventional animal facility. Animals were maintained under controlled temperature (23 ± 2°C), relative humidity (55 ± 5%), air changes (12 times/h), and lighting (12 h light-dark cycle) conditions with free access to a basal diet (CRF-1; Oriental Yeast Co., Ltd, Tokyo, Japan) and tap water. At the end of each experiment, the rats were euthanized by exsanguination via transection of the abdominal aorta under deep anesthesia.

Animal treatments

Experiment I: The effects of a single administration of DEN on the development of GST-P-positive foci were evaluated. A partial hepatectomy (PH) was performed on ten-week-old male F344/NSlc rats (n=5 rats per dose). After 18 h, an intraperitoneal (ip) injection of DEN was administered at doses of 0, 10, 50, and 100 mg/kg. Six weeks after the start of the experiment, the rat livers were fixed in 10% neutral-buffered formalin. The fixed tissues were embedded in paraffin, sectioned and evaluated using immunohistochemistry for the quantitative analysis of GST-P-positive foci.

Experiment II: Changes in the development of GST-P-positive foci over time following administration of PhB after a PH and single dose exposure to DEN were examined. Six-week-old male F344/NSlc rats (n=10 rats per dose) were fed PhB at concentrations of 0 and 500 ppm in their basal diets. This dose was selected based on a previous carcinogenicity test[12]. After 4 weeks, a PH was performed. An ip injection of DEN at a dose of 10 mg/kg was administered 18 h after the PH. The rats continued to feed on a diet containing PhB until they were sacrificed at 10, 12, or 14 weeks after the start of the experiment. The livers were fixed in 10% neutral-buffered formalin, and the tissues were embedded in paraffin, sectioned and evaluated using immunohistochemistry for the quantitative analysis of GST-P-positive foci.

Experiment III: Validation of the animal model was confirmed using genotoxic, non-genotoxic carcinogens and a non-carcinogen. Six-week-old male F344/NSlc-Tg (*gpt* delta) rats (n=15 per dose) were fed 20 ppm 2-AAF, 12000 ppm PBO or 6000 ppm APAP in their basal diets. A control group was fed the basal diet without chemical supplementation. The 2-AAF dose was selected based on a preliminary study in which no toxic effects were observed in rats treated with 20 ppm (data not shown). The doses of PBO and APAP were selected based on previous carcinogenicity tests[13,14]. The animal model was further validated using genotoxic and non-genotoxic carcinogens and a genotoxic non-hepatocarcinogen. Six-week-old male F344/NSlc-Tg (*gpt* delta) rats (n=15 per dose) were fed 20 ppm IQ, 5000 ppm SF or 2400 ppm PHE in their basal diets. The rats treated with AA received 0.3 mg/kg body weight in 1% sodium bicarbonate by gavage once a day. A control group was fed the basal diet without chemical supplementation. The IQ dose was selected based on a preliminary study in which no toxic effects were observed in rats treated with 20 ppm (data not shown). The doses of SF and PHE were selected based on previous carcinogenicity tests[15,16], and the dose of AA was determined based on a previous report in which the *gpt* mutant frequencies (MFs) were increased in rats treated with AA for 4 weeks[17]. The carcinogenic properties of the test chemicals are summarized in Table 1. A PH was performed on all rats after 4 weeks, and an ip injection of DEN at a dose of 10 mg/kg was administered 18 h after the PH. The excised liver tissues were perfused with saline to remove residual blood and stored at –80°C for the *gpt* assay. The rats continued to feed on the basal diets containing the various chemicals. Ten weeks after the start of the experiment, the livers were fixed in 10% neutral-buffered formalin. The fixed tissues were embedded in paraffin, sectioned and evaluated using immunohistochemistry for the quantitative analysis of GST-P-positive foci.

Table 1. Summary of the carcinogenic properties of the test chemicals used in the validation study

Test chemical	Mutagenicity	Carcinogenicity	Principal site of tumor induction	Group
2-AAF	+	+	Liver, Bladder, Zymbal gland	Genotoxic carcinogen
IQ	+	+	Liver, Forestomach, Intestines	
SF	+	+	Liver	
PBO	−	+	Liver	Non-genotoxic carcinogen
PHE	−	±*	Liver	
AA	+	+	Kidney, Urinary tract, Forestomach	Genotoxic non-hepatocarcinogen
APAP	−	−	−	Non-carcinogen

* The carcinogenic activity of PHE is classified as "equivocal evidence" based on studies that have shown a marginal increase in neoplasms that may be related to chemical exposure in a NTP technical report[16].

In vivo mutation assays

6-Thioguanine (6-TG) was used according to the method described in Nohmi *et al.* [18]. Briefly, genomic DNA was extracted from each liver, and the lambda EG10 DNA (48 kb) was rescued in phages by *in vitro* packaging. For 6-TG selection, the packaged phages were incubated with *Escherichia coli* YG6020, which expresses Cre recombinase, and converted to plasmids carrying genes encoding *gpt* and chloramphenicol acetyltransferase. The infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol and 6-TG. To determine the total number of rescued plasmids, the infected cells were poured on plates containing chloramphenicol without 6-TG. The plates were incubated at 37°C for the selection of 6-TG-resistant colonies. Positive colonies were counted on day 3 and collected on day 4. The *gpt* MFs were calculated by dividing the number of *gpt* mutants by the number of rescued phages.

