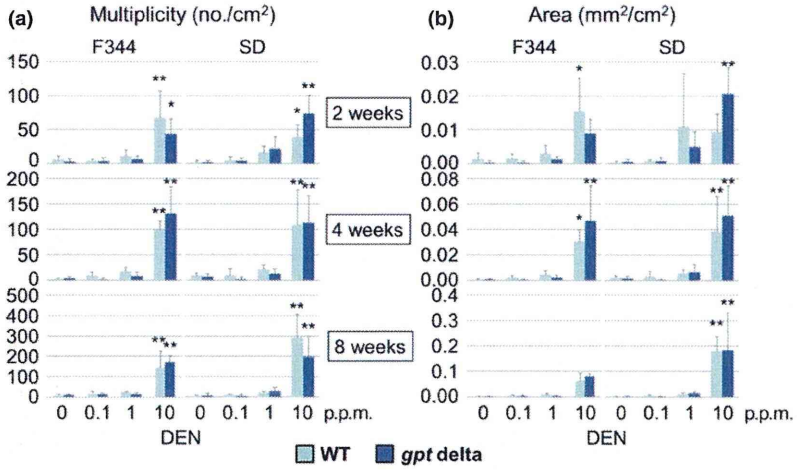

Fig. 4. Immunohistochemical analysis of GST-P-positive foci. The multiplicity (no./cm^2) (a) and area (mm^2/cm^2) (b) of GST-P-positive foci in the livers of *N*-nitrosodiethylamine (DEN)-treated rats. *$P < 0.05$, **$P < 0.01$ versus 0 p.p.m. DEN group (control). SD, Sprague–Dawley; WT, wild type.

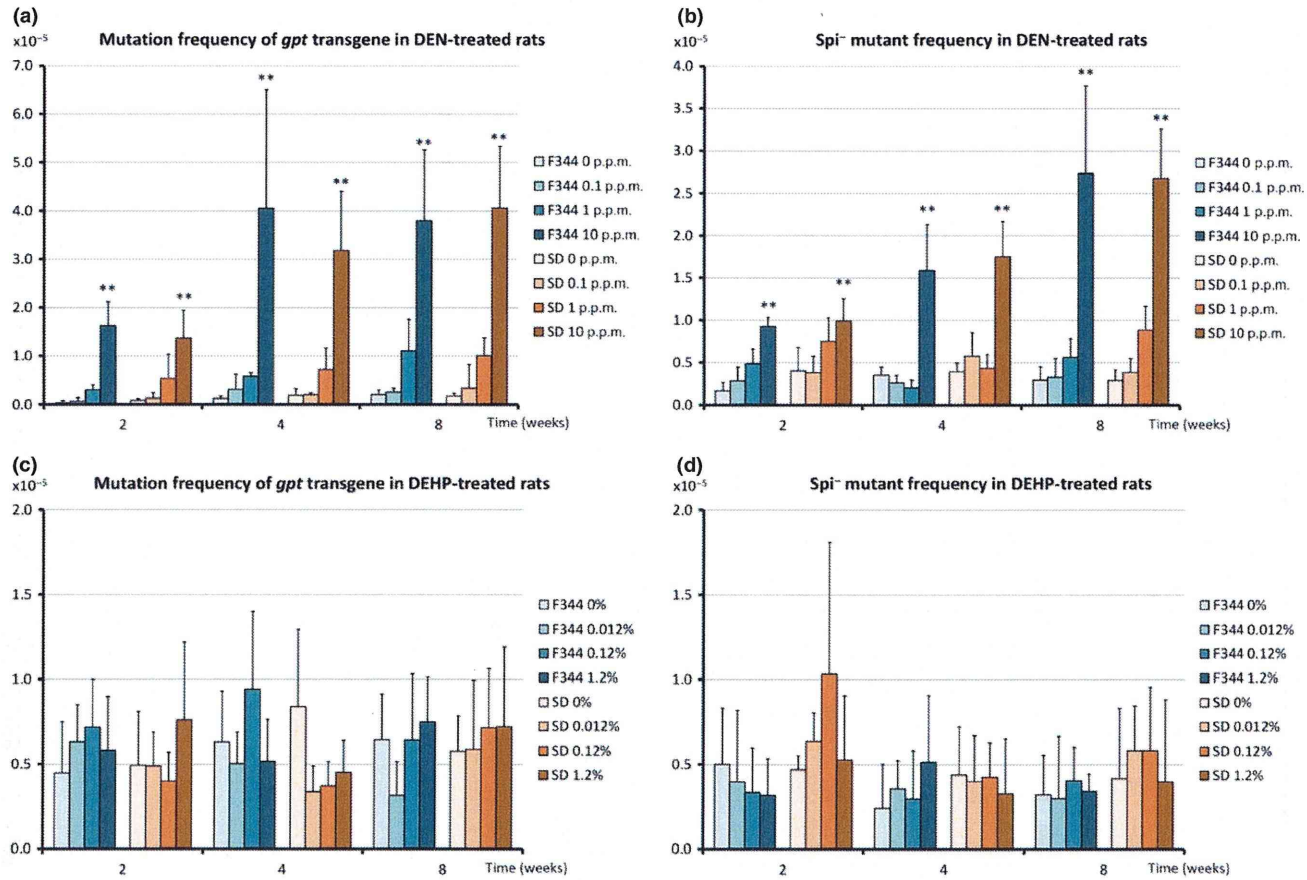

Fig. 5. (a) Frequency of mutations in the *gpt* transgene in the livers of male F344/*gpt* delta and Sprague–Dawley (SD)/*gpt* delta rats administered 0, 0.1, 1, or 10 p.p.m. *N*-nitrosodiethylamine (DEN) in drinking water.(b) Spi$^-$ mutant frequencies in the livers of male F344/*gpt* delta and SD/*gpt* delta rats administered 0, 0.1, 1, or 10 p.p.m. DEN in the drinking water. (c) Frequency of mutations in the *gpt* transgene in the livers of male F344/*gpt* delta and SD/*gpt* delta rats administered 0, 0.012, 0.12, or 1.2% di(2-ethylhexyl)phthalate (DEHP) in the diet. (d) Spi$^-$ mutant frequencies in the livers of male F344/*gpt* delta and SD/*gpt* delta rats administered 0, 0.012, 0.12, or 1.2% DEHP in the diet. **$P < 0.01$ versus 0 p.p.m. DEN group (control).

rats[14] even at lower doses than those used in this study, no significant changes were found in the present histopathological examination of both *gpt* delta and WT rats. This may have been due to the age of the rats at the beginning of administration and/or the administration durations. Although we did observe some statistically significant differences between *gpt* delta and WT rats in serum biochemistry values in the DEN exposure study and in other organ weights and

