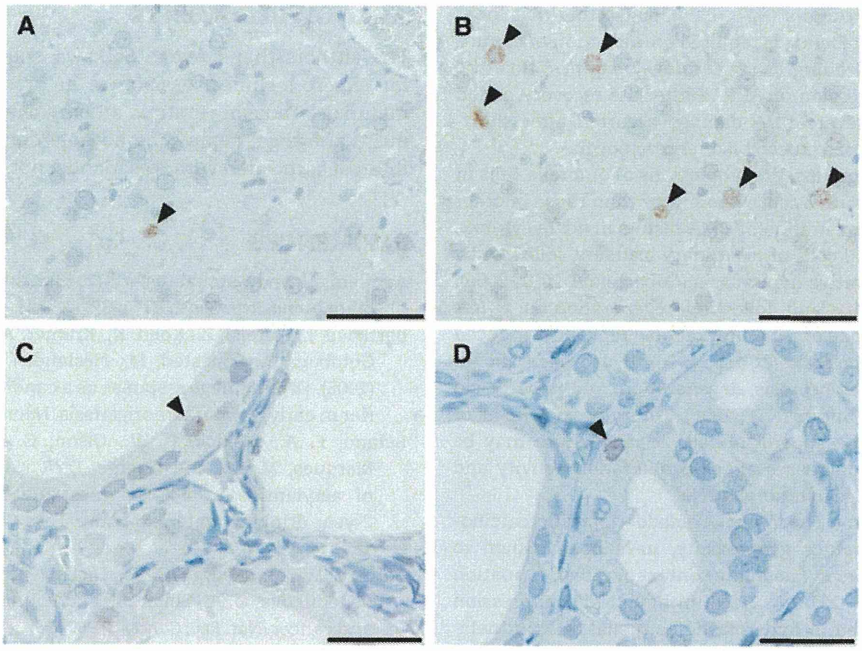

FIG. 6. Representative immunohistochemical findings for γ-H2AX in the liver and thyroid gland of male F344 rats. Bars = 50 μm (A and B) and 25 μm (C and D). A, Liver from a control rat. B, Liver from an N-nitrosodiethylamine (DEN)-treated rat. C, Thyroid gland from a control rat. D, Thyroid gland from an acrylamide-treated rat. γ-H2AX-positive cells (arrowheads) were observed in both hepatocytes (A and B) and follicular epithelial cells (C and D). Note the increased expression of γ-H2AX in the DEN-treated liver (B).

DISCUSSION

In the present study, we investigated the expression profiles of γ-H2AX and Ki67 induced by 12 chemicals exhibiting genotoxic/nongenotoxic or carcinogenic/noncarcinogenic characteristics in the urinary bladder using a standard 28-day repeated-dose toxicity study. Our data clearly demonstrated that all 4 genotoxic bladder carcinogens examined in this study (ie, BBN, 2-NA, 2-AAF, and *p*-cresidine) significantly increased the number of γ-H2AX-positive urothelial cells after 4 weeks of administration, whereas all 3 chemicals that are genotoxic but not carcinogenic to the bladder (ie, glycidol, DEN, and AA) did not induce this change. These results suggested that immunohistochemistry analysis of γ-H2AX expression may be useful for early detection of genotoxic bladder carcinogens.

DSBs are induced by various factors, including direct DNA damage through adduct formation by genotoxic chemicals and indirect interactions, eg, excessive accumulation of reactive oxygen species or deficient repair of non-DSB DNA lesions (Bonner *et al.*, 2008). Therefore, nongenotoxic agents can increase γ-H2AX expression in their target tissues through a combination of frequent errors of DNA replication by abnormally enhanced cell proliferation and induction of oxidative stress. Indeed, in the present study, 2 nongenotoxic agents (melamine and uracil) and a chemical with equivocal genotoxicity (PEITC) induced a slight (nonsignificant) increase in the number of γ-H2AX-positive cells in the urothelium. Because these 3 chemicals cause proliferative lesions in the urinary bladder, as shown by immunohistochemistry for Ki67, changes in γ-H2AX expression levels may reflect the degree of DNA damage induced by indirect DNA injury in the urothelium. Thus, increases in γ-H2AX expression indicate both direct and indirect severe DNA damage. In addition, γ-H2AX expression in rats treated with chemicals that do not target the bladder was similar to that in control rats, indicating that γ-H2AX may also be helpful for the detection of bladder-toxic chemicals.

At the end of 4 weeks of administration, γ-H2AX- and Ki67-positive cells were predominantly found in basal cells in most groups. Basal cells of the bladder urothelium are generated from stem cells located in the basal layer and sequentially differentiate into intermediate and umbrella cells (Ho *et al.*, 2012). As the basal cells are major proliferative cells in bladder urothelium, as shown by immunohistochemistry for Ki67, these basal cells may be highly susceptible to DNA damage directly or indirectly induced by the chemicals. γ-H2AX-positive cells were markedly reduced in number after the recovery period, suggesting the progression of DNA repair and removal of damaged cells by apoptosis. Interestingly, the majority of γ-H2AX-positive cells shifted from basal cells to intermediate and umbrella cells in most groups. There are at least 2 possible explanations for this observation: (1) The basal cells may have a relatively high potential for DNA repair compared with the intermediate and umbrella cells, and (2) some of the damaged basal cells may be able to continue differentiating into these 2 cell populations.

γ-H2AX expression induced by 3 of the 4 genotoxic bladder carcinogens (ie, BBN, 2-NA, and 2-AAF) remained significantly higher than that in control rats, even after 2 weeks of recovery. The remaining DNA damage may contribute to subsequent carcinogenesis because 4 weeks of treatment with 0.05% BBN is sufficient to induce bladder carcinoma in rats (Imaida *et al.*, 1983). On the other hand, γ-H2AX expression in rats treated with *p*-cresidine, another genotoxic bladder carcinogen used in this study, was reduced to levels similar to those observed in control rats after withdrawal. A carcinogenicity study of *p*-cresidine conducted by the National Toxicology Program reported that 51 weeks of dietary administration is required for

detection of bladder tumors (NTP, 1979), suggesting that long-term exposure to *p*-cresidine is required for induction of continuous DNA damage leading to cancer development. Thus, the remaining high expression of γ-H2AX after the recovery period may indicate that severe DNA damage occurred after short-term administration of a crucial bladder carcinogen.