Immunohistochemical staining for GST-P

Immunohistochemical staining was performed using polyclonal antibodies against GST-P (1:1000 dilution; Medical & Biological Laboratories Co., Ltd., Nagoya, Japan). The number and area of GST-P-positive foci consisting of 5 or more nucleated hepatocytes in a crosssection were evaluated using an image analyzer (IPAP, Sumika Technoservice, Hyogo, Japan) [19].

Statistics

The number and area of GST-P-positive foci in experiment I were analyzed using ANOVA followed by Dunnett's multiple comparison test. The number and area of GST-P-positive foci in experiments II, III and IV and the *gpt* MFs in experiments III and IV were analyzed by assessing the variance for homogeneity using the *F*-test. The Student's *t*-test and Welch's *t*-test were used for homogeneous and heterogeneous data, respectively. The *gpt* MFs in the rats treated with SF in experiment IV were analyzed using the Mann-Whitney U test.

Results

Experiment I

Two of the rats in the control group died due to surgical complications of the PH and were eliminated from further evaluation. Treatment with DEN increased the number and area of GST-P-positive foci in a dose-dependent manner compared with the control group (Table 2), although the differences were not significant in the rats that were treated with 10 mg/kg and 50 mg/kg.

Experiment II

Two rats from the 14-week control group, one rat from the 10-week PhB group and one rat from the 12-week PhB group died due to surgical complications of the PH and were eliminated from further evaluation. The number and area of GST-P-positive foci were significantly increased in the rats treated with PhB in each experimental time period (Table 2).

Experiment III

Three rats in the control group, one rat in the group treated with 2-AAF, five rats in the group treated with PBO and one rat in the group treated with APAP died due to surgical complications of the PH and were eliminated from further evaluation. Table 3 shows the MFs in the excised livers of *gpt* delta rats that were treated with 2-AAF, PBO or APAP for 4 weeks. The MFs in the rats treated with 2-AAF were significantly increased compared with the rats in the control group. No significant changes were observed in the rats treated with PBO or APAP. In the *gpt* mutation spectra, GC:TA and GC:CG transversions and single base pair deletions were significantly increased in the rats treated with 2-AAF (Table 4). The number and area of GST-P-positive foci were significantly increased in livers of the rats treated with 2-AAF or PBO and significantly decreased in the livers of the rats treated with APAP (Table 2).

One rat in the control group, four rats in the group treated with IQ, eight rats in the group treated with SF, three rats in the group treated with PHE and two rats in the group treated with AA died due to surgical complications of the PH and were eliminated from further evaluation. Table 5 shows the MFs in the excised livers of *gpt* delta rats that were treated with IQ, SF, PHE or AA for 4 weeks. The MFs in the rats treated with IQ, SF and AA were significantly

Table 2. Quantitative analysis of GST-P-positive foci

Groups	No. of rats	No. of foci (No./cm^2)	Area of foci (mm^2/cm^2)
Experiment I			
Control	3	0.21 ± 0.36	0.002 ± 0.003
DEN 10 mg/kg	5	7.65 ± 3.42	0.072 ± 0.034
DEN 50 mg/kg	5	20.06 ± 3.60	0.326 ± 0.103
DEN 100 mg/kg	5	28.31 ± 5.78**	1.042 ± 0.297**
Experiment II			
10 weeks			
Control	10	5.72 ± 2.47	0.038 ± 0.019
PhB	9	19.81 ± 4.08**	0.153 ± 0.035**
12 weeks			
Control	10	8.59 ± 4.33	0.053 ± 0.028
PhB	9	22.36 ± 4.89**	0.171 ± 0.043**
14 weeks			
Control	8	7.39 ± 2.60	0.053 ± 0.019
PhB	10	26.53 ± 4.41**	0.243 ± 0.048**
Experiment III			
Control	12	4.70 ± 1.53	0.027 ± 0.011
2-AAF	14	24.79 ± 6.15**	0.630 ± 0.315**
PBO	10	7.94 ± 2.22**	0.054 ± 0.015**
APAP	14	0.98 ± 0.42**	0.005 ± 0.002**
Control	14	4.40 ± 1.59	0.025 ± 0.010
IQ	11	7.83 ± 3.33**	0.046 ± 0.019**
SF	7	37.02 ± 10.03**	0.586 ± 0.293**
PHE	12	17.29 ± 5.55**	0.113 ± 0.040**
AA	13	4.70 ± 1.86	0.029 ± 0.015

**Significantly different from the control group at $p< 0.01$.

Table 3. *gpt* MFs in livers of F344 *gpt* delta rats treated with 2-AAF, PBO and APAP