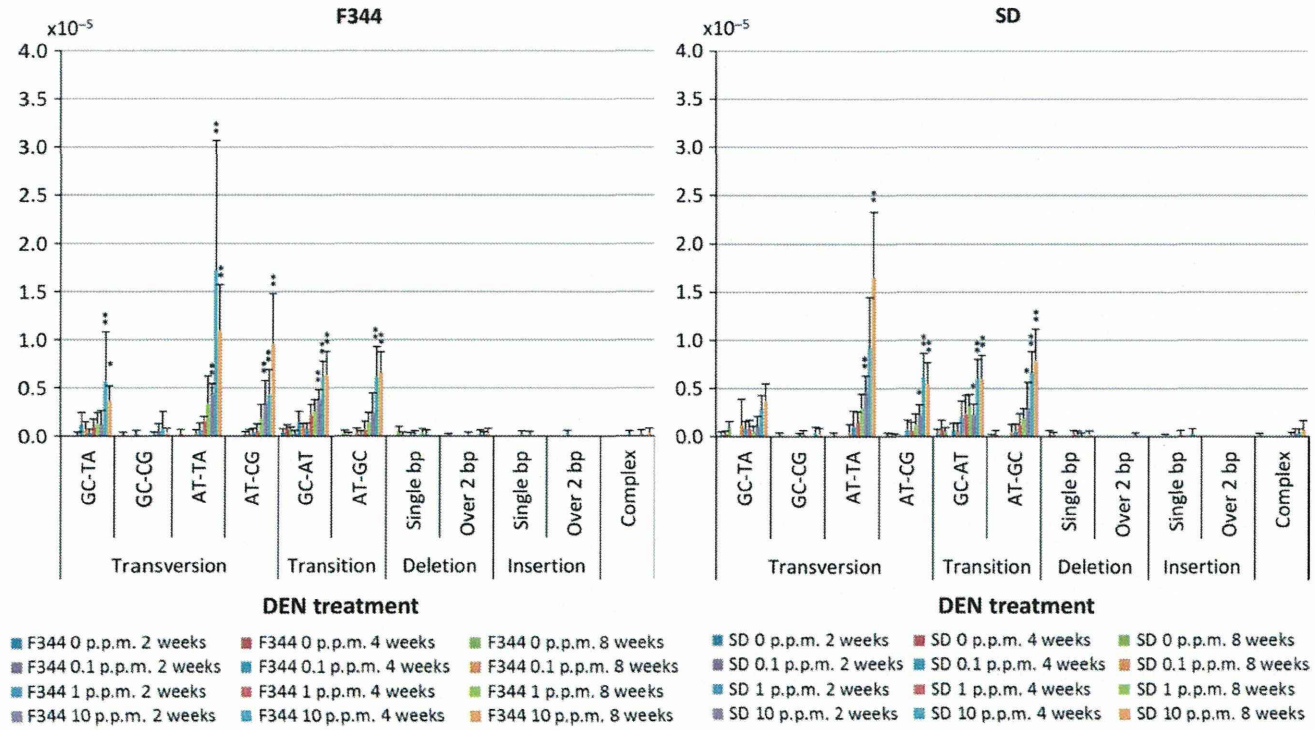

Fig. 6. Mutation spectrum of the *gpt* transgene in the livers of male F344/*gpt* delta and Sprague–Dawley (SD)/*gpt* delta rats administered 0, 0.1, 1, or 10 p.p.m. *N*-nitrosodiethylamine (DEN) in drinking water. **P* < 0.05, ***P* < 0.01 versus 0 p.p.m. DEN group (control).

serum biochemistry values in the DEHP exposure study, no dose-dependent responses were observed, and these differences were sporadic. Thus, we assumed that these differences were incidental. Taken together, our data indicated that *gpt* delta and WT rats (both F344 and SD strains) showed comparable general toxicity responses to DEN and DEHP.

With regard to the comparison of *in vivo* genotoxicity, administration of DEN induced the same level of mutations in F344/*gpt* delta and SD/*gpt* delta rats in both *gpt* and Spi$^-$ assays. The *gpt* mutation spectra were also similar for both strains. Administration of DEN is known to generate various monoalkylated lesions, that is, N^7-ethylguanine, O^4-ethylthymine, N^3-ethyladenine, and O^6-ethylguanine. N^7-ethylguanine further undergoes depurination, resulting in the formation of abasic sites.[15] These lesions cause mutations mainly as consequences of error-prone translesion synthesis. Thus, the most frequent mutations were AT-TA transversions, followed by GC-AT and AT-GC transitions and AT-CG and GC-TA transversions. However, the major mutations found in DEN-induced tumors were GC-AT and AT-GC transitions in the *Ha-ras* gene in C3H/He mice,[16] AT-GC and GC-AT transitions and GC-TA transversions in the *K-ras* gene in A/J mice,[17] and AT-GC transitions in the *H-ras* gene in B6C3F$_1$ mice.[18] The discrepancy in the mutation spectra between these studies and our results could be due to hotspots of examined genes and results of selective amplification. As endogenous *ras* genes are functionally expressed in rats, mutations responsible for clonal expansion would be selectively amplified accompanying tumorigenesis. However, the *gpt* transgene is not expressed in rats, so that it is genetically neutral, which avoids selective pressure *in vivo*.[10] Thus, *gpt* assays can detect a wide spectrum of mutations of the *gpt* transgene that result in loss of its enzymatic activity in *E. coli*. While gene mutation assays are only available in *gpt* delta rats, the equivalent formation of procarcinogenic GST-P-positive foci between *gpt* delta and WT rats implied that similar levels of genotoxicity were induced in both *gpt* delta and WT rats following administration of DEN. In the DEHP treatment group, mutation frequencies of control groups were much higher than that of DEN treatment. The reason for the difference would be that these experiments were independently carried out by different people. We think it is important that the constant protocol and criteria were kept in each experiment so that we are able to compare to mutation frequencies within respective experiments. Although DEHP is generally considered to be non-genotoxic,[12] *lacZ* plasmid-based transgenic mouse mutation assays[19] and several *in vitro* studies have suggested the genotoxic potential of DEHP.[20] Our present results clearly showed that administration of DEHP did not induce gene mutations in either *gpt* mutation frequency or Spi$^-$ mutant assays in both F344 and SD strains of *gpt* delta rats. This is consistent with our previous report.[9] Thus, we concluded that DEHP was non-genotoxic in *gpt* delta rats.

Collectively, these results suggested that *gpt* delta rats (both F344 and SD strains) showed comparable general toxicity responses, including the presence of preneoplastic liver lesions and genotoxicity, to WT rats in response to DEN and DEHP treatment. Therefore, these data supported the validity of the combined assay to detect both general toxicity and genotoxicity simultaneously.