In BMP and DMA groups, the proportions of umbrella cells in γ-H2AX- and Ki67-positive cells were higher than those in other groups. Dietary administration of DMA results in acute cytotoxicity in the superficial cells of rat urinary bladders, followed by necrosis and regenerative hyperplasia (Cohen *et al.*, 2001, 2006). Urothelial cytotoxicity and following regeneration is a frequently-observed mode of action for rat bladder cancer induced by nongenotoxic chemicals (Cohen, 1998). It has been shown that BMP also induces necrosis, degeneration, and hyperplasia in the urinary tracts of rats (NTP, 1996). The cytotoxic effects of BMP and DMA on the superficial cells of urothelium may be associated with the increases of cell proliferation activity and following DNA damage in umbrella cells.

Melamine and uracil, well-known bladder-specific carcinogens that do not induce genotoxicity, have been shown to induce bladder tumors in rats in the context of calculi formation (Fukushima *et al.*, 1992; IARC, 1999). Although Ki67 expression was diffusely detected in hyperplastic epithelial cells in melamine- and uracil-treated rats harboring numerous macroscopic calculi, γ-H2AX-positive cells were relatively scattered as discrete foci in the urothelium. Focal increases in the inflammatory response and cell proliferation activity in response to the physical stimulus of the calculi may be associated with the regional increase of cytotoxicity and following expression of γ-H2AX.

The observed increase in the number of γ-H2AX-positive hepatocytes induced by DEN treatment suggested the potential application of this target for detection of genotoxic liver carcinogens. In contrast, 4-week administration of glycidol and AA, which are both carcinogenic to the rat thyroid gland, did not show such an increase. These findings indicate the organ-specific differences of chemical-induced DNA damage; thus, some organs may require long-term exposure of more than 4 weeks to detect increases in γ-H2AX expression. Importantly, the absence of an increase in γ-H2AX after 4 weeks of treatment does not always indicate that a test chemical is nongenotoxic or noncarcinogenic to the specific organ. Further complementary analyses will be needed for determination of genotoxicity and carcinogenicity in γ-H2AX-negative cases.

In conclusion, in the present study, we demonstrated that γ-H2AX is a potential biomarker for the early detection of genotoxic carcinogens in the urinary bladder. Immunohistochemistry for γ-H2AX can be performed simultaneously with repeated-dose oral toxicity tests and may be readily applied for retrospective analyses using formalin-fixed and paraffin-embedded tissues from previous studies. Although further analysis of the specificity, dose-dependency, and applications of this method to other organs is required, γ-H2AX may also be a useful tool for investigation of the mechanisms of bladder cancer development.

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A medium-term *gpt* delta rat model as an *in vivo* system for analysis of renal carcinogenesis and the underlying mode of action

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ABSTRACT

The kidney is a major target site of chemical carcinogenesis. However, a reliable *in vivo* assay for rapid identification of renal carcinogens has not been established. The purpose of this study was to develop a new medium-term *gpt* delta rat model (the GNP model) to facilitate identification of renal carcinogens. In this model, we carried out an *in vivo* mutation assay using unilaterally nephrectomized kidney tissue and a tumor-promoting assay using residual kidney tissue, with diethylnitrosamine (DEN) as the renal tumor initiator. To clarify the optimal time of DEN injection after nephrectomy, time-dependent changes in bromodeoxyuridine-labeling indices in the tubular epithelium of nephrectomized rats were examined. The optimal dose of DEN injection and sufficient duration of subsequent nitrilotriacetic acid treatment were determined for detection of renal preneoplastic lesions. The standard protocol for the GNP model was determined as follows. Six-week-old female *gpt* delta rats were treated with test chemicals for 4 weeks, followed by a 2-week washout period, and 40 mg/kg DEN was administered intraperitoneally to initiate renal carcinogenesis. Unilateral nephrectomy was performed 48 h before DEN injection, followed by *gpt* assays using excised kidney tissues. One week after DEN injection, rats were further exposed to test chemicals for 12 weeks, and histopathological analysis of renal preneoplastic lesions was performed as an indicator of tumor-promoting activity in residual kidney tissue. Validation studies using aristolochic acid, potassium dibasic phosphate, phenylbutazone, and *d*-limonene indicated the reliability of the GNP model for predicting renal carcinogens and the underlying mode of action.

1. Introduction

Carcinogenicity is a key factor in safety assessments of environmental chemicals because resulting neoplastic lesions can be irreversible and often fatal. The kidney receives an abundant supply of blood in order to perform its vital roles in metabolism and excretion of xenobiotics, which may increase the risk of carcinogen exposure (Radford et al., 2013). In addition, as the kidney possesses phase I and phase II detoxification mechanisms, it is highly probable that DNA damage can be caused by reactive metabolites or oxidative stress generated during chemical metabolism (Choudhary et al., 2005; Mizerovská et al., 2011; Priestap et al., 2012; Kakehashi et al., 2013). Indeed, National Toxicology Program (NTP) background data for lifetime bioassays using rodents demonstrated that the kidney is the second organ targeted by chemical carcinogenesis after the liver (National Toxicology Program, 2014). However, because tremendous amounts of time and large numbers of animals are required in lifetime bioassay using rodents, the International Conference on Harmonisation (ICH) guideline recommends alternative *in vivo* studies, including medium-term rat liver animal models, e.g., the Ito model, or 6-month carcinogenicity models using transgenic mice, such as rasH2 and *p53*-deficient mice (International Conference on Harmonisation, 1997). In particular, quantitative analysis of glutathione *S*-transferase placental form (GST-P) foci, which is used as a preneoplastic hepatocyte marker in the Ito model, is very

Abbreviations: UN, unilateral nephrectomy; PH, partial hepatectomy; GST-P, glutathione *S*-transferase placental form; DEN, diethylnitrosamine; AT, atypical tubule; AH, atypical hyperplasia; BrdU, 5-bromo-2'-deoxyuridine; PCT, proximal convoluted tubule; PST, proximal straight tubule; DT, distal tubule; NTA, trisodium nitrilotriacetic acid; AA, aristolochic acid; PDP, potassium dibasic phosphate; PBZ, phenylbutazone; DL, *d*-limonene; DW, distilled water; MF, mutant frequency; LIs, labeling indices.