Group	Animal no.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R Colonies	MF ($\times 10^{-5}$)	Mean ± SD
Control	101	11.75	5	0.43	0.44 ± 0.10
	102	22.46	6	0.27	
	103	11.07	6	0.54	
	104	8.46	4	0.47	
	105	10.62	5	0.47	
2-AAF	201	8.33	12	1.44	2.07 ± 0.85**
	202	12.20	14	1.15	
	203	7.79	15	1.93	
	204	8.15	21	2.58	
	205	8.96	29	3.24	
PBO	301	7.70	1	0.13	0.49 ± 0.27
	302	8.42	7	0.83	
	303	7.65	5	0.65	
	304	15.03	5	0.33	
	305	8.10	4	0.49	
APAP	401	18.77	4	0.21	0.40 ± 0.14
	402	18.68	7	0.37	
	403	11.39	7	0.61	
	404	15.53	6	0.39	
	405	14.45	6	0.42	

**Significantly different from the control group at $p< 0.01$.

increased compared with the rats in the control group. In the *gpt* mutation spectra, GC:TA transversions, GC:AT transitions and single base pair deletions were significantly increased in the rats treated with IQ, and AT:TA transversions were significantly increased in the rats treated with AA (Table 6). No significant changes were observed in the rats treated with SF. The number and area of GST-P-positive foci were significantly increased in the livers of the rats treated

Table 4. Mutation spectra of *gpt* mutant colonies in livers of F344 *gpt* delta rats treated with 2-AAF, PBO and APAP

	Control		2-AAF		PBO		APAP	
	Number (%)	Mutation frequency (10^{-5})	Number (%)	Mutation frequency (10^{-5})	Number (%)	Mutation frequency (10^{-5})	Number (%)	Mutation frequency (10^{-5})
Transversions								
GC-TA	6[a] (23.1)	0.11 ± 0.08	32 (35.2)	0.72 ± 0.27**	5 (22.7)	0.13 ± 0.16	7 (23.3)	0.01 ± 0.09
GC-CG	1 (3.8)	0.01 ± 0.02	9 (9.9)	0.20 ± 0.17*	1 (4.5)	0.02 ± 0.05	3 (10.0)	0.03 ± 0.05
AT-TA	1 (3.8)	0.02 ± 0.04	8 (8.8)	0.17 ± 0.21	2 (9.1)	0.03 ± 0.06	3 (10.0)	0.04 ± 0.05
AT-CG	1 (3.8)	0.11 ± 0.02	3 (3.3)	0.07 ± 0.15	1 (4.5)	0.02 ± 0.06	1 (3.3)	0.02 ± 0.04
Transitions								
GC-AT	15 (57.7)	0.26 ± 0.08	19 (20.9)	0.39 ± 0.35	9 (40.9)	0.20 ± 0.14	14 (46.7)	0.19 ± 0.09
AT-GC	0	0	4 (4.4)	0.10 ± 0.11	1 (4.5)	0.02 ± 0.05	0	0
Deletion								
Single bp	1 (3.8)	0.02 ± 0.04	12 (13.2)	0.28 ± 0.21*	2 (9.1)	0.04 ± 0.06	2 (6.7)	0.03 ± 0.04
Over 2 bp	0	0	1 (1.1)	0.02 ± 0.05	1 (4.5)	0.02 ± 0.05	0	0
Insertion	1 (3.8)	0.02 ± 0.04	3 (3.3)	0.07 ± 0.07	0	0	0	0
Complex	0	0	0	0	0	0	0	0

[a] Number of colonies with independent mutations.*,**Significantly different from the control group at $p< 0.05$ and $p< 0.01$, respectively.

Table 5. *gpt* MFs in livers of F344 *gpt* delta rats treated with IQ, SF, PHE and AA

Group	Animal no.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies	MF ($\times 10^{-5}$)	Mean ± SD
Control	101	15.1	3	0.20	0.38 ± 0.19
	102	6.8	4	0.59	
	103	15.9	7	0.44	
	104	12.2	2	0.16	
	105	8.1	4	0.50	
IQ	201	8.9	18	2.03	3.35 ± 1.22**
	202	7.2	34	4.69	
	203	6.1	18	2.94	
	204	10.4	26	2.49	
	205	4.4	20	4.58	
SF	301	10.0	8	0.80	1.18 ± 0.74**
	302	5.0	5	1.00	
	303	5.6	14	2.49	
	304	10.1	7	0.69	
	305	5.4	5	0.92	
PHE	401	7.9	3	0.38	0.36 ± 0.26
	402	4.5	1	0.22	
	403	11.4	1	0.09	
	404	5.9	2	0.34	
	405	7.7	6	0.78	
AA	501	8.6	13	1.50	1.18 ± 0.41**
	502	9.8	17	1.73	
	503	12.9	12	0.93	
	504	11.3	9	0.79	
	505	9.5	9	0.95	

**Significantly different from the control group at $p< 0.01$.

with IQ, SF and PHE (Table 2).