In this study, we also examined whether these toxicities could be detected after short durations of administration. Both the general toxicity of 1.2% DEHP and the genotoxicity of 10 p.p.m. DEN could be detected after 2 weeks of administration, suggesting that the combined assay using *gpt* delta rats was capable of detecting both general toxicity and genotoxicity

after at least 2 weeks of administration. Since mutation frequencies in the *gpt* and *red/gam* transgenes at 4 weeks of administration were higher than those at 2 weeks of administration, longer administration may improve the sensitivity of the *in vivo* genotoxicity test. As 4-week repeated-dose toxicity tests have been widely used for risk assessments, as described in OECD Test Guideline 407,[21] we consider the detection capacity of the combined general toxicity and genotoxicity model at 4 weeks to be an advantage of this *gpt* delta rat model for use in risk assessment. In conclusion, we found that the combined repeated-dose toxicity and genotoxicity assay using *gpt* delta rats was applicable for simultaneous detection of genotoxicity with general toxicity, thereby serving to reduce cancer risks in humans from environmental chemicals.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Body weight gain of F344/*gpt* delta rats treated with 0 or 10 p.p.m. *N*-nitrosodiethylamine (DEN) in the additional experiment.

Fig. S2. Immunohistochemical analysis of GST-P-positive foci in the livers of F344/*gpt* delta rats treated with 0 or 10 p.p.m. of *N*-nitrosodiethylamine (DEN) for 8 weeks in the additional experiment.

Table S1. Relative organ weights of F344/wild type (WT), F344 *gpt* delta, Sprague–Dawley (SD)/WT, and SD/*gpt* delta rats treated with *N*-nitrosodiethylamine (DEN).

Table S2. Relative organ weights of F344/wild type (WT), F344 *gpt* delta, Sprague–Dawley (SD)/WT, and SD/*gpt* delta rats treated with di(2-ethylhexyl)phthalate (DEHP).

Table S3. Serum biochemistry of F344/wild type (WT), F344 *gpt* delta, Sprague–Dawley (SD)/WT, and SD/*gpt* delta rats treated with *N*-nitrosodiethylamine (DEN).

Table S4. Serum biochemistry of F344/wild type (WT), F344 *gpt* delta, Sprague–Dawley (SD)/WT, and SD/*gpt* delta rats treated with di(2-ethylhexyl)phthalate (DEHP).

Table S5. Frequency of mutations in the *gpt* gene in the livers of F344/*gpt* delta rats treated with *N*-nitrosodiethylamine (DEN).

Table S6. Frequency of mutations in the *gpt* gene in the livers of Sprague–Dawley (SD)/*gpt* delta rats treated with *N*-nitrosodiethylamine (DEN).

Table S7. Spi$^-$ mutant frequencies in the livers of F344/*gpt* delta rats treated with *N*-nitrosodiethylamine (DEN).

Table S8. Spi$^-$ mutant frequencies in the livers of Sprague–Dawley (SD)/*gpt* delta rats treated with *N*-nitrosodiethylamine (DEN).

Table S9. Mutation spectrum of the *gpt* gene in livers of F344/*gpt* delta rats treated with *N*-nitrosodiethylamine (DEN).

Table S10. Mutation spectrum of the *gpt* gene in livers of Sprague–Dawley (SD)/*gpt* delta rats treated with *N*-nitrosodiethylamine (DEN).

Table S11. Frequency of mutations in the *gpt* gene in livers of F344/*gpt* delta rats treated with di(2-ethylhexyl)phthalate (DEHP).

Table S12. Frequency of mutations in the *gpt* gene in livers of Sprague–Dawley (SD)/*gpt* delta rats treated with di(2-ethylhexyl)phthalate (DEHP).

Table S13. Spi$^-$ mutant frequencies in livers of F344/*gpt* delta rats treated with di(2-ethylhexyl)phthalate (DEHP).

Table S14. Spi$^-$ mutant frequencies in livers of Sprague–Dawley (SD)/*gpt* delta rats treated with di(2-ethylhexyl)phthalate (DEHP).

Table S15. Mutation spectrum of the *gpt* gene in livers of F344/*gpt* delta rats treated with di(2-ethylhexyl)phthalate (DEHP).

Table S16. Mutation spectrum of the *gpt* gene in livers of Sprague–Dawley (SD)/*gpt* delta rats treated with di(2-ethylhexyl)phthalate (DEHP).

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Research Article

Early Detection of Genotoxic Urinary Bladder Carcinogens by Immunohistochemistry for γ-H2AX

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ABSTRACT

DNA double-strand breaks (DSBs) induced by exposure to genotoxic agents are known to cause genome instability and cancer development. To evaluate the applicability of γ-H2AX, a sensitive marker of DSBs, in the early detection of genotoxicity and carcinogenicity of chemicals using animal models, we examined γ-H2AX expression in urinary bladders of rats. Six-week-old male F344 rats were orally treated for 4 weeks with a total of 12 chemicals divided into 4 categories based on genotoxicity and carcinogenicity in the urinary bladder. Animals were sacrificed at the end of administration or after 2 weeks of recovery, and immunohistochemistry for γ-H2AX was performed. At week 4, γ-H2AX expression in bladder epithelial cells was significantly increased by all 4 genotoxic bladder carcinogens as compared with the controls, whereas the 3 chemicals that were genotoxic but not carcinogenic in the bladders did not cause upregulation of γ-H2AX. After the recovery period, γ-H2AX expression was markedly reduced in all groups but remained significantly elevated in rats treated with 3 of the 4 genotoxic bladder carcinogens. Although slight increases in γ-H2AX expression were induced by a weak bladder carcinogen with equivocal genotoxicity (phenethyl isothiocyanate) and 2 nongenotoxic bladder carcinogens (melamine and uracil) at week 4, these differences were not significant and were thought to be associated with activated proliferation by urothelial hyperplasia, as demonstrated by increased Ki67-positive cells. These results suggested that γ-H2AX may be a potential biomarker for the early detection of genotoxic bladder carcinogens.

Key words: urinary bladder; γ-H2AX; genotoxicity; carcinogenicity; rat

Chemical carcinogens are classified into 2 major groups: genotoxic carcinogens and nongenotoxic carcinogens. Although *in vivo* genotoxicity tests using rodent models play an important role in the safety evaluation of chemicals, these assays are limited in that they must be conducted independently of conventional repeated-dose toxicity studies and require specific experimental procedures. Thus, the development of more appropriate analytical methods and procedures for identification of novel biomarkers that can be performed simultaneously with general short-term toxicity studies is needed.