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reliable and contributes to the predictive accuracy of liver tumor promoters (Ito et al., 2003; Tsuda et al., 2010). However, there have been no reports describing the expression of mutational enzymes corresponding to GST-P in renal cells, and therefore, no medium-term animal models to rapidly predict renal carcinogens have been developed. In addition, alternative *in vivo* studies to long-term bioassays are required to gain information about modes of action underlying carcinogenesis, including the participation of genotoxic mechanisms (Cohen and Arnold, 2011).

In vivo mutation assays using reporter gene transgenic rodents can be combined with additional assays to investigate modes of action underlying chemical carcinogenesis, such as the formation of DNA adducts, induction of cell proliferation, and occurrence of oxidative stress (Kuroda et al., 2013; Tasaki et al., 2013; Ishii et al., 2014). In the previous studies, we developed a medium-term *gpt* delta rat model (the GPG model) capable of rapidly detecting *in vivo* mutagenicity and tumor-promoting activity in the liver (Matsushita et al., 2013, 2014). In the GPG model, partial hepatectomy (PH) is performed to collect samples for an *in vivo* mutation assay. Because genotoxic compounds can effectively induce gene mutations under conditions giving rise to cell proliferation (Cohen and Arnold, 2011), treatment with diethylnitrosamine (DEN) for subsequent tumor-promoting analysis is conducted at 18 h after PH (Tsuda et al., 1980; Kobayashi et al., 1997). To apply this concept of the GPG model to a new assay for renal carcinogens (*i.e.*, the GNP model), unilateral nephrectomy (UN) was performed in place of PH. Although UN also induces compensatory cell proliferation in the residual kidney tissue (Mulroney et al., 1996; Mulroney and Pesce, 2000), detailed kinetics of cell proliferation, including sex differences, remain to be fully elucidated. In addition, although expression of specific proteins in renal cells as markers for preneoplastic lesions has not been found, as mentioned above, morphological alterations regarded as preneoplastic lesions, such as atypical tubules (ATs) and atypical tubular hyperplasia (AH), have been identified. Moreover, because about 90% of target sites of environmental renal carcinogens are renal tubules, these lesions could be suitable markers in the GNP model, corresponding to GST-P foci in the liver (Dietrich and Swenberg, 1991a; National Toxicology Program, 2014).

In the present study, the kinetics of cell proliferation in renal tubules of residual kidneys from male and female rats after UN were investigated to determine the optimal timing of DEN treatment. Subsequently, the optimal dose of DEN and optimal duration of exposure to test chemical were determined based on data describing the development incidences and/or multiplicities of AT and/or AH after treatment with DEN followed by trisodium nitrilotriacetic acid monohydrate (NTA-H_2O), a potent tumor promoter of renal carcinogenesis. Finally, the GNP model was validated using a genotoxic carcinogen, nongenotoxic carcinogens and a noncarcinogen.

2. Materials and methods

2.1. Chemicals

DEN was obtained from Tokyo Kasei Kogyo (Tokyo, Japan), and NTA, potassium dibasic phosphate (PDP), and *d*-limonene (DL) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 5-Bromo-2′-deoxyuridine (BrdU), aristolochic acid (AA), and phenylbutazone (PBZ) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. 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 5 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 (2–3 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.

2.3. Experiment I

To determine the optimal timing of DEN injection as a tumor initiator, the time course for cell proliferation in residual kidney tissues after the UN in male and female rats was investigated.

2.3.1. Animal treatment

Three male and female 10-week-old F344 rats in each group were subjected to UN (left kidney) under deep anesthesia. Male rats were sacrificed at 6, 18, 24, or 48 h after UN, and female rats were sacrificed at 6, 18, 24, 48, or 72 h after UN. All rats were injected intraperitoneally (i.p.) with BrdU (100 mg/kg) 2 h before sacrifice. Residual right kidneys were fixed in ice-cold acetone and processed by embedding in paraffin, sectioning (4-μm), and immunostaining for BrdU after histochemical demonstration of γ-glutamyltranspeptidase (γ-GT) activity.

2.3.2. Immunohistochemical staining for BrdU

For immunohistochemical staining of BrdU, sections were treated sequentially with normal goat serum, monoclonal mouse anti-BrdU (1:100 dilution; Becton Dickinson, Franklin Lakes, NJ, USA), and high polymer stain (HISTOFINE Simple Stain, Nichirei Bioscience Inc., Tokyo, Japan) after denaturation of DNA with 4*N* HCl. Before the denaturation step, sections were processed histochemically for demonstration of γ-GT activity based on previously reported methods (Rutenburg et al., 1969) using L-glutamyl-4-methoxy-β-naphthylamide (Polysciences, Ltd., Warrington, PA, USA) as a substrate in order to distinguish among the three types of tubules, as previously described (Umemura et al., 2004, 2009). The sites of peroxidase binding were demonstrated by incubation with 3,3′-diaminobenzidine tetrahydrochloride. At least 3000 tubule cells of the proximal convoluted tubule (PCT), proximal straight tubule (PST), and distal tubule (DT) were counted for each kidney, and BrdU-labeling indices (LIs) were calculated as the percentages of cells positive for BrdU incorporation.