Discussion

Chemical carcinogenesis involves multiple gene alterations, which can be divided into initiation and promotion phases. A medium-term rat liver bioassay involving the quantitative analysis of GST-P-positive foci following cell proliferative stimuli via PH was established to detect the tumor promoting activities of various chemicals. Reporter gene mutation assays using transgenic animals have been developed to detect *in vivo* mutagenicity. Because this assay can be performed under conditions that are similar to the conventional long-term bioassay, the results may represent the tumor initiation phase of chemical carcinogenesis. GST-P-positive foci have been analyzed in *gpt*delta rats[7,20,21]. The GPG46 animal model described in this study can detect the tumor-initiating and tumor-promoting activities of vari-

Table 6. Mutation spectra of *gpt* mutant colonies in livers of F344 *gpt* delta rats treated with IQ, SF, PHE and AA

	Control		IQ		SF		PHE		AA	
	Number (%)	Mutation frequency (10^{-5})	Number (%)	Mutation frequency (10^{-5})	Number (%)	Mutation frequency (10^{-5})	Number (%)	Mutation frequency (10^{-5})	Number (%)	Mutation frequency (10^{-5})
Transversions										
GC-TA	5[a] (25.0)	0.11 ± 0.09	50 (43.1)	1.40 ± 0.41**	13 (33.3)	0.41 ± 0.38	4 (30.8)	0.10 ± 0.17	11 (18.3)	0.21 ± 0.09
GC-CG	1 (5.0)	0.01 ± 0.03	4 (3.5)	0.11 ± 0.25	6 (15.4)	0.17 ± 0.13	1 (7.7)	0.03 ± 0.06	1 (1.7)	0.02 ± 0.05
AT-TA	0	0	6 (5.2)	0.20 ± 0.18	3 (7.7)	0.09 ± 0.09	0	0	29 (48.3)	0.55 ± 0.30**
AT-CG	0	0	1 (0.9)	0.03 ± 0.06	2 (5.1)	0.06 ± 0.08	0	0	0	0
Transisions										
GC-AT	8 (40.0)	0.14 ± 0.11	14 (12.1)	0.40 ± 0.16*	6 (15.4)	0.17 ± 0.14	6 (46.2)	0.16 ± 0.15	7 (11.7)	0.15 ± 0.13
AT-GC	3 (15.0)	0.07 ± 0.13	0	0	4 (10.3)	0.13 ± 0.15	1 (7.7)	0.03 ± 0.08	2 (3.3)	0.04 ± 0.09
Deletion										
Single bp	3 (15.0)	0.04 ± 0.04	39 (33.6)	1.17 ± 0.58*	3 (7.7)	0.10 ± 0.17	1 (7.7)	0.03 ± 0.08	8 (13.3)	0.16 ± 0.16
Over 2 bp	0	0	1 (0.9)	0.02 ± 0.04	0	0	0	0	0	0
Insertion	0	0	1 (0.9)	0.02 ± 0.05	2 (5.1)	0.06 ± 0.08	0	0	2 (3.3)	0.04 ± 0.06
Complex	0	0	0	0	0	0	0	0	0	0

[a] Number of colonies with independent mutations. *,**Significantly different from the control group at $p< 0.05$ and $p< 0.01$, respectively.

ous chemicals by combining the reporter gene mutation assay and the medium-term liver bioassay.

In this animal model, *gpt* delta rats were exposed to chemicals, and a PH was performed to collect liver samples for an *in vivo* mutation assay. The rats were subsequently administered a single ip injection of DEN, and the tumor promoting activity of the chemical was evaluated based on the development of GST-P-positive foci. The World Health Organization (WHO) guidelines state that 4 weeks of exposure is sufficient for detecting mutations in the reporter gene[6], which is supported by additional data[8,22]. Therefore, the period of exposure prior to PH in this study was determined to be 4 weeks. Initial exposure to a potent genotoxic carcinogen is necessary to detect tumor promoting activities over a short period of time. In this model, DEN was selected because correlations between the administration of DEN and the induction of GST-P foci in the rat liver have been extensively reported[23–26]. However, the dose of DEN should be as low as possible to avoid any effects on the metabolism of the test chemical because DEN has been shown to influence various parameters, including the induction of cytochrome P450 (CYP) and glutathione S-transferase[27,28]. We took advantage of the rapid induction of cell proliferation following PH because genotoxic compounds can effectively induce gene mutations under conditions of high cell proliferation[4]. Tsuda *et al.* [29] reported that the initiator should optimally be administered 18 h after PH to effectively enhance initiation. Based on these data, appropriate dosages of DEN were investigated in a dose-response study consisting of single ip injections of DEN 18 h after PH at doses of 10 mg/kg and higher. The optimal dosage of DEN was established as 10 mg/kg based on the quantitative analysis of GST-P-positive foci. PhB, a liver tumor promoter in rodents[30], was used to determine the optimal duration of exposure following a PH in experiment II. The results of this study demonstrate that treatment with PhB at 500 ppm in the diet for 6 weeks is