DNA damage and repair play key roles in carcinogenesis, and DNA double-strand breaks (DSBs) have been shown to cause genome instability (Aguilera and Garcia-Muse, 2013; Bonner *et al.*, 2008). H2AX is a variant of the histone 2A family and is involved in DNA packing and DNA repair. Rapid phosphorylation of H2AX at serine 139, yielding γ-H2AX, occurs in response to DSBs (Pilch *et al.*, 2003). γ-H2AX is then rapidly accumulated over a large region of chromatin surrounding DSBs (Rogakou *et al.*, 1999), leading to the aggregation of repair proteins; this process can be microscopically detected by immunofluorescence and immunohistochemistry using specific primary antibodies (Redon *et al.*, 2012). Since its discovery in 1998 (Rogakou *et al.*, 1998), γ-H2AX has been applied as a useful tool in multiple scientific fields, such as the *in vitro* assessment of preclinical drugs, evaluation of DNA damage, and genotoxicity screening for chemical materials (Nikolova *et al.*, 2014; Redon *et al.*, 2012).

Urinary bladder cancer is the seventh most common cancer in men worldwide, and smoking and occupational exposure are considered the major risk factors for development of transitional cell carcinoma (Jemal *et al.*, 2011). Previous studies have demonstrated the expression of γ-H2AX in bladder cancer and its correlation with recurrence (Bartkova *et al.*, 2005; Cheung *et al.*, 2009). However, few reports have investigated γ-H2AX expression during early-stage bladder carcinogenesis, including that in normal urothelium after exposure to genotoxic agents. We have recently demonstrated that γ-H2AX expression is induced in bladder epithelial cells of rats treated with N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN), a genotoxic bladder carcinogen, for 4 weeks (Toyoda *et al.*, 2013). γ-H2AX-positive cells were detected not only in BBN-induced hyperplasia and carcinoma but also in the normal urothelium at 33 weeks after treatment, suggesting that DNA damage occurring during the administration period may have long-term effects on bladder carcinogenesis.

In the present study, we performed immunohistochemical analysis of γ-H2AX expression in chemical-treated rats to evaluate the potential application of γ-H2AX as a biomarker for genotoxicity and carcinogenicity in the urinary bladder.

MATERIALS AND METHODS

Test chemicals. We selected 4 potent bladder carcinogens with genotoxicity (BBN, 2-nitroanisole [2-NA], 2-acetylaminofluorene [2-AAF], and *p*-cresidine), 2 weak bladder carcinogens with equivocal genotoxic properties (2,2-bis(bromomethyl)-1,3-propanediol [BMP] and phenethyl isothiocyanate [PEITC]), 3 nongenotoxic bladder carcinogens (dimethylarsinic acid [DMA], melamine, and uracil), and 3 genotoxic agents that are not carcinogenic in the urinary bladder (glycidol, N-nitrosodiethylamine [DEN], and acrylamide [AA]; Table 1). The suppliers and purities of the chemicals were as follows: BBN (Sigma-Aldrich, St Louis, Missouri; Lot No. SLBF3806V, 98.5%), 2-NA (Wako Pure Chemical Industries, Osaka, Japan; Lot No. DCF1232, 99.7%), 2-AAF (Tokyo Chemical Industry, Tokyo, Japan; Lot No. 243BD, 99.8%), *p*-cresidine (Sigma-Aldrich; Lot No. BCBF1417V, 99.5%), BMP (Wako; Lot No. WEE4772, 99.3%), PEITC (Sigma-Aldrich; Lot No. 05311LHV, 99.3%), DMA (Sigma-Aldrich; Lot No. BCBJ3595V, 100%), melamine (Wako; Lot No. WEN1818, 100%), uracil (Wako; Lot No. LAE4468, 100%), glycidol (Wako; Lot No. PDM3910, 97.0%), DEN (Tokyo Chemical Industry; Lot No. FBMVM, 99.9%), and AA (Wako; Lot No. PDJ0711, 100%). The chemicals were previously assessed for the genotoxicity (Delker *et al.*, 2000; IARC, 1994, 2000; Kassie and Knasmuller, 2000; Kirkland *et al.*, 2005; Kligerman *et al.*, 2003; Moore *et al.*, 1997; Musk *et al.*, 1995; Noda *et al.*, 2002; Stoll *et al.*, 2006; Zhang *et al.*, 2011).

Experimental animals. A total of 140 male-specific pathogen-free rats (F344/DuCrlCrlj, 5 weeks old) were purchased from Charles River Japan (Yokohama, Japan) and used after 1 week of acclimatization. The animals were housed in polycarbonate cages with soft chip bedding (Sankyo Labo Service, Tokyo, Japan) in a room with a barrier system controlled for the light/dark cycle (12 h), ventilation (air exchange rate 18 times/h), temperature (23°C ± 2°C), and relative humidity (55% ± 5%). The cages and chip bedding were exchanged twice a week. All animals had free access to a basal diet and water with or without the test chemicals.

Study design. The present study was performed as 2 divided experiments using the same protocols (experiments 1 and 2). At the beginning of the experiments, the animals were randomly allocated to 7 groups of 10 rats each based on their body weights measured just before starting chemical treatment. Animals were administered 0.05% BBN (Vasconcelos-Nobrega *et al.*, 2012), 1.8% 2-NA (Irwin *et al.*, 1996a), 2.0% BMP (Dunnick *et al.*, 1997), 0.1% PEITC (Ogawa *et al.*, 2001), 3.0% melamine (Ogasawara *et al.*, 1995), or 3.0% uracil (Fukushima *et al.*, 1992) in experiment 1 and 0.025% 2-AAF (Wilson *et al.*, 1941), 1.0% *p*-cresidine (NTP, 1979), 0.02% DMA (Wei *et al.*, 2002), 0.04% glycidol (Irwin *et al.*, 1996b), 0.001% DEN (IARC, 1978), and 0.005% AA (Beland *et al.*, 2013) in experiment 2 in their drinking water with light-shielded bottles (BBN, DMA, glycidol, DEN, and AA) or basal diet (CRF-1; Oriental Yeast, Tokyo, Japan) for 4 weeks. We set the administration doses as the carcinogenic doses reported in previous studies. The diet and water were changed once and twice per week, respectively. Five rats in each group were sacrificed at the end of administration, and the remaining animals were maintained an additional 2 weeks as a recovery period.