2.4. Experiment II

To determine the optimal dose of DEN and experimental period to detect renal preneoplastic lesions in the residual kidney, rats were treated with two different doses of DEN, followed by treatment with NTA as a tumor promoter.

2.4.1. Animal treatment

The experimental design is described in Fig. 1. A total of 180 female 6-week-old F344 rats were administered NTA solution at a concentration of 0 or 1000 ppm in drinking water for 4 weeks ($n = 90$ per dose), and UN was then performed in all rats under deep anesthesia. The dose of NTA was determined based on a previous 2-year carcinogenicity study (Goyer et al., 1981). The rats treated with 0 ppm NTA were given distilled water (DW). At 48 h after UN, an i.p. injection of DEN was administered at doses of 20 and 40 mg/kg in

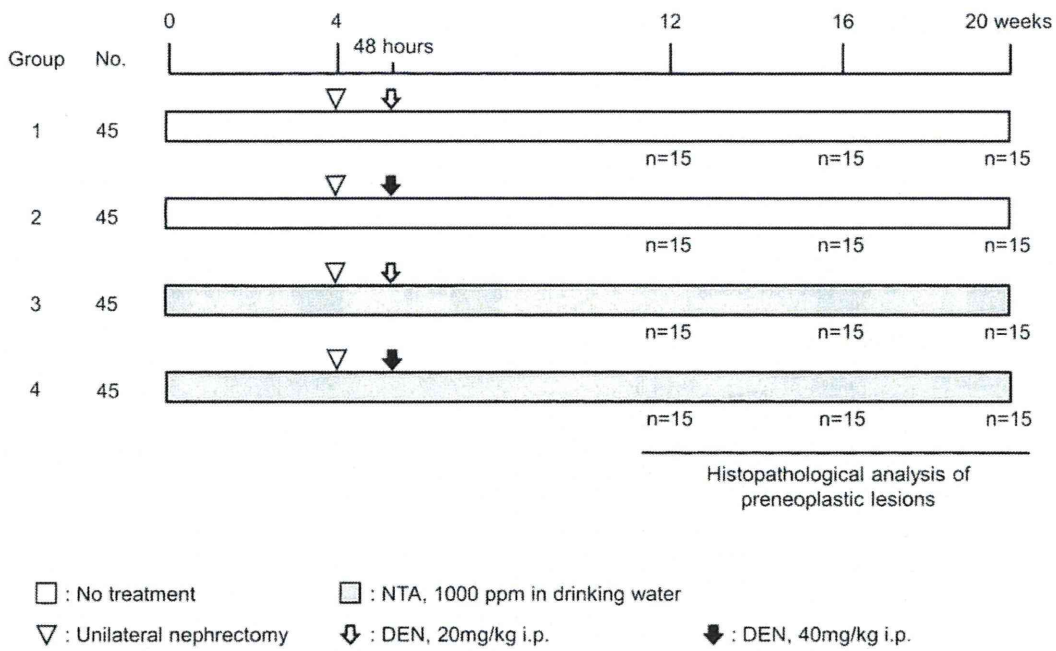

Fig. 1. Treatment protocol for experiment II. Animals were 6-week-old female F344 rats. Development of preneoplastic lesions was evaluated histopathologically in residual kidneys at 12, 16, and 20 weeks after the start of the experiment, *i.e.*, at 8, 12, and 16 weeks after the unilateral nephrectomy.

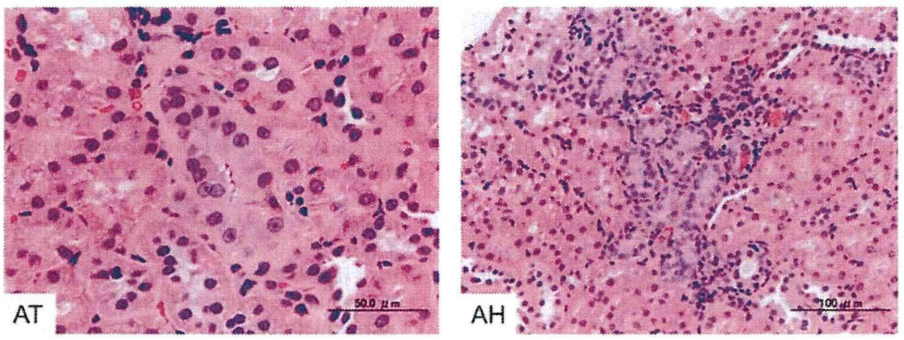

Fig. 2. Representative photographs of atypical tubules (ATs) and atypical hyperplasia (AH) in the kidney of rats. HE stain.

rats treated with 0 or 1000 ppm NTA, respectively. The rats continued to consume water containing NTA until they were sacrificed at 8, 12, or 16 weeks after UN ($n = 15$ per time point). The residual right kidneys were fixed in 10% neutral-buffered formalin, and four pieces were taken from each kidney, routinely processed by embedding in paraffin, sectioning (4-μm), and H&E staining. Renal tubular lesions of AT and AH (Fig. 2) were diagnosed as preneoplastic lesions according to generally accepted guidelines (Dietrich and Swenberg, 1991a).

2.5. Experiment III

The animal model was validated using a genotoxic renal carcinogen, two nongenotoxic renal carcinogens, and a noncarcinogen.