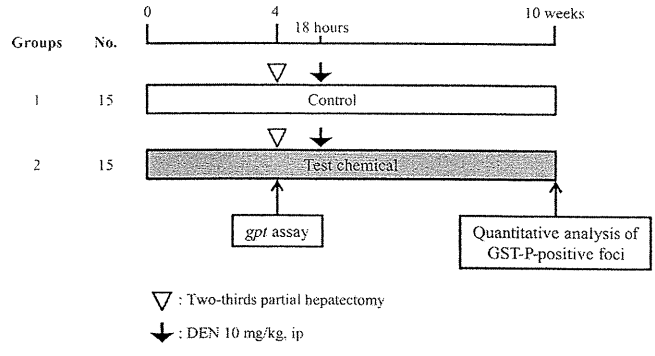

Fig. 1. Tentative protocol for the GPG46 model. Six-week-old male F344 *gpt* delta rats were exposed to various chemicals for 10 weeks. A partial hepatectomy (PH) was performed at week 4, and the rats were administered a single ip injection of 10 mg/kg diethylnitrosamine (DEN) 18 h after PH. The *gpt* assay, which is an indicator of tumor initiation, was performed using the liver samples excised via PH at week 4. Tumor promoting activities were evaluated based on the development of GST-P-positive foci induced by DEN at week 10.

effective in detecting the effects of tumor promotion. The tentative protocol for the GPG46 animal model is shown in Fig. 1.

The animal model was validated in experiment III. 2-AAF, IQ and SF are genotoxic murine liver carcinogens that produce deoxyguanine adducts via metabolic activation and play a key role in liver carcinogenesis[31–34]. A significant increase in the MFs of the *gpt* genes in the rats treated with 2-AAF, IQ and SF was shown using the GPG46 model. Spectrum analysis in the *gpt* mutant colonies revealed that guanine-related mutations and single base pair deletions were induced by 2-AAF and IQ, but not SF, which is in agreement with previous reports[35–37]. In the conventional medium-term bioassay, 2-AAF, IQ and SF exposure induced a marked in-

crease in the development of GST-P-positive foci[38], implying that these chemicals also exert a strong tumor promoting action. The GPG46 animal model showed that the development of GST-P-positive foci at 10 weeks was markedly increased in the livers of rats treated with these carcinogens. PBO and PHE were reported to act as hepatocarcinogens in F344 rats fed a diet containing 12000 ppm and 2400 ppm for 2 years, respectively[13,16]. These compounds are classified as non-genotoxic carcinogens based on the results of various genotoxicity studies[16,39]. An increase in the development of GST-P-positive foci was observed in rats treated with PBO or PHE in a conventional medium-term bioassay[38,40]. Treatment with PBO and PHE at the carcinogenic dose in the GPG46 animal model did not increase the *gpt* MF, although the development of GST-P-positive foci was significantly increased. APAP was not reported to behepatocarcinogenic in F344 rats fed a diet containing 6000 ppm for 2 years[14]. In the present study, treatment with APAP in the GPG46 model at a dose of 6000 ppm did not increase the *gpt* MF and inhibited the development of GST-P-positive foci. Ito *et al.*[38] showed that APAP had an inhibitory effect on the development of GST-P-positive foci in a conventional medium-term bioassay. AA has been reported to be carcinogenic in the kidney and the stomach of rodents[41]. In an *in vivo* genotoxicity study in Big Blue transgenic rats, AA exposure elevated *cII* MFs and produced AA-specific deoxyadenine and deoxyguanine adducts in the kidney and the liver[42]. A significant increase in *gpt* MFs in rats treated with AA was observed in the GPG46 model, and AT:TA transversions were the predominant mutation in the mutation spectra analysis, which is similar to a previous report[42]. AA did not have an enhancing effect on the development of GST-P-positive foci, which may reflect the fact that AA exerts initiation activity, but not carcinogenicity, in the liver.

Overall, the validation results show the possibility of developing a new animal model using *gpt* delta rats. However, a possible limitation of the tentative protocol is that the test chemicals are co-administered simultaneously with DEN. Although there did not appear to be any mutual effects between DEN and the test chemicals, this treatment regimen may modify the detoxification or metabolic activation of DEN. Several isoforms of CYP have been reported to participate in the metabolic activation of DEN, with CYP2E1 in particular playing an essential role[43]. Because many liver tumor promoters in rodents can induce several types of CYPs and/or modify the expression of phase II enzymes, we are working toward improving the timing of the regimen to avoid the possibility of mutual effects. Validation studies of the revised protocol based on changes in the timing of chemical administration are currently in progress.

In conclusion, the potential development of a GPG46 medium-term animal model to evaluate the tumor-initiating and tumor-promoting activities of various chemicals in a single study was demonstrated. In this assay, additional analyses, such as quantification of DNA modifications, the activities of metabolic enzymes and the mRNA levels of tumor-associated genes, are valuable for understanding the modes of action of various test chemicals.