TABLE 1. Chemicals Used in This Study

Chemicals	CAS No.	Carcinogenicity in Rat Urinary Bladder	Genotoxicity	Ames Test	MLA	CA *in vitro*	MN	
							in vitro	*in vivo*
BBN	3817-11-6	+	+	+[a]				
2-NA	91-23-6	+	+	+[a]	+[a]	+[a]		
2-AAF	53-96-3	+	+	+[a]	+[a]	+[a]	+[a]	
p-Cresidine	120-71-8	+	+	+[a]				+/−[b,c]
BMP	3296-90-0	+/−	+/−	+/−[d]		+[d]		+[d]
PEITC	2257-09-2	+/−	+/−	+[e]		+[f]	+[e]	−[g]
DMA	75-60-5	+	−	−[h]	+[i]			−[j]
Melamine	108-78-1	+	−	−[a]	−[a]	−[a]		−[k]
Uracil	66-22-8	+	−	−[a]				
Glycidol	556-52-5	−	+	+[a]	+[a]	+[a]		
DEN	55-18-5	−	+	+[a]	+[a]	+[a]	+[a]	
AA	79-06-1	−	+	−[l]	+[a]	+[a]		+[l]

MLA, mouse lymphoma assay; CA, chromosomal aberration test; MN, micronucleus test; BBN, N-butyl-N-(4-hydroxybutyl)-nitrosamine; 2-NA, 2-nitroanisole; 2-AAF, 2-acetylaminofluorene; BMP, 2,2-bis(bromomethyl)-1,3-propanediol; PEITC, phenethyl isothiocyanate; DMA, dimethylarsinic acid; DEN, N-nitrosodiethylamine; AA, acrylamide.

Source: [a]Kirkland et al. (2005); [b]Stoll et al. (2006); [c]Delker et al. (2000); [d]IARC (2000); [e]Kassie and Knasmuller (2000); [f]Musk et al. (1995); [g]Suzuki I, Cho YM, Toyoda T, Akagi J, Nishikawa A, Ogawa K. (unpublished data); [h]Kligerman et al. (2003); [i]Moore et al. (1997); [j]Noda et al. (2002); [k]Zhang et al. (2011); [l]IARC (1994).

The date of sacrifice was assigned to each rat at the beginning of experiments. The animals were subjected to laparotomy with excision of the urinary bladder under deep anesthesia caused by inhalation of isoflurane. The liver and thyroid gland were also collected for immunohistochemical analysis. The experimental design was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences, Japan, and the animals were cared for in accordance with institutional guidelines as well as the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, June 1, 2006).

Histopathology and immunohistochemistry. For histopathological and immunohistochemical examination, the urinary bladders were inflated with 10% neutral-buffered formalin and carefully removed for immersion fixation. After fixation for 24 h, the bladders were sliced into 6 strips of equal width along the longitudinal axis and embedded in paraffin. Serial sections (4 μm thick) were prepared and stained with hematoxylin and eosin for histological observation. For immunohistochemistry, the sections were deparaffinized, hydrated through a graded series of ethanol, and immersed in 3% H_2O_2/methanol solution for inactivation of endogenous peroxidase activity. For antigen retrieval, all sections were autoclaved in 10 mM citrate buffer (pH 6.0) for 15 min at 121°C. After blocking nonspecific reactions with 10% normal goat serum, the sections were incubated with primary antibodies for γ-H2AX (diluted 1:50; anti-phosphohistone H2A.X [Ser139] antibody; Cell Signaling Technology, Danvers, Massachusetts) and Ki67 (diluted 1:500; anti-Ki67 antibody [SP6]; Abcam, Cambridge, UK) for 12 h at 4°C. Visualization of antibody binding was performed using a Histofine Simple Stain Rat MAX PO kit (Nichirei Corporation, Tokyo, Japan) and 3,3′-diaminobenzidine. All sections were counterstained with hematoxylin. γ-H2AX- and Ki67-labeled bladder epithelial cells were counted under a light microscope in nonblinded fashion. Whole epithelial cells in each strip were sequentially counted, and percentage of labeled cells in 1000 cells was calculated based on a minimum total count of 3000 cells from each animal. In addition, γ-H2AX- and Ki67-positive cells were classified into 3 categories: basal, intermediate, and umbrella cells, which reflected the level of urothelial differentiation based on cell morphology and localization (Ho *et al.*, 2012). γ-H2AX-positive hepatocytes per 1000 cells were also calculated in the left lobe of liver of rats treated with 2-AAF and DEN.

Statistical analysis. Statistical analysis was separately performed in the 2 experiments. Variance in data was checked by Bartlett's test for homogeneity. When the data were homogeneous, 1-way analysis of variance was conducted. In heterogeneous cases, the Kruskal-Wallis test was applied. When statistically significant differences were indicated, Tukey's test was employed for comparisons between the controls and other groups. Differences with P values of less than .05 were considered statistically significant.

RESULTS

In-life parameters. One rat treated with 0.02% DMA died on day 20, and severe tubular necrosis of the kidney was histopathologically observed. Because this was considered to be a toxic effect of DMA, the administration dose was changed to 0.01%, which is still in the range of the carcinogenic dose (Wei *et al.*, 2002), for the final week.

Body weight gain was significantly reduced in rats receiving 2-NA, BMP, melamine, uracil, 2-AAF, *p*-cresidine, DMA, or glycidol as compared with that of the controls at week 4 (Table 2). After 2 weeks of recovery, body weights of rats in the 2-NA, BMP, melamine, uracil, and 2-AAF groups remained significantly lower than those of rats in the control group. Daily food intake was slightly lower in rats in the 2-NA, melamine, uracil, and 2-AAF groups than in rats in the control group.

TABLE 2. Body Weight and Chemical Intake Data for Male F344 Rats

Experiment	Group	Body Weight (g)			Daily Consumption (to Week 4)		Chemical Intake
		Initial	Week 4	Week 6	Diet/Water	(g/rat/day)	(mg/kg bw/day)
Experiment 1	Control 1	120.8 ± 3.7	228.2 ± 8.6	262.4 ± 13.7	Diet	15.9	—
					Drinking water	22.1	—
	0.05% BBN	120.3 ± 5.2	231.0 ± 9.0	266.8 ± 8.5	Drinking water	25.8	68.3
	1.8% 2-NA	120.4 ± 5.2	121.2 ± 12.2**	205.7 ± 5.5**	Diet	6.5	1048.1
	2.0% BMP	120.5 ± 5.4	179.2 ± 15.2**	222.7 ± 10.0**	Diet	16.6	2127.6
	0.1% PEITC	120.9 ± 5.0	227.1 ± 9.0	263.4 ± 6.1	Diet	14.8	77.2
	3.0% Melamine	120.4 ± 8.1	143.5 ± 13.1**	218.1 ± 9.7**	Diet	9.5	2089.2
	3.0% Uracil	120.7 ± 3.3	148.9 ± 6.0**	221.9 ± 7.7**	Diet	8.0	1813.7
Experiment 2	Control 2	120.5 ± 5.5	244.2 ± 9.5	277.8 ± 11.2	Diet	17.3	—
					Drinking water	22.1	—
	0.025% 2-AAF	120.3 ± 4.8	153.5 ± 7.3‡	223.9 ± 8.8‡	Diet	9.2	16.1
	1.0% *p*-Cresidine	120.6 ± 5.0	223.2 ± 7.2‡	266.0 ± 12.6	Diet	14.2	768.8
	0.02%/0.01% DMA[a]	120.6 ± 5.2	221.2 ± 9.5‡	272.1 ± 8.9	Drinking water	32.0	28.7
	0.04% Glycidol	120.5 ± 4.8	230.9 ± 5.4†	266.2 ± 7.2	Drinking water	24.0	49.5
	0.001% DEN	120.6 ± 5.6	239.9 ± 11.6	274.3 ± 20.9	Drinking water	22.6	1.2
	0.005% AA	120.3 ± 3.4	239.8 ± 6.2	271.5 ± 5.9	Drinking water	23.4	6.0

Values are means ± SDs.