2.5.1. Animal treatment

Six-week-old F344/NSlc-Tg (*gpt* delta) rats ($n = 15$ per dose) were fed 50,000 ppm PDP or 2500 ppm PBZ in their basal diets. The rats treated with AA received 1% sodium bicarbonate at a dose of 0.3 mg/kg body weight by gavage once a day. The rats given DL were administered 600 mg/kg in corn oil by gavage once a day. The doses of PDP and PBZ were determined based on previous reports in which these chemicals exerted tumor-promoting effects in rat kidneys (Hiasa et al., 1992; Maekawa et al., 1987). The dose of AA was selected based on a previous report in which the *gpt* mutant frequencies (MFs) were increased in rats treated with AA for 4 weeks (Kawamura et al., 2012). The dose of DL was based on a previous carcinogenicity test (National Toxicology Program, 1990a). A control group was fed the basal diet without chemical supplementation. After 4 weeks, test chemical treatment was interrupted in all animals. At 6 weeks, an i.p. injection of DEN at a dose of 40 mg/kg was administered, and UN was performed under deep anesthesia 48 h before DEN administration in all rats. The excised left kidney tissues were perfused with saline to remove residual blood and stored at −80 °C for the *gpt* assay. Test chemical exposure resumed at 7 weeks, and animals were sacrificed at 19 weeks. The experimental procedures and sample preparation after the end of the experiment were the same as for experiment II.

2.5.2. In vivo mutation assay

6-Thioguanine (6-TG) was used according to previously described methods (Nohmi et al., 2000). Briefly, genomic DNA was

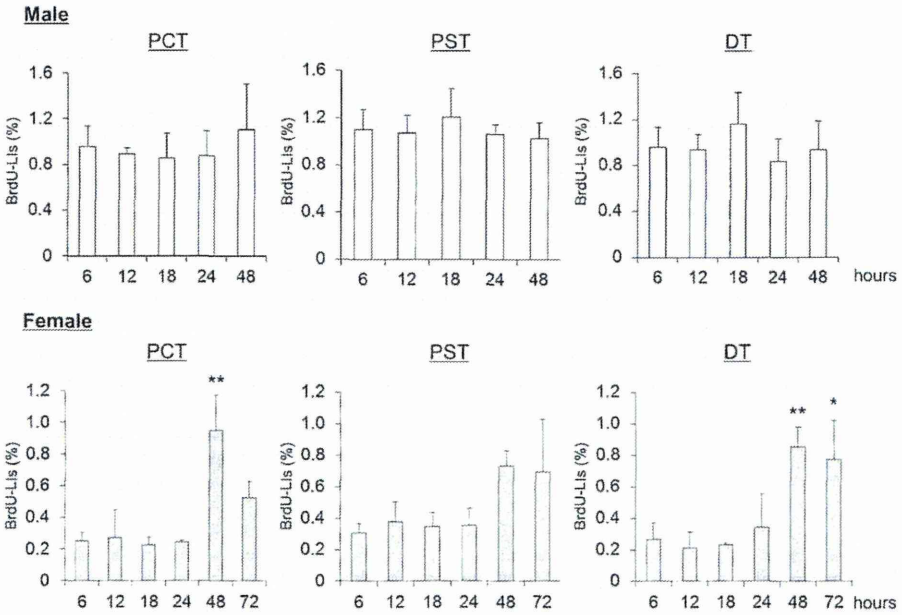

Fig. 3. BrdU-LIs in the proximal convoluted tubule (PCT), proximal straight tubule (PST), and distal tubule (DL) of residual kidneys of male (white column) and female (gray column) F344 rats after unilateral nephrectomy ($n = 3$). Values are means ± SDs. *,** Significantly different from the 6-h group at $p < 0.05$ and $p < 0.01$, respectively.

extracted from each kidney, and 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.

2.6. Statistical analysis

The data for BrdU-LIs, multiplicity of preneoplastic lesions in experiment III, and *gpt* MFs were analyzed with analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. The data for multiplicity of preneoplastic lesions in experiment II were analyzed with the Student–Welch test. The incidence of preneoplastic lesions was compared with Fisher's exact probability test.

3. Results

3.1. Experiment I

Fig. 3 illustrates changes in BrdU-LIs for each tubule type in residual kidney tissues after UN in male and female rats. In female rats, BrdU-LIs in the PCT, PST, and DT were highest at 48 h after UN, with significant changes in the PCT at 48 h and in the DT at 48 and 72 h. However, no obvious changes in BrdU-LIs were observed in all types of tubules in male rats.

3.2. Experiment II

Two rats from each of the groups of rats treated with 20 mg/kg DEN followed by 12 weeks of DW treatment or 40 mg/kg DEN followed by 16 weeks of DW treatment and one rat from each of the groups of rats treated with 40 mg/kg DEN followed by 8 weeks of NTA treatment, 20 mg/kg DEN followed by 12 weeks of NTA treatment, 40 mg/kg DEN followed by 12 weeks of NTA treatment, 20 mg/kg DEN followed by 16 weeks of DW treatment, or 20 mg/kg DEN followed by 16 weeks of NTA treatment died due to surgical complications during UN and were eliminated from further evaluation. The incidences and multiplicities of renal preneoplastic lesions in rats exposed to NTA and DEN are given in Fig. 4. DEN induced preneoplastic lesions in the kidneys during each experimental period, and more lesions were observed in rats treated with 40 mg/kg DEN than in rats treated with 20 mg/kg DEN. NTA treatment enhanced the formation of preneoplastic lesions for both doses of DEN in each experimental period. In rats treated with 40 mg/kg DEN followed by NTA treatment, the incidence and multiplicity of preneoplastic lesions at 12 weeks after UN and the multiplicity of preneoplastic lesions at 16 weeks after UN were significantly increased compared to those in rats treated with 40 mg/kg DEN alone.