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In Vivo Genotoxicity of Methyleugenol in *gpt* Delta Transgenic Rats Following Medium-Term Exposure

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Methyleugenol (MEG), which is commonly used as a fragrance and flavoring agent, has been shown to induce hepatocellular tumors in rodents. However, the role of genotoxicity as a possible mechanism of action is not fully understood even though the DNA-reactive metabolite of MEG has been identified. In this study, a *gpt* delta transgenic rat model was used to clarify whether genotoxic mechanisms are involved in MEG-induced hepatocarcinogenesis following medium-term exposure. F344 *gpt* delta rats were subjected to repeated oral administration of MEG at dosages of 0, 10, 30, or 100 mg/kg (a carcinogenic dose) for 13 weeks. The relative weight of the liver of the male and female rats that were administered 100 mg/kg MEG and the absolute weight of the liver of the male rats that were administered 100 mg/kg MEG were significantly increased. In addition, the number and area of glutathione S-transferase placental form (GST-P) positive foci and proliferating cell nuclear antigen (PCNA) positive cell ratios in the hepatocytes were significantly increased in the male and female rats that were administered 100 mg/kg MEG compared with the control animals. In the *in vivo* mutation assays, a significant increase in the *gpt* and Spi$^-$ mutant frequencies was observed in both sexes at the carcinogenic dose. These results suggest the possible participation of genotoxic mechanisms in MEG-induced hepatocarcinogenesis.

Key Words: **methyleugenol; *in vivo* genotoxicity; *gpt* delta rats.**

Methyleugenol (3,4-dimethoxyallylbenzene; MEG) is a natural flavor present in many herbs and spices consumed at low levels in the human diet and has been approved for commercial use as a fragrance and flavoring agent. MEG is a member of the alkoxy-substituted allylbenzene family of chemicals, which include other naturally occurring substances, such as estragole and safrole. MEG is a hepatocarcinogen in F344 rats and B6C3F$_1$ mice at doses higher than 37 mg/kg, which is similar to the carcinogenic dose of other alkoxy-substituted allylbenzenes (National Toxicology Program [NTP], 2000). At the 69th meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), a safety assessment of flavoring substances, including alkoxy-substituted allylbenzenes, was reported to be pending because the underlying mechanisms of their carcinogenic effects remained unclear (FAO/WHO, 2008). Data regarding the underlying modes of action in MEG-induced hepatocarcinogenesis are important for establishing an overall risk assessment of the flavoring agents classified as alkoxy-substituted allylbenzenes.

Zhou *et al.* (2007) reported that MEG forms adducts in human cellular DNA. Other alkoxy-substituted allylbenzenes, such as safrole and estragole, also form specific DNA adducts (Ishii *et al.*, 2011; Miller and Miller, 1983), which may trigger genotoxicity in the rat liver (Jin *et al.*, 2011; Suzuki *et al.*, 2012). As such, it is highly probable that MEG has the potential to be genotoxic *in vivo* in the rat liver. However, MEG was not shown to be mutagenic in *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537 (NTP, 2000). Several MEG metabolites have been reported to be activated to DNA-reactive mutagens in *S. typhimurium* following incorporation of increased sulfation capacity and may be involved in carcinogenicity, organ toxicity, and immune reactions (Cartus *et al.*, 2012; Herrmann *et al.*, 2012). The results of conventional genotoxicity tests, such as sister chromatid exchange (SCE), the micronucleus test, and the comet assay, have not been consistent (Ding *et al.*, 2011; NTP, 2000).

The *gpt* delta transgenic rat developed by Nohmi *et al.* (1996) is recognized as an *in vivo* mutation assay model that is capable of detecting point mutations by 6-thioguanine (6-TG) selection and deletion mutations by Spi$^-$ selection (Hayashi *et al.*, 2003; Masumura *et al.*, 2003; Umemura *et al.*, 2009). Quantitative analyses of glutathione S-transferase placental form (GST-P) positive foci can be performed in *gpt* delta transgenic rats and conventional non-transgenic rats (Toyoda-Hokaiwado *et al.*, 2010). The *gpt* delta rat animal model may be a promising tool for investigating the comprehensive toxicities, including the genotoxicity and carcinogenicity, of chemical agents in target organs. We confirmed the availability of a medium-term animal model using *gpt* delta rats that demonstrated hepatotoxicity, hepatocarcinogenicity, and genotoxicity following exposure to safrole in the target organ (Jin *et al.*, 2011).

In this study, the *gpt* delta rat animal model was used to determine the comprehensive carcinogenicity and *in vivo* genotoxicity of MEG at three different doses, including the reported carcinogenic dose, following medium-term exposure.

MATERIALS AND METHODS

Chemicals. MEG and methylcellulose were purchased from Wako Pure Chemical Industries (Osaka, Japan). The MEG solutions were prepared in 0.5% aqueous methylcellulose.