BBN, N-butyl-N-(4-hydroxybutyl)-nitrosamine; 2-NA, 2-nitroanisole; BMP, 2,2-bis(bromomethyl)-1,3-propanediol; PEITC, phenethyl isothiocyanate; 2-AAF, 2-acetylaminofluorene; DMA, dimethylarsinic acid; DEN, N-nitrosodiethylamine; AA, acrylamide.

[a]The administration dose was changed to 0.01% for the last week due to 1 death, which occurred because of the toxic effects of the chemical.

**Significantly different from the control 1 at $P < .01$. † and ‡; significantly different from the control 2 at $P < .05$ and $< .01$, respectively.

Immunohistochemistry for γ-H2AX in rat urinary bladders. γ-H2AX expression in bladder epithelial cells of chemical-treated rats was investigated by immunohistochemistry (Figure 1). After 4 weeks of treatment, γ-H2AX-positive cells with characteristic intranuclear dot-like foci were distributed throughout the bladder epithelium in rats in the BBN, 2-NA, 2-AAF, and *p*-cresidine groups, whereas γ-H2AX expression was rarely observed in rats in the glycidol, DEN, AA, and control groups (Figs. 1A–E and 1K–M). PEITC treatment induced simple hyperplasia of the urothelium with occasional expression of γ-H2AX (Figure 1G). In rats in the BMP and DMA groups, γ-H2AX expression was sporadically observed, particularly in the luminal layer of the urothelium (Figs. 1F and 1H). Moreover, diffuse simple and papillary hyperplasia of the bladder mucosa with focal areas containing γ-H2AX-positive cells were observed in rats in the melamine and uracil groups (Figs. 1I and 1J).

Quantitative analysis revealed that all 4 genotoxic bladder carcinogens (ie, BBN, 2-NA, 2-AAF, and *p*-cresidine) induced significant increases in the numbers of γ-H2AX-positive epithelial cells compared with the controls at the end of administration (Figure 2). Slight increases in γ-H2AX-positive cells were detected in rats in the PEITC, melamine, and uracil groups at week 4, but these differences were not significant. Two weeks after withdrawal, although γ-H2AX-positive cells were markedly reduced in all groups, the number of γ-H2AX-positive cells remained significantly higher in rats treated with the genotoxic chemicals BBN, 2-NA, and 2-AAF.

Immunohistochemistry for Ki67 in rat urinary bladders. To assess the potential relationship between DNA damage and cell proliferation activity, Ki67 expression was examined in bladder epithelial cells by immunohistochemistry (Figure 3). After 4 weeks of treatment, Ki67-positive cells were increased by all 4 genotoxic bladder carcinogens (Figure 4). Similarly, the weak bladder carcinogen PEITC and 2 nongenotoxic bladder carcinogens (melamine and uracil) tended to induce high expression levels of Ki67 in association with diffuse hyperplasia of the urothelium. Probably because of large SD values in these 3 groups of the experiment 1, statistical significance was detected only in 2-AAF and *p*-cresidine groups of the experiment 2. After 2 weeks of recovery, Ki67 expression was markedly reduced in all groups.

Classification of γ-H2AX- and Ki67-positive cells based on the degree of differentiation. The bladder epithelium is histologically classified as transitional epithelium composed of 3 layers of basal, intermediate, and umbrella (superficial) cells (Ho *et al.*, 2012). These cell populations may reflect the differentiation level of bladder epithelial cells and may be associated with the development of bladder tumors (Shin *et al.*, 2014; Van Batavia *et al.*, 2014). Therefore, we classified γ-H2AX- and Ki67-positive cells

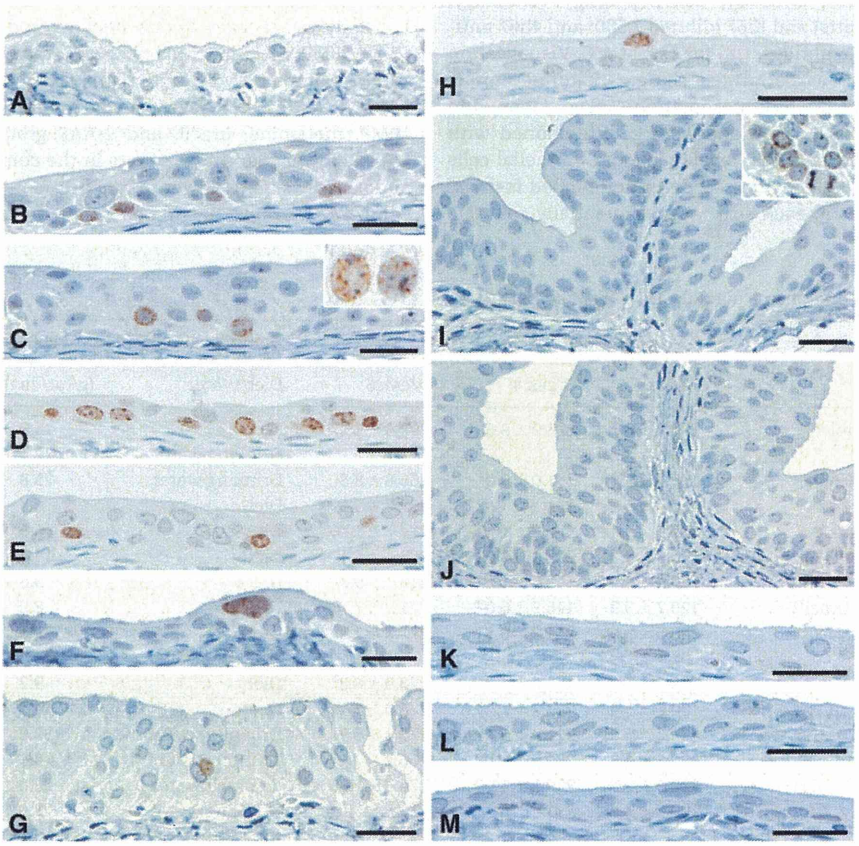

FIG. 1. Representative immunohistochemical findings for γ-H2AX in the urinary bladder of male F344 rats at week 4. Bars = 50 μm. A, Untreated control. B, N-butyl-N-(4-hydroxybutyl)-nitrosamine. C, 2-Nitroanisole. D, 2-Acetylaminofluorene. E, *p*-Cresidine. F, 2,2-Bis(bromomethyl)-1,3-propanediol. G, Phenethyl isothiocyanate. H, Dimethylarsinic acid. I, Melamine. J, Uracil. K, Glycidol. L, N-nitrosodiethylamine. M, Acrylamide. γ-H2AX-positive cells showed characteristic dot-like intranuclear expression (C, inset). γ-H2AX expression was mainly observed in the basal layer of the urothelium (B–E), and in the intermediate (G) and superficial layers (F and H). Note the regional expression of γ-H2AX in the melamine-treated group (I, inset).