3.3. Experiment III

One rat from each of the AA, PDP, PBZ, and DL treatment groups died due to surgical complications during UN and were eliminated from further evaluation. Table 1 shows the MFs in the excised kidneys of *gpt* delta rats treated with AA, PDP, PBZ, or DL for 4 weeks followed by a 2-week washout period. The MFs in rats exposed to AA were significantly increased compared with that in rats in the control group. There were no significant changes in MFs in the rats treated with PDP, PBZ, or DL. In the *gpt* mutation spectra, AT:TA transversions were significantly increased in rats treated with AA (Table 2). The results of histopathological analyses of preneoplastic lesions are illustrated in Fig. 5. The incidence of preneoplastic lesions was significantly increased in the kidneys of rats treated with AA and PDP, and the multiplicity of preneoplastic lesions was significantly increased in kidneys of rats treated with AA, PDP, and PBZ. No significant changes were observed in the rats exposed to DL. Representative photographs of the kidney of rats treated with PDP and DL were illustrated in Fig. 6. Calcium depositions were

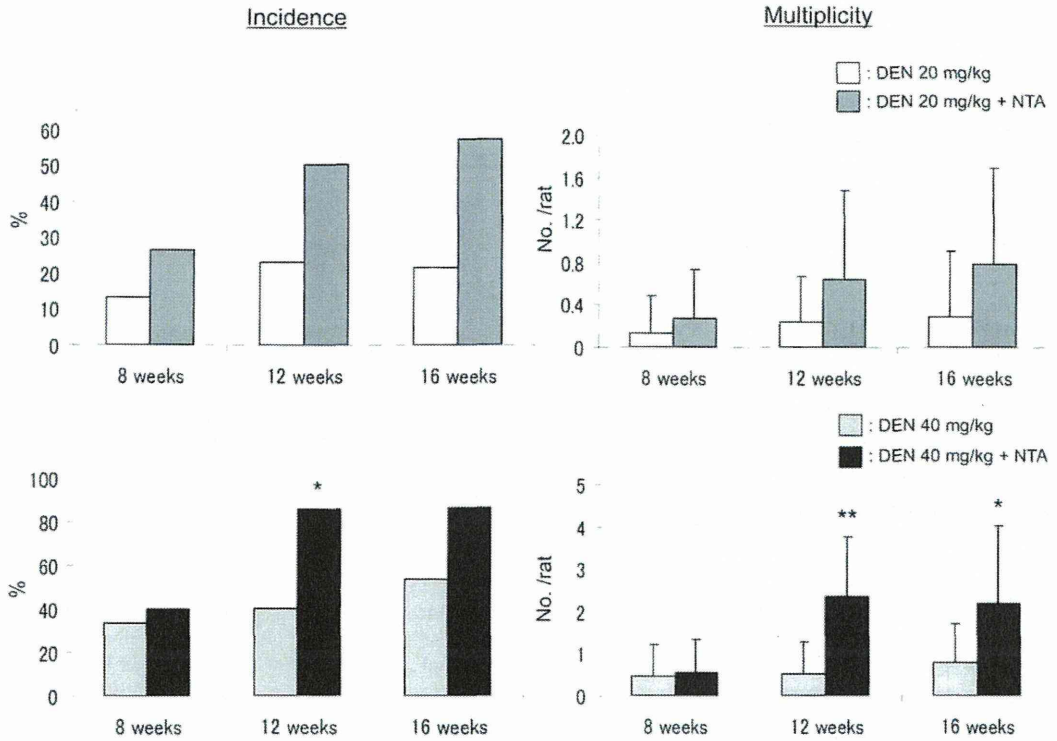

Fig. 4. Incidence and multiplicity of renal preneoplastic lesions in the residual kidneys of female F344 rats treated with DEN and NTA ($n = 13–15$). Values of multiplicity are means ± SDs. The horizontal axes represent the treatment period after unilateral nephrectomy. *,** Significantly different from the DEN 40 mg/kg group at $p < 0.05$ and $p < 0.01$, respectively.

Table 1
gpt MFs in kidneys of F344 *gpt* delta rats treated with AA, PDP, PBZ and DL.

Group	Animal no.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies	MF ($\times 10^{-5}$)	Mean ± SD
Control	101	3.7	2	0.54	0.43 ± 0.22
	102	10.0	3	0.30	
	103	8.9	2	0.22	
	104	9.5	3	0.32	
	105	7.9	6	0.76	
AA	201	3.7	4	1.07	1.20 ± 0.42**
	202	6.9	5	0.73	
	203	5.5	10	1.81	
	204	6.2	6	0.97	
	205	6.3	9	1.42	
PDP	301	5.9	1	0.17	0.57 ± 0.32
	302	4.8	5	1.05	
	303	7.5	4	0.54	
	304	6.4	4	0.63	
	305	6.2	3	0.48	
PBZ	401	5.5	3	0.55	0.48 ± 0.32
	402	5.4	1	0.19	
	403	7.4	2	0.27	
	404	7.8	7	0.90	
	405	3.6	0[a]	–	
DL	501	5.1	4	0.78	0.47 ± 0.29
	502	4.4	1	0.23	
	503	6.9	5	0.72	
	504	7.2	1	0.14	
	505	6.2	3	0.48	

** Significantly different from the control group at $p < 0.01$.
[a] No mutant colonies were detected on the plate, with this data being excluded from the calculation of MF.

Table 2
Mutation spectra of *gpt* mutant colonies in kidneys of F344 *gpt* delta rats treated with AA, PDP, PBZ and DL.

	Control		AA		PDP		PBZ		DL	
	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	1[a] (6.3)	0.02 ± 0.05[b]	3(8.8)	0.11 ± 0.12	5(29.4)	0.17 ± 0.14	2(11.8)	0.06 ± 0.10	3(21.4)	0.11 ± 0.17
GC-CG	2(12.5)	0.05 ± 0.11	1(2.9)	0.03 ± 0.07	2(11.8)	0.08 ± 0.18	0	0	0	0
AT-TA	0	0	12(35.3)	0.43 ± 0.39*	0	0	1(5.9)	0.02 ± 0.04	0	0
AT-CG	0	0	2(5.9)	0.06 ± 0.14	0	0	0	0	2(14.3)	0.07 ± 0.10
Transitions										
GC-AT	11(68.8)	0.31 ± 0.17	8(23.5)	0.28 ± 0.08	6(35.3)	0.18 ± 0.15	10(58.8)	0.28 ± 0.13	5(35.7)	0.17 ± 0.11
AT-GC	1(6.3)	0.03 ± 0.06	4(11.8)	0.13 ± 0.14	1(5.9)	0.04 ± 0.08	1(5.9)	0.02 ± 0.04	1(7.1)	0.03 ± 0.07
Deletion										
Single bp	1(6.3)	0.02 ± 0.05	3(8.8)	0.10 ± 0.14	3(17.6)	0.09 ± 0.09	3(17.6)	0.10 ± 0.10	3(21.4)	0.09 ± 0.14
Over 2 bp	0	0	0	0	0	0	0	0	0	0
Insertion	0	0	1(2.9)	0.05 ± 0.12	0	0	0	0	0	0
Complex	0	0	0	0	0	0	0	0	0	0

[a] Number of colonies with independent mutations.
[b] Mean ± SDs.
* Significantly different from the control group at $p < 0.05$.