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 (Tokyo, Japan). Five-week-old male and female F344 *gpt* delta rats carrying approximately five tandem copies of the transgene lambda EG10 per haploid genome were obtained from Japan SLC (Shizuoka, Japan). The rats were housed in polycarbonate cages (three or four rats per cage) with hardwood chips for bedding in a conventional animal facility. The animals were maintained in a controlled environment with constant temperature (23±2°C), humidity (55±5°C), air changes (12 times per hour), and lighting (12 h light/dark cycle). The animals were allowed free access to a basal diet of CRF-1 commercial pellets (Charles River Japan, Kanagawa, Japan) and tap water.

Experimental design. After a 1-week acclimatization period, the animals were divided into four groups consisting of 10 male and 10 female F344 *gpt* delta rats per group. The rats were administered an intragastric dose of 0, 10, 30, or 100 mg/kg MEG daily for 13 weeks.

The clinical signs and general appearance of the rats were observed once per day. Body weight and food consumption were measured once per week. At the end of each 13-week treatment cycle, the animals were euthanized under deep anesthesia. The left lobes of the liver were fixed in neutral-buffered formalin for histopathological and immunohistopathological examination. The remaining liver was stored at −80°C for *in vivo* mutation assays.

In vivo *mutation assays.* 6-TG and Spi⁻ (insensitive P2 interference) selection assays were performed as previously described (Nohmi *et al.*, 1996, 2000). Briefly, genomic DNA was extracted from the liver tissue, and lambda EG10 DNA (48 kb) was rescued as the lambda phage through *in vitro* packaging. For 6-TG selection, the packaged phage was incubated with *Escherichia coli* YG6020, which expresses Cre recombinase and converted to a plasmid carrying *gpt* and chloramphenicol acetyltransferase. Infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol and 6-TG. To determine the total number of rescued plasmids, the infected cells were poured onto plates containing chloramphenicol without 6-TG. The plates were incubated at 37°C for selection of 6-TG-resistant colonies, and the *gpt* mutant frequency (MF) was calculated by dividing the number of *gpt* mutants after clonal correction by the number of rescued phages. To characterize the *gpt* mutations, a 739-bp DNA fragment containing the 456-bp coding region of the *gpt* gene was amplified using PCR as previously described. The PCR products were analyzed with an Applied Biosystems 3730x1 DNA Analyzer (Applied Biosystems, Japan Ltd).

For Spi⁻ selection, the packaged phage was incubated with *E. coli* XL-1 Blue MRA for survival titration and *E. coli* XL-1 Blue MRA P2 for mutant selection. The infected cells were mixed with molten lambda-trypticase agar plates. The following day, plaques (Spi⁻ candidates) were punched out with sterilized glass pipettes, and the agar plugs were suspended in SM buffer. To confirm the Spi⁻ phenotype of the candidates, the suspensions were spotted on three types of plates containing XL-1 Blue MRA, XL-1 Blue MRA P2, or WL95 P2 strains and spread with soft agar. The number of mutants that exhibited clear plaques on each plate was counted as confirmed Spi⁻ mutants. The Spi⁻ MF was calculated by dividing the number of Spi⁻ mutants by the number of rescued phages. In all of the *in vivo* mutations assays, positive DNA samples were included to ensure that the assay was functioning properly.

Immunohistochemical staining. Immunohistochemical staining was performed using polyclonal antibodies against GST-P (1:1000 dilution; Medical and Biological Laboratories Co., Ltd, Nagoya, Japan), which is a marker of preneoplastic lesions in the rat liver, and monoclonal anti-mouse proliferating cell nuclear antigen (PCNA) antibodies (1:100; Dako, Glostrup, Denmark) to evaluate cell proliferation activity using the avidin–biotin peroxidase complex method. The number (No./cm²) and area (mm²/cm²) of the GST-P-positive foci (> 0.01 mm²) and the total area of each liver section were measured using an IPAP image analyzer (Sumika Technos, Osaka, Japan) (Watanabe *et al.*, 1994). The numbers of PCNA-positive cells per 600–800 intact liver cells from 10 different areas per animal were counted to determine the PCNA-positive ratio.

Statistics. The body weight measurements, food and water consumption, weight of the liver, GST-P-positive foci, PCNA-LI, *gpt* and Spi⁻ MFs, and various mutation frequencies in the spectrum analysis were expressed as the mean ± SD. Significant differences between the control and treated groups were determined using Dunnett's multiple comparison test (Dunnett, 1955) after ANOVA. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Body and Liver Weights and Histopathological Examination

The final body and liver weights are shown in Table 1. There was no suppression of body weight gain in the treated groups

TABLE 1
Final Body and Liver Weights of *gpt* Delta Rats Administered MEG for 13 Weeks