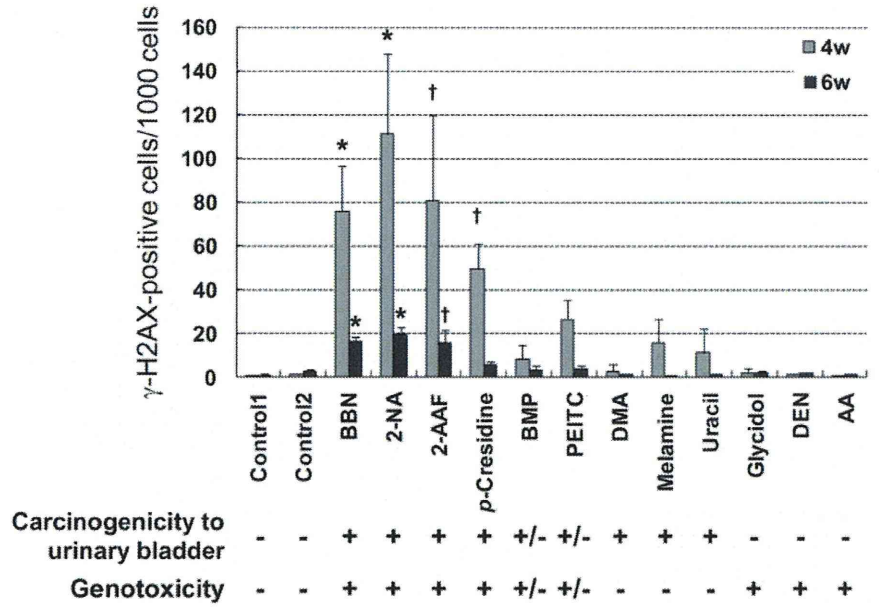

FIG. 2. Quantitative analysis of γ-H2AX expression in the urothelium of chemical-treated rats. γ-H2AX staining was evaluated by determining the average number of γ-H2AX-positive epithelial cells per 1000 cells. Values are the means ± SDs. * and †: Significantly different at $P < .01$ compared with controls 1 and 2, respectively. BBN, N-butyl-N-(4-hydroxybutyl)-nitrosamine; 2-NA, 2-nitroanisole; 2-AAF, 2-acetylaminofluorene; BMP, 2,2-bis(bromomethyl)-1,3-propanediol; PEITC, phenethyl isothiocyanate; DMA, dimethylarsinic acid; DEN, N-nitrosodiethylamine; AA, acrylamide.

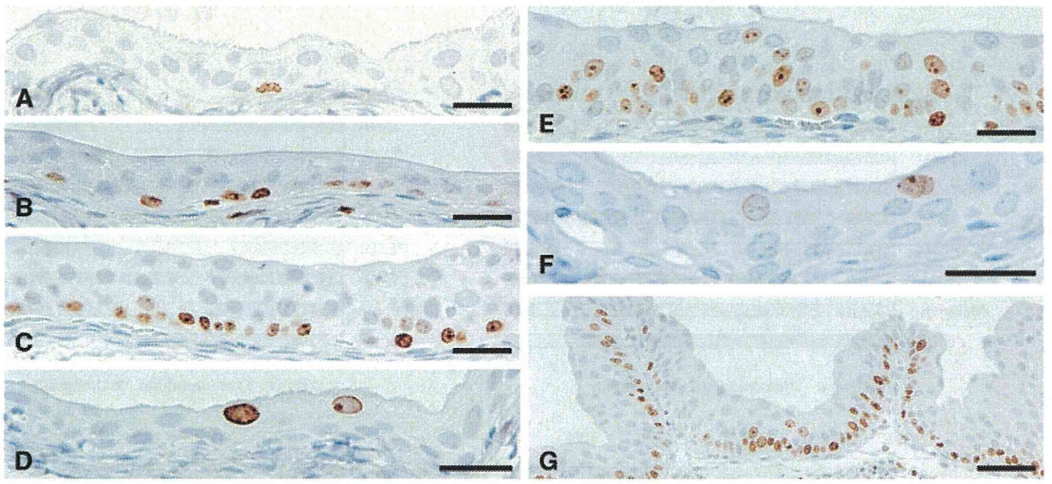

FIG. 3. Representative immunohistochemical findings for Ki67 in the urinary bladder of male F344 rats at week 4. Bars = 50 μm. A, Untreated control. B, N-butyl-N-(4-hydroxybutyl)-nitrosamine. C, 2-Nitroanisole. D, 2,2-Bis(bromomethyl)-1,3-propanediol. E, Phenethyl isothiocyanate. F, Dimethylarsinic acid. G, Uracil. Ki67 expression was mainly observed in the basal layer of the urothelium (A–C and G) and in the intermediate (E) and superficial layers (D and F).

into these 3 categories (Figure 5). Intranuclear γ-H2AX expression was observed in all 3 cell populations in the bladder urothelium. Although most chemicals induced γ-H2AX expression mainly in basal cells at week 4, BMP/DMA and PEITC caused increased expression of γ-H2AX in umbrella cells and intermediate cells, respectively.

Although Ki67 was expressed mainly in the basal cells of all groups at week 4, Ki67-positive umbrella and intermediate cells were observed in relatively large numbers in BMP/DMA and PEITC groups, respectively. After 2 weeks of recovery, the ratios of both γ-H2AX- and Ki67-positive basal cells were reduced in most groups, and the proportions of intermediate and/or umbrella cells increased.

Immunohistochemistry for γ-H2AX in rat liver and thyroid gland. We also examined γ-H2AX expression in the livers and thyroid glands of rat to explore the possibility of application of this method to other organs (Figure 6). γ-H2AX expression was occasionally observed in both hepatocytes in the liver and follicular epithelial cells in the thyroid gland of the control group (Figs. 6A and 6C). Increased numbers of γ-H2AX-positive hepatocytes per 1000 cells were detected in the livers of rats treated with 2-AAF (211.0 ± 87.4, $P < .01$) and DEN (61.4 ± 28.0, $P < .05$), potent hepatic carcinogens, compared with the controls (17.0 ± 10.0) at week 4 (Figure 6B); however, γ-H2AX expression in follicular epithelial cells following treatment with glycidol or AA, which are carcinogenic to the thyroid gland, was comparable with that in control rats (Figure 6D).