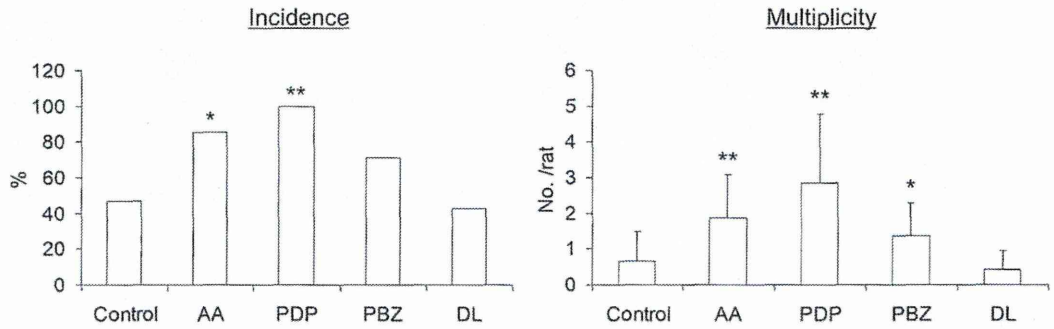

Fig. 5. Incidence and multiplicity of renal preneoplastic lesions in the residual kidneys of female F344 rats treated with AA, PDP, PBZ, and DL ($n = 14–15$). Values of multiplicity are means ± SDs. *,**Significantly different from the control group at $p < 0.05$ and $p < 0.01$, respectively.

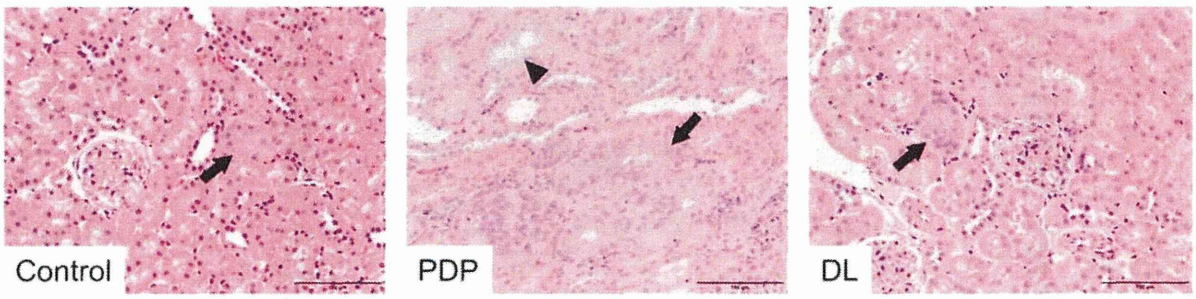

Fig. 6. Representative photographs of the residual kidneys of female F344 *gpt* delta rats treated with PDP and DL in the validation study. Calcium depositions were observed in the proximal tubules of rats treated with PDP (arrowhead). There were no hyaline droplets indicating accumulation of α_{2u}-globulin in the proximal tubular epithelium in DL-treated rats. Arrows represent preneoplastic lesions in renal tubules. HE stain.

observed in the proximal tubules of rats treated with PDP. There were no hyaline droplets in the proximal tubular epithelium of rats treated with DL.

4. Discussion

Although the kidney is a major organ targeted by chemical carcinogenesis, reliable medium-term animal models for identification of renal carcinogens have not been established. In this study, we applied the concepts of the GPG model (Matsushita et al., 2013, 2014) to develop the GNP model, which was able to detect the *in vivo* mutagenicity and tumor-promoting activity of renal carcinogens. In the GPG model, *gpt* delta rats are subjected to PH to harvest samples for an *in vivo* mutation assay. Subsequently, DEN is administered at 18 h after PH to effectively initiate hepatocyte carcinogenesis for subsequent analysis of tumor-promoting effects by taking advantage of the induction of compensatory cell proliferation after PH. In the GNP model, UN is performed in place of PH. However, since the detailed kinetics of cell proliferation in the residual kidney tissue after UN remained unclear, clarifying the time to reach peak cell proliferation after UN was needed to determine the optimal time for initiation of renal cell carcinogenesis.

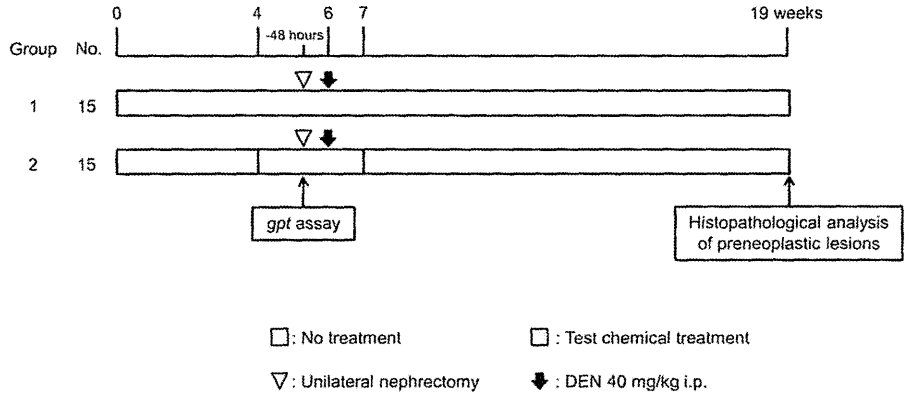

Fig. 7. Standard protocol for the GNP model. Animals were 6-week-old female F344 *gpt* delta rats. The *gpt* assay was performed in excised kidney samples as an indicator of *in vivo* mutagenicity. Tumor-promoting activities were evaluated based on the enhancement of preneoplastic lesions induced by DEN in residual kidney samples.