Sex	Groups	Number of animals	Final body weight (g)	Liver weight	
				Absolute (g)	Relative (g/100 g bw)
Male					
	Control	10	300.0±17.4[a]	8.09±0.49	2.45±0.07
	10 mg/kg MEG	10	325.0±10.4	8.22±0.41	2.53±0.08
	30 mg/kg MEG	10	322.4±13.7	8.54±0.41	2.65±0.08
	100 mg/kg MEG	10	310.2±35.4	9.43±0.71*	3.09±0.45*
Female					
	Control	10	189.0±5.6	4.26±0.22	2.26±0.12
	10 mg/kg MEG	9	190.3±8.3	4.34±0.22	2.28±0.14
	30 mg/kg MEG	9	187.6±5.3	4.29±0.22	2.29±0.11
	100 mg/kg MEG	9	176.5±9.7	4.38±0.29	2.48±0.08**

[a]Mean ± SD.
*Significantly different from the male control group at the levels of $p < 0.001$ (Dunnett's test).
**Significantly different from the female control group at the levels of $p < 0.001$ (Dunnett's test).

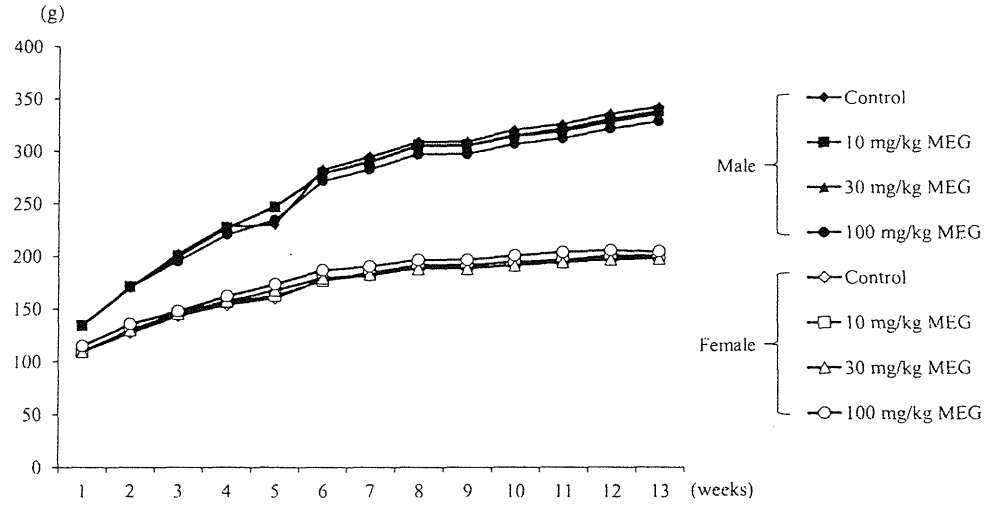

FIG. 1. Body weights of male and female *gpt* delta rats treated with MEG for 13 weeks.

TABLE 2
***gpt* MFs in the Liver of Male *gpt* Delta Rats Administered MEG for 13 Weeks**

Sex	Groups	Animal No.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies	MF ($\times 10^{-5}$)	Mean ± SD
Male	Control	1	3.4	2	0.59	0.53±0.18
		2	4.5	2	0.44	
		3	4.5	3	0.67	
		4	2.9	2	0.69	
		5	7.6	2	0.27	
	10 mg/kg MEG	11	9.5	4	0.42	0.79±0.34
		12	4.8	6	1.25	
		13	3.0	2	0.66	
		14[a]	1.9	2	1.03[a]	
		15	5.3	3	0.57	
	30 mg/kg MEG	21	2.5	1	0.40	0.79±0.42
		22	5.5	8	1.45	
		23	4.9	3	0.61	
		24	7.3	7	0.96	
		25	3.6	2	0.55	
	100 mg/kg MEG	31	6.0	12	2.20	1.35±0.60*
		32	4.1	3	0.73	
		33	8.3	8	0.96	
		34	3.5	4	1.14	
		35	3.6	6	1.69	

[a]Data from animal No. 14 was excluded for the calculation of MF because of the poor packaging efficiency of the transgene.
*Significantly different from the control group at $p < 0.05$ (Dunnett's test).

during the experiment (Fig. 1). The relative weight of the liver of the male and female rats treated with 100 mg/kg and the absolute weight of the liver of the males treated with 100 mg/kg MEG were significantly increased compared with the rats in the control group. There were no histopathological changes observed in the liver of the rats treated with MEG.

In Vivo *Mutation Assays*

The *gpt* and Spi^- MFs in the liver of male and female *gpt* delta rats treated with MEG for 13 weeks are summarized in Tables 2–5. A significant increase in the *gpt* and Spi^- MFs was observed in the male and female rats treated with the carcinogenic dose of 100 mg/kg. We determined the *gpt* mutation spectra in the *gpt* mutant colonies to characterize the types of *gpt* mutations caused by exposure to MEG. A GC→CG transversion mutation in the treated male rats and an AT→TA transversion mutation in the treated female rats were observed, but the incidence rates were not statistically significant compared with the controls (Table 6).

Effects of MEG on GST-P-Positive Foci and Cell Proliferation

Under immunohistochemical examination, treatment with MEG increased the number and area of GST-P-positive foci