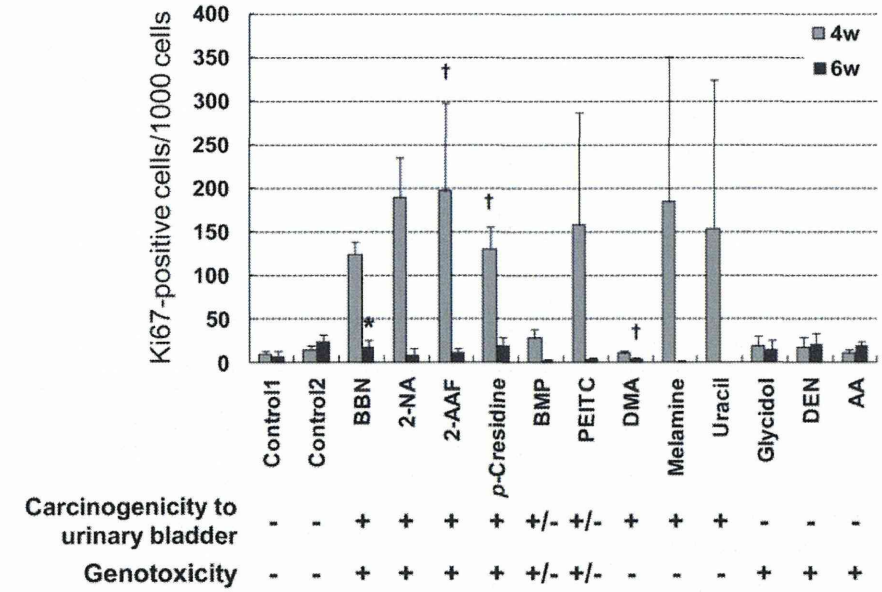

FIG. 4. Quantitative analysis of Ki67 expression in the urothelium of chemical-treated rats. Ki67 staining was evaluated by determining the average number of Ki67-positive epithelial cells per 1000 cells. Values are means ± SDs. Significantly different at $^{*}P < .05$ compared with control 1 and $\dagger P < .01$ compared with control 2. BBN, N-butyl-N-(4-hydroxybutyl)-nitrosamine; 2-NA, 2-nitroanisole; 2-AAF, 2-acetylaminofluorene; BMP, 2,2-bis(bromomethyl)-1,3-propanediol; PEITC, phenethyl isothiocyanate; DMA, dimethylarsinic acid; DEN, N-nitrosodiethylamine; AA, acrylamide.

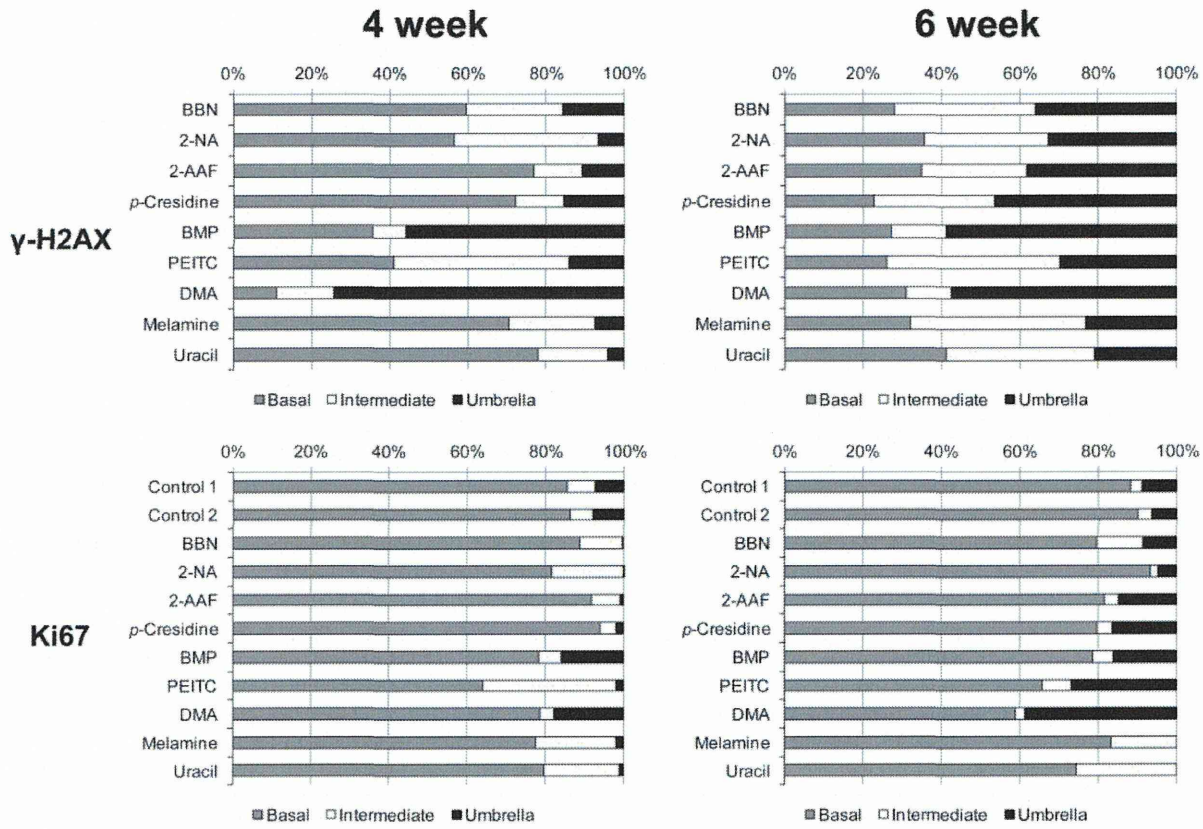

FIG. 5. Percentage of total γ-H2AX- and Ki67-positive cells in the urothelial cell population in chemical-treated rats. Data for the glycidol, N-nitrosodiethylamine, and acrylamide groups and γ-H2AX in the controls are not shown because of low total numbers of Ki67- and/or γ-H2AX-positive cells. BBN, N-butyl-N-(4-hydroxybutyl)-nitrosamine; 2-NA, 2-nitroanisole; 2-AAF, 2-acetylaminofluorene; BMP, 2,2-bis(bromomethyl)-1,3-propanediol; PEITC, phenethyl isothiocyanate; DMA, dimethylarsinic acid.