Thereafter, BrdU-LIs in three types of tubules located at the cortex and outer stripe of outer medulla in the residual kidney were examined following UN in male and female rats. Our data demonstrated that BrdU-LIs increased, reaching a peak at 48 h after UN in female rats, although there were no remarkable changes in male rats. Compensatory mechanisms in the residual kidney tissue following UN have been reported to involve sex differences, *i.e.*, hypertrophic responses are observed in male rats, while hyperplastic responses are observed in female rats (Mulroney et al., 1996; Mulroney and Pesce, 2000). Thus, our cell kinetic study revealed that female rats were suitable for use in the GNP model. Additionally, the use of female rats is expected to yield an additional advantage, *i.e.*, it will be possible to eliminate the interference of male rat-specific renal carcinogens, so-called α_{2u}-globulin-mediated carcinogens, in terms of the risk of human cancer. Because DEN is capable of inducing renal tubular tumors as well as hepatocyte tumors (Nogueira, 1987; Athar and Iqbal, 1998; Umemura et al., 2000), we performed a study to investigate optimal timing of DEN administration to induce renal tumors. We found that female *gpt* delta rats should be administered DEN at 48 h after UN in the GNP model. No reports have identified a reliable marker for renal preneoplastic lesions, such as specific enzymes corresponding to GST-P in the liver. However, characteristic tubular lesions, such as ATs and AH, are known to appear early, prior to tumor formation. ATs are normal in size, but contain epithelial cells showing atypia. In contrast, AH consists of aggregations of proliferating atypical cells that are single- or multilayered. These lesions are believed to represent preneoplastic lesions of renal tubular cell tumor (Dietrich and Swenberg, 1991a).

For the second half of the GNP model protocol, we determined the optimal dose of DEN and duration of treatment with the renal cell tumor promoter. Two doses of DEN (20 and 40 mg/kg) were applied using the tentative standard protocol described above, and rats were then treated with NTA, a typical promoter of renal tubular cell tumors, for 8, 12, or 16 weeks in order to select optimal conditions for achieving the tumor-promoting effects of NTA. Since development of preneoplastic lesions was significantly enhanced in rats treated with 40 mg/kg DEN followed by NTA treatment for 12 and 16 weeks as compared to rats treated with DEN alone, the dose of DEN was chosen as 40 mg/kg, and the duration of test chemical treatment was chosen as 12 weeks. In addition, as in the GPG model (Matsushita et al., 2014), we added 2- and 1-week washout periods before and after DEN injection, respectively, to avoid interaction between DEN and the test chemical. From these studies, we developed the standard protocol for the GNP model as follows. Female *gpt* delta rats were treated with the test chemical for 4 weeks, followed by a 2-week washout period, and i.p. injection of DEN was subsequently performed at a dose of 40 mg/kg. UN was carried out 48 h before DEN administration, and the *gpt* assay was performed using excised kidney samples. At 1 week after DEN administration, test chemical treatment was resumed. The incidences and/or multiplicities of preneoplastic lesions were evaluated in residual kidney samples at 12 weeks after resuming test chemical treatment (Fig. 7).

To validate the GNP model established above, we applied 1 genotoxic renal carcinogen, 2 nongenotoxic renal carcinogens, and 1 noncarcinogen to the model. The genotoxic renal carcinogen AA was reported to produce AA-specific DNA adducts, and 7-(deoxyadenosine-N^6-yl) aristolactum I (AAI-dA) was found to induce the most persistent DNA adducts *in vivo* (Mengs et al., 1982; Mei et al., 2006). In the present study, we observed a significant increase in the MFs of *gpt* in rats treated with AA, and frequencies of AT:TA transversion mutations were predominantly elevated in spectrum analysis. Accordingly, it is highly probable that AA-specific deoxyadenine adducts may be responsible for AA-induced gene mutations (Mei et al., 2006; Xing et al., 2012). Ishii et al. (2014) demonstrated that *gpt* delta rats could be powerful tools not only for examination of *in vivo* genotoxicity but also for investigation of the relationship between DNA base modifications and gene mutations. Thus, in the GNP model, it is possible to measure chemical-specific DNA modifications using excised or residual kidney samples, which could be helpful for further understanding the causes of chemical-induced gene mutations. In addition, the GNP model showed that AA was capable of exerting tumor-promoting effects on the kidney. This was inconsistent with the negative results of AA in the liver using the GPG model (Matsushita et al., 2014), and this difference may be explained by the observation that the carcinogenic effects of AA target the kidney (Mengs et al., 1982).

PDP has been reported to exert its tumor-promoting effects in the kidneys of rats (Hiasa et al., 1992; Konishi et al., 1995). Additionally, PBZ has carcinogenic effects on the rat kidney and is classified as a nongenotoxic carcinogen based on negative results in various *in vivo* genotoxicity tests (National Toxicology Program, 1990b; Kari et al., 1995; Machemer and Hess, 1971; Müller and Strasser, 1971; Rathenberg and Müller, 1972; Gebhart and Wissmüller, 1973; Charles and Leonard, 1978). In the GNP model, while exposure to neither PDP nor PBZ elevated the MFs of *gpt* delta rats, significant enhancements in the frequencies of preneoplastic lesions were observed in both treatment groups. In addition, calcium deposition was detected in the proximal tubules of rats treated with PDP, and this was considered the mechanism through which PDP exerted its tumor-promoting effects. DL has been reported to act as a renal carcinogen in male rats only through a mechanism mediated by α_{2u}-globulin (Dietrich and Swenberg, 1991b). Treatment